KISSPEPTINS: AIRWAY REMODELING IN ASTHMA

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State

University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Asthma is a chronic respiratory disorder that affects people of all ages. Sex and gender disparity in asthma is recognized and suggests a modulatory role for sex steroids, particularly estrogen. However, there is a dichotomous role of 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. Kisspeptins (Kp) are a novel peptide existing upstream of sex steroids and function as a crucial regulator of puberty. Kp via KISS1R are further implicated in the sex steroid biogenesis via action on the gonadotrophins. Therefore, this research hypothesizes that kisspeptin (Kp)/KISS1R signaling serves this role, thereby regulating airway remodeling and airway hyperresponsiveness (AHR).

Airway smooth muscle (ASM) is a key structural cell type that contributes to remodeling in asthma. In the first aim, I report 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.

My second aim discusses the functional mechanisms of Kp/KISS1R signaling on majorly proliferation, and in part ECM deposition studies. Proliferation studies show that Kp-10, mitigates PDGF-induced ASM proliferation. Pharmacological inhibition and shRNA knockdown of KISS1R increased basal ASM proliferation, which was further amplified by PDGF. The anti-proliferative effect of Kp-10 in ASM was mediated by inhibition of MAPK/ERK/Akt pathways, with altered expression of PCNA, C/EBP-α, Ki-67, cyclin D1, and cyclin E leading to cell cycle arrest at G_0/G_1 phase. ECM studies show that administration of Kp-10 can mitigate PDGF- and TGFβ- induced increase in ECM remodeling gene and protein expression, such as collagens and fibronectins.

To corroborate my *in vitro* findings, I have further performed *in vivo* studies utilizing Kp-10 (a cleaved peptide of parent kisspeptin) in mixed allergen-induced mouse models of asthma. I found that Kp-10 was able to mitigate asthma by significantly improving airway structural, morphological and lung function parameters. Overall, I demonstrate the importance of Kp/KISS1R signaling in the ASM as a potential therapeutic avenue to blunt remodeling in asthma.

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DEDICATION

Dedicated to God

My Family

And Me

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LIST OF ABBREVIATIONS

AbAm...Antibiotic Antimycotic

AF ....Alexa Fluor

AHR ....Airway Hyperresponsiveness

Akt..Ak Strain Transforming

AR ....Androgen Receptor

ARDS ....Acute Respiratory Distress Syndrome

aSMA ....Alpha-Smooth Muscle Actin

ASM..Airway Smooth Muscle

BSA...Bovine Serum Albumin

BPD...Bronchopulmonary Dysplasia

cAMP ....Cyclic AMP

CDC ....Centers for Disease Control and Prevention

cDNA ....Complementary DNA

COPD..Chronic Obstructive Pulmonary Disease

COMT..Catechol-O-Methyltransferase

CLD...Chronic Liver Disease

Crs...Compliance

CYP...Cytochrome P450 Enzymes

CYP11A1...Cytochrome P450, Family 11, Subfamily A, Polypeptide 1

CYP17A1...Cytochrome P450, Family 17, Subfamily A, Polypeptide 1

CYP19A1...Cytochrome P450, Family 19, Subfamily A, Polypeptide 1

CYP1A2...Cytochrome P450, Family 1, Subfamily A, Polypeptide 2

CYP1B1 ....Cytochrome P450, Family 1, Subfamily B, Polypeptide 1

CYP3A4 ....Cytochrome P450, Family 3, Subfamily A, Polypeptide 4

DAPI ....4',6-diamidino-2-phenylindole

DC ....Dendritic Cell

DMEM ....Dulbecco's Modified Eagle's Medium

E ....Estrogen

E_2 ....17β-estradiol

EDTA ....Ethylenediaminetetraacetic Acid

ER ....Estrogen Receptor

ERα ....Estrogen Receptor alpha

ERβ ....Estrogen Receptor beta

ERE ....Estrogen Response Elements

Ers ....Elastance

ECM ....Extracellular Matrix

ELISA ....Enzyme Linked Immunosorbent Assay

ERK...Extracellular Signal-Regulated Kinase

FBS ....Fetal Bovine Serum

FSH ....Follicle Stimulating Hormone

GnRH ....Gonadotropin Hormone-Releasing hormone

GPCR ....G Protein Coupled Receptor

GPER ....G Protein Estrogen receptor

GPR54...G-Protein Coupled receptor 54

3β-HSD ....3β-hydroxysteroid dehydrogenase

17β-HSD ....17β-hydroxysteroid dehydrogenase

NANC ....Nonadrenergic, Noncholinergic

NIH ....National Institutes of Health

NPY...Neuropeptide Y

2-OHE$_2$...2-hydroxyestradiol

4-OHE$_2$...4-hydroxyestradiol

16-OHE$_1$...16-hydroxyestrone

OVA..Ovalbumin

OVX..Ovariectomized

P ....Progesterone

PAS ....Periodic Acid Schiff's Reagent

PIP$_2$ ....Phosphatidylinositol 4,5-bisphosphate

PR...Progesterone Receptor

P450scc ....Cholesterol Side-Chain Cleavage Enzyme

pAkt...Phosphorylated Protein Kinase B

pERK..Phosphorylated Extracellular Signal-Regulated Kinase

PAH...Pulmonary Arterial Hypertension

PDGF ....Platelet Derived Growth Factor

PBS ....Phosphate Buffered Saline

POMC ....Proopiomelanocortin

PVDF ....Polyvinylidene Fluoride

qRT-PCR...Quantitative Real Time Polymerase Chain Reaction

RDS...Respiratory Distress Syndrome

Rrs..Resistance

SFM...Serum Free Media

T ....Testosterone

TBS ....Tris Buffered Saline

TBST..Tris Buffered Saline with Tween-20

TCR..T Cell Receptor

Th ....T Helper Cell

Th1 ....Naïve T helper Cell

Th1 ....T Helper Type 1

Th2 ....T Helper Type 2

Th9 ....T Helper Type 9

Th17 ....T Helper Type 17

Th22 ....T Helper Type 22

TIMPs ....Tissue Inhibitors of Matrix Metalloproteinases

TGFβ...Transforming Growth Factor beta

TNFα...Tumor Necrosis Factor alpha

WT ....Wild Type

LIST OF SYMBOLS

°C ....Degree Celsius

[C(t)]..Cycle Threshold Value

g..Relative Centrifugal Force

h..Hour/s

i.p..Intraperitoneal

i.n..Intranasal

kg..Kilogram

mg...Milligram

mL ....Milliliter

µg ....Microgram

µL ....Microliter

µM ....Micromolar

min ....Minute/s

ng..Nanogram

nM ....Nanomolar

pH..Potential of Hydrogen

1. GENERAL INTRODUCTION[1]

1.1. Asthma

Asthma is a common chronic respiratory disease afflicting more than 300 million people worldwide[1]. Hallmark asthma symptoms include chest tightness, shortness of breath, and coughing, all of which fluctuate over time, leading to increased respiratory distress and worsening of symptoms, which are characteristic features of severe asthmatics[2, 3]. For example, the incidence, prevalence, and severity of asthma are higher in females (compared with males) after puberty, suggesting a role for sex steroids[4, 5]. Here, sex steroids (estrogens, progesterone, and testosterone) play a major modulatory role in all the life stages. Given the heterogeneity and wide spectrum of asthma, the standard biologics investigated have been disappointing and still need improvement. Similarly, a prototype medicine recognizing a single target will apply to only a small patient pool out of the vast population that shows different endotypes of asthma. Therefore, a major factor in our understanding of sex-based physiology and pathophysiology are sex steroids. The effects of sex steroids depend on their concentration, time of day, and site of action, as they vary across the life span of an individual. This phenomenon is highly relevant and evident in pulmonary biology, especially in asthma.

Overall, a huge female-skewed ration to men of several lung diseases, especially asthma also hinting at worse health implications for women, emphasizes the need to understand the mechanism and the role of inherent sex differences versus how endogenous sex steroids act in asthma pathophysiology. Despite experimental evidence from several in vitro studies and animal models, the specific mechanisms of action for sex steroids to promote or prevent lung diseases remains unclear. In this regard, research is actively being pursued to consider sex steroid mechanisms via differential receptor signaling to gain insight into asthma pathophysiology. In totality, the present clinical and bench data showcase a contrasting nature of sex steroids, wherein there is no clarity on the nature of sex steroids pathophysiologically. Therefore, a better understanding of the biological role of sex steroids in mediating asthma is needed.

1.1.1. Pathophysiology of Asthma

Airway inflammation, together with airway hyperresponsiveness (AHR) and airway structural remodeling, is one of the most noticeable features of asthma[7]. The spectrum of asthma severity varies from mild to moderate to severe uncontrolled disease depending on the inflammatory cell types involved[8]. Typically, asthma is characterized by two major predominant phenotypes: eosinophilic vs. neutrophilic and T helper type 1 (Th1) vs. Th2, or even mixed eosinophilic/neutrophilic inflammatory patterns[9]. However, newer variants of the T-cell immune response, such as T helper type 9 (Th9), T helper type 17 (Th17) and T helper type 22 (Th22) have also been shown to contribute to immune cell responses in asthma[10, 11]. Eosinophilic inflammation can be associated with the spectrum of asthma severity, ranging from mild to moderate to severe uncontrolled disease, whereas neutrophilic inflammation occurs typically in more severe asthma. Eosinophilic asthma includes Th2 cell-derived cytokines that cause allergic or nonallergic phenotypes, while neutrophilic asthma is characterized by Th17

cell-inflammatory mechanisms. Severe asthma is characterized by a polarized Th2 mediated-immune response, exaggerated in the case of females[12, 13]. Unfortunately, targeting this disease with immune cell type selective therapies has proven to be extremely disappointing, necessitating a need to re-evaluate the therapeutic strategies used to modulate, if not, treat this disease. There are several hallmark characteristics of asthma: epithelial basement membrane thickening, an increased (hyper-proliferated) ASM mass, and enhanced numbers of fibroblasts[14, 15]. Thus, what is seen in asthma as a single cellular and inflammatory response to allergens, is more complex and involves a complicated interplay between the sex of an individual, sex steroid regulation characterized by estrogen vs. testosterone effects, the structural and formed elements of the airway and last but not the least, the immune reactivity to these confounding factors. An overview of the various T-cell subsets involved in asthma pathogenesis has bene described in Figure 1.
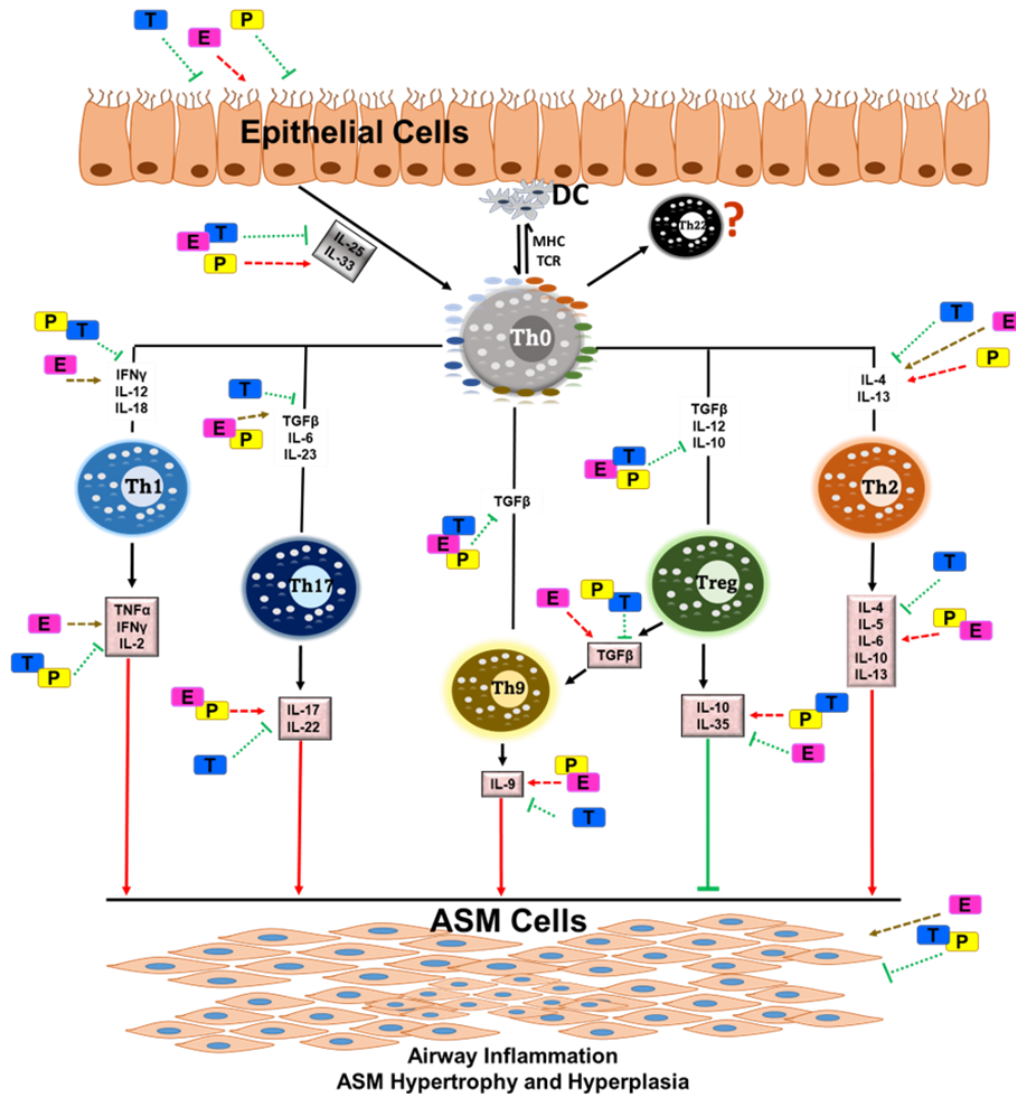
Figure 1: Schematic of asthma pathophysiology.
It is well known that asthma is a multifaceted respiratory disorder consisting of allergic triggers on both the immune system and alterations in the structural cells of the airway. Overall, airway inflammation is mainly orchestrated by the T cell subsets and the regulation of T cell subsets from naïve (Th0) cells may regulate cytokine release towards specific T-cell endotypes. These T cells and their cytokine release are in turn influences by sex steroids such as estrogen (E), testosterone (T) and progesterone (P). All these T cell subsets play key roles in recruiting, activating, and promoting the survival of multiple cell types along with altered pro- and anti-inflammatory cytokines in the airways, subsequently leading to ASM inflammation and remodeling. This is greatly influenced in the presence of circulating sex steroids. Pathways/cytokines aggravated in asthma are marked by red arrows (single and dotted), whereas pathways inhibiting airway inflammation in asthma are marked by green arrows (single and dotted). Yellow arrows denote unknown effects. Abbreviations: DC, dendritic cells; MHC, major histocompatibility complex; TCR, T-cell receptor; Th0, T helper cell (naïve); IFNϒ, interferon gamma; IL, interleukin; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha; E, estrogen; T, testosterone; P, progesterone.

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1.2. Role of Structural Cells in the Airway

The airways consist of a functional equilibrium between the three cell layers: bronchial epithelial cells, airway smooth muscle cells and fibroblasts. During homeostasis, there is equilibrium functionality between these three cell layers to maintain the airway tone.

The epithelial cell layer acts as a frontline defense against viral particles, pollution, respiratory pathogens and infectious agents. They contribute to the production of mediators and other growth factors. The epithelial cell layer consists of three main cell types- cilia cells, goblet cells and basal cells. The ciliated cells are situated across the apical surface and facilitates the movement of mucus across the airways. The goblet cells in turn produce and secrete mucus to trap pathogens and debris that may be freely traversing the airways. Goblet cell differentiation is the major feature noted in asthmatic airways which undergo hypertrophy and hyperplasia in more severe cases of asthma. The basal cells are progenitor cells that can differentiate into other cell types within the epithelium, the second major cell type in the airways. The ASM cells constitute the major contractile cells of the airways which regulate airway tone, structure and function. As a result, much attention has been given to studying the ASM cell in lung pathologies. Fibroblasts are found abundantly in the lung interstitium. Fibroblasts comprise the cells contributing to production of extracellular matrix components such as collagens and glycoproteins such as fibronectins. These proteins help maintain the structural integrity of the airways. Lung fibroblasts respond to lung tissue injury and are essential in the repair and remodeling process. Interestingly, it has been reported that fibroblast migration to the site of injury is crucial to effective tissue repair[16]. Inadequate or excessive accumulation of fibroblasts to the site of injury could result in abnormal tissue function.

1.2.1. Airway Smooth Muscle

Although the airways consist of multiple cell types, the ASM cell is one of the major structural components of the airways and a significant histological indicator of airway remodeling in an asthmatic patient. It is believed that increased ASM cell mass in asthma is a consequence of both hypertrophy (increased size of ASM cells) and hyperplasia (increased proliferation of ASM cells), both are hallmarks of severe asthma. Several studies have shown that ASM from asthmatics generate more force, and therefore, contract greater extent, thereby resulting in exaggerated airway narrowing or AHR[17, 18]. Several studies have characterized the increase in smooth muscle mass to be due to stimulation by pro-inflammatory mediators and other growth factors, such as PDGF[19-21]. For example, other studies have also shown that insulin and other mitogenic agents (such as growth factors and inflammatory cytokines) upregulate the expression of contractile markers and ECM-derived molecules which subsequently alter the phenotype of ASM cells[22]. However, the mechanism by which these growth factors modulate airway hypertrophy and hyperplasia remains unclear. The ASM is also the main cell type involved in AHR. Moreover, the ASM cell also displays immunomodulatory and pro-inflammatory functions by expressing a wide range of cell surface molecules, integrins, costimulatory molecules and toll-like receptors.

What is clear, though, is that the ASM is a potential target and effector cell type in the airways responsible for airway inflammation, hyperresponsiveness and remodeling. ASM cells constitute: 1) a part of the inflammatory process in asthma, 2) have contractile, secretory and proliferative functions, 3) give rise to different asthmatic phenotypes, 4) have remodeling properties and 5) are a viable target to treat asthma.

1.3. Gender Inequality in Asthma

Asthma as a disease is characterized by sex difference. These sex differences are influenced by the interplay between sex chromosomes, sex steroids and the immune system[5]. The female bias in asthma is well established in comparison to a male predominance before puberty (from infancy to childhood) suggesting a role of sex chromosomes in the incidence and prevalence of asthma during the early stages of life. This epidemiological fact suggests that the influence of sex chromosomes in asthma pathophysiology is independent of sex steroids effects. The four-core genotype (FCG) mouse model is a novel technique used widely to study the role of XX versus XY genes separately from the sex steroidal effects[23]. According to previous reports, the incidence and severity of asthma is higher in boys under the age of 13, whereas females suffer more from asthma as compared to adult men[24]. Interestingly, these sex differences are observed across clusters of asthmatics, with a common focal point of female predominance. Moreover, in the case of females, factors such as menstrual cycle, pregnancy, use of oral contraceptives (OCPs), and menopause also exist. Women generally suffer greater respiratory discomfort and exacerbation of asthma symptoms during the menstrual cycle primarily due to fluctuations in the sex steroid levels throughout menses[25, 26]. On the other hand, women on birth control pills or OCPs experience fewer asthma attacks and are at a lesser risk of developing asthma[27]. Additionally, women on hormone replacement therapies also experience increased as well as decreased risk of asthma depending on various factors. Interestingly, asthmatic symptoms worsen during pregnancy and vary in each trimester[28]. Menopausal females may experience worsened or improved asthmatic symptoms, adding more complexity to this scenario. This could be attributed to unbalanced levels of female sex

steroids. Here, sex hormones are key mediators of these differences in asthma prevalence across the sexes from childhood to old age as shown in Figure 2.

According to a recent report published by the American Thoracic Society, in a workshop by the National Heart, Lung, and Blood Institute, the National Institutes of Health (NIH) Office of Research on Women's Health, and the NIH Office of Rare Diseases Research ("Female Sex and Gender in Lung/Sleep Health and Disease," clear emphasis was placed on the use of sex as a biological variable in both bench side-based laboratory research practices and clinical settings[29]. This report critically reviewed the present understanding of the comprehensive implications of female sex on lung and sleep health and disease, addressing research gaps and envisioning better health outcomes by means of a specific and accurate management plan.
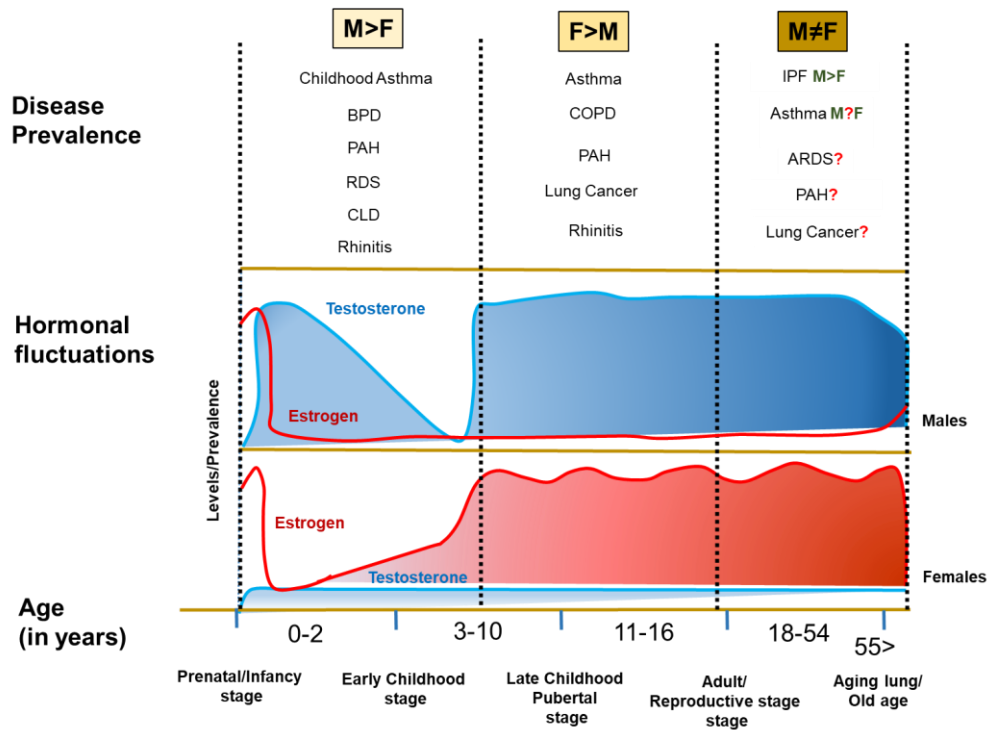
Figure 2: Illustration of the levels of estrogen (female) and testosterone (male) in the prevalence of lung diseases throughout the lifespan in males and females.
Sex steroid fluctuations throughout the lifespan of an individual cause predispositions to certain lung diseases, particularly asthma as individuals age. Similarly, other lung diseases are not affected by sex steroid levels with no differences in their incidence and severity. Abbreviations: E, estrogen; T, testosterone; P, progesterone.

1.4. Sex Steroid Biogenesis

Cholesterol from food sources is used by different cells in tissues of gonadal and nongonadal origin to synthesize the common precursor, pregnenolone. This cascade's end is a synthesis of androgens and estrogens used to elicit a biological effect. The gonads are responsible for the production of sex steroids under the influences of LH and FSH. LH and FSH are secreted by gonadotrophs, located in the anterior pituitary gland. The HPG axis can be regarded as the command center of the endocrine system, regulating the synthesis and secretion of hormones from other glands through the kisspeptin–neurokinin B–dynorphin (KNDy) neuronal network and GnRH-producing neurons. The absence of ERα on GnRH neurons raises the possibility of an intermediate signaling pathway that may be activated to mediate sex steroid feedback mechanisms. Kisspeptins act as a crude proxy for GnRH, stimulating robust induction by integrating endocrine and metabolic signals via classic feedback loops, thus controlling the downstream HPG axis throughout the lifespan of an individual (Figure 3). Although a role of kisspeptins has been implicated in the estrogen positive feedback mechanism, it is unclear how kisspeptin signaling would be affected by the same hormonal stimulus[30-32].

Kisspeptins are increasingly recognized as a critical factor for normal puberty, secretion of gonadotropin, and reproduction. Kisspeptins are involved in both the positive and the negative feedback regulation of GnRH, which has been highlighted in many of the initial studies[31].

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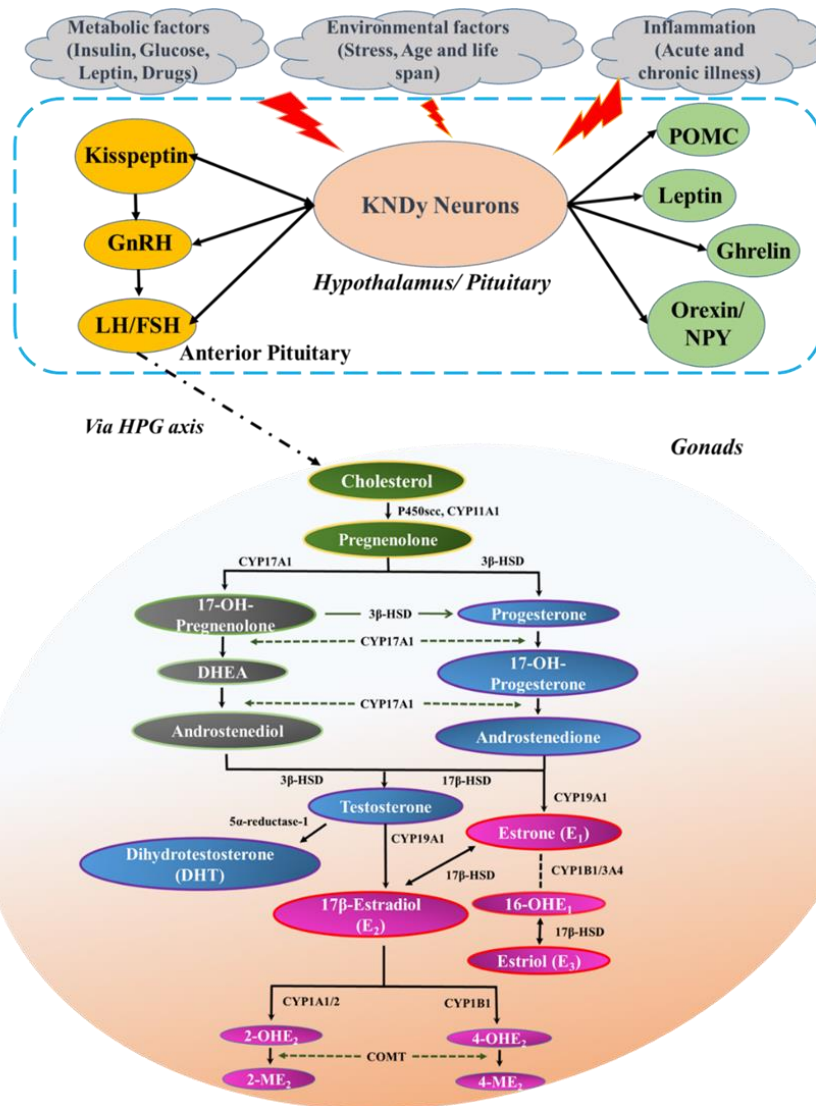
Figure 3: Sex steroid metabolism and regulation.

A simplified overview of steroidogenesis, starting with the synthesis of estrogen, progesterone, and testosterone from cholesterol. Here, steroid hormones (luteinizing hormone [LH], follicle-stimulating hormone [FSH], and kisspeptin) regulate the release of the gonadal sex steroids, mainly by the hypothalamic–pituitary–gonadal axis. Cytochrome P450 (CYP) enzymes are particularly important and are a key modulator of the levels of sex steroids. Abbreviations: Gonadotropin-releasing hormone (GnRH), neuropeptide Y (NPY), proopiomelanocortin (POMC), cholesterol side-chain cleavage enzyme (P450scc), cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) gene, 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1) gene, 17β-hydroxysteroid dehydrogenase (17β-HSD), cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1) gene, 16-hydroxyestrone (16-OHE$_1$), 2-hydroxyestradiol (2-OHE$_2$), 2-methoxyestradiol (2-ME$_2$), 4-hydroxyestradiol (4-OHE$_2$), 4-methoxyestradiol (4-ME$_2$), cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) gene, cytochrome P450, family 11, subfamily A, polypeptide 2 (CYP1A2) gene, cytochrome P450, family 11, subfamily

B, polypeptide 1 (CYP1B1) gene, cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4) gene, and catechol-O-methyltransferase (COMT).

1.5. Kisspeptins and KISS1R

The kisspeptin (Kiss1) gene was first identified in humans in 1997 as a tumor suppressor in melanoma cells[33]. The Kiss1 gene is located on chromosome 1 and reported to encode a 145 amino acid preprotein. This parent protein is further processed to smaller cleaved peptides-Kp-10, Kp-13, Kp-14 and Kp-54, all of which share the biologically active decapeptide sequence which is demonstrated to activate the transmembrane G-Protein Coupled Receptor, KISS1R or the GPR54[34, 35], discovered later in 2003. GPR54 or KISS1R is a member of the rhodopsin family of GPCR's with sequence homologies to members of the galanin receptor family. Although galanin does not bind to KISS1R, endogenous ligands with a common RF-amide C terminus, derived from the kisspeptins bind to and activate KISS1R (Figure 4)[36, 37].

Kp and its cleaved peptides have often been used interchangeably with the term "metastin", coined so because of its role as a tumor suppressor[38]. Studies in endocrinology and oncology suggest a crucial role for Kp/KISS1R signaling in different cell types with a pivotal contribution to the onset of puberty and suppression of cancer metastasis via inhibition of proliferation[39-41]. This provides us an idea that a range of physiological systems may be impacted by kisspeptins.

Interestingly, kisspeptins exists in a sex-specific manner in the hypothalamus. In mammals, including humans, kisspeptin signaling has been implicated as the key event representing initiation of pubertal process[41]. In support of these findings, the importance of kisspeptin signaling was first shown by reports indicating that Kiss1R KO mice do not undergo puberty, while also suffering from major reproductive function deficits[42]. Mutations in the

KISS1 and KISS1R genes are linked to hypogonadotropic hypogonadism, a condition where gonads lose their ability to produce sex steroids[43].
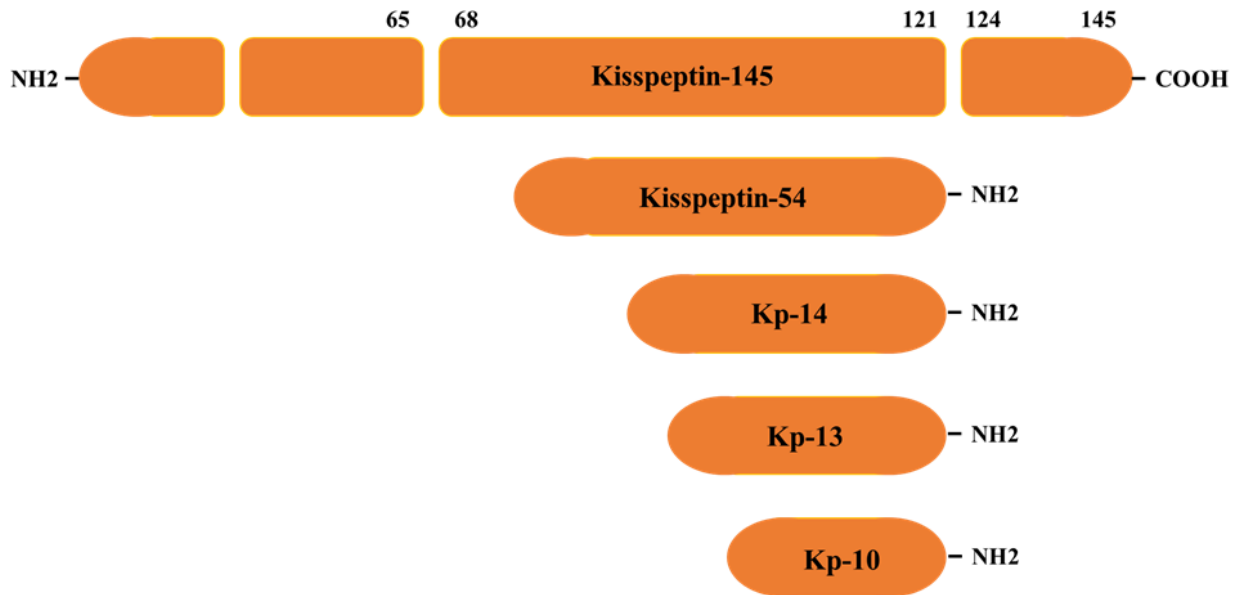
Figure 4: Cleaved peptide fragments of Kisspeptin (Kp)
Kp is cleaved into shorter fragments; Kp-54, Kp-14, Kp-13 and Kp-10. Different kisspeptins are generated by the cleavage from a common precursor, the prepro-kisspeptin. The prepro-kisspeptin contains 145 amino acids, with a central 54-amino acid region, kisspeptin-54 (Kp-54). Further cleavage of metastin generates kisspeptins of lower molecular weight: kisspeptin-14 (Kp-14), Kp-13, and Kp-10. All kisspeptins contain the RF-amide motif containing the biologically active Kp-10 fragment that binds to and activate the kisspeptin receptor or KISS1R.

Later, in 2005, the next important milestone in understanding the role of kisspeptins in reproductive physiology was uncovered by Dhillo *et al*, where they showed dose-dependent increases of LH, FSH and testosterone hormones in response to exogenous administration of kisspeptin-54 infusions to healthy male volunteers[44]. Overall, Dhillo *et al's* work revolutionized the field of kisspeptins in reproductive physiology by demonstrating that kisspeptins can increase circulating levels of gonadotropins and sex steroids in normal males. Most importantly, this scientific evidence was nothing short of a miracle, since there were no adverse events reported. Subsequently, Kp and its receptor, KISS1R were thrust into various niches of cancer and reproductive physiology to answer long-standing questions within the

fields[45-47]. All this previous literature by endocrinologists, reproductive physiologists and clinicians alike shows that administration of kisspeptins can be used as a potential manipulator of sex steroids in humans and therefore raises many new possibilities in the field of opportunistic drug discovery in other organ systems and disease models[48, 49].

In sharp contrast to the amount of studies directed at elucidating the regulatory functions of the Kp/KISS1R signaling in the reproductive system and cancer, only a limited number of studies have been primarily focused on the intracellular signaling pathways activated by kisspeptins via KISS1R. Initial functional analyses of the kisspeptin system demonstrated that the all kisspeptin bind to and activate the KISS1R, with maximal activity shown by Kp-10 at the receptor level[34]. Upon binding to and activating the KISS1R, the major intracellular pathway includes the activation of phospholipase C and PIP_2, followed by the accumulation of IP_3, Ca^{2+} mobilization, arachidonic acid release and phosphorylation of ERK and P38 MAP kinases (Figure 5)[34].
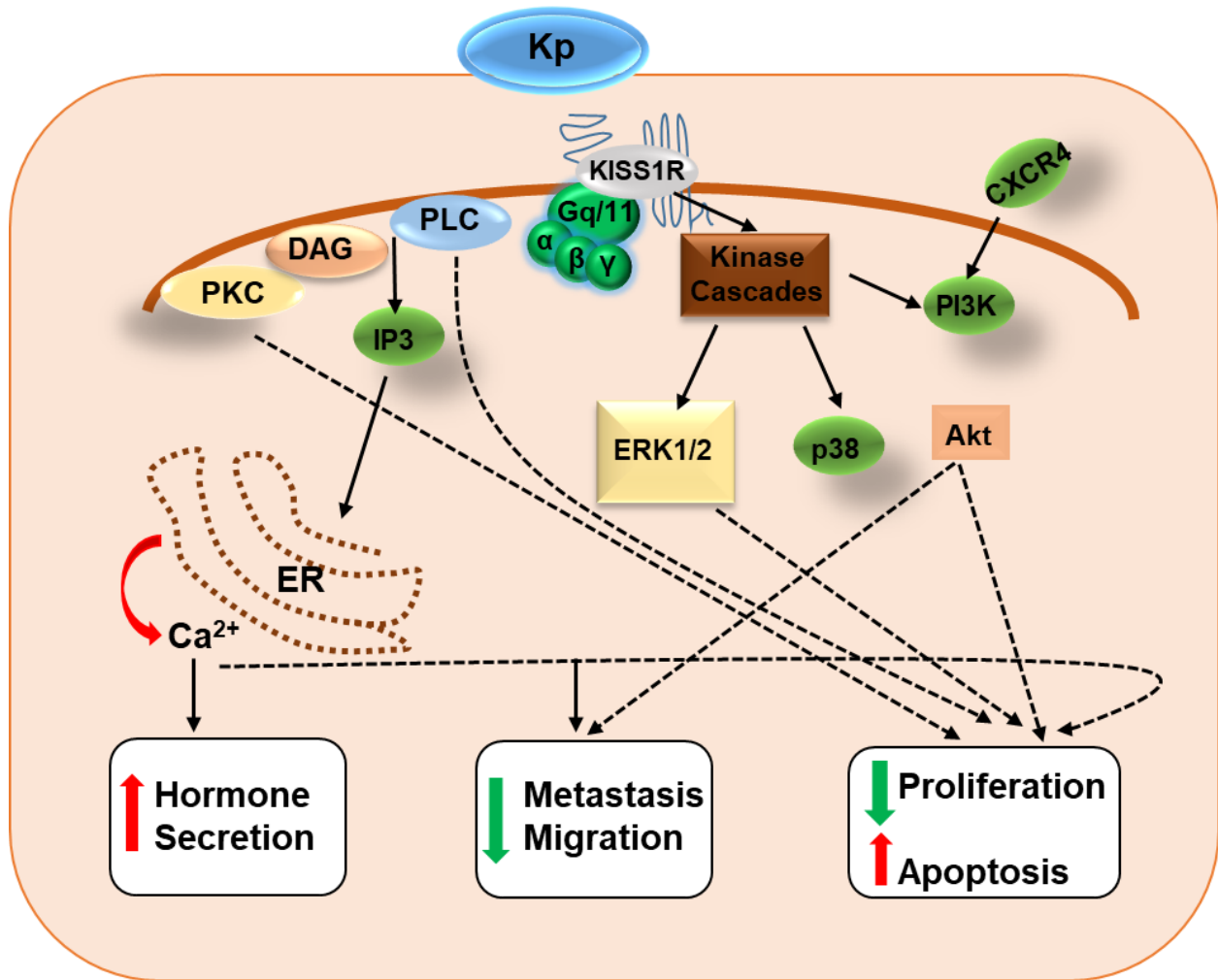
Figure 5: Proposed intracellular signaling pathways activated by Kp/KISS1R in different cell systems.
Schematic diagram illustrating the main signal transduction pathways that have been reported and/or proposed to be involved in kisspeptin actions upon KISS1R activation. Although, the mechanism appears to be same, they are highly cell-, tissue- and organ specific in each individual cell system. Solid arrows stand for actions that have been clearly demonstrated and are considered important in Kp/KISS1R mediated effects. Dotted arrows reflect pathways whose potential involvement in Kp/KISS1R actions have been proposed but are still to be clearly proven or that are considered marginal with respect to other more important routes. Figure adapted from[50].

Although, kisspeptins previous identity was in the field of cancer biology, the fresh

discovery of numerous loss-of-function mutations in its receptor, KISS1R, has cast new light on

the implications of the Kp/KISS1R signaling pathway.

1.6. Research Gaps

Lack of clarity on the precise role of sex steroids in asthma pathophysiology prompted me to investigate upstream of sex steroids. While there are differential effects of estrogen and testosterone reported in asthma, a clear consensus of how those effects take place are not known. Also, it is well known that there are hormonal fluctuations exist during the lifespan of an individual and therefore, there are multiple factors that could be regulating asthma as a disease. Furthermore, estrogen fluctuations in perimenopausal females suggest a role for other pathways during the luteal period. This brings up a possibility for other mechanisms upstream to or independent of estrogen to play a role in the observed sex differences in asthma. Several pieces of evidence in the central nervous system 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[51]; B) Kp regulates gonadotropin-releasing hormone and gonadal steroids[47]; C) administration of kisspeptin receptor (KISS1R) agonist has been investigated as a potential treatment for sex steroid-dependent diseases[52]. Accordingly, Kp/KISS1R's in the airways may be important to understand towards identifying novel pathways that contribute to asthma per se, and potentially the noted sex differences. Studies in endocrinology and oncology suggest a crucial role for Kp/KISS1R signaling in different cell types with a pivotal contribution to the onset of puberty and suppression of cancer metastasis via inhibition of proliferation[39]. Kp/KISS1R influences p38 mitogen-associated protein kinases (MAPK) signaling pathways[50], and can thus modulate inflammation and proliferation, two aspects also important for ASM in asthma. However, to the best of our knowledge, there is no information on Kp/KISS1R in the lung, but data in other systems raise the question of whether Kp/KISS1R can regulate airway remodeling in the context

of asthma. I therefore, set the following specific aims to achieve the overall objective of my research.

- **Specific Aim 1: To determine the expression of Kp/KISS1R in human male vs. female and non-asthmatic vs. asthmatic airway smooth muscle cells.** There is currently no information of the expression of Kp/KISS1R in the lungs, let alone the airway smooth muscle cell. Moreover, to investigate upstream of sex steroids, I performed a sample screening of human airway smooth cells to evaluate the differential expression between males vs. females and non-asthmatics vs. asthmatics.

- **Specific Aim 2: Kp/KISS1R signaling in proliferation and extracellular matrix production and deposition in human airway smooth muscle cells.** Previous data demonstrated a ubiquitous, yet sex- and disease-differentiated expression of Kp/KISS1R in human ASM, as determined in Specific Aim 1. The mitogenic role of PDGF, TNFα and TGFβ on human airway smooth muscle cells is well established. In this aim, using these mitogenic factors, I have looked at the effect of Kp/KISS1R activation on ASM cell proliferation and extracellular matrix production and deposition. The observation in this aim will help shed more insight on the mechanistic basis of Kp/KISS1R-induced mitigation of ASM-specific remodeling changes.

- **Specific Aim 3: In a mouse model of mixed allergen-induced asthma, determine the role of Kp/KISS1R in airway hyperresponsiveness and remodeling.** In this aim, to investigate the role of chronic kisspeptin exposure on lung health, I used different populations (male, female and OVX) of mixed-allergen induced wild-type mice to check for airway hyperresponsiveness and remodeling.

This research will set the stage for future exploratory research in kisspeptins in the airways and in other pathologies. It will also serve as a potentially new avenue for several sex-dependent pathologies, such as in the case of asthma and allow researchers to disseminate a new therapeutic strategy using the kisspeptins.

2. KISSPEPTIN AND ITS RECEPTOR, KISS1R ARE EXPRESSED IN THE HUMAN AIRWAYS[1]

2.1. Introduction

Asthma shows age- and sex-related differences in epidemiology and clinical manifestation, where prepubertal boys are more likely to have asthma compared to girls, but following puberty, adult women show greater incidence, frequency, and severity of asthma compared to men, a difference that decreases following menopause[53]. 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 as some studies suggest that estrogens enhance inflammation, while others associate estrogens with an asthma-mitigating role. Specific to ASM, we and others have found that differential effects of 17 beta-Estradiol (E_2) depend on the contributions of estrogen receptor subtypes (ERα vs. ERβ) in regulating ASM structure and function, thus adding to complexity of sex steroid signaling in asthma pathophysiology[19, 54]. Furthermore, estrogen fluctuations in perimenopausal females suggest a role for other pathways during the luteal period. This brings up a possibility for other mechanisms upstream to or independent of estrogen to play a role in the observed sex differences in asthma.

Kisspeptin refers to the family of neuropeptides, resulting due to the cleavage of a 145 amino acid precursor peptide, encoded by the KISS gene[34, 35]. The first observation of kisspeptins anti-metastatic property occurred in melanoma cells. Soon after, several other evidences of kisspeptin expression were found in other peripheral organs. Years after the initial discovery of kisspeptin, the kisspeptin receptor, also called as the KISS1R or GPR54 was discovered as a G-Protein coupled receptor having strong binding affinity to kisspeptin. The KISS1R is expressed predominantly within regions in the hypothalamus. Although reports suggest the expression and function of other sex steroid receptors, namely Erα, Erβ and AR in the human airways, there is no known literature suggesting the expression of Kp/KISS1R in the airways.

2.2. Materials and Methods

2.2.1. Chemicals, Drugs/Inhibitors and Antibodies

Cell culture reagents, Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), Antibiotic-Antimycotic (AbAm), and 0.25% trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA, USA). Charcoal stripped fetal bovine serum (FBS) was procured from Sigma-Aldrich (St. Louis, MO, USA). Protease and phosphatase inhibitor cocktail (Cat# P178445) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies for Kp (Kisspeptin, Cat# sc-101246), was obtained from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Primary antibody for KISS1R (Cat# PA5-27179) was obtained from Thermo Fisher Scientific. Human Kisspeptin ELISA Kit (Cat# MBS3803580) was procured from MyBiosource (San Diego, CA, USA) and LDH-Cytotoxicity Colorimetric Assay Kit (Cat# K311) from BioVision (Milpitas, CA, USA). Beta-actin antibody (Cat# G043) was purchased from ABM biological materials (Richmond, BC, Canada). IRDye® Goat anti-mouse and goat anti-rabbit

secondary antibodies were used for scanning in Li-Cor Odyssey CLX (Li-Cor Systems, Omaha, NE, USA). Other chemicals and reagents were procured from Sigma-Aldrich unless otherwise indicated.

2.2.2. Tissue and ASM Cell

The procedure for acquiring human lung samples and isolating primary human ASM cells has been described previously[17, 18, 56]. Formalin-fixed, paraffin-embedded human lung tissue sections were used for immunofluorescence studies. Airway samples denuded of epithelium and ASM tissue were enzymatically dissociated as per the manufacturer's instructions (Worthington Biochemical, Lakewood, NJ, USA) to generate ASM cells. For cells, cultures (<5th passage) were maintained under standard conditions of 37°C (5% CO_2, 95% air) using DMEM/F12 supplemented with 10% FBS and 1% AbAm. ASM phenotype was verified periodically by western analysis for expression of α-smooth muscle actin, transgelin, calponin and caldesmon as shown in Figure 6[57].
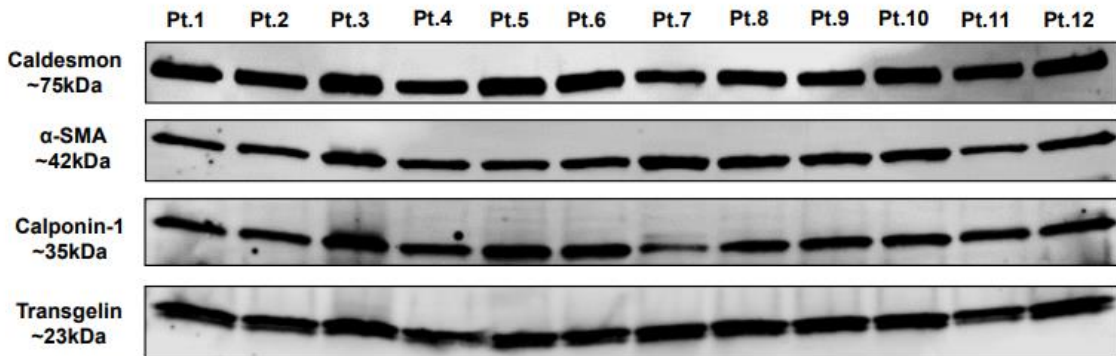
Figure 6: Validation of differentiation state of primary human ASM cells.
The human ASM cells utilized for functional studies were validated for their actual differentiation state by confirming presence of smooth muscle specific marker proteins such as caldesmon, alpha smooth muscle actin (α-SMA), calponin-1 and transgelin (N = 12).

2.2.3. Immunofluorescence Studies

Standard immunofluorescence techniques were applied to 6 µm thick human lung sections. Briefly, sections were baked (56°C for 2 h), 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 phosphate-buffered saline (PBS), blocked with 10% goat serum, and incubated with antibodies against Kp, KISS1R, and alpha-SMA. AlexaFluor-488 for alpha-SMA and AlexaFluor-647 for Kp and KISS1R were used as secondary antibodies with DAPI-AF408 counterstaining for nuclei. High-resolution Z-stack images were captured on a confocal microscope (Zeiss-LSM900 with Airyscan2)[58].

For immunofluorescence detection in human ASM cells, cells were fixed with 4% paraformaldehyde in PBS pH 7.2, washed twice with PBS, and permeabilized using 0.05% Triton X-100 in PBS (permeabilization step was omitted for KISS1R), washed twice with PBS, and blocked with 10% goat serum. Followed by blocking, cells were incubated overnight at 4°C with polyclonal rabbit anti-KISS1R and monoclonal mouse anti-Kp antibodies in different wells. Primary antibodies were detected with AlexaFluor-647 secondary antibodies 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.

2.2.4. qRT-PCR Analysis

Cells were washed with RNA-grade PBS before trypsinization and proceeded for RNA isolation. RNA cell isolation was performed using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA) and OneScript cDNA Synthesis Kit (ABM biological materials, Richmond, BC, Canada) were used for complementary DNA (cDNA) synthesis using a

minimum of 500 ng of RNA for each sample. Genomic DNA contamination was avoided by using DNAse I treatment. BrightGreen 2X qPCR Master Mix (Applied Biological Materials, Cat# MasterMix-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: hKISS1 (forward 5'-CAA GCC TCA AGG CAC TTC TA-3'; reverse 5'-AAA GTG GGT GGC ACA GAG-3'), hKISS1R (forward 5'-ATC GGA ATT CAC CAT GCA CAC CGT GG-3'; reverse 5'-ATC ATC TAG AAC AGA AGA TAG CCG C-3') and hS16 (forward 5'-CAA TGG TCT CAT CAA GGT GAA CGG-3'; reverse 5'-CTG ACG GAT AGC ATA AAT CTG GGC-3'). Cycle threshold [C(t)] values of target mRNA were normalized to reference gene s16. 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.

2.2.5. Western Blot Analysis

Human ASM cell lysates for individual treatment groups were prepared using cell lysis buffer as previously described. 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 (BioRad, Hercules, CA, USA). 30 µg equivalent protein from respective treatment groups were loaded on 4-15% 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. 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h at room temperature was used as a blocking buffer and membranes were incubated overnight at 4°C with specific primary antibodies of interest. Following three washes for 8 min/wash with Tris-buffered saline containing 0.1% tween (TBST), blots were incubated with LiCOR near-red conjugated secondary antibodies at room

temperature for 1 h. Beta-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 beta-actin.

2.2.6. Measurement of Kisspeptin Levels

To measure the endogenous ASM Kp secretion, the media were collected after 48 h serum deprivation and concentrated equally to 500 µL using Amicon Ultra-15 Centrifugal Filter Units (Cat# UFC901024). The endogenous Kp levels in the concentrated conditional media was measured using the Kp ELISA kit as per the manufacturer's protocol.

2.2.7. Statistical Analysis

Human ASM cells from at least five donors (males and females; non-asthmatics and asthmatics) were used for all experiments. For expression studies, cell lysates for Western analysis and cDNA for qRT-PCR were obtained from at least five different individual donor samples. Statistical analysis was performed using a one-tailed unpaired t-test or one-way/two-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 of $p<0.05$ level. All values are expressed as mean ± SEM.

2.2.8. Study Approval

Human bronchi from third- to sixth generations were isolated from lung specimen's 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 COPD) were considered to have an otherwise normal lung function and classified as non-asthmatics (de-identified

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pulmonary conditions of the donors). The protocols were approved by the Mayo Clinic

Institutional Review Board (IRB#08-002518).

2.3. Results

2.3.1. Expression of Kp and KISS1R in Human Lung Tissue and ASM Cells

Immunohistochemistry 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 7A and B), and by

multicolor immunofluorescence staining of isolated human ASM cells (Figure 8A and B).
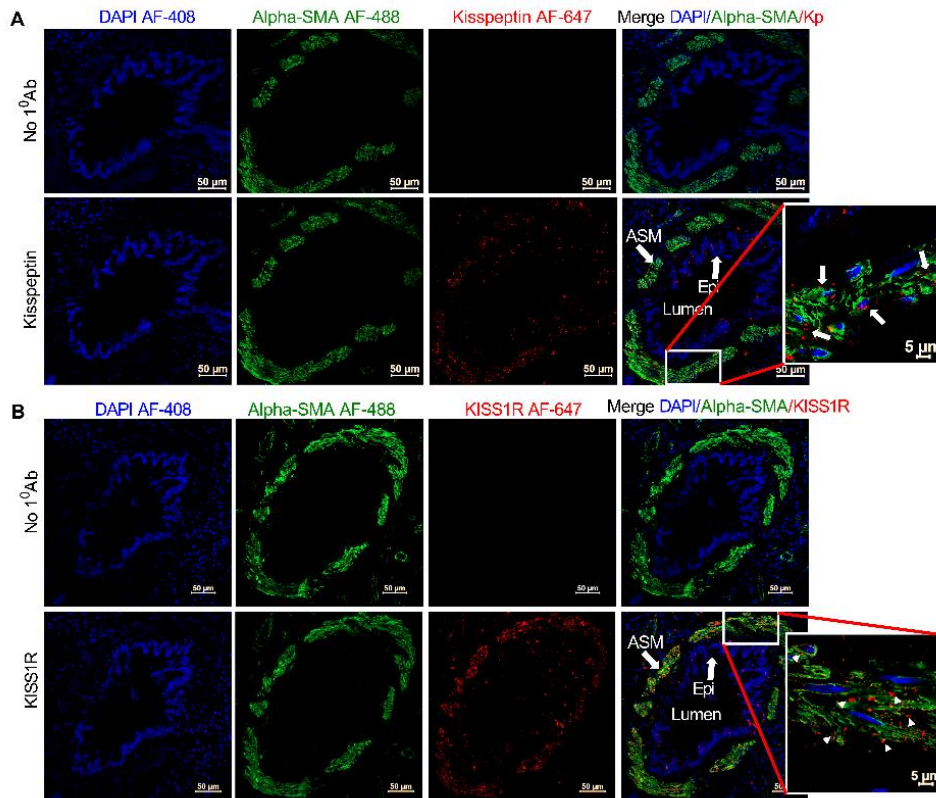
Figure 7: Kisspeptin (Kp) and its receptor, KISS1R are expressed in the human airway as shown by immunohistochemistry (IHC).
Human lung sections were immunostained for Kp (A, AF-647) and KISS1R (B, AF-647) with alpha-smooth muscle actin (alpha-SMA, AF-488) as a reference marker for smooth muscle-specific 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 µm (insert: 5 µm). Representative images from N = 5 independent non-asthmatic donor samples.
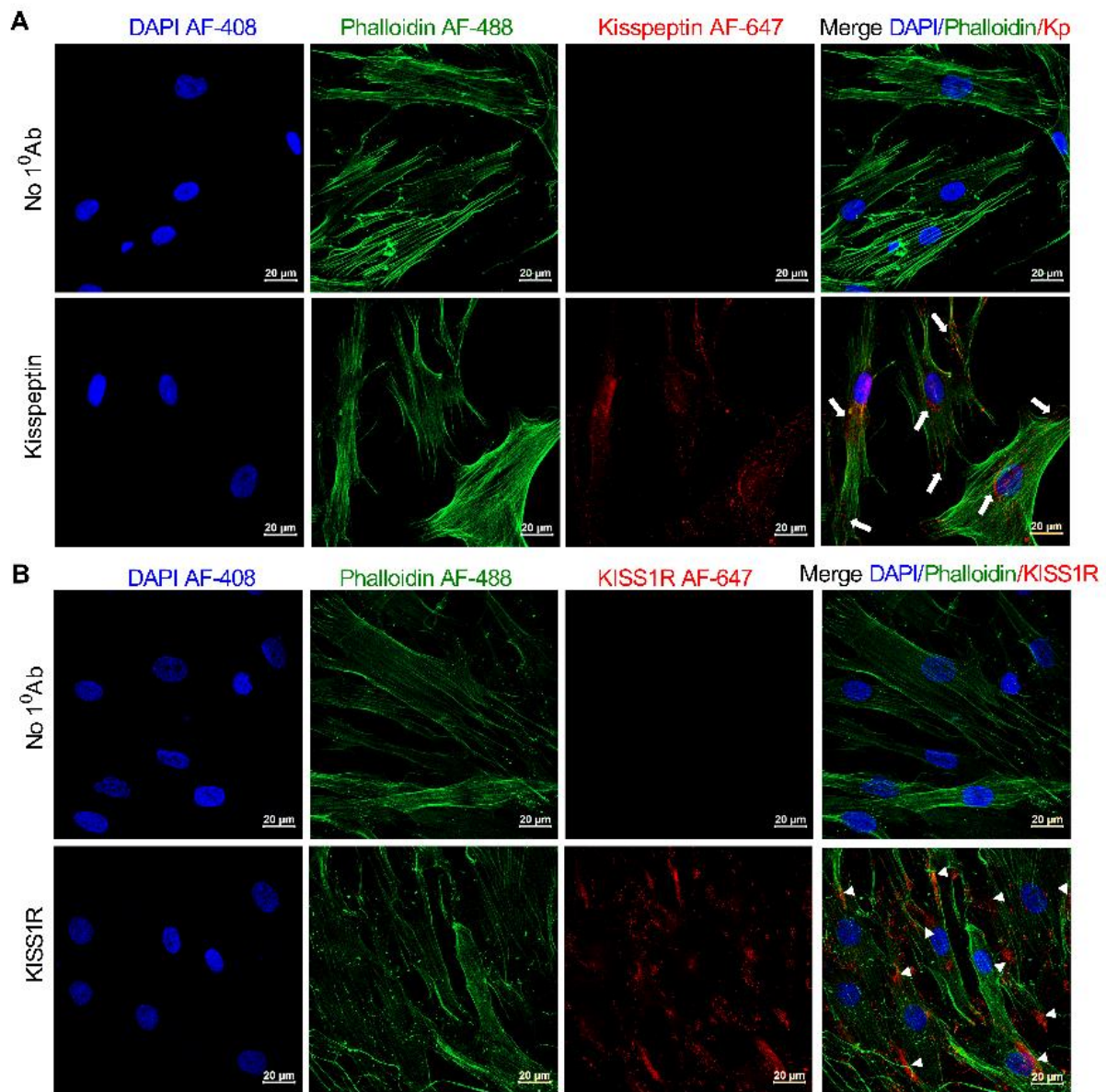
Figure 8: Kp and KISS1R expression in isolated human airway smooth muscle (ASM) cells as shown by immunocytochemistry (ICC).

Paraformaldehyde fixed human ASM cells were immunostained with Kp (A) and KISS1R (B). Kp and KISS1R were probed with AF-647 secondary antibodies 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 µm. Representative images from N = 5 independent non-asthmatic donor samples.

2.3.2. Sex Differences in ASM Expression of Kp and KISS1R

Baseline mRNA and protein expression of KISS1 (mRNA)/Kp (protein) and KISS1R in primary non-asthmatic human ASM cells from male vs. female donors showed both KISS1 (P<0.01; Figure 9A)/Kp (P<0.001; Figure 9C) and KISS1R were significantly lower in females compared to males for both mRNA (P<0.01; Figure 9B) and protein (P<0.001; Figure 9D).
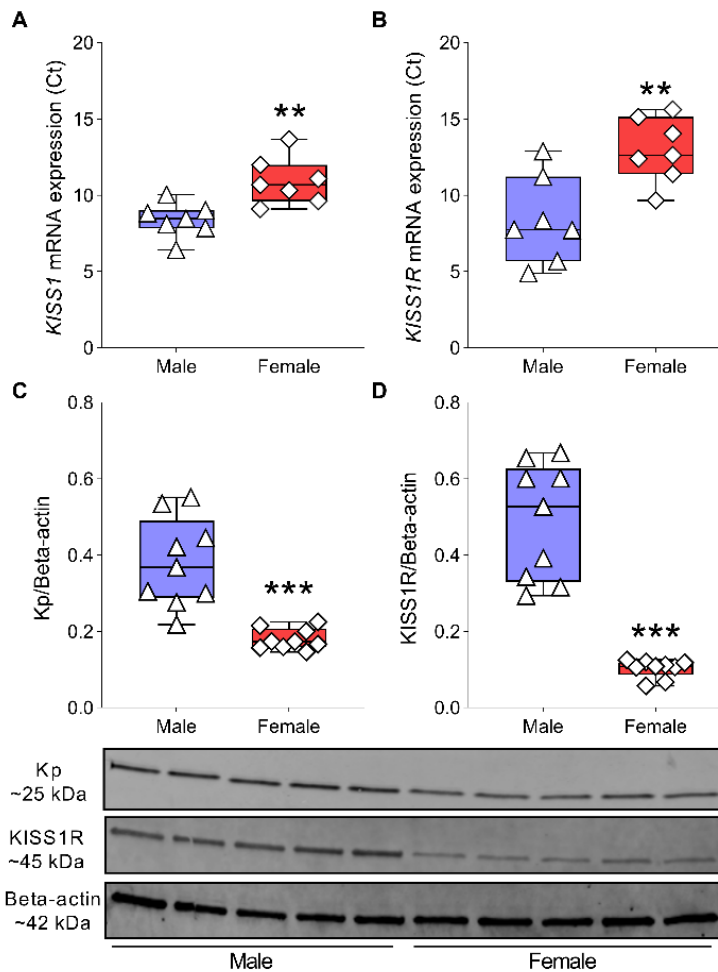
Figure 9: Kp and KISS1R expression in primary human ASM cells from males and females. qRT-PCR and Western analysis data show lower baseline Kp (A, C) and KISS1R (B, D) expression in ASM cells from females compared to males. mRNA expression of KISS1 (A) and KISS1R (B) normalized with housekeeping gene s16 and 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 represented as a minimum to maximum of 7-9 individual non-asthmatic male or female donor samples and analyzed using a one-tailed unpaired t-test. **P<0.01, ***P<0.001 vs. males.

2.3.3. Kp and KISS1R in Asthmatic ASM

mRNA expression of KISS1 was significantly lower in asthmatic ASM from both males (P<0.01) and females (P<0.05) compared to non-asthmatic counterparts (Figure 10A). This was confirmed by Western analysis for Kp protein expression (P<0.001; Figure 10C). Significant difference was noted for KISS1R mRNA in asthmatic ASM from males and females (P<0.001; Figure 10B) compared to non-asthmatics, again confirmed by analysis for KISS1R protein (P<0.001 for males and P<0.01 for females, Figure 10D).
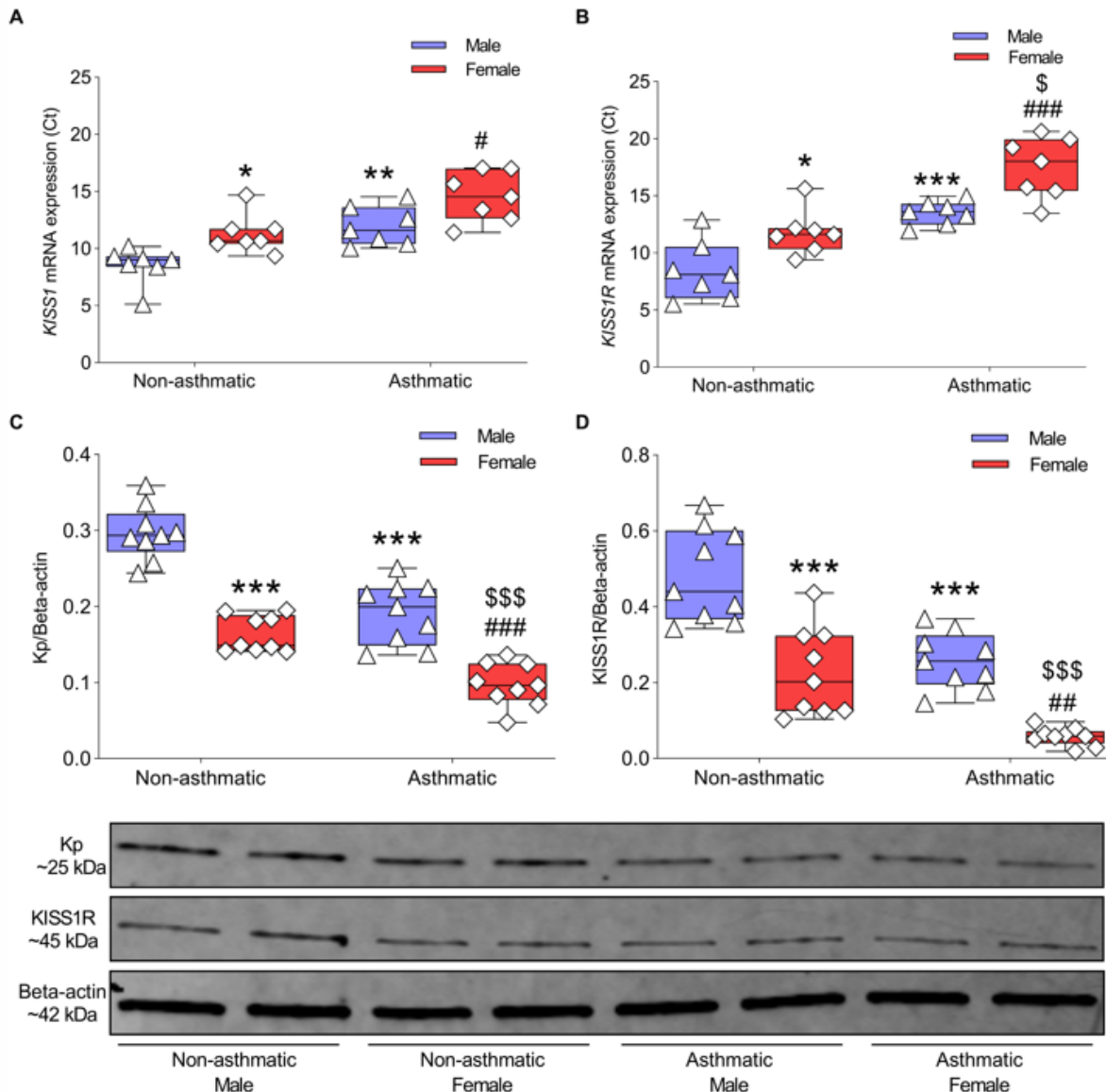
Figure 10: Kp and KISS1R expression in non-asthmatic and asthmatic human ASM cells.
qRT-PCR and Western analysis data showed lower baseline Kp (A, C) and KISS1R (B, D) expression in asthmatic ASM cells compared to non-asthmatics. Interestingly, Kp and KISS1R expression were found to be low in both male and female asthmatic ASM cells compared to non-asthmatic ASM cells. mRNA expression of KISS1 (A) and KISS1R (B) normalized with housekeeping gene s16 and represented as Ct. The protein expression of Kp (C) and KISS1R (D) were determined by Western analysis in human ASM cells and normalized with beta-actin as a loading control. Data are represented as a minimum to maximum of 7-9 individual donor ASM samples from male and female and analyzed using two-way ANOVA followed by Tukey's post-hoc test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. non-asthmatic male; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs. non-asthmatic female; \$$P<0.05$, \$\$\$$P<0.001$ vs. asthmatic male.

2.3.4. Kp is Released by the ASM

To confirm whether the ASM cell itself releases Kp, we measured and found endogenous ASM Kp secretion in the conditional media of both non-asthmatic and asthmatic ASM samples (Figure 11), with significantly lower secretion in asthmatics ($P<0.05$).
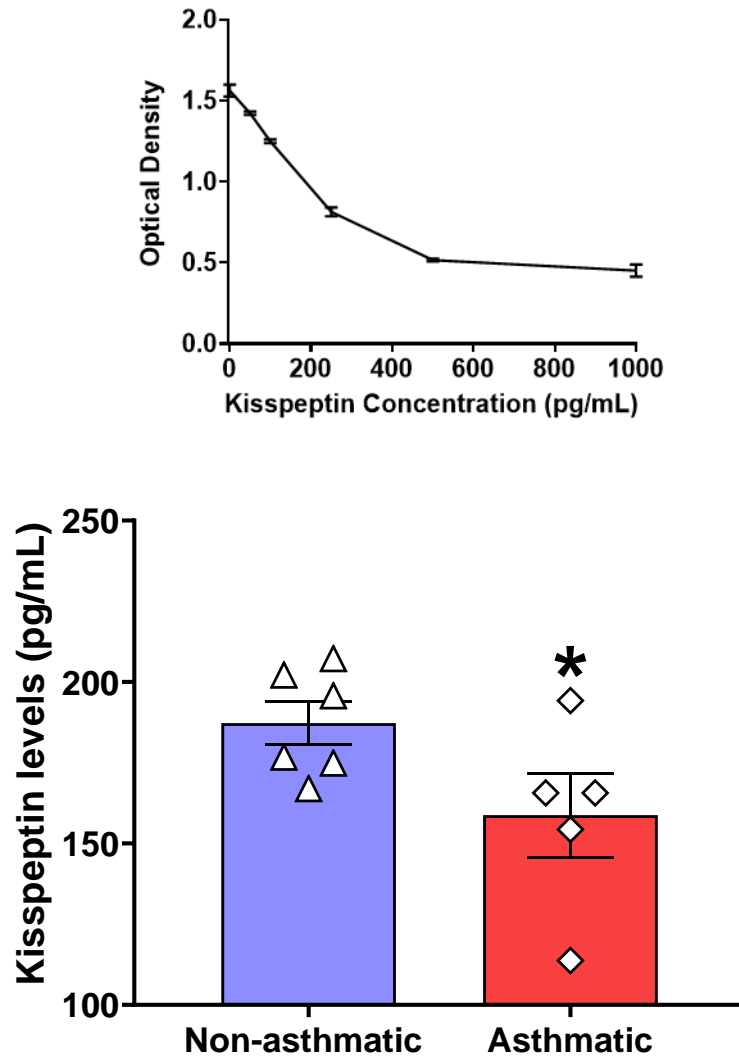
Figure 11: The endogenous Kp secretion of ASM was measured by ELISA using conditioned media.
The calibration of ELISA was confirmed by a standard curve of Kp (top panel). ASM cells from asthmatics showed reduced Kp secretion in conditioned media compared to non-asthmatics (bottom panel). Data are represented as a minimum to maximum of 5-6 individual donor ASM samples from non-asthmatic/asthmatic and analyzed using one-tailed unpaired t-test. *$P<0.05$ vs. non-asthmatic vehicle.

2.4. Discussion

Data on asthma prevalence from the CDC reported that almost 11.2% of population suffering from asthma lie between the age range of 12-14 years[1]. Furthermore, population studies on adult asthma indicated a higher prevalence of asthma in women compared to men. With regards to adult asthma, another study conducted in China reported that poor asthma control and female sex are risk factors for asthma[59]. Moreover, another study focused on the unequal prevalence of asthma in men and women to re-iterate the "sex-shift" of asthma towards females' post-puberty[60]. Therefore, it is clear from multiple studies that sex differences remain an integral risk factor in the predominance of asthma. Thus, much attention has been paid, understandably to studying the role of sex steroids in asthma pathophysiology. Despite this conscious understanding and several evidence, the specific mechanisms of action for sex steroids to promote or prevent asthma is still not clearly known.

Dysregulated ASM proliferation contributes to airway remodeling and AHR in asthma[61, 62]. Exploring the mechanisms underlying ASM remodeling is important, given the current therapies including corticosteroids are not effective in alleviating or reversing airway remodeling in asthma. The current study highlights an entirely novel and potentially targetable mechanism that modulates ASM proliferation in the context of asthma: Kp/KISS1R signaling. Most of the information regarding Kp derives from studies in the central nervous system, where Kp regulates puberty and hormonal function. Earlier studies using rat receptors suggested that all the cleaved Kps (Kp-10, Kp-13, Kp-14, and Kp-54) possess similar affinity and efficacy in activating the KISS1R[34]. However, data from endocrinology and oncology suggest that the differential effects of Kp fragments are a major aspect of understanding Kp biology.
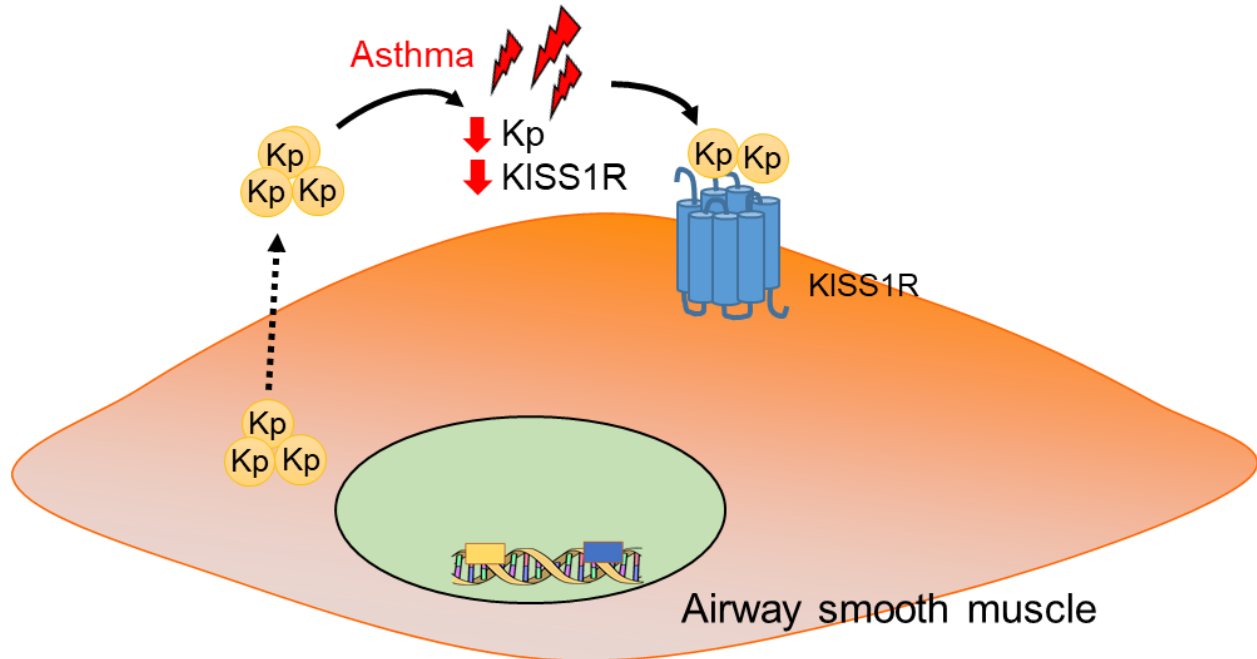
Figure 12: Schematic overview of observations in Chapter 2.
As elaborated earlier, Kp/KISS1R signaling potentially impacts multiple downstream signaling pathways, which may further be affected under a lack of Kp/KISS1R expression in ASM cells. This chapter provides novel evidence of a loss of Kp/KISS1R expression in the ASM, thereby affecting downstream functions of kisspeptin signaling.

Asthma or severe asthma has long been defined as not a disease, but rather an umbrella term identifying individuals showcasing distinct clinical, biological and inflammatory characteristics. Therefore, the obstacle that current biologics face is that these are not targeted to asthma in general, but rather to specific asthma phenotypes. As described earlier, it is evident that the ASM thickening is a prominent pathological feature in asthma that contributes to increased airway hyperreactivity in asthmatic individuals. We, ourselves show novel evidence of abundant Kp/KISS1R expression in the ASM cell. There may be a strong possibility that loss of Kp/KISS1R may trigger in a decline of protective pathways, thereby resulting in promoting ASM remodeling. Furthermore, loss of Kp/KISS1R may hamper downstream signaling pathways that participate in multifunctional roles with respect to Kp/KISS1R in ASM.

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There is currently no information on the expression of Kp or KISS1R in the airways. Our novel 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 lower expression of Kp and KISS1R in ASM from asthmatics, suggesting possible reduced or loss of intrinsic Kp/KISS1R signaling in asthma. Overall, our findings suggest that Kp/KISS1R is abundantly expressed in the human airways, but is lower in asthmatic human ASM. we show for the first time the lower expression of Kp/KISS1R in females as compared to males, which may underlie intrinsic differences and endogenous effects in vitro (Figure 12)[63, 64]. We, therefore, lay the foundation for further exploratory studies on Kp/KISS1R signaling in the airways.

3. KISSPEPTIN INHIBITS HUMAN AIRWAY SMOOTH MUSCLE CELL REMODELING[1]

3.1. Introduction

Airway remodeling is defined as structural changes in the airways, which includes the epithelium, glands, blood vessels, extracellular matrix and the ASM. These changes play a crucial role in narrowing the airway as well as limiting the airflow, thereby leading to airflow obstruction. Despite the discovery of the concept of ASM remodeling by Huber and Koessler 90 years ago, the underlying mechanism is still not well known[65]. However, multiple literature evidence suggests that increase in ASM mass correlates with the severity of asthma[62, 66]. Anomalous proliferation of ASM cells directly contributes to ASM hyperplasia. However, the molecular mechanisms controlling ASM cell proliferation are not entirely understood. Proliferation in ASM cells is induced by a wide spectrum of mitogens (example; growth factors, cytokines, inflammatory mediators and allergens) has been proposed as a primary mechanism underlying ASM mass. However, there is scanty information regarding the intrinsic mechanisms controlling these pathways which get dysregulated in ASM cells during asthma.

Altered extracellular matrix (ECM) deposition is another key hallmark feature contributing to airway wall remodeling in asthma[67]. In turn, ECM proteins promote the survival, proliferation, cytokine synthesis, migration, and contraction of human ASM cells. In addition to increased synthesis of ECM proteins such as collagens and fibronectins, ASM cells are also involved in downregulation of matrix metalloproteinases (MMPs) and upregulation of tissue inhibitors of metalloproteinases (TIMPs), thereby contributing to alterations in ECM[22, 68]. Thus, the intertwined relationship of ASM with ECM dynamics in asthma is an important regulator of airway structural changes as described in Figure 13.
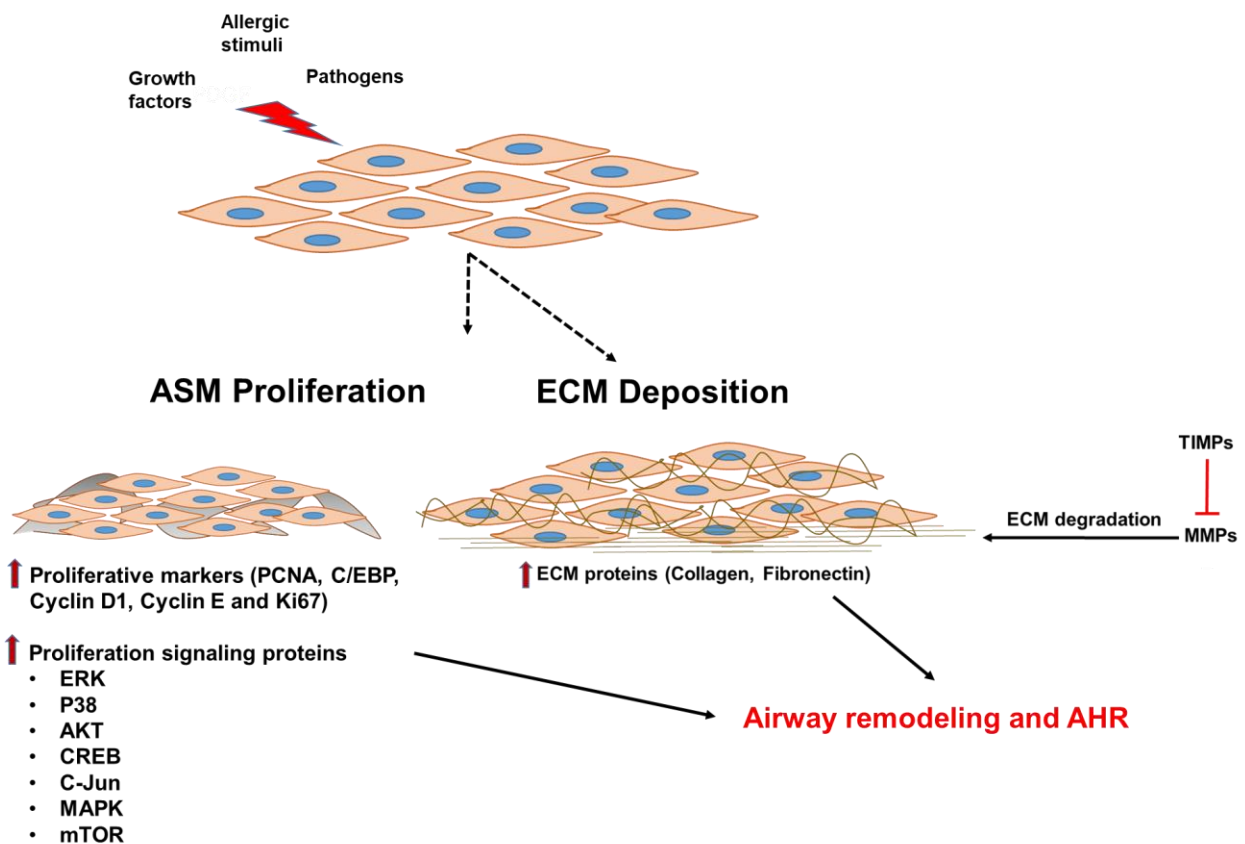
Figure 13: Schematic representation of ECM-ASM dynamics in the ASM cell.
Interaction of PDGF-BB with PDGFR induces dimerization which is essential for the activation of tyrosine kinase receptors. Subsequently, PDGFR undergoes phosphorylation in specific tyrosine residues further activating intracellular signaling transduction molecules such as Ras/Rac, small GTPases, MAPK, PI3K, STAT and Src signaling.

3.2. Materials and Methods

3.2.1. Chemicals, Drugs (Agonist/Antagonist) and Antibodies

Human recombinant Platelet-derived growth factor-BB (PDGF-BB; Cat# PHG0046) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Pro-inflammatory cytokine TNFα (Cat# 210-TA) and Recombinant human TGFβ (2240-B) were purchased from RnD Biosystems. Kp-10 (Metastin 45-54, KISS1R agonist, Cat# sc-221884), Control shRNA lentiviral particles-A (Cat# sc-108080), GPR54 shRNA lentiviral particles (Cat# sc-60747-V), primary antibodies for PCNA (Cat# sc-25280), C/EBP-alpha (Cat# sc-365318), Cyclin-D1 (Cat# sc-8396), Cyclin E (Cat# sc-377100), p38 (Cat# sc-271120), Akt1/2/3 (Cat# sc-81434) and p-ERK1/2 (Tyr204) (Cat# sc-7383) were obtained from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Primary antibody for collagen I (ab34710) was purchased from abcam. Kisspeptin-234 trifluoroacetate (Kp-234, KISS1R inhibitor, KI, Cat# 3881) was purchased from Tocris Bioscience (Minneapolis, MN, USA). Kp-13 (Cat# 50-194-6708) was procured from Bachem (Torance, CA, USA). Kp-14 (Cat# crb1000930) was purchased from Discovery Peptides (Belasis, UK). Kp-54 (Cat# 1443) was obtained from R&D Systems (Minneapolis, USA). LDH-Cytotoxicity Colorimetric Assay Kit (Cat# K311) was purchased from BioVision (Milpitas, CA, USA). p-p38 (Thr180/Tyr182) (Cat# 4511S), p-Akt (Ser473) (Cat# 4060S), ERK1/2 (Cat#9102S) were procured from Cell Signaling Technologies (Beverly, MA, USA). IRDye goat anti-rabbit and goat anti-mouse secondary antibodies were used, followed by scanning in a Li-Cor Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). Other chemicals and reagents were procured from Sigma-Aldrich unless otherwise indicated.

3.2.2. 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 100mm culture petri plates (for RNA, Protein, and flow cytometry) or 96 well plates (~7000 cells/well, for proliferation and ECM studies). Cells were allowed to adhere overnight and washed twice with PBS. Post-washing, serum medium was replaced with serum-free medium for 24 h to synchronize cell growth. 1% serum medium was used as a vehicle to maintain the quiescent phase for the proliferation study. Efficacy and ASM toxicity of Kp-10 was determined using 3-log concentrations (0.1μM, 1μM, and 10μM), alone and in the presence of PDGF (2ng/mL) (13,14). ASM cells were treated with different concentrations of Kp-10 or KI 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 as per the manufacturer's protocol. Human ASM cells were exposed to Kp-10 or KI in the presence/absence of 2ng/mL PDGF, added after 2 h of pre-incubation with respective treatment groups, and incubated for a total time of 6 h (RNA) and 24 h (proteins). For ECM studies, PDGF and TNFα (20ng/mL) added after 2 h of pre-incubation with respective treatment group and incubated for a total time of 6 h for RNA. TGFβ was used at a concentration of 1ng/mL to perform western blot analysis (24 h) for determining the expression of ECM proteins.

3.2.3. shRNA Lentiviral Particle Transduction

Non-asthmatic and asthmatic human ASM cells were cultured in 6-well plates to approximately 50-60% confluence. Transfection was achieved using a 20 µl viral stock containing $1X10^5$ infectious units of the virus (IFU) for control and KISS1R/GPR54 shRNA lentiviral particles. Once cells reached 60-70%, serum medium was replaced with fresh 5%

serum medium (no antibiotics) and with polybrene (Santa Cruz Cat# sc-134220). Lentiviral

particles were thawed, added and the cells incubated overnight. The medium was replaced with a

5% serum medium containing 1% AbAm without polybrene and further incubated for 24 h. After

ensuring growth, the medium was replaced with a 10% serum medium with antibiotics for 48 h.

For the selection of cells stably transfected with shRNA, the medium was replaced with a 10%

serum medium containing 5µg/mL puromycin and incubated for 48-72 h. Cells were replenished

with medium every 3-4 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

analysis.

3.2.4. Cell Proliferation Assays

3.2.4.1. Brightfield Cell Count

After 24 h 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 (LFX; BioTek Instruments, Winooski, VT, USA).

3.2.4.2. MTT Cell Proliferation Assay

Following 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 h.

The medium was aspirated followed by the addition of 100 µL dimethylsulfoxide (DMSO), and

cells were maintained with gentle shaking for 15 min at room temperature. Absorbance at 570

nm was then measured using a Synergy HTX Multi-Mode Plate Reader (BioTek Instruments,

Winooski, VT, USA).

3.2.5. Flow Cytometry Analysis

12-well culture plates were seeded with non-asthmatic and asthmatic ASM cells and treated with appropriate treatments for 24 h. Cells were trypsinized and fixed with ice-cold ethanol followed by centrifugation at 2,000g. Pellets were washed and resuspended in PBS followed by propidium iodide (PI; 3 µM) staining for 15 min. PI-stained cells were analyzed using BD Accuri® C6 Flow cytometer (BD Biosciences, San Jose, CA USA). A minimum of 40,000 events were captured per sample and cells were analyzed for G0/G1, S, and G2/M phase using BD Accuri™ C6 Plus software.

3.2.6. qRT-PCR Analysis

The following primers were used for qRT-PCR analysis: hPCNA (forward 5′-GCC TGA ATG GCG AAT GGA-3′; reverse 5′-GAA GGG AAG AAA GCG AAA GGA-3′), hKi67 (forward 5′-GCT TAC TCC GAC GAT GAT TTC T-3′; reverse 5′-GCC GAT GCT TGC AAT AGT TTA G-3′), hC/EBP-alpha (forward 5′-ATT GGA CCC AGA GAA GTT GAC-3′; reverse 5′-TCA GAC CAT TTA AGT CTT CAG AGA T-3′), hCyclin-D1 (forward 5′-GAC GGG TAG AAC CTC AGT AAT C -3′; reverse 5′-CTC CGG GTG TTT CCC TAT AAT C -3′), hCyclin E (forward 5′-GTC CTG GCT GAA TGT ATA CAT GC-3′; reverse 5′-CCC TAT TTT GTT CAG ACA ACA TGG C-3′), hprocollagen I (forward 5′-AGA GTG CAG AGT ACT GGA TTG A – 3′; reverse 5′-GTT GGG ATG GAG GGA GTT TAC –3′), hprocollagen III (forward 5′-CTA CTT CTC GCT CTG CTT CAT C–3′; reverse 5′-CCT TGA GGT CCT TGA CCA TTA G–3′), hFibronectin (forward 5′-ACC ACT ATA CCC TCC CTC TTT–3′; reverse 5′-GGG CTC TCC TCT TAC CAA TAA C–3′), hMMP-2 (forward 5′-CAG GTG ATC TTG ACC AGA ATA CCA TC–3′; reverse 5′-AGC AAA GGC ATC ATC CAC TGT CTC TG–3′), hMMP-9 (forward 5′-TGG CAG AGG AAT ACC TGT ACC GCT AT–3′; reverse 5′-CTC AAA GGT TTG GAA

TCT GCC CAG GTT–3′) and hS16 (forward 5'-CAA TGG TCT CAT CAA GGT GAA CGG-3'; reverse 5'-CTG ACG GAT AGC ATA AAT CTG GGC-3'). Cycle threshold [C(t)] 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.

3.2.7. Western Blot Analysis

Human ASM cell lysates for individual treatment groups were prepared using cell lysis buffer as previously described. 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 (BioRad, Hercules, CA, USA). 30 µg equivalent protein from respective treatment groups were loaded on 4-15% 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. 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h at room temperature was used as a blocking buffer and membranes were incubated overnight at 4°C with specific primary antibodies of interest. Following three washes for 8 min/wash with Tris-buffered saline containing 0.1% tween (TBST), blots were incubated with LiCOR near-red conjugated secondary antibodies at room temperature for 1 h. Beta-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 beta-actin.

3.2.8. Statistical Analysis

Human ASM cells from at least five donors (males and females; non-asthmatics and asthmatics) were used for all experiments. For expression studies, cell lysates for Western analysis and cDNA for qRT-PCR were obtained from at least five different individual donor samples. Statistical analysis was performed using one-way/two-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 of $p<0.05$ level. All values are expressed as mean ± SEM.

3.3. Results

3.3.1. Cleaved Kp's and ASM Cell Proliferation

Previous studies have shown that all cleaved forms of Kps show comparable KISS1R receptor binding affinity but have different downstream potency in terms of cellular effect. Thus, Kp effects may be cell- and context-specific. To determine which of the cleaved Kps may be involved in regulating ASM proliferation, I evaluated the effects of 1µM Kp-10, Kp-13 Kp-14, and Kp-54 on basal and PDGF-stimulated proliferation of non-asthmatic ASM cells using MTT assay (Figure 14). 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 to vehicle. PDGF substantially increased ASM proliferation ($P<0.001$) compared to 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 to PDGF alone.
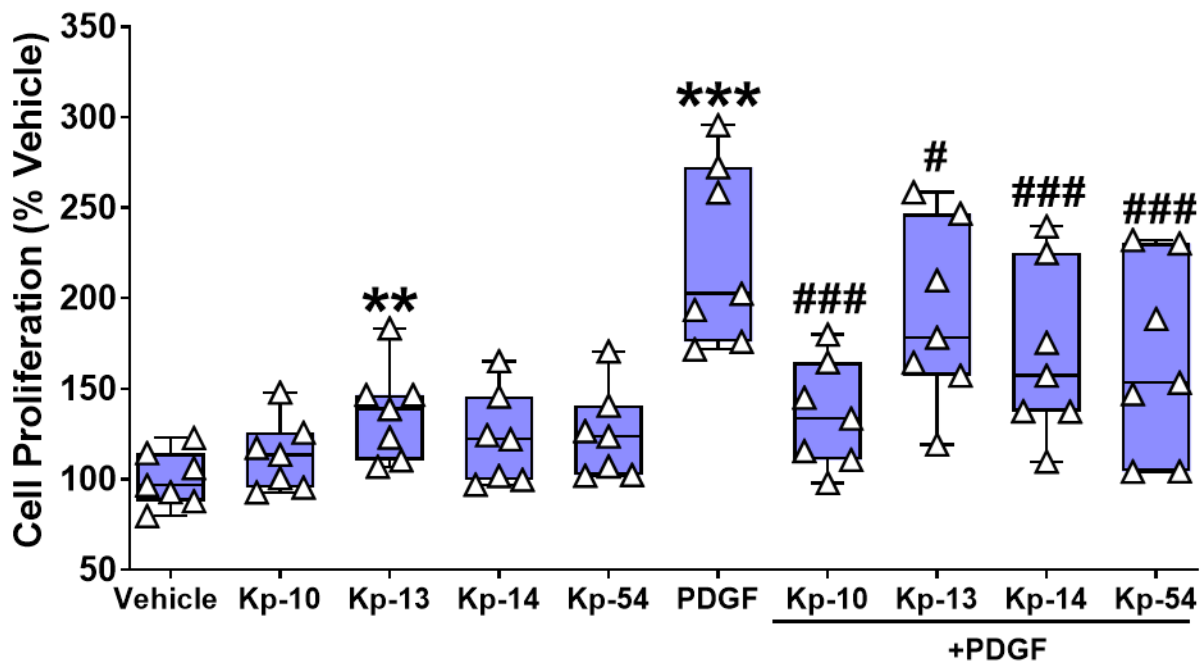
Figure 14: Kisspeptins and human ASM cell proliferation.
The effect of different Kisspeptins (Kp-10, Kp-13 Kp-14, and Kp-54) on basal and PDGF-induced cell proliferation in non-asthmatic human ASM cells was evaluated using MTT assay. Amongst the three forms of Kisspeptins (1 µM), Kp-10 significantly blunted PDGF-induced ASM cell proliferation. **$P<0.01$, ***$P<0.001$ vs. Vehicle; #$P<0.05$, ###$P<0.001$ vs. PDGF.

Studies showed that among the Kp fragments, Kp-10 was the most effective in modulating ASM proliferation, accordingly subsequent studies focused on this fragment. Based on these data, I selected Kp-10 as the cleaved form of Kp to further explore. I first performed a concentration dependence study at 100nM, 1µM, and 10µM for PDGF-induced human ASM proliferation using non-asthmatic human ASM cells and found 1µM and 10µM Kp-10 significantly ($P<0.001$) inhibited proliferation (Figure 15). Accordingly, I selected 1µM Kp-10 for subsequent studies.
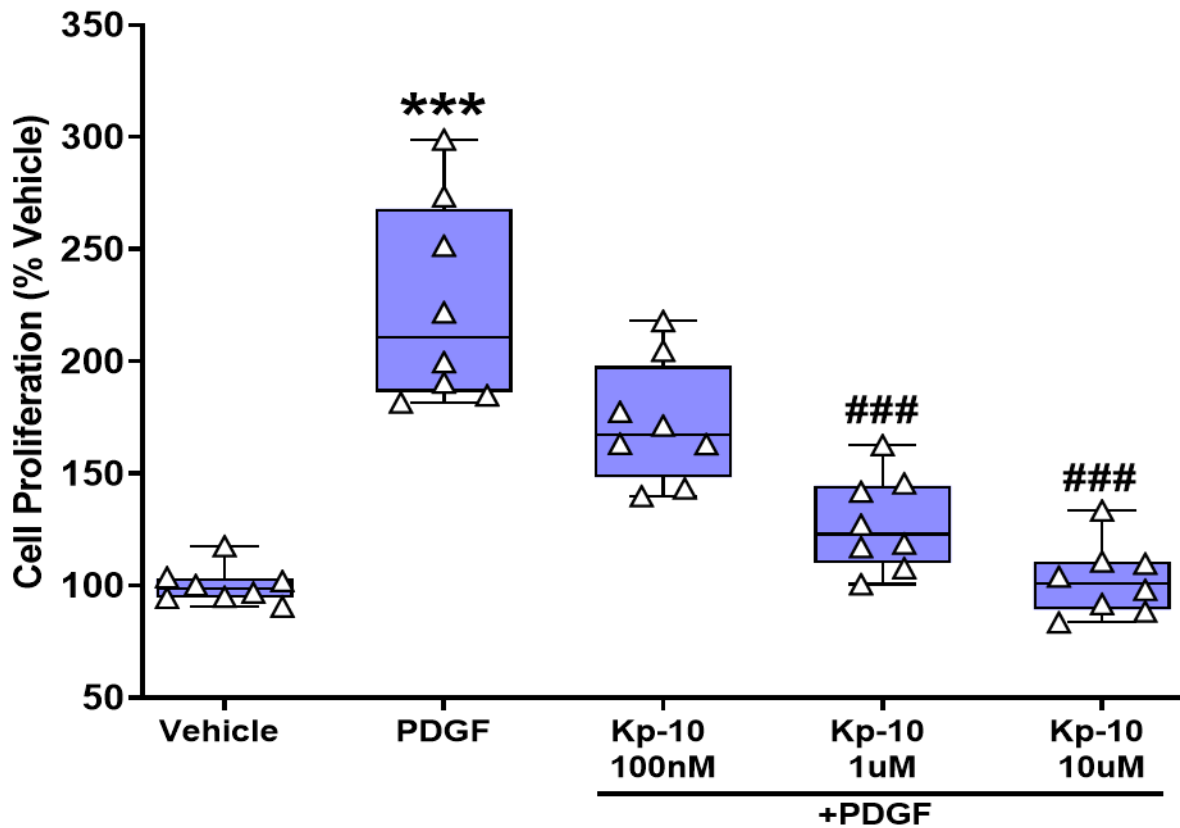
Figure 15: Dose optimization of Kp-10.
Further, the effect of different log concentrations of Kp-10 (100nM, 1μM, and 10μM) on regulating PDGF-induced ASM proliferation was determined. ***P<0.001 vs. Vehicle; ###P<0.001 vs. PDGF.

To verify the lack of cytotoxicity from Kp-10, I performed an LDH assay (Figure 16) with serum-free media as a negative control and 0.1 % Triton-X 100 as positive control and found no cytotoxicity with any of the treatment groups.
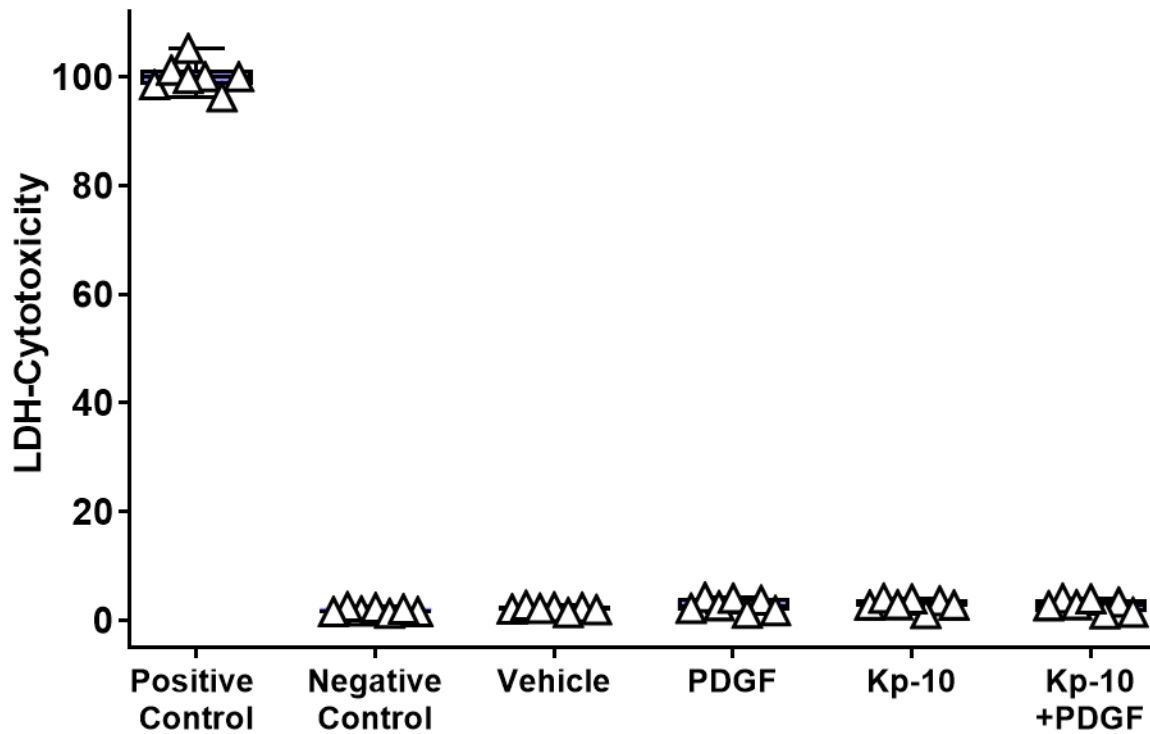
Figure 16: Estimation of cytotoxicity using lactate dehydrogenase (LDH) assay.
The relative level of lactate dehydrogenase enzyme was measured in ASM cell supernatants to evaluate the cytotoxicity of the selected concentration (1 µM) of Kp-10.

3.3.2. Kp-10 Effects on ASM Cell Proliferation

I performed Brightfield (BF) and MTT assays in both non-asthmatic and asthmatic ASM cells with/without PDGF (Figure 17 and 18). In addition, I incorporated a KISS1R inhibitor (KI, Kp-234 trifluoroacetate; 100nM) as a treatment group to confirm the role of this receptor. As expected, I observed significantly higher proliferation in asthmatic ASM cells compared to non-asthmatics (P<0.05). At baseline, Kp-10 did not significantly alter proliferation in either non-asthmatic or asthmatic ASM. Interestingly, inhibition of KISS1R with KI significantly increased in the basal proliferation of non-asthmatic (P<0.01 in BF assay and P<0.05 in MTT assay) and asthmatic (P<0.01 in BF assay and P<0.05 in MTT assay) ASM cells. Further, ASM cells exposed to PDGF showed a significant (p<0.001 for both BF and MTT assays) increase in

proliferation for both non-asthmatic and asthmatic cells with a greater effect on asthmatic ASM (P<0.05 in BF assay and P<0.001 in MTT assay). Pretreatment with Kp-10 significantly blunted the pro-proliferative effect of PDGF in both non-asthmatic (P<0.001) and asthmatic (P<0.001) ASM cells. 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 either non-asthmatic or asthmatic cells, overall highlighting the importance of KISS1R in mediating Kp-10 effects.
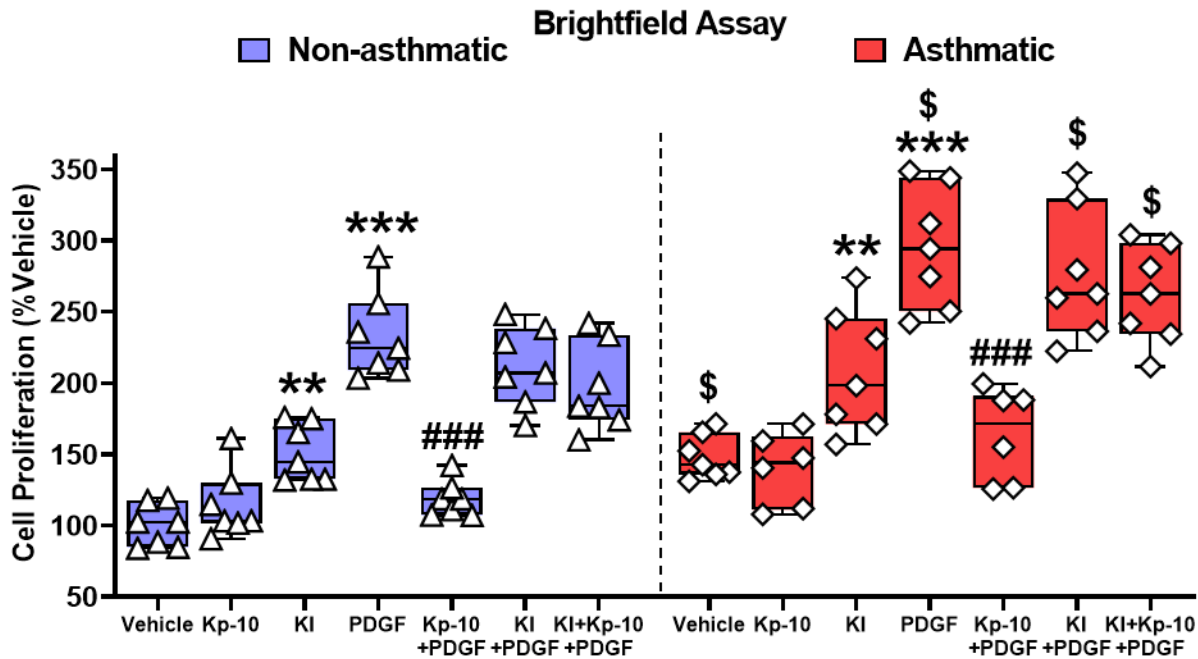
Figure 17: KISS1R activation and PDGF-induced proliferation of human ASM cells by BF assay.
KISS1R agonist, Kp-10 significantly blunted the mitogenic effect on PDGF in non-asthmatic and asthmatic human ASM cells as evaluated by high contrast brightfield assay. **P<0.01, ***P<0.001 vs. respective vehicle; ###P<0.001 vs. respective PDGF; $P<0.05 vs. respective group without asthma.
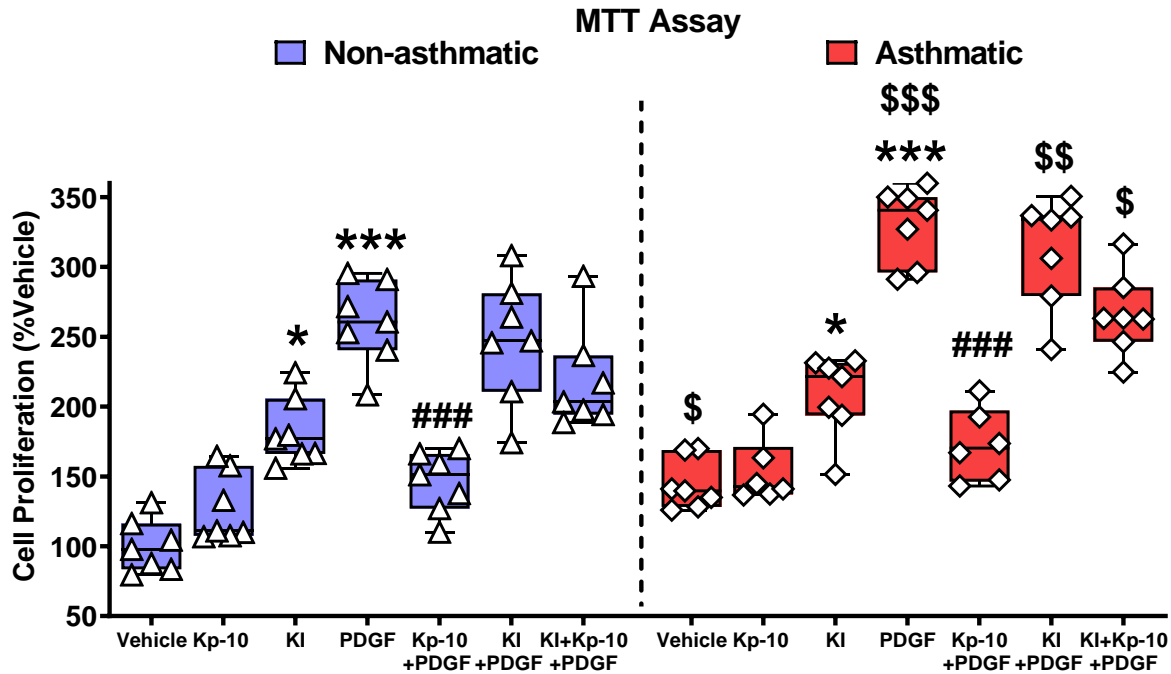
Figure 18: KISS1R activation and PDGF-induced proliferation of human ASM cells by MTT assay.

KISS1R agonist, Kp-10 significantly blunted the mitogenic effect on PDGF in non-asthmatic and asthmatic human ASM cells as evaluated by MTT assay. *$P<0.05$, ***$P<0.001$ vs. respective vehicle; ###$P<0.001$ vs. respective PDGF; \$$P<0.05$, \$\$$P<0.01$, \$\$\$$P<0.001$ vs. respective group without asthma.

To further confirm the role of KISS1R in Kp-10 effect, I used KISS1R shRNA knockdown (with a scrambled negative shRNA as control). The efficiency of shRNA transduction was confirmed by Western analysis for both non-asthmatic and asthmatic transduced ASM samples ($P<0.001$; Figure 19). 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 non-asthmatic and asthmatic cells ($P<0.001$ for both BF and MTT assays): effects that were amplified in KISS1R knockdown non-asthmatic and asthmatic ASM cells ($P<0.001$ both BF and MTT assays) (Figure 20 and 21). Pretreatment with Kp-10 significantly lowered PDGF-induced ASM proliferation in negative shRNA transduced

non-asthmatic and asthmatic ASM cells (P<0.001 for both BF and MTT assays), but not in

KISS1R shRNA knockdown cells.

Figure 19: Western blot analysis was performed to confirm the transduction efficacy of KISS1R shRNA in non-asthmatic and asthmatic ASM cells.
Human ASM cells transduced with negative or KISS1R shRNA were validated for successful transduction by western blot analysis. ***P<0.05 versus respective negative shRNA.

Figure 20: Effect of KISS1R shRNA transduced cells on ASM cell proliferation by Brightfield assay.
Human ASM cells transduced with KISS1R specific shRNA show increased basal ASM proliferation compared to negative shRNA. Further, 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 brightfield assay. ***P<0.001 vs. respective neg. shRNA; ###P<0.001 vs. respective PDGF; $$P<0.01, $$$P<0.001 vs. respective neg.shRNA group.

Figure 21: Effect of KISS1R shRNA transduced cells on ASM cell proliferation by MTT assay. Human ASM cells transduced with KISS1R specific shRNA show increased basal ASM proliferation compared to negative shRNA. Further, we did not observe any significant reduction in PDGF-induced proliferation after Kp-10 treatment in KISS1R shRNA transduced cells as measured by MTT assay. ***P<0.001 vs. respective vehicle; ###P<0.001 vs. respective PDGF; $$P<0.01 vs. respective neg.shRNA group.
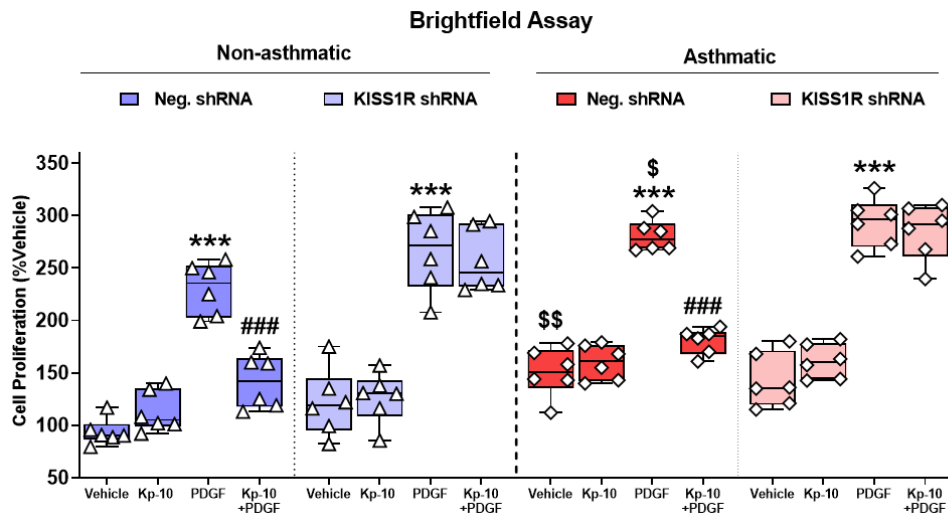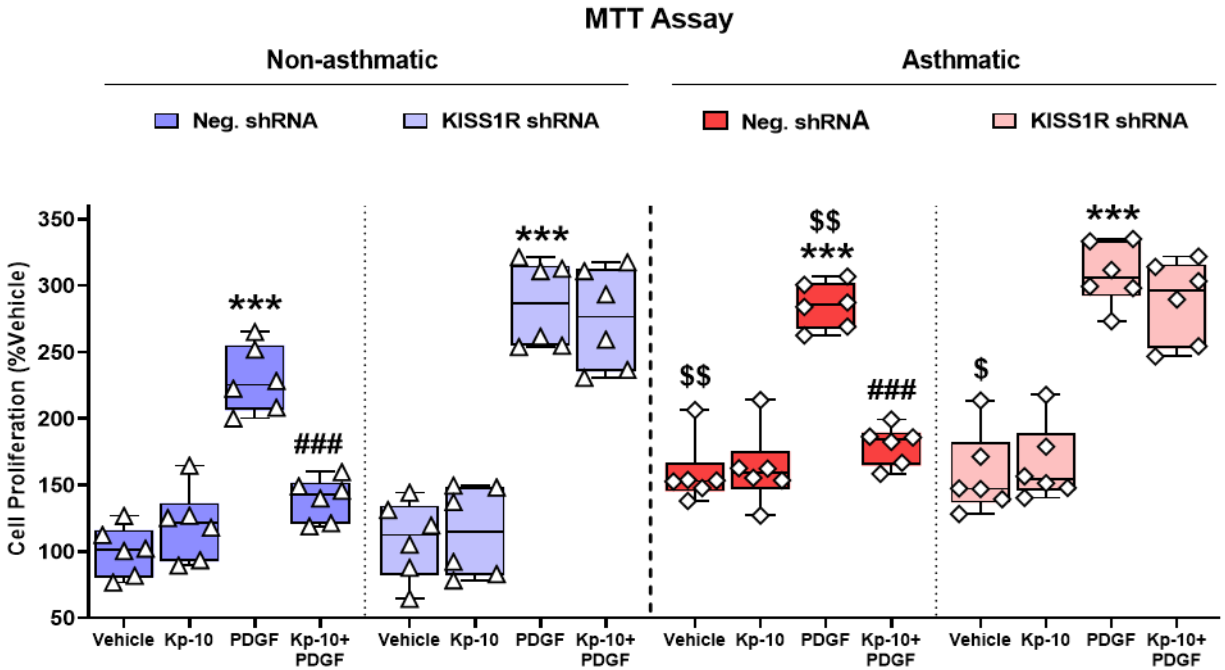
3.3.3. Effect of Kp-10 on Markers of Proliferation

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 to vehicle in both non-asthmatic (P<0.05) and asthmatic ASM (P<0.01, Figure 23), consistent with an increase in proliferation. This mitogenic effect of PDGF was significantly inhibited by Kp-10 pretreatment in both non-asthmatic (P<0.05) and asthmatic (P<0.01) ASM cells. Furthermore, pretreatment with Kp-10 significantly downregulated PDGF induced cell entry into the G2/M phase in both non-asthmatic and asthmatic ASM (P<0.01, Figure 23). Additionally, Kp-10 pretreatment increased the numbers of cells in G0/G1 phase compared to PDGF in both non-

asthmatic and asthmatic ASM cells (P<0.01, Figure 23), suggesting the arrest of cell cycle

progression in the G0/G1 phase, and again consistent with inhibition of proliferation.
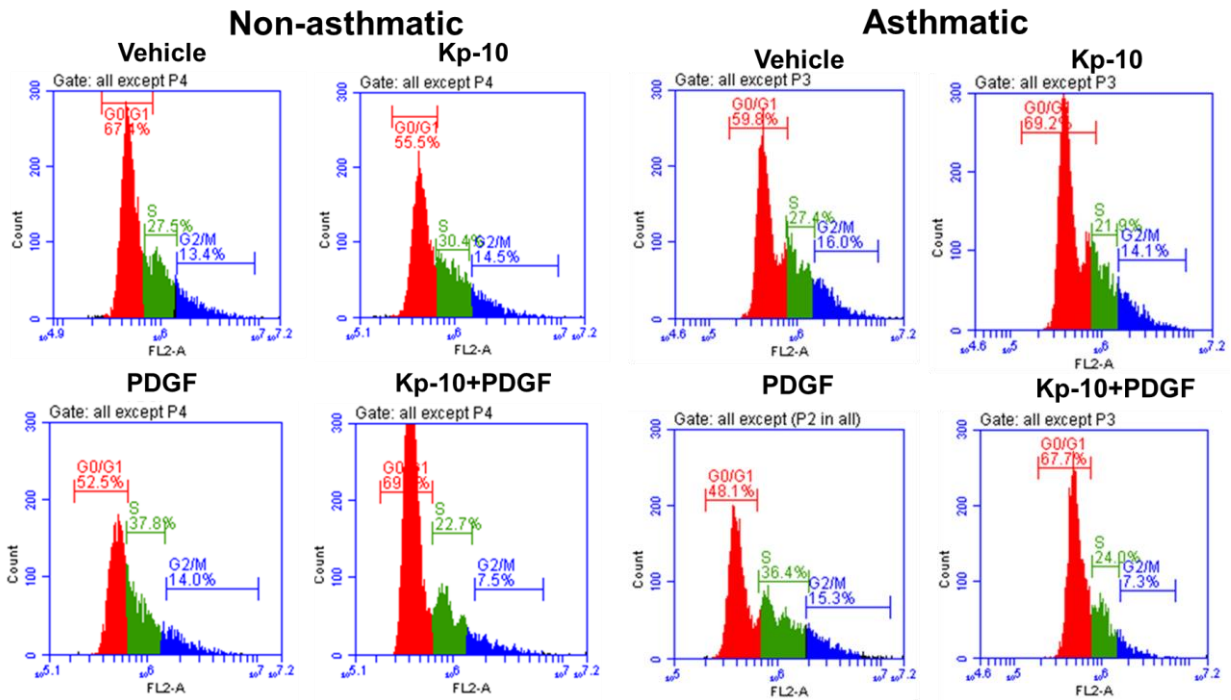
Figure 22: Representative Flow profiles for individual treatment groups belonging to non-asthmatic and asthmatic ASM.
The representative flow profiles of ASM cell cycle distributions of individuals without asthma (left panel) and with asthma (right panel) exposed to various treatment groups are depicted.
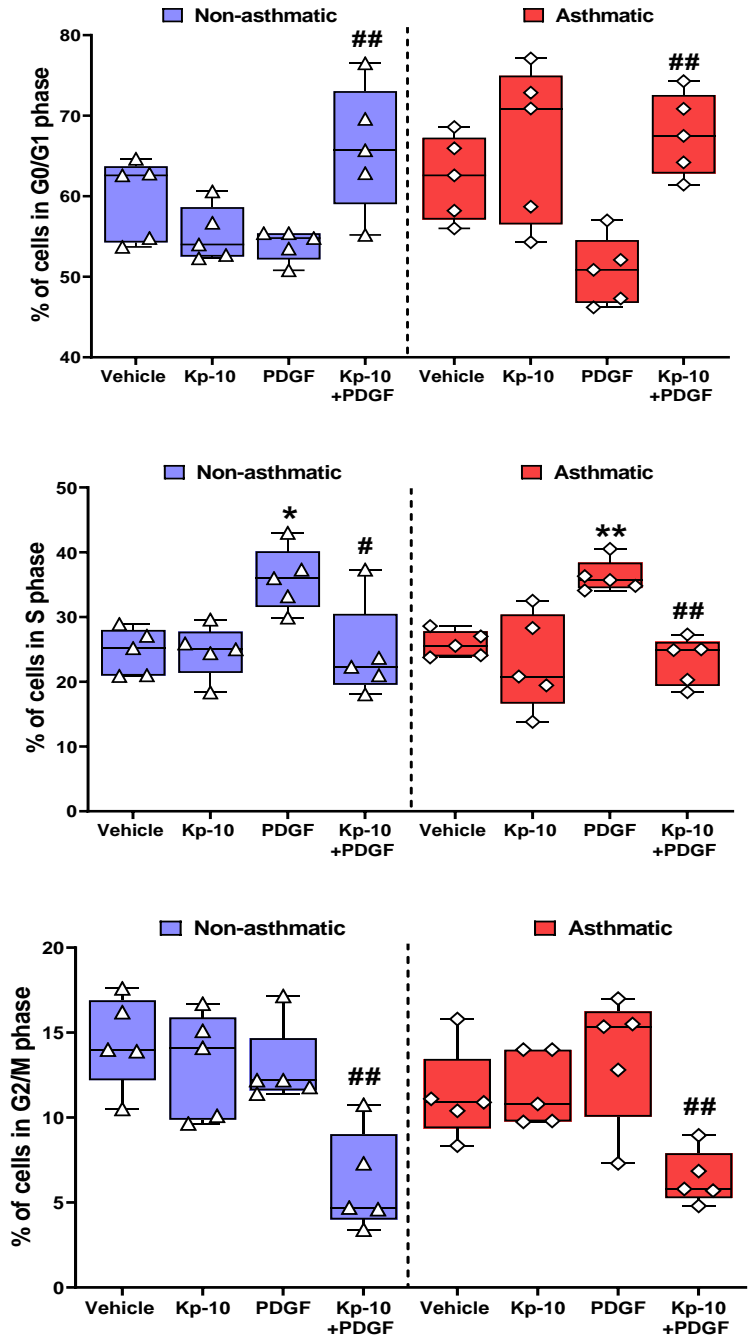
Figure 23: Effect of Kp-10 on G0/G1, S and G2/M phases of human ASM cell cycle studied using flow cytometry.

Cell cycle analysis showed decreased cell population in G0/G1 phase and increased cell population in S and G2/M phases. With PDGF treatment. Kp-10 treatment significantly reversed the PDGF effect and resulted in reduced cell populations in S and G2/M phases, suggesting the arrest of cell cycle progression in the G0/G1 phase. Data are reported as a minimum to maximum of 5 individual samples from donors with and without asthma and analyzed using one-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.

I evaluated the expression of proliferative markers, PCNA and C/EBPα using qRT-PCR and Western analyses, as well as changes in Ki-67 expression by qRT-PCR and nuclear localization by immunofluorescence analysis. PDGF significantly increased the mRNA expression of PCNA ($P<0.01$) (Figure 24), C/EBPα ($P<0.01$ for non-asthmatics and $P<0.001$ for asthmatics) (Figure 25), and Ki-67 ($P<0.001$ for non-asthmatics and asthmatics) (Figure 26). Kp-10 treatment inhibited PDGF-induced increase in mRNA expression of PCNA ($P<0.05$ for non-asthmatics and $P<0.01$ for asthmatics), C/EBPα ($P<0.01$ for non-asthmatics and $P<0.05$ for asthmatics), and Ki-67 ($P<0.001$). Consistently, PDGF significantly upregulated protein expression for PCNA ($P<0.01$ for non-asthmatics and $P<0.05$ for asthmatics) and C/EBPα ($P<0.001$ for non-asthmatics and $P<0.05$ for asthmatics). Nuclear localization of Ki-67 was significantly increased with PDGF ($P<0.001$), which was further inhibited by Kp-10 ($P<0.05$ for non-asthmatics and $P<0.001$ for asthmatics), also as shown in Figure 27 IF representatives.

Figure 24: Effect of Kp-10 on PDGF-induced human ASM cell proliferative marker, PCNA. Non-asthmatic and asthmatic human ASM cells showed increased mRNA and protein expression of PCNA, following PDGF exposure, an effect substantially blunted by Kp-10. *P<0.05, **P<0.01 vs. respective vehicle; #P<0.05, ##P<0.01 vs. respective PDGF.

Figure 25: Effect of Kp-10 on PDGF-induced human ASM cell proliferative marker, C/EBPα. Non-asthmatic and asthmatic human ASM cells showed increased mRNA and protein expression of C/EBPα, following PDGF exposure, an effect substantially blunted by Kp-10. *P<0.05, **P<0.01, ***P<0.001 vs. respective vehicle; #P<0.05, ##P<0.01 vs. respective PDGF.

Figure 26: Effect of Kp-10 on PDGF-induced human ASM cell proliferative marker, Ki67. Non-asthmatic and asthmatic human ASM cells showed increased mRNA and nuclear localization of Ki67, following PDGF exposure, an effect substantially blunted by Kp-10. ***P<0.001 vs. respective vehicle; #P<0.05, ###P<0.001 vs. respective PDGF.

Ki-67 Immunofluorescence

Non-asthmatic
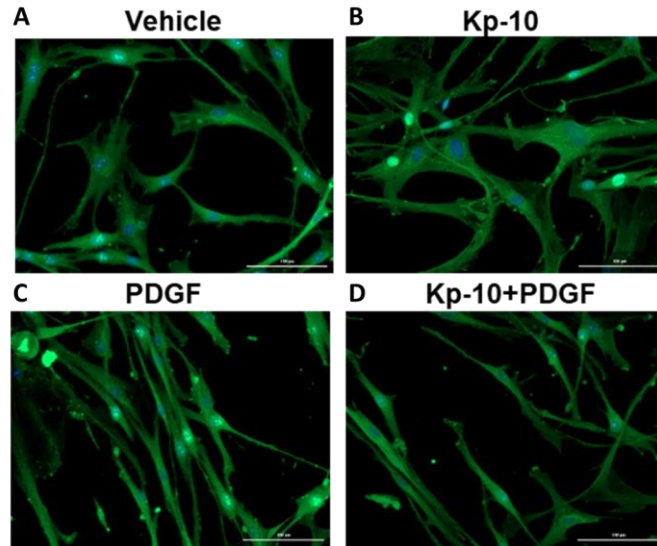
Asthmatic
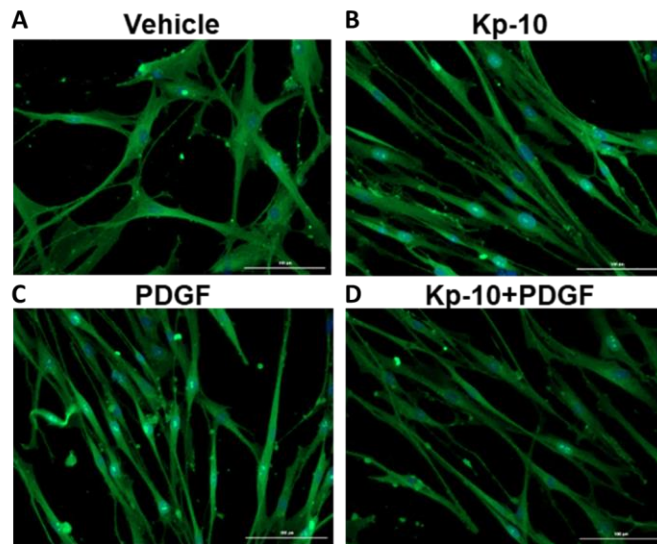
Figure 27: Ki67 IF images for ASM cells.
ASM cells belonging to both non-asthmatic (top; A,B,C,D) and asthmatic (bottom; A,B,C,D) individuals were seeded and imaged for Ki-67 IF with DAPI. As expected, PDGF-induced Ki-67 nuclear localization was significantly reduced upon Kp-10 treatment. Scale bar:100μM.

PDGF also significantly increased mRNA expression of the cell cycle proteins Cyclin-D1 ($P<0.05$) and Cyclin E ($P<0.001$ for non-asthmatics and $P<0.01$ for asthmatics; Figure 28 and 29 respectively): effects significantly inhibited in both non-asthmatic ($P<0.05$) and asthmatic ASM ($P<0.01$ for Cyclin-D1 and $P<0.05$ for Cyclin E) pretreated with Kp-10. Consistent results for Cyclin D1 and Cyclin E proteins were observed. PDGF effects on the cell cycle proteins were significantly blunted with Kp-10 pretreatment in both non-asthmatic ($P<0.001$ for Cyclin-D1 and $P<0.01$ for Cyclin E) and asthmatic ($P<0.05$ for both Cyclin-D1 and Cyclin E) ASM cells.
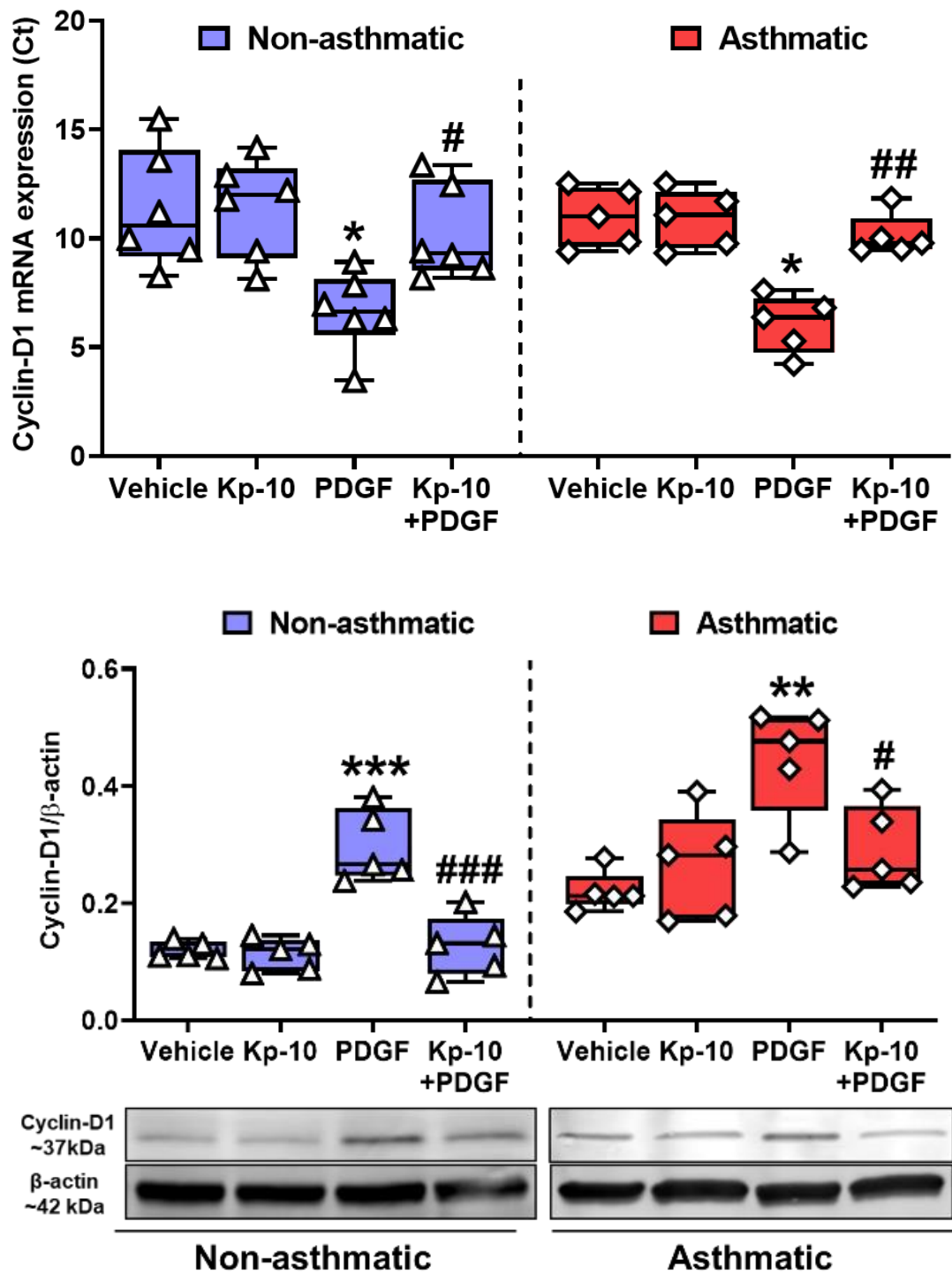
Figure 28: Effect of Kp-10 on PDGF-induced human ASM cell proliferative marker, Cyclin-D1. Non-asthmatic and asthmatic human ASM cells showed increased mRNA and protein expression of Cyclin-D1, following PDGF exposure, an effect substantially blunted by Kp-10. *P<0.05, **P<0.01, ***P<0.001 vs. respective vehicle; #P<0.05, ##P<0.01, ###P<0.001 vs. respective PDGF.
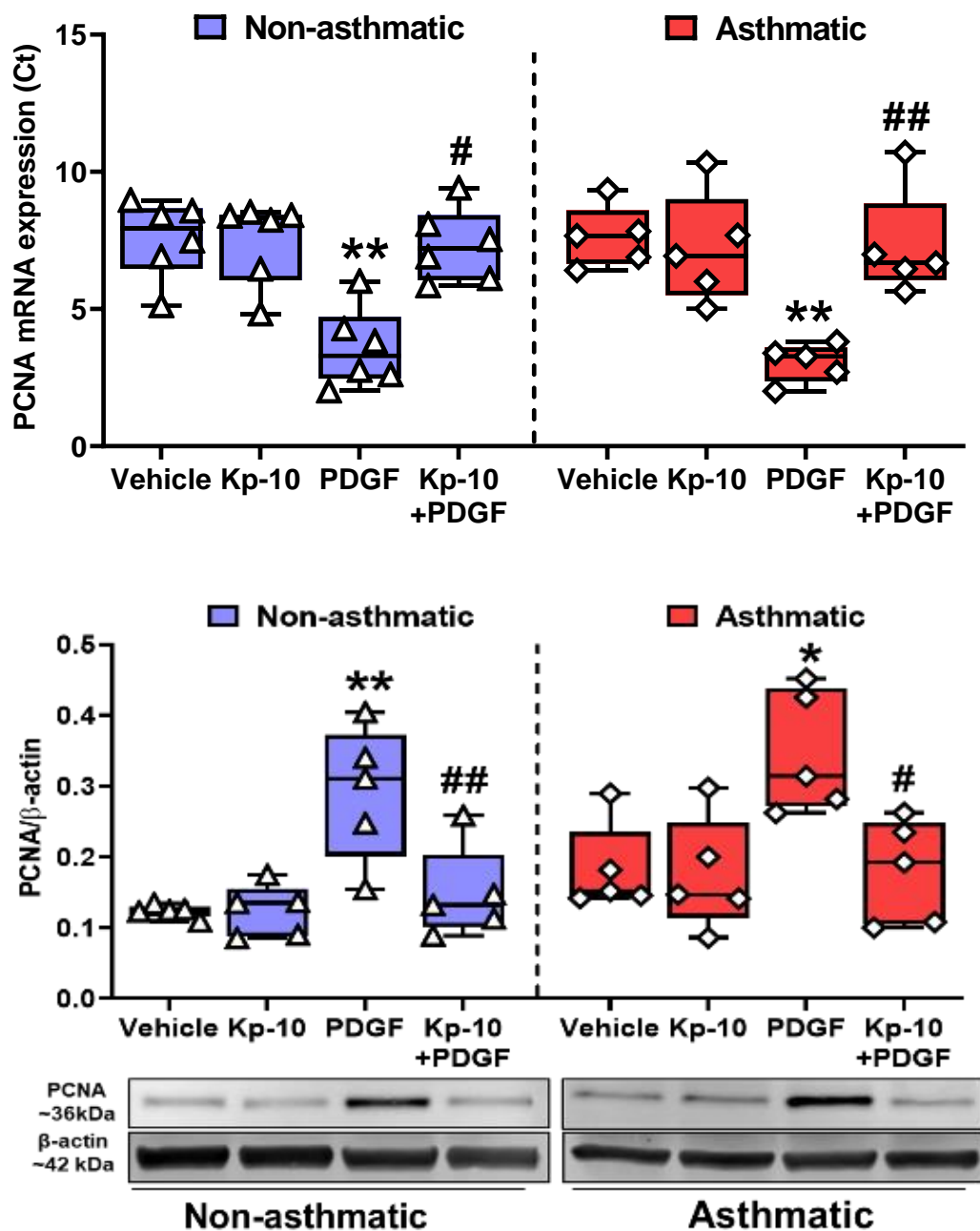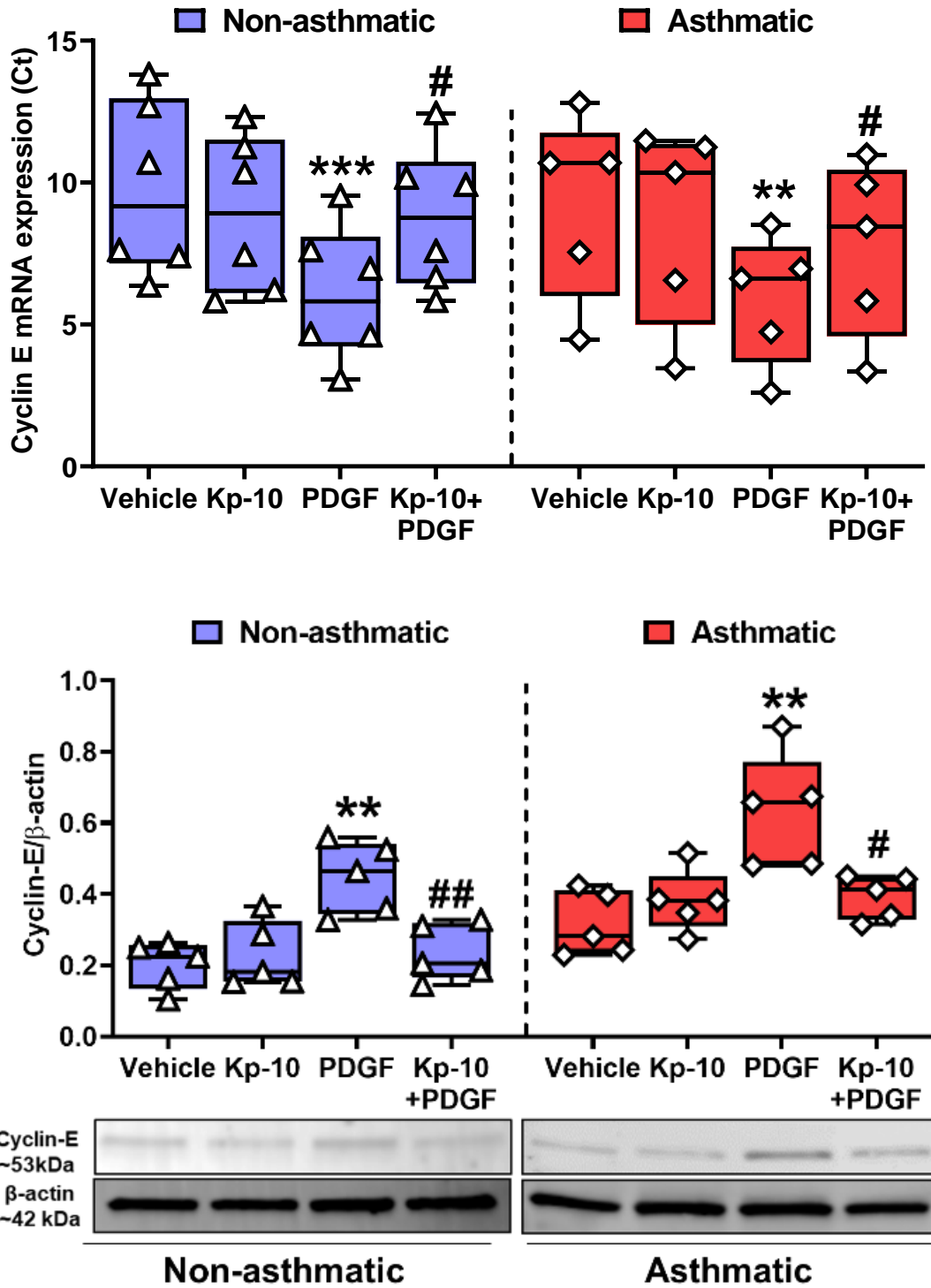
Figure 29: Effect of Kp-10 on PDGF-induced human ASM cell proliferative marker, Cyclin-E. Non-asthmatic and asthmatic human ASM cells showed increased mRNA and protein expression of Cyclin-E, following PDGF exposure, an effect substantially blunted by Kp-10. **P<0.01, ***P<0.001 vs. respective vehicle; #P<0.05, ##P<0.01 vs. respective PDGF.
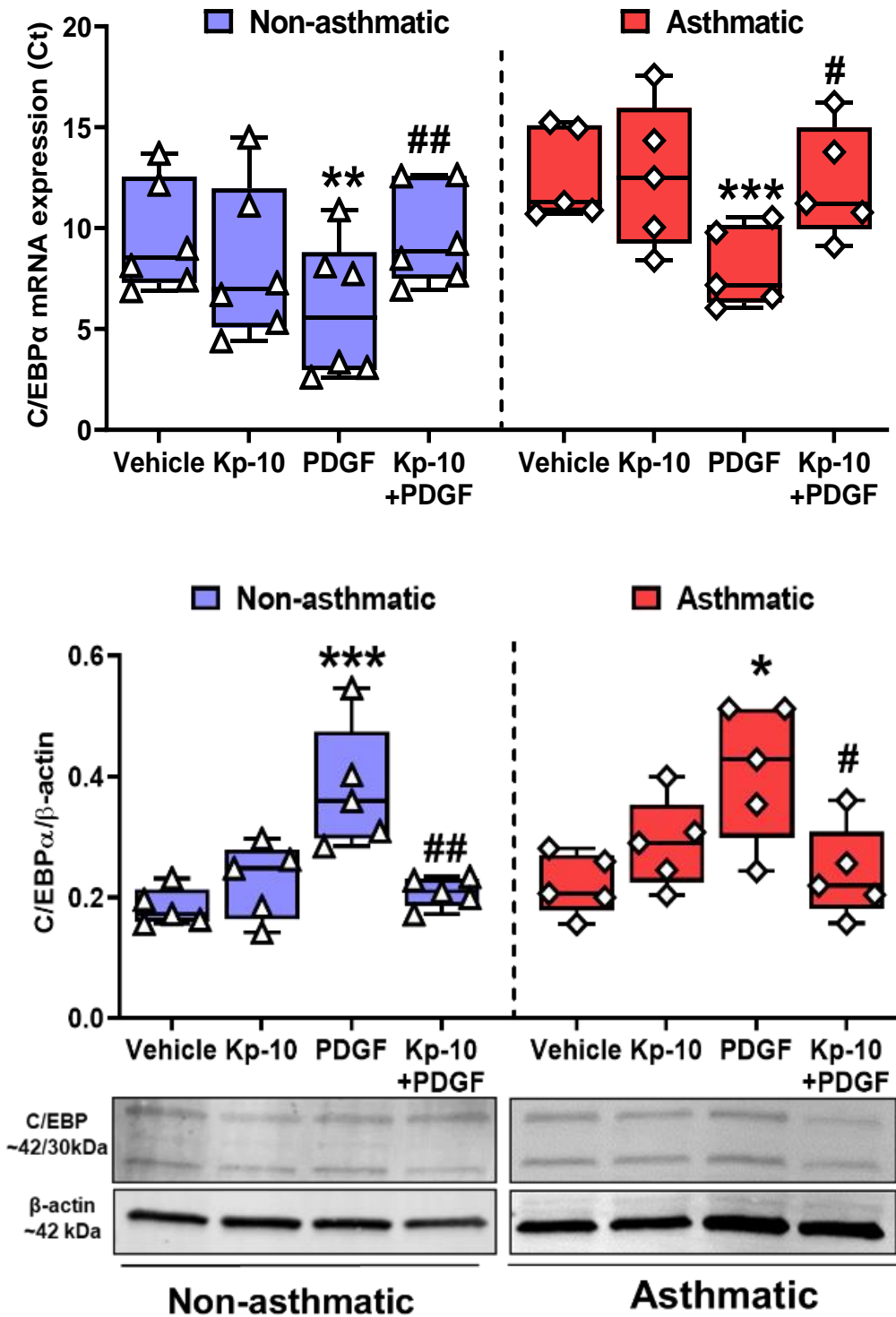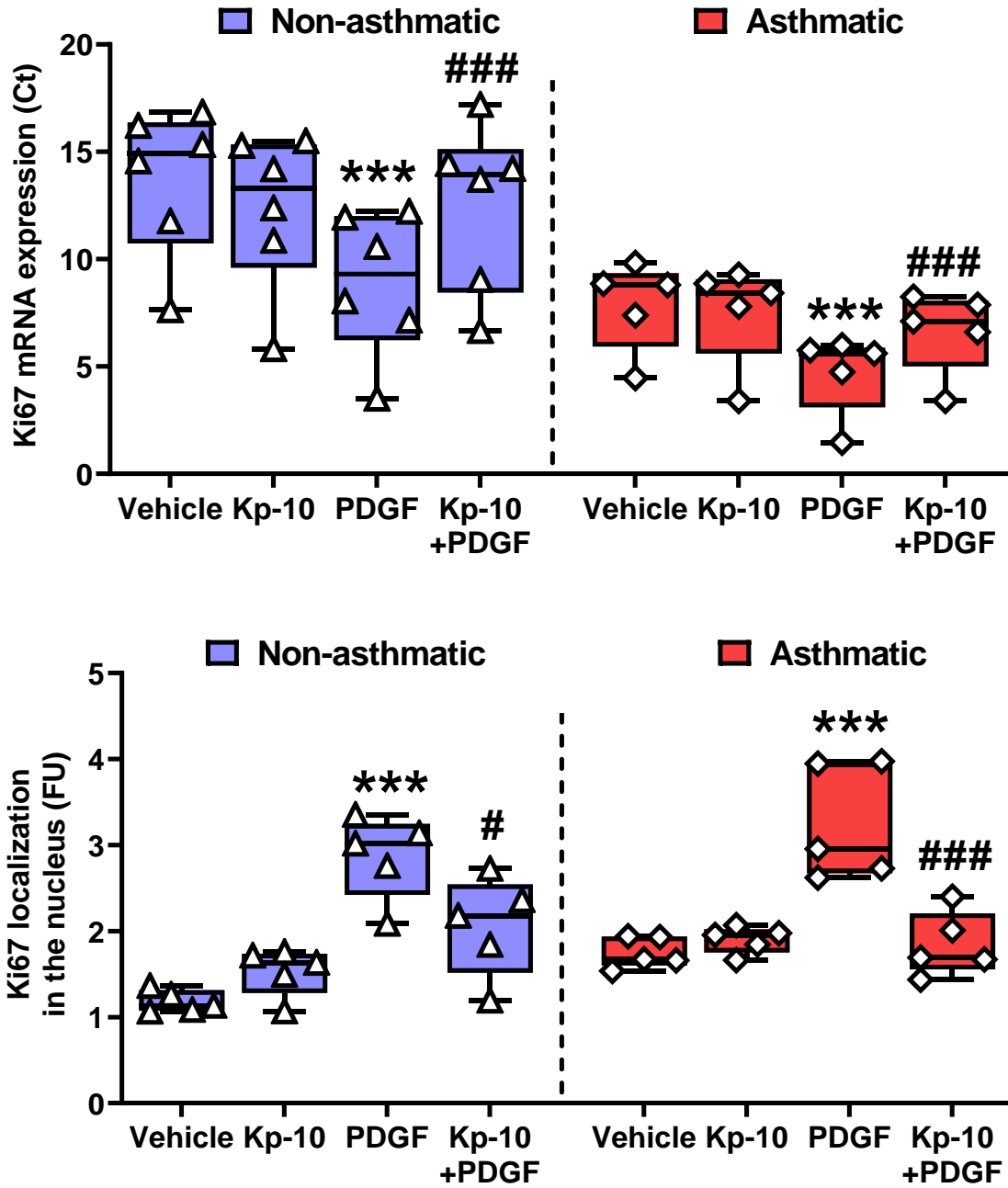
3.3.4. Effect of Kp-10 on ASM Proliferative Signaling Pathways

I also evaluated phosphorylated and total p38 MAPK (Figure 30), Akt (Figure 31), and ERK1/2 (Figure 32) in human ASM cells. PDGF significantly increased phosphorylation of p38 MAPK, Akt and ERK1/2 ($P<0.05$) compared to the vehicle group. Pretreatment with Kp-10 significantly reduced PDGF-induced phosphorylation of p38 MAPK, Akt ($P<0.05$), and pERK1/2 ($P<0.01$).
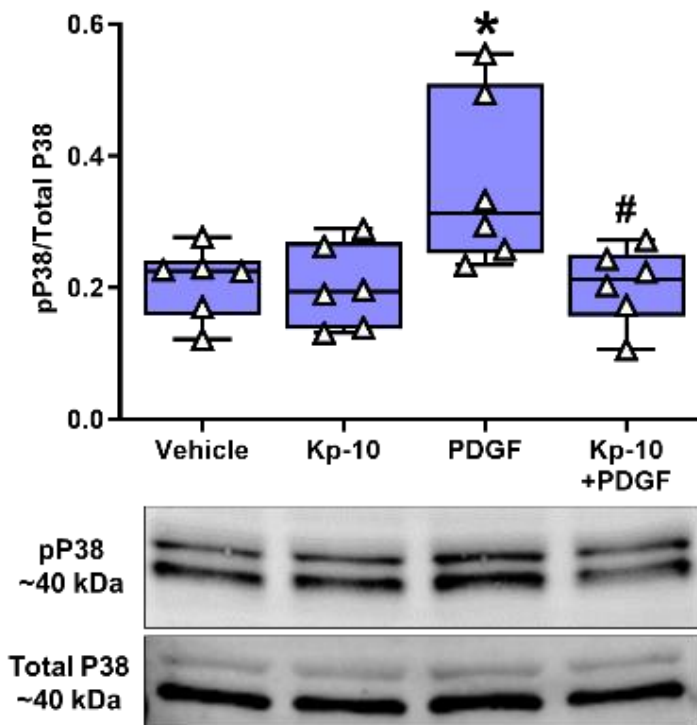
Figure 30: Effect of Kp-10 on human ASM P38 signaling pathway.
Western analysis showed PDGF-induced activation of p38 in human ASM cells. Pretreatment with Kp-10 significantly reduced the PDGF-induced phosphorylation of p38. Data are represented as a minimum to maximum of 6 individual samples from non-asthmatics and analyzed using one-way ANOVA followed by Tukey's post-hoc test. *$P<0.05$ vs. Vehicle; #$P<0.05$ vs. PDGF-exposed group.
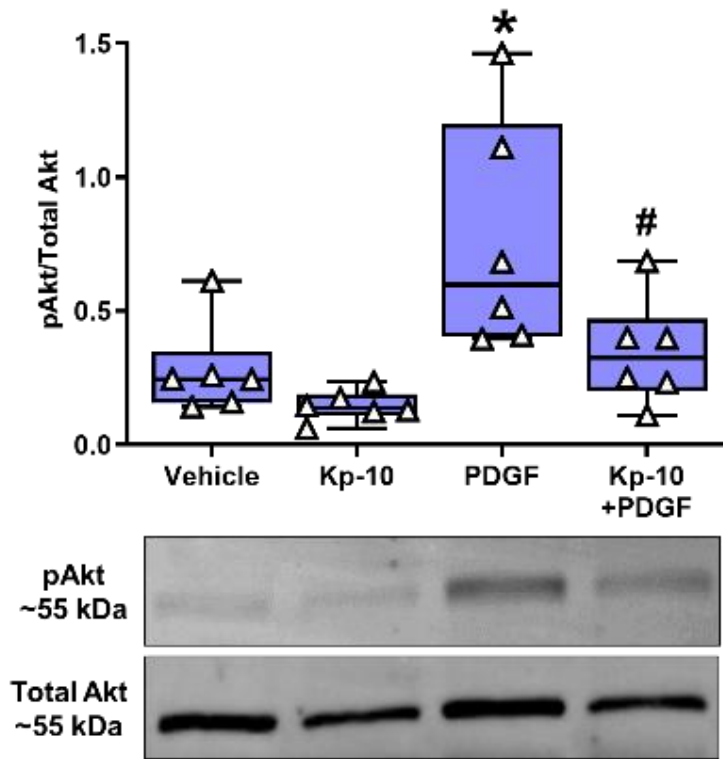
Figure 31: Effect of Kp-10 on human ASM Akt signaling pathway.
Western analysis showed PDGF-induced activation of Akt in human ASM cells. Pretreatment with Kp-10 significantly reduced the PDGF-induced phosphorylation of Akt. Data are represented as a minimum to maximum of 6 individual samples from non-asthmatics and analyzed using one-way ANOVA followed by Tukey's post-hoc test. *$P<0.05$ vs. Vehicle; #$P<0.05$ vs. PDGF-exposed group.
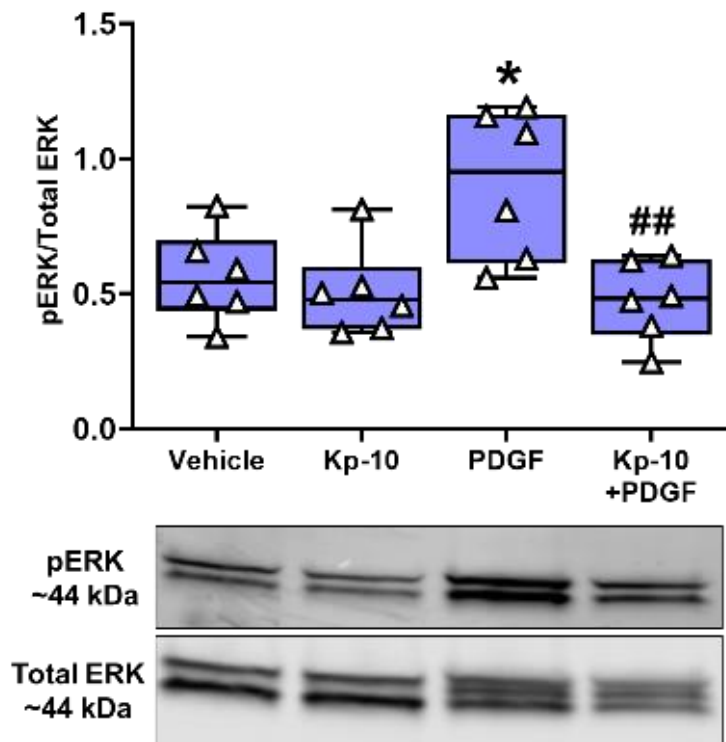
Figure 32: Effect of Kp-10 on human ASM ERK signaling pathway.
Western analysis showed PDGF-induced activation of ERK in human ASM cells. Pretreatment with Kp-10 significantly reduced the PDGF-induced phosphorylation of ERK. Data are represented as a minimum to maximum of 6 individual samples from non-asthmatics and analyzed using one-way ANOVA followed by Tukey's post-hoc test. *$P<0.05$ vs. Vehicle; ##$P<0.01$ vs. PDGF-exposed group.

3.3.5. ECM Proteins and Their Regulators

The ECM serves as a structural support and stabilizes the lung to facilitate a homeostatic environment for the cellular responses. Alterations of the extracellular matrix composition is one of the key features in asthma. Growth factors such as TGFβ and others after being released from the airway walls promote the synthesis of collagens and fibronectins in the ASM cells, suggesting a bi-directional relationship between the ASM and the ECM. Additionally, an increase in the synthesis of ECM proteins also alteration sin MMPs and TIMPs, which eventually contribute to alterations in the ECM. Thus, despite the ECM being a key pathological finding, the mechanism behind ECM production and deposition and its relation to asthma are

poorly understood. The newer data suggests that targeting the ECM might be an alternative for the treatment of chronic lung diseases such as asthma

Considering all the above factors, I investigated whether Kp-10 plays any role in ECM production and deposition. Furthermore, to isolate the influence of ECM dynamics with proliferation, I have used SFM as the control media in my ECM experiments. Likewise, all the treatments were also prepared in SFM for the ECM based studies.

3.3.6. Effect of Kp-10 on MMP's mRNA Expression

TNFα- and PDGF- exposures significantly increased the mRNA expression of MMP-2 (Figure 33) and MMP-9 (Figure 34). As expected, both PDGF- and TNF α- treated ASM cells showed significant increases in MMP-2 and MMP-9 mRNA expression, albeit more in asthmatic ASM cells. This increase was observed to much more robust in the case of non-asthmatic and asthmatic ASM cells exposed to PDGF, compared to TNFα. Non-asthmatic and asthmatic ASM cells exposed to Kp-10 alone did not show any significant differences compared to SFM or vehicle group. Kp-10 treatment showed significant reductions in PDGF-induced increased mRNA expression of MMP2 and MMP9 as compared to PDGF-alone treated groups. Interestingly, Kp-10 pretreatment tends to reduce TNFα-induced expressions, although not significant for both MMP-2 and MMP-9. In the case of asthmatic ASM cells, Kp-10 pre-treatment also showed more significance in reducing PDGF-induced increase in MMP-2 and MMP-9 mRNA expression as compared to non-asthmatic ASM cells.
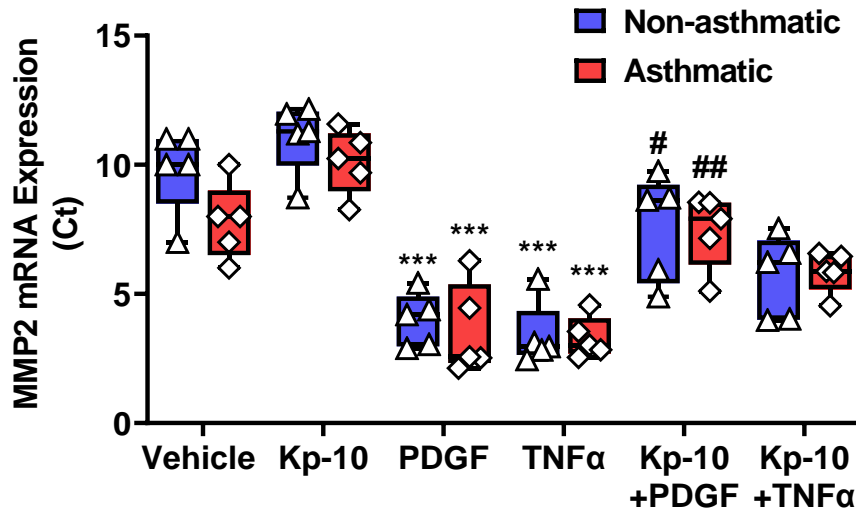
Figure 33: Effect of Kp-10 on mRNA expression of MMP-2 in non-asthmatic and asthmatic ASM.

Kp-10 exposures significantly reduced PDGF-induced mRNA expression of MMP-2, albeit not significantly for TNFα. ***$P<0.001$ vs. Vehicle, #$P<0.05$, ##$P<0.01$ vs. PDGF-exposed group. N=5 non-asthmatic and asthmatic ASM
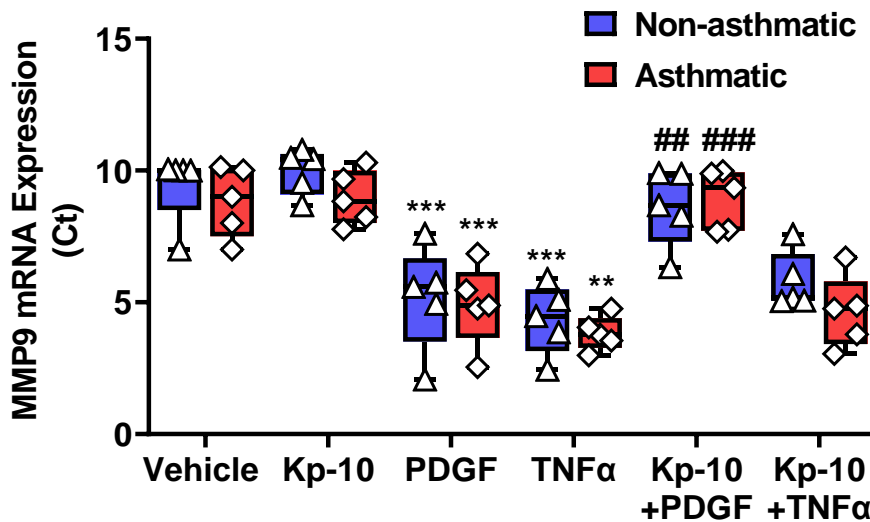
Figure 34: Effect of Kp-10 on mRNA expression of MMP-9 in non-asthmatic and asthmatic ASM.

Kp-10 exposures significantly reduced PDGF-induced mRNA expression of MMP-9, but not significant for TNFα. **$P<0.01$, ***$P<0.001$ vs. Vehicle, ##$P<0.01$, ###$P<0.001$ vs. PDGF-exposed group. N=5 non-asthmatic and asthmatic ASM

3.3.7. Effect of Kp-10 on Collagens and Fibronectin mRNA Expression

As expected, TNFα- and PDGF- exposures significantly increased the mRNA expression of procollagen I (Figure 35), procollagen III (Figure 36) and fibronectin (Figure 37). Kp-10 alone treated non-asthmatic and asthmatic ASM cells were comparable to SFM or vehicle alone group. Kp-10 treatment showed significant reductions in PDGF-induced increased mRNA expression of collagens and fibronectin as compared to PDGF-alone treated groups. Interestingly, Kp-10 pretreatment tends to show reduced collagen and fibronectin mRNA expression, however this reduction is not significant.
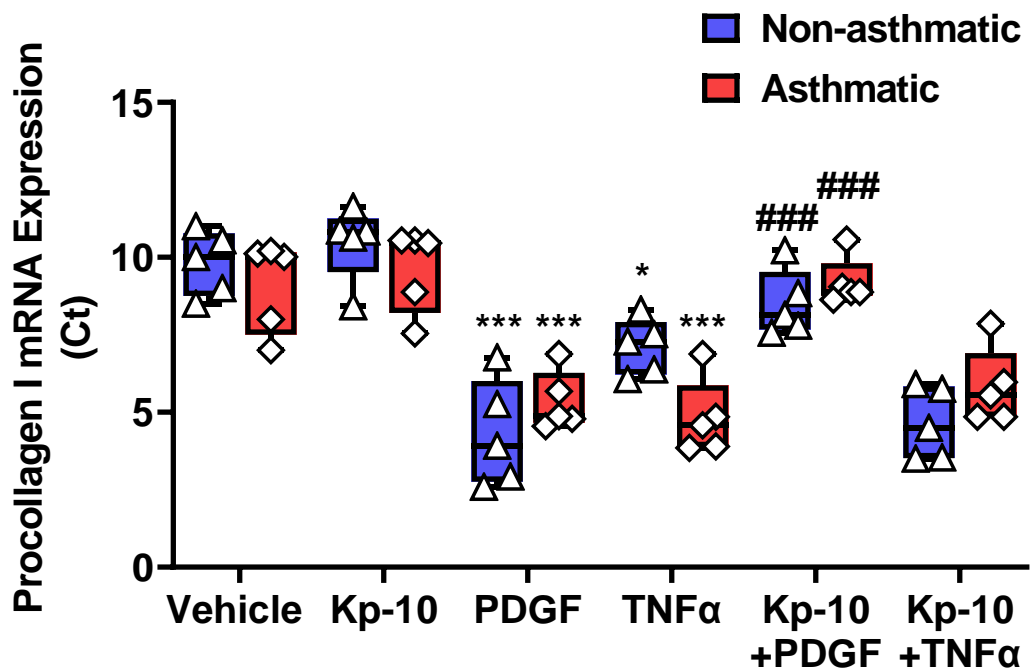
Figure 35: Effect of Kp-10 on mRNA expression of procollagen I in non-asthmatic and asthmatic ASM.
Kp-10 exposures significantly reduced PDGF-induced mRNA expression of procollagen I, albeit not significantly for TNFα. *P<0.05, ***P<0.001 vs. Vehicle, ###P<0.001 vs. PDGF-exposed group. N=5 non-asthmatic and asthmatic ASM.
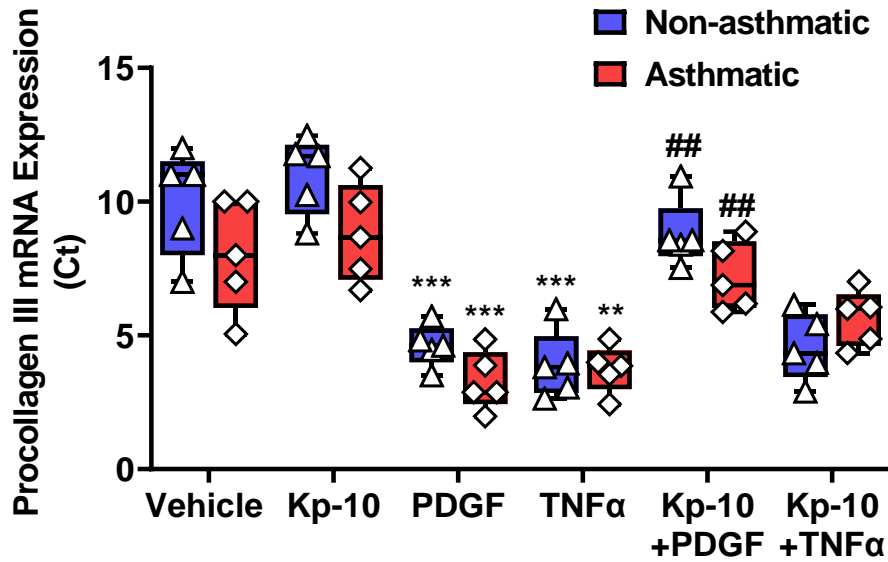
Figure 36: Effect of Kp-10 on mRNA expression of procollagen III in non-asthmatic and asthmatic ASM.

Kp-10 exposures significantly reduced PDGF-induced mRNA expression of procollagen III, albeit not significantly for TNFα. ***P<0.001 vs. Vehicle, ###P<0.001 vs. PDGF-exposed group. N=5 non-asthmatic and asthmatic ASM.
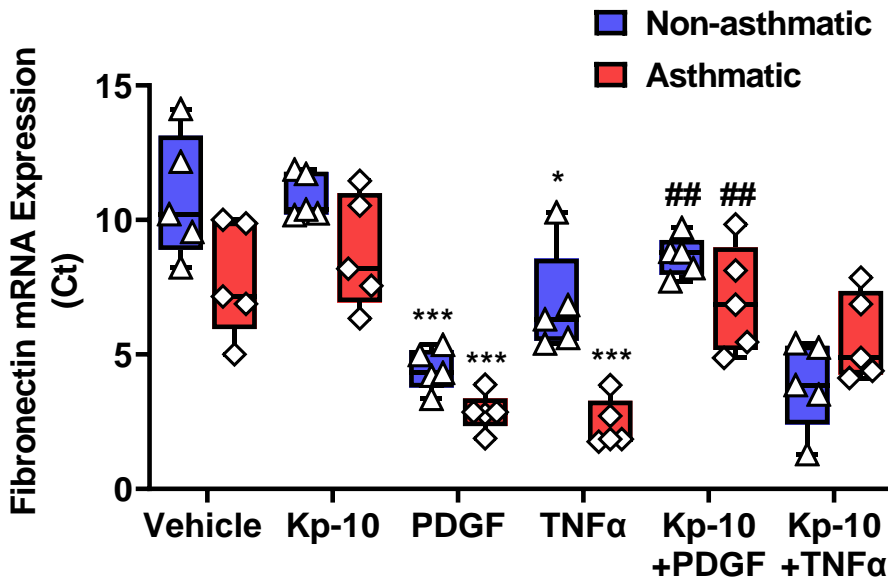
Figure 37: Effect of Kp-10 on mRNA expression of fibronectin in non-asthmatic and asthmatic ASM.

Kp-10 exposures significantly reduced PDGF-induced mRNA expression of fibronectin, albeit not significantly for TNFα. *P<0.05, ***P<0.001 vs. Vehicle, ##P<0.01 vs. PDGF-exposed group. N=5 non-asthmatic and asthmatic ASM

3.3.8. Effect of Kp-10 on PDGF-induced Expression of Collagen I

To further confirm the observed mRNA expression of corresponding ECM proteins under PDGF induction, I performed western blot analysis. Consistent with mRNA data, I found a significant increase in expression of Collagen I in human ASM cells isolated from non-asthmatics alone when exposed to PDGF. Pretreatment with Kp-10 significantly inhibited PDGF-induced increased protein expression of Collagen I (Figure 38).
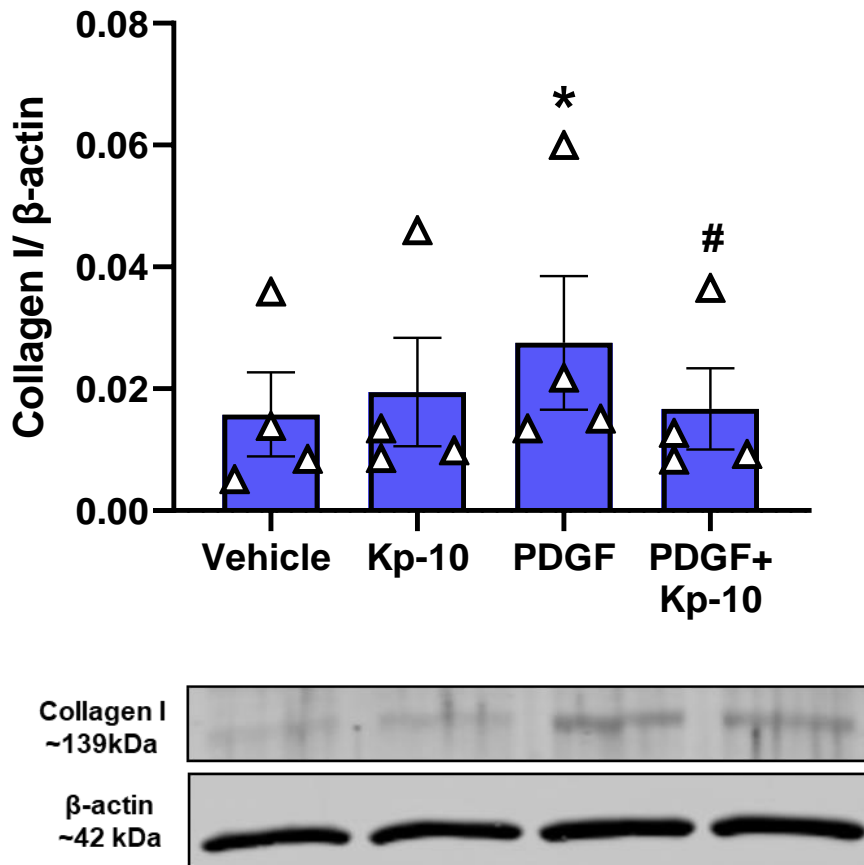
Figure 38: Effect of Kp-10 on PDGF-induced expression of collagen I in non-asthmatic ASM cells.
Kp-10 pre-exposure downregulated PDGF-induced expression of collagen I as represented in the western blot image *$P<0.05$, vs. Vehicle, #$P<0.05$ vs. PDGF-exposed group, N=4.

3.3.9. Effect of Kp-10 on TGFβ-induced Expression of Collagen I

Given the ineffectiveness of Kp-10 in mitigating TNFα-induced increase in mRNA expressions of remodeling genes, I sought to determine whether other mitogenic agents such as TGFβ (1ng/mL) stimulation of remodeling would be affected by KISS1R activation. Similarly, to confirm whether Kp-10 had any role in mitigating TGFβ-induced increase in protein expression of Collagen I, I performed western blot analysis for human non-asthmatic ASM cells exposed to TGFβ alone and TGFβ in the presence of Kp-10. I observed significant upregulation in Collagen I protein expression when exposed to TGFβ (Figure 39). This increase seemed much more significant as compared to PDGF alone treated non-asthmatic ASM samples, indicating the high tendency of TGFβ to induce remodeling-associated changes, as evidenced by previous studies. Interestingly, Kp-10 in the presence of TGFβ was able to significantly inhibit TGFβ-induced remodeling changes as observed by the reduction in Collagen I protein expression.
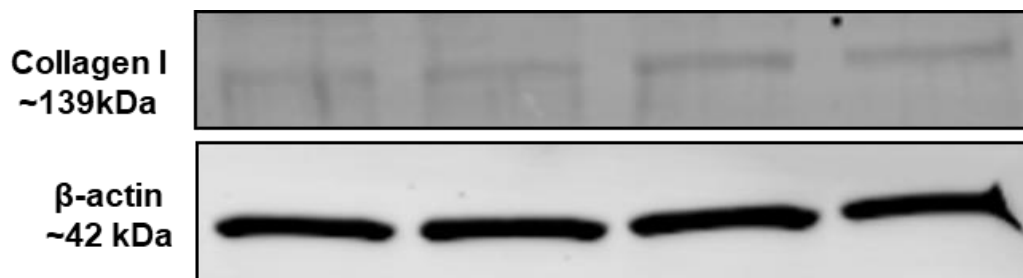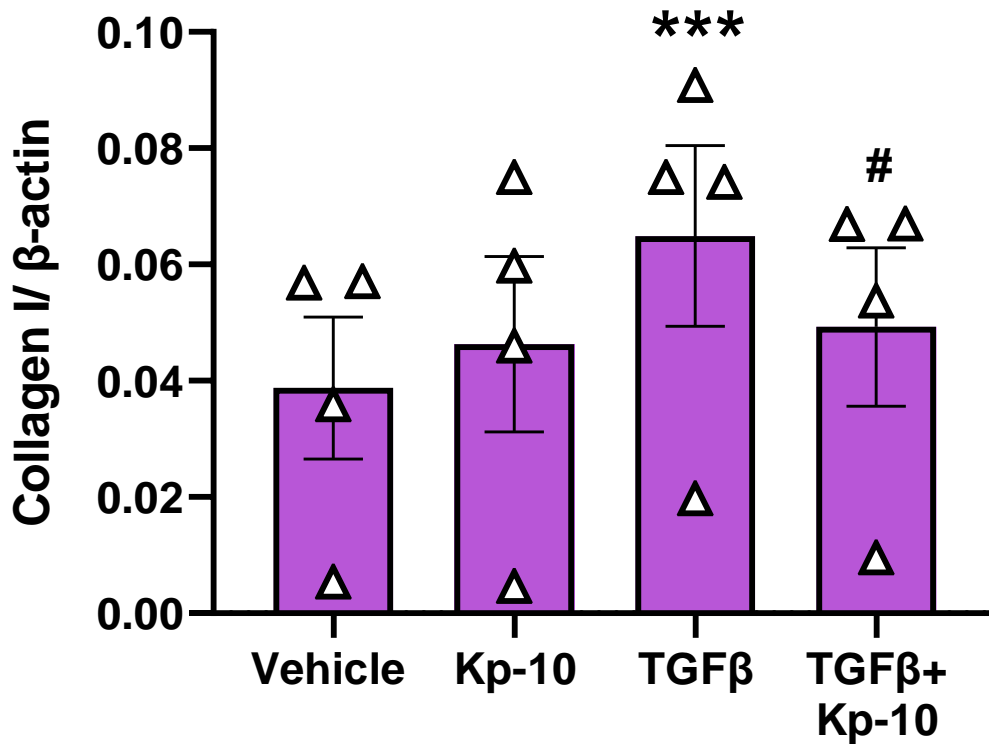
Figure 39: Effect of Kp-10 on TGFβ-induced expression of collagen I in non-asthmatic ASM cells.
Kp-10 pre-exposure downregulated TGFβ -induced expression of collagen I as represented in the western blot image ***P<0.001, vs. Vehicle, #P<0.05 vs. PDGF-exposed group, N=4.

3.4. Discussion

In the present aim, I show an increase in the proliferation and ECM deposition of proteins such as collagen-I, collagen-III and fibronectin upon mitogenic stimulation. Collagen-I and collagen-III are the major fibrillar proteins in the ASM. Several studies in the past have shown increase in collagen-I and -III in ASM from asthmatic individuals. Fibronectins are also

abundant in the ASM and has been previously reported to be increased in asthmatics airways. Differential expression of MMPs has also been well established in asthma pathophysiology. Apart from their role in degrading ECM components, MMPs are also involved in inflammatory cell trafficking, host defense mechanisms and tissue repair. MMPs are also expressed in the structural cells of the airways, especially the ASM. Taken together, these findings indicate that proliferative and ECM changes reflect alterations in airway structure and function. These functional changes ultimately depend on the relative contribution of each protein in the structural cell of the airway.

Additionally, I investigated the functional effect of KISS1R activation on human ASM proliferation and ECM deposition and production. It is well known that dysregulated ASM cell proliferation and ECM production and deposition contribute to airway remodeling and AHR in asthma. Therefore, exploring the mechanisms underlying ASM remodeling are very important, especially since current therapies fall short of alleviating or reversing airway remodeling in asthma. The observations in this aim highlight a novel and potentially targetable mechanism of Kp-10 in the context of ASM remodeling. To stimulate proliferation in ASM cells, I used PDGF as a mitogenic agent given its well-known effects in human ASM. PDGF and its receptor (PDGF-R) have been established to play significant roles in airway proliferation and remodeling. Additionally, in earlier studies, we have shown that PDGF at a concentration of 2ng/mL significantly induces ASM proliferation via activation of intracellular signaling pathways such as ERK1/2, p38, and Akt that are relevant to both asthma and Kp biology. The increased proliferation observed with PDGF in this study is consistent with our earlier studies. In cell proliferation studies, Kp-10, Kp-14 and Kp-54 showed 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. An interesting observation was that Kp-10 effects on proliferation were similar between non-asthmatic and asthmatic ASM 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 asthmatic ASM. Regardless, what is novel and potentially relevant is the data showing that ASM from both asthmatics and non-asthmatics 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 G0/G1 phase, consistent with reports from other cell types, which find that Kps initiate cell cycle arrest via KISS1R/GPR54 dependent mechanisms. PCNA and C/EBPα are established markers for proliferation as they play a crucial role during the DNA synthesis phase of mitosis. PDGF upregulated the expression of PCNA and C/EBPα 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/EBPα expression in asthma, whereas my observed data shows increased C/EBPα expression with PDGF exposure in ASM cells from both non-asthmatics and asthmatics. The reason for this discrepancy is unclear and warrants further exploration. Ki-67 is a crucial marker for mitosis as it is exclusively detected in the nucleus during the G1, S, and G2 phases of the cell cycle and absent in the G0 phase. PDGF-induced nuclear translocation of Ki-67 in the nucleus was blunted by Kp-10 pre-exposure, further strengthening my findings from ASM cell cycle analysis.

Further, the importance of MAPK/ERK signaling pathways in cell growth, proliferation, and differentiation of human ASM cells is well established. In other cell systems, KISS1R activation is known to inhibit the phosphorylation of MAPK signaling via beta-arrestin-1[69]. Additionally, KISS1R via regulating alpha sub-unit of Gq/11 inhibits the phosphorylation of PI3K/Akt and subsequently regulates cell growth. Similarly, my findings reported increased phosphorylation of ERK1/2 and p38 MAPK upon PDGF exposure, which was inhibited by Kp-10 pretreatment. Further, PDGF exposure upregulated the PI3K/Akt signaling which activates mTOR signaling, which functions as a serine/threonine-protein kinase that regulates ASM cell proliferation. These effects of Kp-10 via KISS1R activation are consistent with data from other cell types. The inhibition of Akt phosphorylation by Kp-10 further indicated the importance of Kp/KISS1R regulation of ASM proliferative pathways.
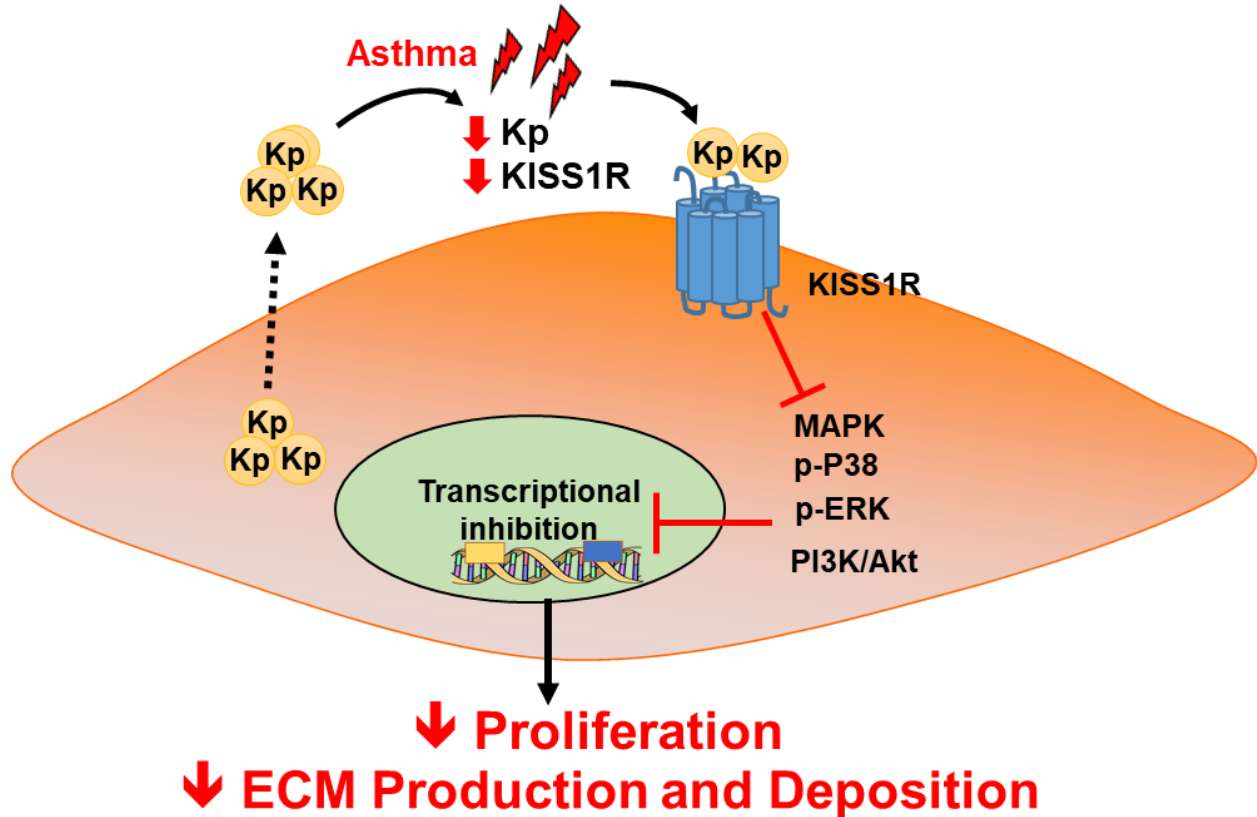
Figure 40: Schematic overview of observations in Chapter 3.
As shown here, KISS1R activation using KISS1R agonist, Kp-10 mitigates proliferation and ECM production, thereby reducing AHR and airway remodeling.

Overall, my findings suggest that KISS1R activation plays a protective role in regulating ASM remodeling, which is lost during asthma, thereby potentially permitting exacerbated ASM remodeling. The 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/EBPα, Ki-67, Cyclin-D1, and Cyclin-E. ECM production is also significantly inhibited by KISS1R activation for both PDGF- and TGFβ-induced ECM remodeling changes.

4. IN A MURINE MODEL OF MIXED ALLERGEN-INDUCED ASTHMA, KISSPEPTIN ATTENUATES AIRWAY REMODELING[1]

4.1. Introduction

Asthma, as we already know is an intricately complex disease with a multifactorial origin. Asthma is a common chronic airway disorder resulting because of airway inflammation, hyperreactivity and remodeling, affecting almost 300 million people worldwide[1, 70]. Asthma is also a sex-skewed disease due to confounding influences of endogenous sex steroids[2, 71]. The influence of sex steroids on asthma *per se* remains still under investigation with multiple groups researching on sex steroid-receptor based effects on asthma.

Factors such as sex, age, disease status and smoking play a critical role in exacerbating asthma. Asthma affects people of all ages, albeit it is more common in females as compared to males as outlined by multiple studies[53, 72, 73]. Although several research groups have addressed these concerns in multiple clinical and epidemiological studies, the underlying factors behind these differences is still lagging. A crucial finding in all the previous studies suggests the post-pubertal reversal of asthma occurrence with women showing increased susceptibility to asthma as compared to men. Therefore, it is understandable that mechanisms regulating these sex differences in asthma originate upstream to or independent of sex steroids, especially estrogen.

Here, it is important to highlight the role of kisspeptins (Kp) as a potential regulator of asthma. Various evidences in the CNS further justify my rationale to investigate Kp as a potential upstream modulator of sex steroids that could be relevant to the airways: A) Kp is a critical initiator of puberty ovulation via central control of the hypothalamic-pituitary-gonadotropic axis[74, 75]; B) Kp regulates release and secretion of gonadotropin-releasing-hormone and gonadal sex steroids and[40, 41]; C) Kp receptor agonist, metastin has been used a treatment option for sex steroid dependent diseases[76]. Kp encoded by the KISS1 gene, is located on chromosome 1 near q32, which upon proteolytic cleavage produces multiple cleaved peptides of shorter amino acid lengths. These smaller peptides share the same C terminus 10 amino-acid sequence and are members of the single precursor Kp. The G-protein coupled receptor 54 or KISS1R serves as the receptor for Kp[34, 35]. Multiple studies have elucidated the role of Kp/KISS1R in different cell types. In Chapter 2 of this proposal, I reported the expression of Kp and KISS1R in the human lung, specifically in the airway smooth muscle (ASM) suggesting a potential role of Kp/KISS1R signaling in airway biology[55]. Interestingly, I observed lesser expression of Kp and KISS1R in asthmatic ASM. I further showed (in the previous aims) that KISS1R agonist, Kp-10 ablates Platelet-Derived Growth Factor (PDGF)-induced ASM cell proliferation via ERK and P38/MAPK signaling pathways[55].

In this aim, I explore the effect of Kp-10 in regulating lung mechanics parameters during airway hyperresponsiveness (AHR) and remodeling in a chronic mixed allergen (MA) induced model of asthma. I have consciously included ovariectomized (OVX) mice along with male and female mice in the study population to disintegrate the effects of endogenous estrogen. In this study, I show for the first time the importance of Kp/KISS1R signaling *in vivo*, thus

corroborating my findings in vitro. Thus, this study provides a valuable insight in Kp/KISS1R

signaling in airway edifice and function in the context of asthma.

Therefore, I have used a well-established and robust mixed-allergen induced mouse

model of asthma to best replicate the underlying pathophysiology and intricate mechanisms

characteristic of asthma[77-79]. I chose to use a mouse as the animal model as it is economical,

has a short gestation period, easily manageable, convenient to breed and relatively easy to work

with manipulated mouse models[80]. The effect of chronic kisspeptin in a mouse model of

mixed allergen induced asthma was not known. Several studies have shown differential effects of

sex steroids on pulmonary function and AHR in various allergic mouse models[81]. These

differences have been attributed to both sex and the receptor involved in the study. Since, there

are no studies to date which have investigated the effect of kisspeptin in regulating AHR and

remodeling, the goal of this aim is to investigate the role of Kp-10 in regulating AHR and

remodeling.
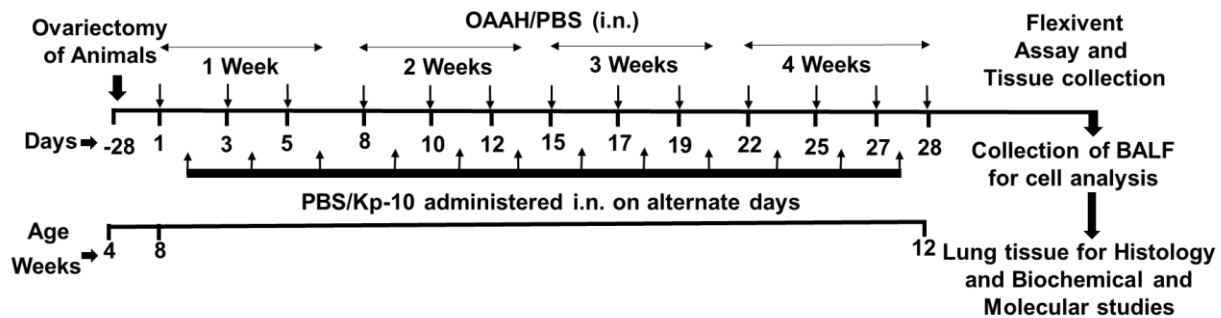
Figure 41: Schematic showing the experimental design of the *in vivo* study.
Mice were sensitized with mixed allergen (MA, OAAH: 10 µg each of Ovalbumin, *Alternaria Alternata, Aspergillus Fumigatus, and Dermatophagoides farinae* (house dust mite)) or phosphate-buffered saline (PBS, vehicle) intranasally (i.n.) on alternate days for 28 days. From day 02, mice received PBS or Kisspeptin-10 (Kp-10) to respective groups via intranasal administration. Ovariectomy (OVX) surgery was performed at the age of 4-weeks.

4.2. Materials and Methods

4.2.1. Mixed Allergens and Reagents

Ovalbumin from chicken egg white was procured from Sigma-Aldrich, USA. The allergens including Alternaria Alternata, Aspergillus Fumigtus, and Dermatophagoides Farinae (House dust mite) were purchased from Greer laboratories, USA. Dulbecco's Phosphate Buffered Saline (PBS) and protease and phosphatase inhibitor (PPI) cocktail were purchased from Thermo Scientific, USA.

4.2.2. Experimental Animals

All animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at North Dakota State University and experiments were conducted as approved under the guidelines of "National Institutes of Health Guide for the Care and Use of Laboratory Animals". Wild-type (WT) breeding pair of C57BL/6J background female and male mice were procured from The Jackson Laboratory. I carried out the in-house mice breeding, and the resultant mice were used in the current study in the age range of 6-8 weeks. Age-matched C57BL/6J background OVX mice were obtained from the Jackson Laboratory. All the animals were housed under appropriate temperatures with 12-12 h light and dark cycles. The animals were provided with food and water ad libitum. Mice from male, female and OVX populations were divided with a minimum of 6 mice per treatment group: 1) Vehicle (PBS-challenged); 2) MA-Challenged; 3) Kp-10 (1.2 mg/mice/day (30)); 4) Kp-10+MA.

4.2.3. Mixed Allergen Challenge and Treatments

Mice from all three populations (male, female, and OVX) were assigned to MA groups and were administered intranasally with a mixture of four allergens containing 10 µg each of ovalbumin, Alternaria alternata, Aspergillus fumigatus and Dermatophagoides farina in 25 µL of

PBS on every even day for 4 weeks. Animals in the vehicle group received an equal volume (25 µL) of PBS alone for the same time durations. Additionally, mice from all three populations were administered intranasally with Kp-10 in 25µl of PBS on every odd day. Please refer schematic in Figure 41 for additional details.

4.2.4. Lung Function Analysis

Mice were subjected to flexiVent (Scireq, Montreal, Canada) system on day 29, and parameters such as respiratory resistance (Rrs), compliance (Crs), and elastance (Ers) were recorded. Ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) were used for anesthesia. Rrs, Crs, and Ers were recorded at baseline (0 mg/mL Methacholine, MCh) and increasing doses of nebulized MCh (6.25 - 50.0 mg/mL). Rrs, Crs, and Ers values at two doses of MCh 25mg/mL and 50mg/mL were used for comparison between treatment groups across the three study populations.

4.2.5. Bronchoalveolar Lavage Fluid (BALF) Processing

Post lung function analysis, BALF was performed by injecting 1.0 mL of PBS (supplemented with PPI cocktail) via the tracheal cannula with about ~600 µl of recovery. To clear the blood from the airways, the lungs were perfused with 10 mL of PBS via the left ventricle of the heart before collecting each lobe. The left lobe of the lung of mice was stored in Carnoy's solution (100 % Ethanol, Chloroform, and Glacial acetic acid in a ratio of 6:3:1 with ferric chloride) followed by replenishing the solution with 70% ethanol after a few hours and utilized for histology studies.

4.2.6. Cytokine ELISA Multiplex Assay in BALF

BAL fluid collected from mice were centrifuged briefly and then aliquoted into 05-0.6 mL microcentrifuge tubes for further analysis. Samples were subsequently processed as per the manufacturer's protocol (Eve Technologies Pvt. Ltd.).

4.2.7. Histopathology Analysis using Hematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS), and Masson's Trichrome Stains

The stored left lungs fixed in paraffin block and sections were processed for hematoxylin and eosin (Sigma, USA), Periodic acid-Schiff (EMS, USA) and Masson's trichrome (Chrondex, Inc., USA) stains, using previously established techniques. The slides were then scanned using Epredia Pannoramic MIDI II 20x (3DHISTECH, Hungary). To determine the inflammatory cells recruitment score in H&E staining, goblet cell hyperplasia with epithelial thickening in PAS staining, and collagen formation in Masson's trichrome staining, I performed scoring by two individuals on a scale of 1-5 (1 as good and 5 as worst).

4.2.8. Western Analysis of Lung Tissue Homogenates

Fresh lung tissue samples were collected for protein (10 mg) and homogenized in sucrose buffer (100 µL) in the presence of a PPI cocktail. Total protein was estimated and at least 40 µg of protein was loaded in each well/sample. Western analyses were carried out as per the standard protocols. The blots were probed with Vimentin (Sigma Aldrich, cat# V5255), α-smooth muscle actin (α-SMA, Sigma Aldrich. Cat# A2547), and β-actin (Applied Biological Materials, cat# G043) followed by scanning in LI-COR near infra-red imaging system. The LI-COR NIR labeled secondary antibodies were used to determine the intensity and densitometric analysis was performed using Image studio software. The data are represented as a ratio of respective proteins with β-actin.

4.2.9. Laser-Capture Microdissection (LCM) Assisted ASM Isolation and qRT-PCR

LCM was performed as per the previously established technique in our laboratory using a Zeiss-Axio Imager Z1 PALM Microbeam laser capture microscope system (Zeiss, Thornwood, NY). Airway smooth muscle cells were identified and marked using Palm-Robo software and a laser cut was performed to catapult the specific ASM cells into the RNAase-free microtube containing single cell PicoPure RNA extraction buffer. RNA was isolated from the collected cells using the PicoPure® RNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA) and stored at −80°C for further studies. Please refer Table 1 for additional primer details.

Table 1: Primer sequences used for qRT-PCR analysis in LCM-assisted ASM isolated samples.

Primer	Forward Sequence	Reverse Sequence
ms16	GATGAAGTCGGAGCTGGTAAA	GGAGTGACAGGCAACTATGAA
mKiss1	CCTCCCAGAATGATCTCAATGG	GTGGATCCAGGCTTCACTTT
mKISS1r	CCCTCTGAGTCTCACCTAGAAA	CCTTGCTCCCAAAGAGGTTAG

4.2.10. Soluble Collagen Assay

Lung tissue lysates obtained from different treatment groups were used to measure the level of soluble collagen as an important marker of airway remodeling. This assay was performed according to the manufacturer's protocol (Sircol Soluble Collagen Assay, Biocolor Life Science Assay, UK) and previously described technique. Final absorbance values were measured at 556nm in a Synergy HTX Plate Reader (Biotek, USA).

4.2.11. Statistical Analysis

All the experimental groups consisted of a minimum of four mice. "n" values represent the number of animals. Statistical analysis was performed using an unpaired t-test or two-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons using GraphPad Prism version 8.1.0 for Windows (GraphPad Software, San Diego, California USA) as applicable. Data are expressed as mean ± SEM with statistical significance tested at a minimum of $p < 0.05$ level.

4.3. Results

4.3.1. Mouse Kp/KISS1r Protein and mRNA Expressions in the Lung

The experimental design of the study represented in Figure 41. Laser capture microdissection (LCM) was used to separate airway smooth muscle (ASM) layers and respective treatment group RNA was isolated. The qRT-PCR analysis showed a significant reduction in ASM-specific Kiss1 mRNA ($P<0.01$ for male and female; $P<0.05$ for OVX, Figure 42) and KISS1r mRNA ($P<0.01$ for male and female; $P<0.05$ for OVX, Figure 42) expression in MA-challenged mice from all the study populations compared to respective PBS challenged mice. Additionally, the protein expression of Kp and KISS1R in the lung tissue homogenate was measured by Western analysis. The results showed a significant reduction in the expression of Kp ($P<0.05$ for male and OVX; $P<0.01$ for female, Figure 43) and KISS1R ($P<0.001$ for male, $P<0.05$ for female and OVX, Figure 43) in the lung homogenate of mice from MA-challenged groups in all three study populations with compared to respective PBS-challenged mice.
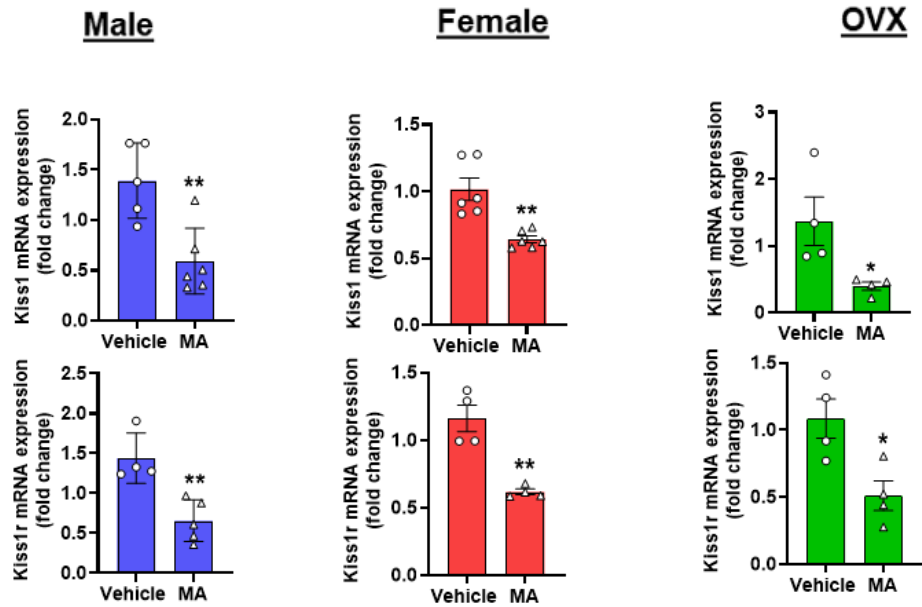
Figure 42: Laser capture microdissection-assisted qRT-PCR analysis of Kiss1 and Kiss1r.
ASM bundle was isolated from the lung using laser capture microdissection-assisted microscopy from the respective treatment groups and RNA was isolated. Subsequently, specific ASM Kiss1 and Kiss1r mRNA expression was measured using qRT-PCR in male (A and D), female (B and E), and OVX (C and F) mice. Data were analyzed using a two-tailed unpaired student's t-test. Data represented as min to max showing all points of at least four mice per group; *P<0.05, **P<0.01 vs. respective vehicle group.

Figure 43: Kisspeptin (Kp) and KISS1R expression in mixed-allergen sensitized mouse lung. Kisspeptin (Kp) and KISS1R protein expressions were observed to be significantly lower in whole lung tissue of MA-challenged mice across all three populations in male (A and D), female (B and E), and OVX (C and F) mice compared to respective PBS group. All proteins are normalized to β-actin. Data were analyzed using a two-tailed unpaired student's t-test. Data represented as min to max showing all points of at least four mice per group; *P<0.05, **P<0.01, ***P<0.001 vs. respective vehicle group.

4.3.2. Effect of Kp-10 on Airway Resistance

I assessed the chronic effect of exogenous kisspeptin (Kp-10) *in vivo* using the forced oscillation technique by flexiVent FX1 module to measure airway resistance (Rrs), which represents airway constriction. Male, female, and OVX mice showed an increase in Rrs in response to inhaled increased methacholine (MCh; 0-50 mg/mL) in the MA-challenged group compared to PBS-challenged mice (Figure 44A-C). To compare the effectiveness of different

treatments, I used Rrs at the dose of 25 and 50 mg/mL of MCh. Mice treated with Kp-10 alone did not show any significant changes in Rrs in all three study populations compared with mice from the respective PBS group. However, MA-challenged mice, treated with Kp-10 showed a significant reduction in Rrs at 25 mg/mL of MCh in male and female mice ($P<0.01$), with no significant changes in the OVX mice. Interestingly, at the higher concentration of MCh (50 mg/mL), I observed a profound effect of Kp-10 in the reduction of MA-induced Rrs in male (Figure 44D and G) and female (Figure 44E and H) mice ($P<0.001$), with moderate reduction ($P<0.05$) of Rrs in OVX mice (Figure 44F and I). In the comparison of all three study populations, female mice tend to show a better effect of Kp-10 on the reduction in MA-induced Rrs, while OVX mice showed minimal effects.
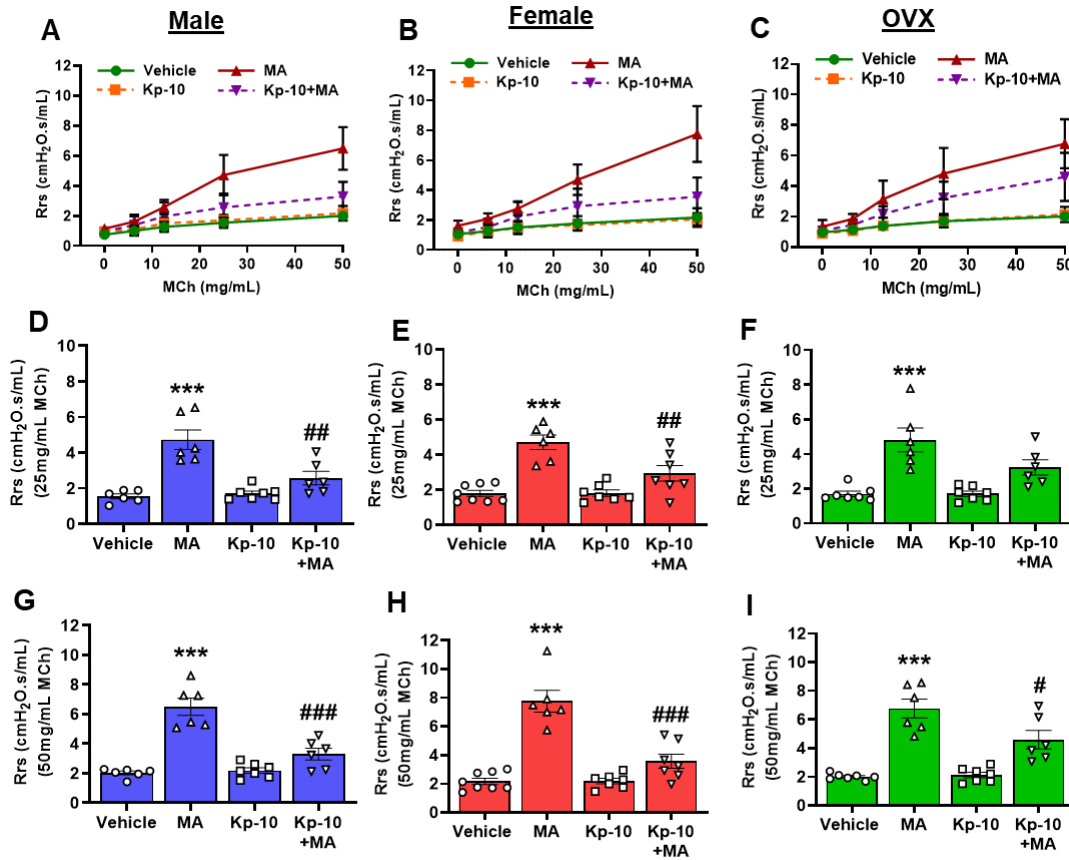
Figure 44: Effect of Kp-10 on respiratory resistance (Rrs) in a MA model of asthma in male (A), female (B), and OVX (C) mice.

The response to inhaled various methacholine doses (MCh) (0-50 mg/mL) was measured in anesthetized animals using a flexiVent system (A-C). Rrs at the doses of 25 and 50 mg/mL of MCh were used to compare the effectiveness of the different treatment groups of male (D and G), female (E and H), and OVX (F and I) mice. Male, female and OVX mice challenged with MA showed a robust increase in Rrs compared to the baseline value (PBS challenge). Kp-10 treatment significantly lowered the effect of MA in all three study populations. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least six to sev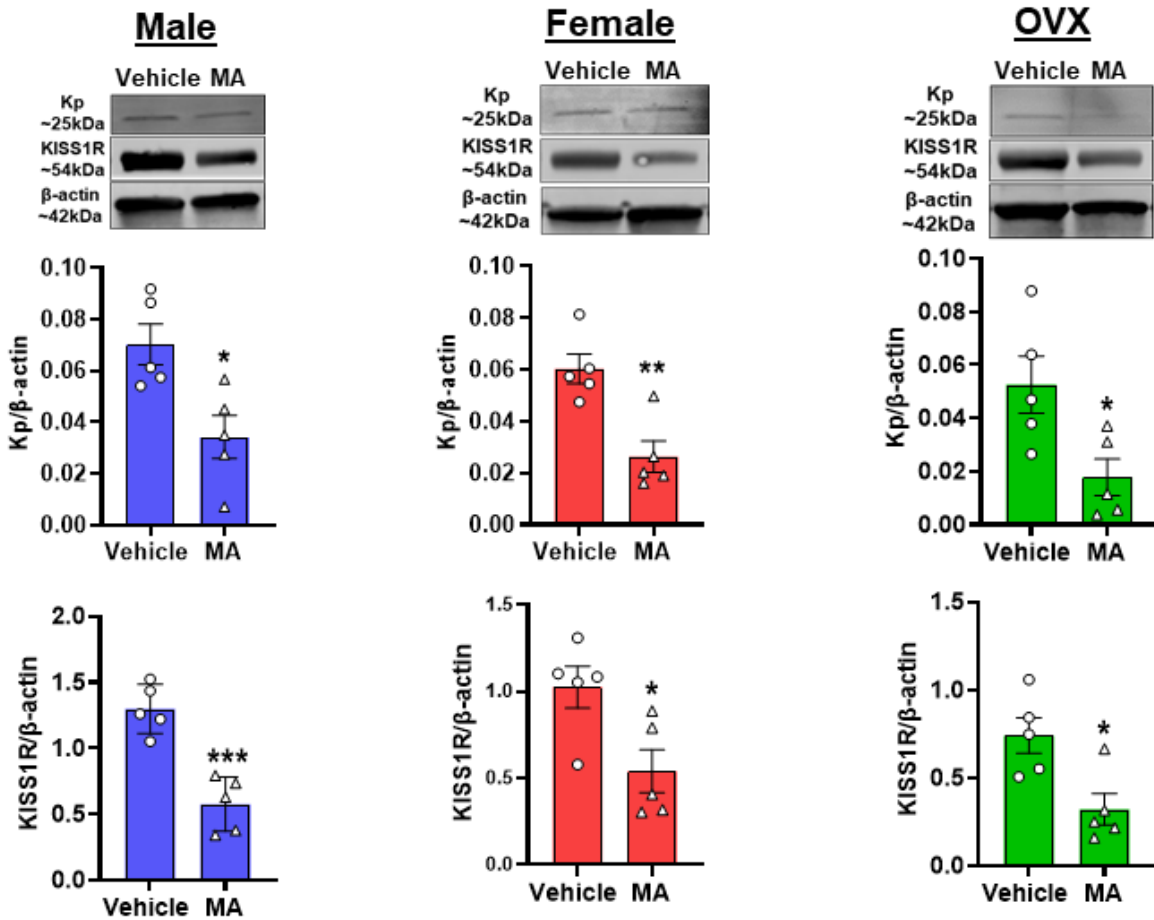en mice per group; ***$P<0.001$ vs. respective vehicle group; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs. respective MA challenged group.

4.3.3. Effect of Kp-10 on Airway Compliance

Further, the airway compliance (Crs) which represents the ease of breathing was measured in the lungs of mice exposed to MA and the effect of Kp-10 was measured in the respective treatment groups. Mice exposed to the MA showed a significant decrease in compliance in all the study populations compared with PBS-treated mice (Figure 45A-C). Crs in

response to 25 and 50 mg/mL of MCh were used to compare the effectiveness of Kp-10 in the different study populations. Mice treated with Kp-10 alone did not show any significant changes in Crs in all three study populations compared to the respective PBS group. MA-challenged mice, treated with Kp-10 showed a significant increase in Crs in female mice at 25 mg/mL ($P<0.01$) (Figure 45E) of MCh challenge. However, male and OVX mice tend to show improvement in the MA-altered Crs, albeit not significant (Figure 45D&F). Similarly, at 50 mg/mL of MCh challenge, I observed a significant increase in MA-altered Crs after Kp-10 treatment in female mice ($P<0.05$, Figure 45H), while no significant changes in male and OVX mice (Figure45G&I).
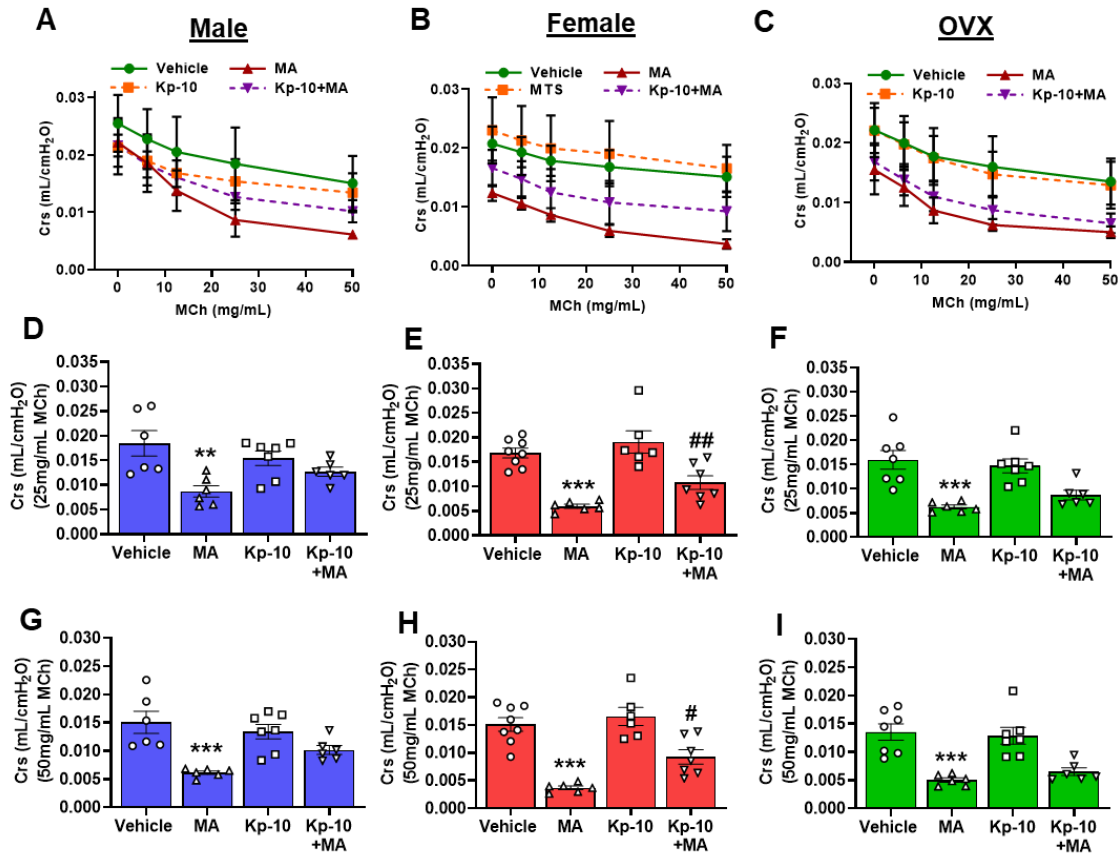
Figure 45: Effect of Kp-10 on airway compliance (Crs) in a MA model of asthma in male (A), female (B), OVX (C) mice.

Crs at increasing MCh log dose concentrations from 0-50 mg/mL were measured in anesthetized animals using a flexiVent system (A-C). Crs at Mch concentrations (25 and 50 mg/mL) were used to compare the effectiveness of the different treatment groups of male (D and G), female (E and H), and OVX (F and I) mice. Male, female and OVX mice challenged with MA showed a significant reduction in Crs compared to the baseline value (PBS challenge). This effect of MA was alleviated in mice treated with Kp-10 in all three study populations. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least six to seven mice per group; $**P<0.01$, $***P<0.001$ vs. respective vehicle group; $\#P<0.05$, $\#\#P<0.01$ vs. respective MA challenged group.

4.3.4. Effect of Kp-10 on Airway Elastance

The airway elastance (Ers) was measured after the exposure of linear doses of MCh in the respective treatment groups. I noticed a significant increase in Ers in male, female, and OVX MA exposed mice with increasing doses of MCh compared with PBS (Figure 46A-C). Kp-10 alone treated mice from all three study populations did not show any significant changes in the

Ers. However, MA-challenged mice treated with Kp-10 showed significant reduction in MA-induced Ers in male (P<0.05), female (P<0.001) and OVX (P<0.05) mice at 25 mg/mL of MCh (Figure 46D-F). Whereas, at the higher dose of MCh (50 mg/mL), Kp-10 showed higher effect in the reducing MA-induced Ers in male and female (P<0.001) mice, with lesser changes in OVX (P<0.05) mice (Figure 46G-I).
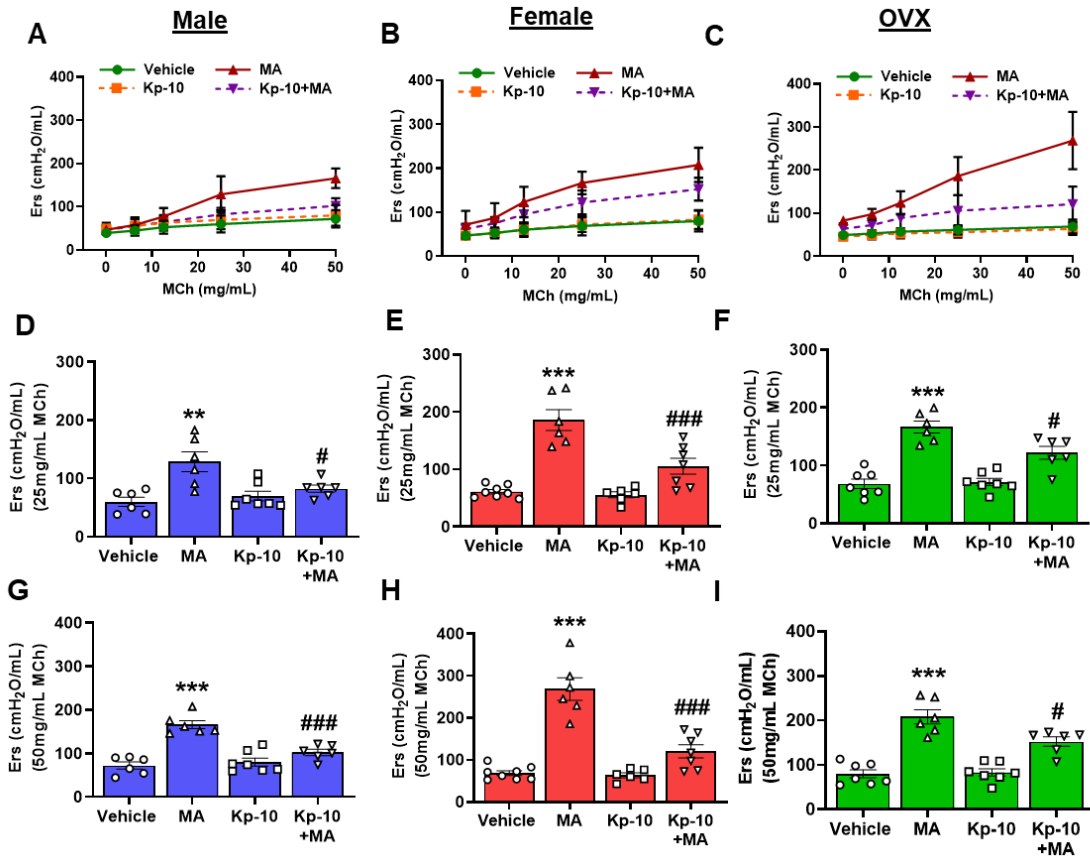
Figure 46: Effect of Kp-10 on respiratory elastance (Ers) in a MA model of asthma in male (A), female (B), OVX (C) mice.
The inhaled response of methacholine (0-50 mg/mL) was measured in anesthetized animals using flexiVent system (A-C). Ers between the different treatment groups of male (D and G), female (E and H) and OVX (F and I) mice were compared at the concentration of 25 and 50 mg/mL of MCh were to determine the effectiveness of Kp-10. All MA challenged mice showed a significant increase in Ers compared to baseline value (PBS challenge) whereas the effect of MA was alleviated in mice treated with Kp-10 in all three study populations. Data analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least six to seven mice per group; **P<0.01, ***P<0.001 vs. respective PBX of male/female/OVX; #P<0.05, ###P<0.001 vs. respective MA of male/female/OVX.

4.3.5. Effect of Kp-10 on the Cytokine Milieu in BALF

Activation of Th2 cells due to allergen exposure leads to the production of inflammatory cytokines, which subsequently contribute to AHR. To correlate the inflammatory milieu with the observed lung mechanics, I measured the cytokines levels in the BALF from respective treatment groups from the lungs of male, female and OVX mice. MA-challenged mice showed a significant increase in the cytokine levels in all three study populations, as evidenced by increased levels of IL-1α ($P<0.001$ for male and female, $P<0.01$ for OVX; Figure 47A), IL-1β ($P<0.001$, for all three study populations; Figure 47B), IL-2 ($P<0.001$ for male and female, $P<0.05$ for OVX; Figure 47C), IL-4 ($P<0.001$ for all three study populations; Figure 47D), IL-5 ($P<0.001$ for all three study populations; Figure 47E), IL-6 ($P<0.001$ for all three study populations; Figure 47F), KC ($P<0.001$ for all three study populations; Figure 47G), and TNF-α ($P<0.001$ for all three study populations; Figure 47H). Furthermore, mice treated with Kp-10 showed a significant suppression of MA-induced increase in cytokine levels for IL-1α ($P<0.05$ for male and OVX, $P<0.01$ for female; Figure 47A), IL-1β ($P<0.05$ for male, $P<0.001$ for female, $P<0.01$ for OVX; Figure 47B), IL-2 ($P<0.01$ for male, $P<0.001$ for female, $P<0.05$ for OVX; Figure 47C), IL-4 ($P<0.01$ for male and female, $P<0.001$ for OVX; Figure 47D), IL-5 ($P<0.001$ for male and female, $P<0.01$ for OVX; Figure 47E), IL-6 ($P<0.01$ for male, $P<0.001$ for female and OVX; Figure 47F), KC ($P<0.001$ for female; Figure 47G), and TNF-α ($P<0.01$ for male, $P<0.001$ for female and OVX; Figure 47H). Notably, Kp-10 treatment alone did not show any significant changes in the different cytokines measured in the BAL fluid compared with PBS treated groups. Overall, these data demonstrate that MA-challenge significantly altered the BAL fluid cytokine levels in all three study populations. Here, Kp-10 treatment significantly ablated MA-induced increase in cytokine levels in all three study populations, with female mice

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tending to show a better effect of Kp-10 in reducing MA-induced upregulation in cytokine

levels.
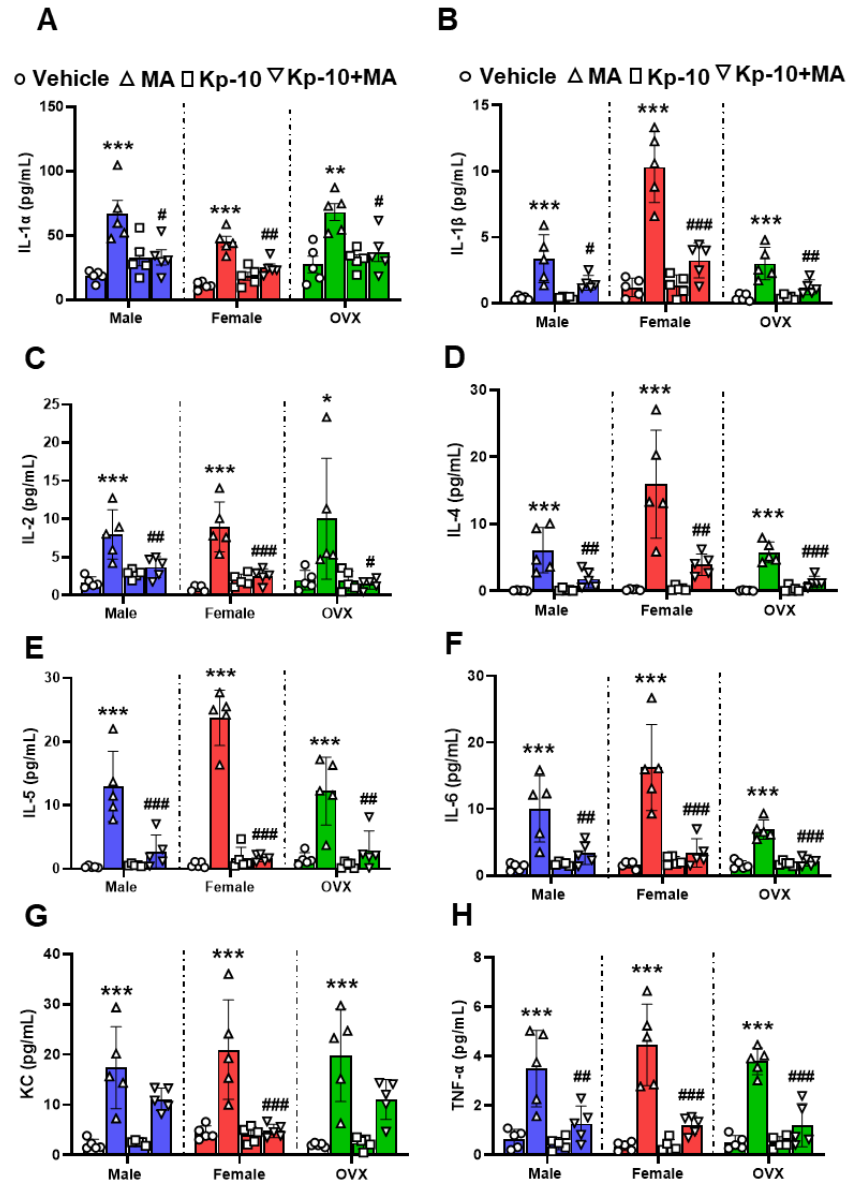
Figure 47: Effect of Kp-10 on cytokine levels in bronchoalveolar lavage (BAL) fluids.
MA-challenge showed an increase in IL-1α (A), IL-1β (B), IL-2 (C), IL-4 (D), IL-5 (E), IL-6 (F), KC (G) and TNF-α (H) levels in a BAL fluid collected from all three study populations compared to respective vehicle (PBS challenged mice). This effect of MA was found to be Th2 biased. Kp-10 via KISS1R activation resulted in decrease in MA induced Th2 cytokines in all three study populations (male, female and OVX). Data analyzed using two-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least five mice per group; *P<0.05, **P<0.01, ***P<0.001 vs. respective vehicle group; #P<0.05, ##P<0.01, ###P<0.001 vs. respective MA challenged mice.

4.3.6. Effect of Kp-10 on Airway Structure and Morphology

To integrate the observed changes in the lung mechanics and cytokine milieu with airway structural changes, I have performed histological analysis using hematoxylin and eosin (H&E; Figure 48), periodic acid-Schiff (PAS; Figure 49), and Masson's Trichrome stains (Figure 50). H&E-stained sections showed a significant increase in inflammatory cells recruitment in the airways of MA-challenged male ($P<0.001$), female ($P<0.001$), and OVX ($P<0.001$) mice compared with respective PBS challenged mice (Figure 48). Mice with MA-challenge and treated with Kp-10, showed a significant reduction in the recruitment of inflammatory cell ($P<0.01$ for male, female and OVX) compared to MA treated group.
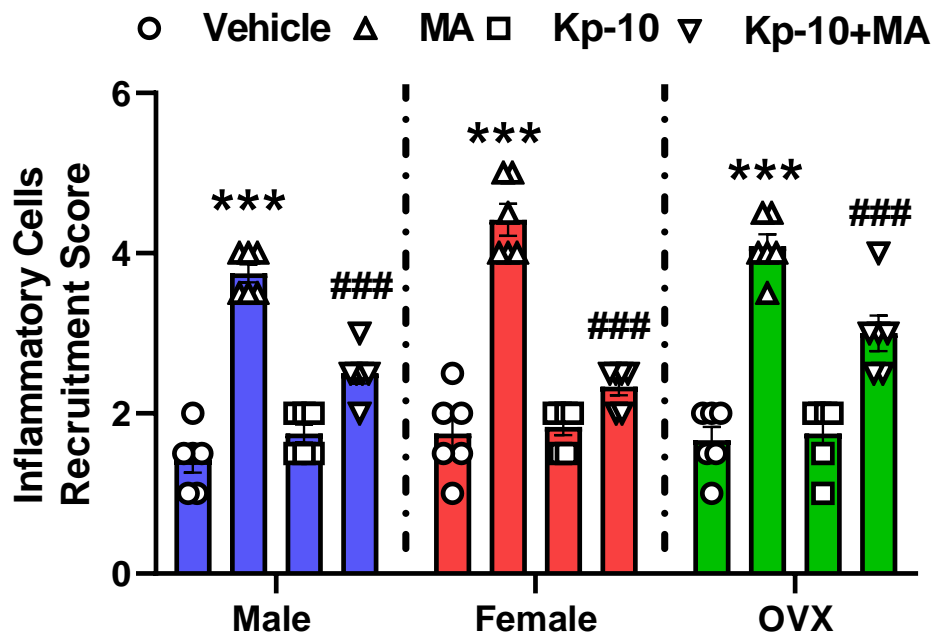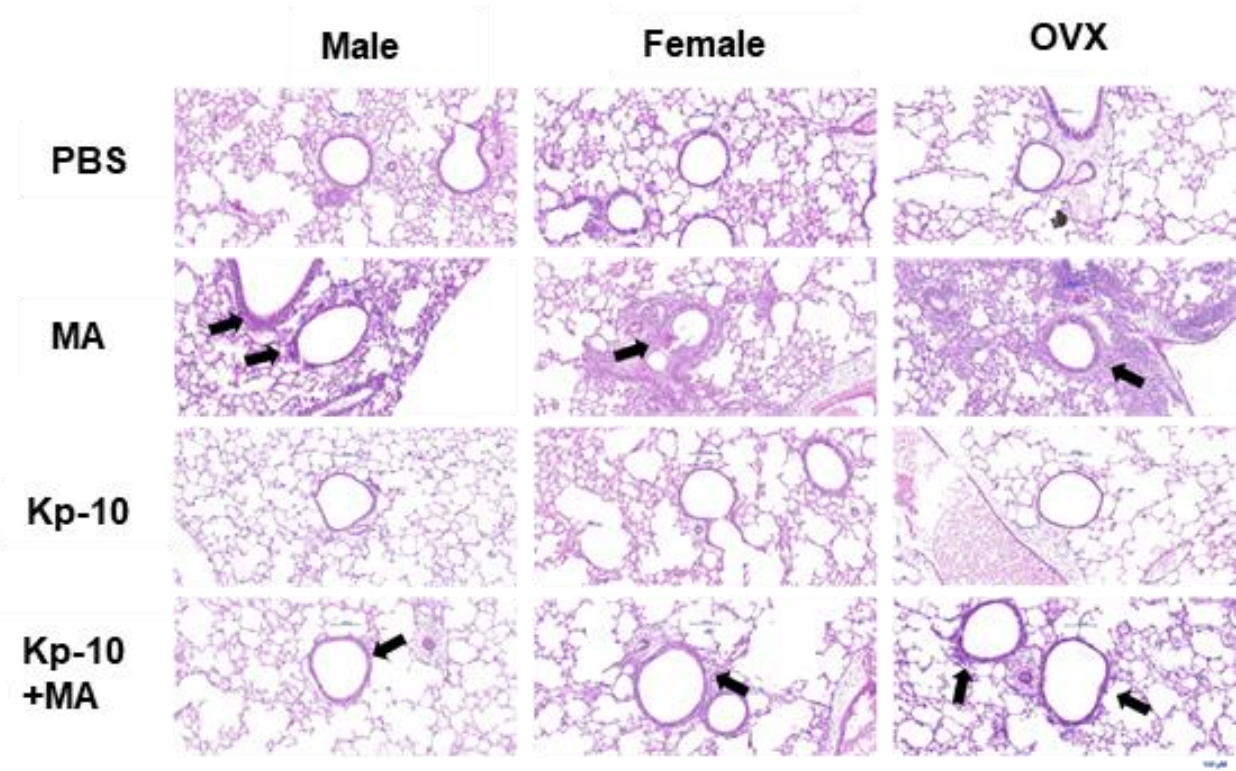
Figure 48: Airway histology and histology scoring using H&E staining.
Data presented as representative images for at least six to seven mice per group; scale bars: 50 μm. Data analyzed using two-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least six mice per group; ***$P<0.001$ vs. respective vehicle group; ###$P<0.001$ vs. respective MA challenged group.

In addition, PAS-stained sections revealed increased epithelial thickening and goblet cell hyperplasia in the airway lining of MA-challenged mice (P<0.01 for male, female, and OVX) compared with respective PBS-challenged mice (Figure 49). Notably, MA-induced epithelia thickening, and goblet cell hyperplasia was reduced upon Kp-10 treatment in mice from all three study populations (P<0.05 for male, P<0.001 for female and OVX).
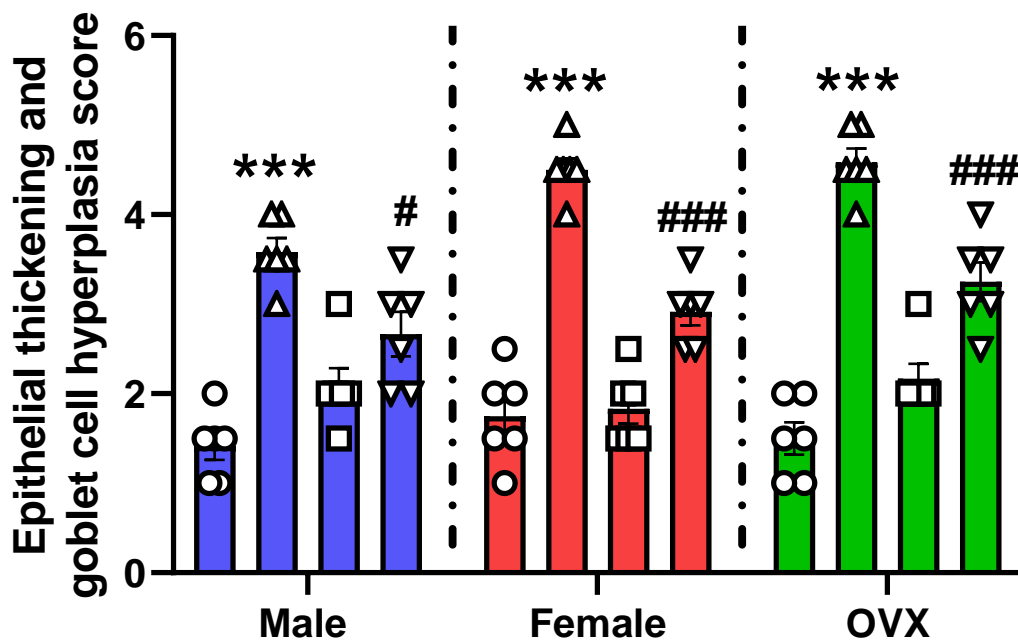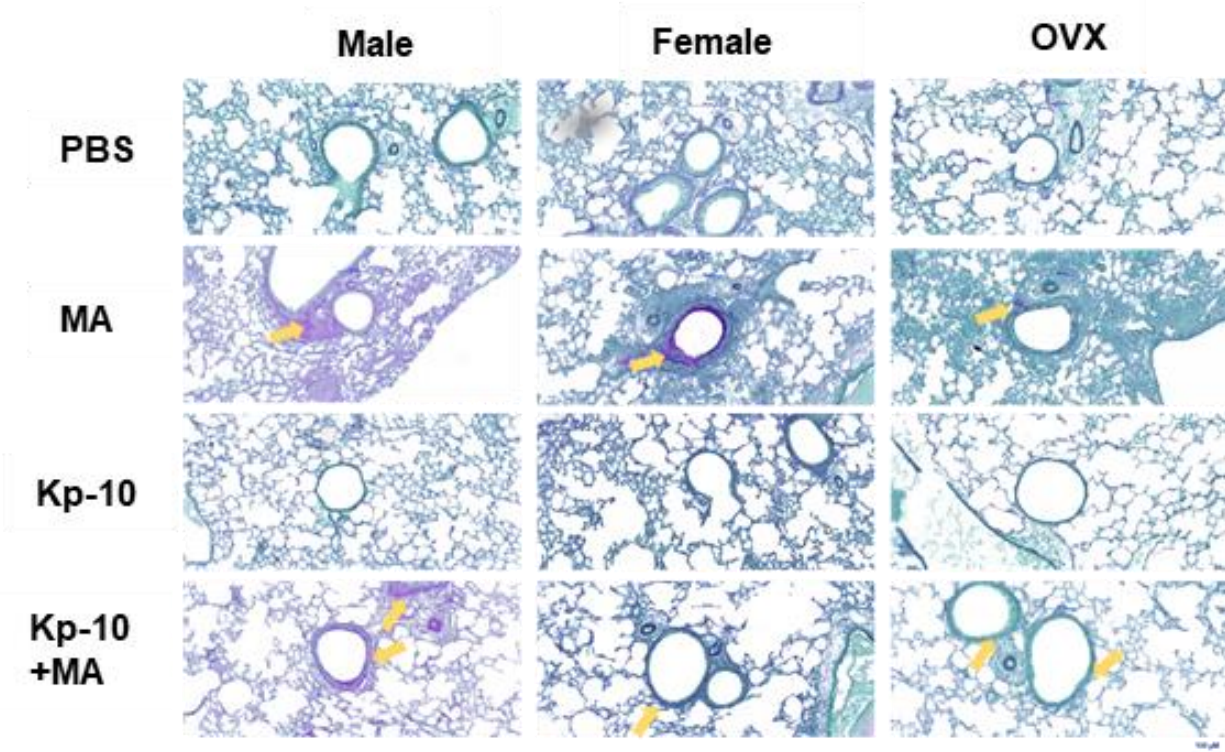
Figure 49: Airway histology and histology scoring using PAS staining.
Data presented as representative images for at least six to seven mice per group; scale bars: 50 μm. Data analyzed using two-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least six mice per group; ***$P<0.001$ vs. respective vehicle group; #$P<0.05$, ###$P<0.001$ vs. respective MA challenged group.

Furthermore, Masson's Trichrome stained sections showed increased collagen deposition around the airways in MA-challenged mice (P<0.01 for male, female and OVX) compared with respective PBS-challenged mice (Figure 50). Here, MA-challenged mice treated with Kp-10 showed reduction in the collagen deposition around the airways in the lung sections of all the study populations (P<0.01 for male, female and OVX).
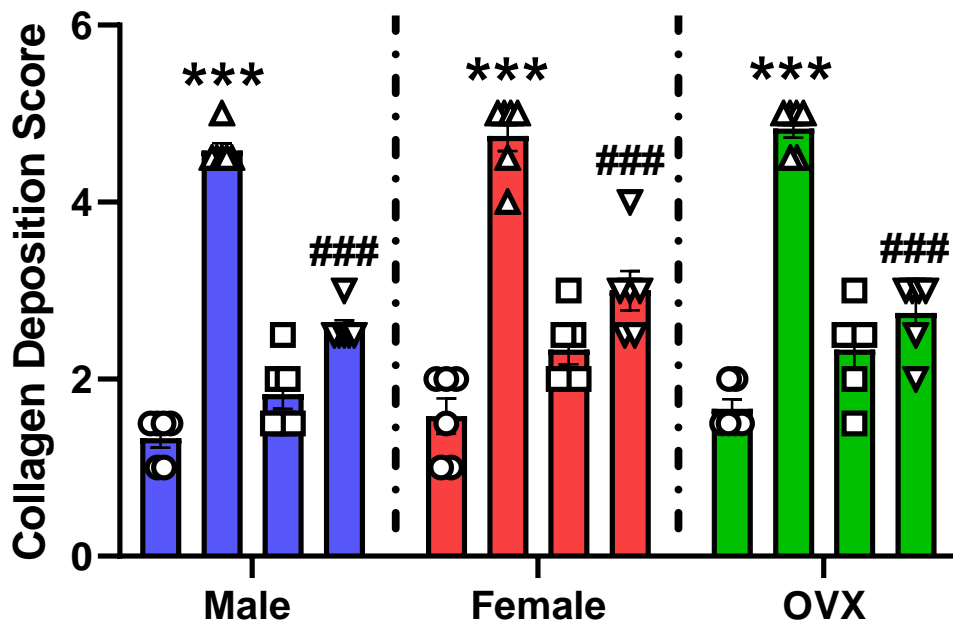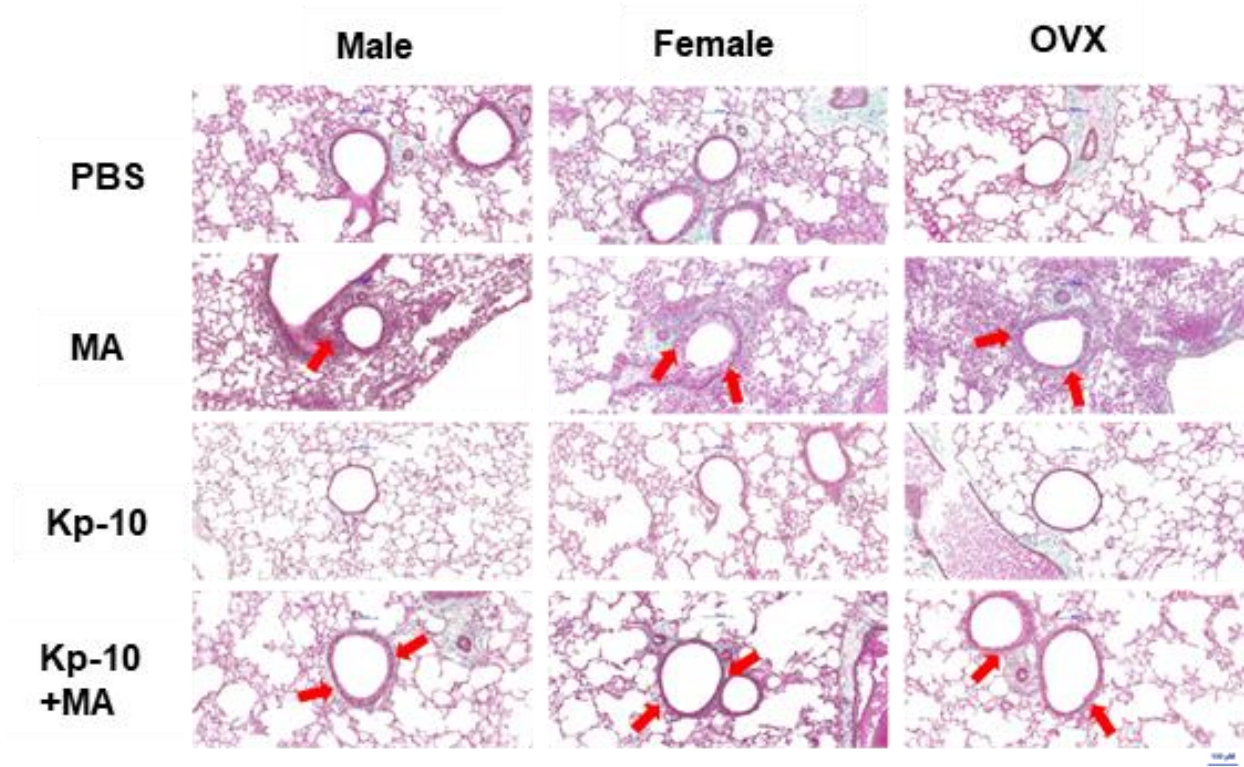
Figure 50: Airway histology and histology scoring using Masson's Trichrome staining.
Data presented as representative images for at least six to seven mice per group; scale bars: 50 μm. Data analyzed using two-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least six mice per group; ***$P<0.001$ vs. respective vehicle group; ###$P<0.001$ vs. respective MA challenged group.

4.3.7. Effect of Kp-10 on α-Smooth Muscle Actin (α-SMA) and Vimentin in the Lung

α-SMA and vimentin are often considered characteristic markers for smooth muscle remodeling and fibrosis respectively during the phenotype changes in the lungs. Western analysis using lung homogenate from MA-challenged mice showed a significant increase in the expression of α-SMA ($P<0.001$ for male and OVX, $P<0.05$ for female; Figure 51) and vimentin ($P<0.001$ for male and OVX, $P<0.05$ for female; Figure 52) expression in all three study populations compared to respective PBS-challenged mice. There were no significant changes in α-SMA and vimentin protein expression with Kp-10 alone exposure. Interestingly, MA-challenged mice treated with Kp-10 showed significant downregulation of α-SMA ($P<0.01$ for male and OVX; $P<0.05$ for female).
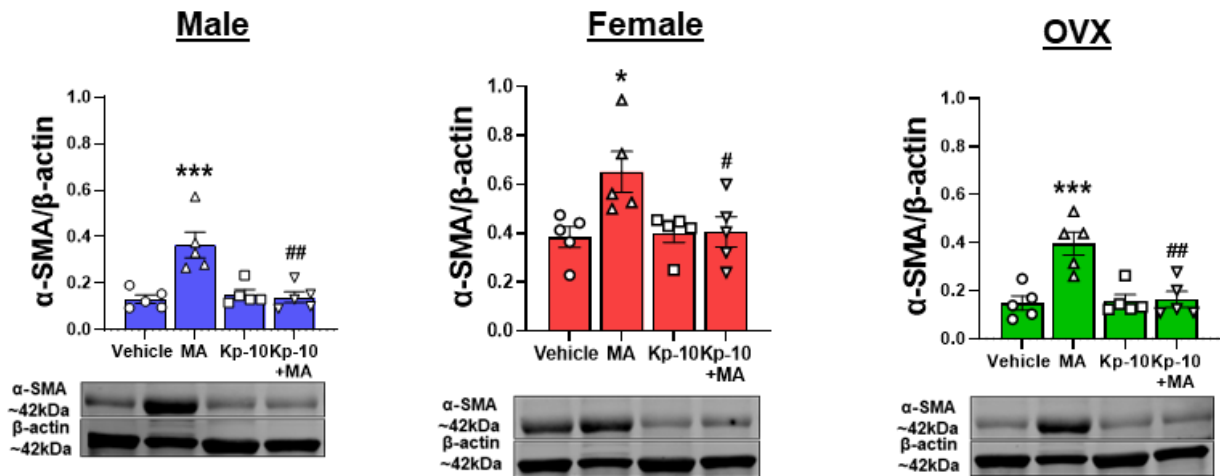
Figure 51: Effect of Kp-10 on α-smooth muscle actin (α-SMA) in the lung.
The expression of α-SMA was higher in MA challenged mice, and this effect was reversed by Kp-10 exposure in male, female and OVX mice lung homogenate. Data analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least five mice per group; *$P<0.05$, ***$P<0.001$ vs. respective vehicle; #$P<0.05$, ##$P<0.01$ vs. respective MA challenged group.
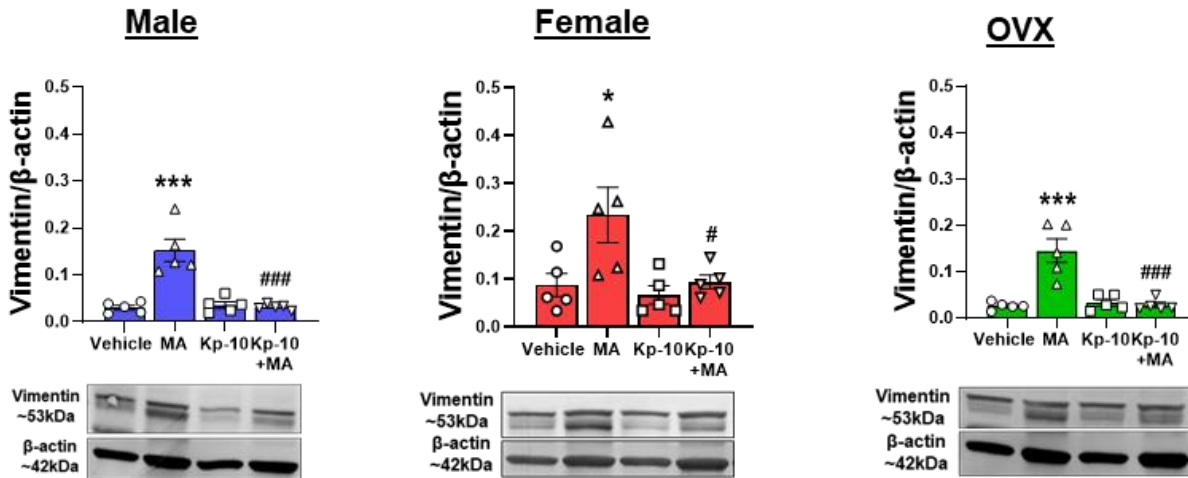
Figure 52: Effect of Kp-10 on vimentin in the lung.
The expression of vimentin was higher in MA challenged mice, and this effect was reversed by Kp-10 exposure in male, female and OVX mice lung homogenate. Data analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least five mice per group; *$P<0.05$, ***$P<0.001$ vs. respective vehicle; #$P<0.05$, ###$P<0.001$, vs. respective MA challenged group.

4.3.8. Effect of Kp-10 on Soluble Collagen Content in Whole Lung Tissues

The accumulation of collagen in the lungs of different treatment groups were determined using Sircol Soluble Collagen Assay (Figure 53). In MA-challenged mice, I observed a significant increase in the soluble collagen content in the lungs of male ($P<0.01$), female ($P<0.001$) and, OVX ($P<0.001$) mice compared with respective PBS-challenged mice. Kp-10 alone treatment showed no significant changes in soluble collagen content compared with the respective PBS-challenged group across. MA-challenged mice treated with Kp-10 significantly reduced the soluble collagen content ($P<0.05$ for male, $P<0.001$ for female and $P<0.01$ for OVX) compared to respective MA-treated groups.

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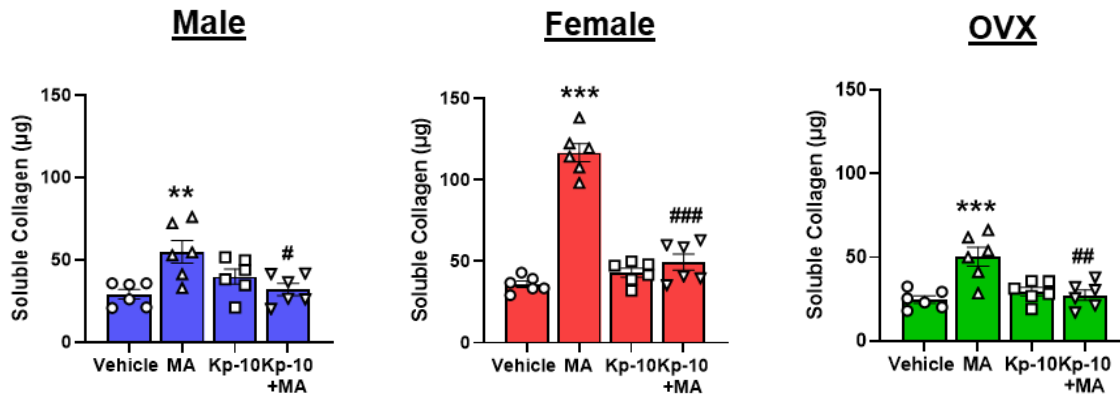
Figure 53: Effect of Kp-10 on soluble collagen levels in murine male, female and OVX lungs. The increase in soluble collagen levels were significantly reduced in mice treated with Kp-10 in the presence of MA. Data analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data represented as min to max showing all points of at least five mice per group; *p<0.05, **P<0.01, ***P<0.001 vs. respective vehicle; #P<0.05, ##P<0.01, ###P<0.001 vs. respective MA challenged group.

4.4. Discussion

Asthma is a heterogeneous disorder of airways mainly associated with chronic airway inflammation, lowered lung functions, tissue remodeling, and AHR[53, 62, 72]. Several studies have performed in exploring the causative factors and the underlying mechanisms responsible for airway inflammation[57, 58, 82]. In contrast, limited studies focused on airway remodeling associated with ASM hypertrophy, which results in chronic airway obstructive changes. Here, ASM is very crucial as considering its role in controlling the secretion of inflammatory cytokines, extracellular matrix (ECM)/remodeling and other growth mediators[83, 84].

In the previous aims, I have investigated the role of Kp via activation of its receptor, KISS1R in regulating ASM proliferation and observed that cleaved forms of Kp, mainly Kp-10, alleviated PDGF-induced ASM proliferation by regulating the p38 mitogen-associated protein kinases (MAPK) signaling pathways. Overall, providing the first evidence of Kp/KISS1R signaling playing an important role in airway remodeling. Thus, paved the foundation for my current *in vivo* study by utilizing a MA-induced murine model of asthma. Kp is a family of

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neuropeptides formed by proteolysis of 145 amino acid precursor peptides encoded by the KISS1 gene and it is expressed in the hypothalamus[85-87], gonads[88-92], placenta[93], liver[88, 94] and heart[95]. Numerous studies using other cell systems delineated the anti-metastatic and tumor-suppressant role of Kp/KISS1R signaling pathways by inhibiting NF-kB and MAPK signaling pathways.

In the current study, I specifically chose the C57BL/6J mouse strain because of its genetic integrity with human and it is widely used mouse model to investigate the pathophysiology of asthma. Further, I used the MA-induced murine model of asthma, which is a robust and effective asthma model in animals that resembles Th2-biased eosinophilic inflammation, airway remodeling, and similar pathological manifestations as observed in human asthma. Data from my previous studies have reported that 17β-estradiol (E_2) regulates Kp/KISS1R signaling through E_2-dependent transcriptional activation of the K1SS1 gene, which controls the KISS1 gene expression[32]. Estrogen has been shown to mediate a decrease in kisspeptin in the arcuate nuclear region of the hypothalamus, while increase kisspeptin expression in the atrioventricular region in the brain. This inhibitory effect of estrogen on KISS1 expression is mediated via ERα. ERα further binds to the estrogen response elements present in the KISS1/KISS1R genes to control gene expression. This finding suggests that estrogen causes negative feedback action on various CNS stimuli, especially kisspeptin. Additionally, other studies show that KISS1 KO rats showed undetectable levels of LH and FSH. Moreover, KISS1 KO rats also lacked estrogen-induced LH surge. Therefore, to isolate the effect of Kp/KISS1R signaling from endogenous E_2, I used OVX mice in our study.

I observed reduced Kp and KISS1R expression in lung tissue homogenates from all MA-challenged mice, implying the altered or impaired protective Kp/KISS1R signaling in the lungs

during asthmatic conditions. This aligns well with my previous *in vitro* studies, where I observed decreased expression of Kp and KISS1R in asthmatic ASM cells. Lung mechanics studies on the flexiVent indicated a higher Rrs and Ers, with reduced Crs in MA-challenged mice from all three study populations, mimicking the experimental animal model of asthma as shown previously. Interestingly, with a reduced lung KISS1R expression observed in the asthmatic condition, exogenous Kp-10 exposure mitigated MA-induced AHR, emphasizing the crucial role of Kp-10 in lung pathophysiology. Interestingly, here I observed the greater Kp-10 effect in mitigating MA-induced asthma in females compared to males or OVX mice. This may be due to the involvement and/or activation of other estrogen related intrinsic mechanisms by Kp/KISS1R downstream signaling, which warrants further investigation.

Multiple evidence suggests that during severe lung inflammation, activation of numerous inflammatory/structural cells occurs, and each secretes cytokines or chemokines to modulate the lung structure and function[6, 96]. These effects may be due to release of inflammatory cytokines from different effector T cells such as Th1/Th2 and Th17. As shown, I measured the inflammatory cytokines in BALF fluid from the MA-challenged mice with or without Kp-10 treatment. As expected, I observed upregulated levels of inflammatory cytokines such as IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, TNF-α, and KC in asthmatic mice. Here, female mice elicited higher Th2 cytokines levels, whereas no change in Th1 cytokine levels compared to MA-challenged male and OVX mice, might be due to the differential regulation of Th1/Th2 cytokines production by estrogens, which are consistent with previously reported studies. Interestingly, the decreased expression of Th2 cytokine levels from allergic male mice signifies the predominant role of androgens in the suppression of Th2 dominant inflammatory responses. The cytokine secretion levels of MA-challenged OVX mice tend to be lower compared to other groups,

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possibly due to the impact of the absence of female sex hormones over the immune system in OVX mice led to an impairment of effector T cell activation. Moreover, I noticed that in all MA-challenged groups there is upregulation of IL-1α and IL-1β levels, which are the predominant IL-1 cytokines that activate the key transcription factors related to inflammatory immune responses. It has been reported that IL-6 is a multifunctional cytokine, a prominent indicator of ongoing inflammation in the airways, and an important regulator of effector CD4+ T cells fate. In the cytokine study, I observed that MA-challenged mice induced significantly higher IL-6 expression and KC, which are clinically relevant and suggest that both cause lung injury by increasing the infiltration of neutrophils. Kp-10 treated mice showed suppression of these notable pro-inflammatory cytokines in all the study populations, suggesting the significant role of Kp/KISS1R signaling in regulating the inflammatory milieu in the lungs. Furthermore, I observed a more pronounced effect of Kp-10 in alleviating the MA-induced inflammatory response in the presence of endogenous estrogen (in female mice), suggesting estrogen signaling is required in combination with exogenous Kp-10 to enhance the protective mechanism to regulate the inflammatory milieu.

The prolonged exposure to allergens increases the inflammatory responses in the airways that cause airway structural changes associated with increased collagen deposition, immune cell recruitments, and airway thickening. Considering the importance of inflammatory cytokines in regulating airway remodeling and AHR, I observed a strong correlation between increased cytokines and histology changes like ASM and epithelial thickening, collagen deposition, and goblet cell hyperplasia in MA-challenged mice. In the histology studies, I observed that MA-challenged mice showed a significant deterioration in the airway structures, which align with the concept of AHR and remodeling. Collagen is one of the primary structural components of airway

ECM important for regeneration and repair. The higher accumulation of collagen in the airway led to an increase in the stiffness of the airways that drive excessive airway constriction. In my data, I observed elevated soluble collagen levels in MA-challenged mice, which correlate with airway remodeling and AHR. However, this effect was ablated with Kp-10 treatment, suggesting that Kp-10's anti-remodeling effects via inhibition of collagen in the lungs, corroborated with Kp's anti-fibrotic activity in bleomycin-induced pulmonary fibrosis mouse model. Vimentin, a molecular marker for fibrosis and α-SMA, which is a smooth muscle-specific marker were also found to be increased in asthmatic lung, signifying the airway structural changes during remodeling in the airways. These changes were reversed upon Kp-10 treatment in MA-challenged mice, suggesting that Kp-10 has a promising potential in regulating remodeling-associated changes in the airways.

To summarize, the results of the present study show that MA significantly altered lung mechanics, thereby resulting in AHR and airway remodeling. The administration of Kp-10 alleviated these MA-induced airway inflammation and remodeling, thereby AHR. Furthermore, I observed the protective role of Kp/KISS1R signaling in asthmatic OVX mice, suggesting an estrogen-independent effect of Kp/KISS1R signaling[97, 98]. All these novel findings suggest a crucial protective role for Kp/KISS1R activation in regulating AHR and airway remodeling.

5. CONCLUSIONS, CLINICAL RELEVANCE, LIMITATIONS AND FUTURE SCOPE

5.1. Conclusions

How sex steroids lead to gender and sex differences is relevant to more than just normal cellular biology and physiology. Sex steroids have fascinating roles that go far beyond the anatomical and physiological realms. The paraphrase "Sex matters to every cell of the body" emphasizes the critical role of sex steroid signaling in the structure and function of lungs at different life stages. Even though steroidogenesis is similar across both sexes, the subsequent molecular pathways each of the sex steroids undertakes vary between males and females, leading to complex and scientifically unexplained actions and interactions with the lungs. Sex steroids typically regulate cellular processes by genomic/slow and nongenomic/rapid actions via nuclear and membrane-associated receptors, respectively. The classic sex steroid receptors—namely, ER (α or β), PR (A or B), and AR—are all nuclear receptors. Depending on their similarities and differences, crosstalk between these receptors can occur at multiple levels. For example, there can be inter-receptor communication, as well as downstream activation. Although the exact mechanism by which sex steroids exert effects through these divergent receptor-mediated actions is not yet fully characterized, it is evident that all the major cell types in the lungs are affected by these sex steroids. Moreover, the presence of tissue-specific metabolism and conversion in the lungs means that the qualitative effects of these sex steroids are radically different from the observed multifactorial effects. Overall, the importance of sex steroids in the lungs and their clinical implications are clearly established, yet not explored and entirely understood mechanistically in asthma.

What is known, however, is that the effects of these sex steroids are, as mentioned previously, time-, context-, dose-, and site-specific. From a molecular perspective, understanding

the numerous pathways begun orchestrated independently or dependently by these sex steroids and their interactions is just the beginning of trying to understand this complex panoply of effects. As much as it is important to understand the individual contributions of sex steroids in the pathogenesis of lung diseases, it is equally important to emphasize that organisms and individuals are integrated entities fulfilling their specific functions. Therefore, even though it is important to classify the role of each sex steroid, the need of the hour is to be able to identify, as well as implement, the major players that cause the overall sex differences in various lung diseases. As more and more experiments are conducted on males and females, underlying sex differences in the etiology of lung diseases are being highlighted, which will enable scientists to untangle alterations and develop specific therapeutic strategies against asthma.

Kisspeptin and its analogues are being evaluated in several clinical trials with regards to endocrine, metabolic and reproductive disorders[99, 100]. Although several groups have postulated theories on kisspeptin signaling via its G-protein coupled receptor, KISS1R, including our groups, still a lot seems to be left in investigating Kp/KISS1R in the lung, particularly in asthma. Moreover, it is well known that the nonadrenergic, noncholinergic (NANC) nerves modulate airway tone via action of several neurotransmitters and peptide activity. The NANC nerves innervate the ASM cells causing associated airway structural changes, characteristic of asthma. It remains to be known whether Kp/KISS1R are expressed on these airway neurons, thereby regulating airway tone. Furthermore, the contribution of NANC nerves in asthma pathophysiology has not been well reported and future studies are required to define Kp/KISS1R neuronal modulation.

The results from the present study demonstrate for the first time the presence of Kp and KISS1R in the human and murine airway smooth muscle cell. Immunofluorescence imaging

shows that Kp and KISS1R are expressed in the human airway smooth muscle bundles and localized in the airway smooth muscle cell. Moreover, expression studies further demonstrated that both Kp and KISS1R are expressed in a sex-differentiated manner with males exhibiting significantly higher expression of both Kp and KISS1R as compared to females, suggesting the lack of a protective ability of kisspeptins in females, thereby resulting in increased asthma in women.

Further, proliferation studies to determine the anti-proliferative activity of cleaved fragments of kisspeptins revealed Kp-10 as the most effective in inhibiting PDGF-induced ASM cell proliferation. Moreover, I evaluated Kp-10's anti-proliferative effect in the presence as well as absence of PDGF in non-asthmatic and asthmatic ASM cells. Activation of KISS1R mitigated PDGF-induced ASM cell proliferation in negatively transduced as well as KISS1R shRNA transduced ASM cells, belonging to both non-asthmatic and asthmatic individuals. Furthermore, KISS1R activation also inhibits phosphorylation and subsequent activation of key signaling molecules related to cell function. My ECM studies further reconfirmed Kp's anti-remodeling capacity in presence of PDGF and TGFβ.

Moreover, my *in vivo* studies shed more light on the effect of chronic kisspeptin administration in a mixed-allergen induced mouse model of asthma. The results from my in vivo studies provide evidence that kisspeptins may be a potential therapeutic option against asthma. The in vivo study demonstrates that chronic kisspeptin administration regulated lung mechanics and subsequent inflammatory pathways, thereby mitigating AHR and airway remodeling. The observations from these functional studies provide novel mechanistic details regarding Kp/KISS1R activation, which can be utilized in the future as a potential therapeutic strategy.

5.2. Clinical Relevance

We are now entering an era of individualized medicine, which requires a clear understanding of our therapeutic interventions to account for the large, heterogeneous patient populations widely seen in asthma. Moreover, we need to consider several factors affecting the patient population such as gender, comorbidities, lifestyle habits as well as influence of endogenous sex steroids pertaining to sex of an individual. The present study not only generates an alternative medication for asthma, but also answers the relevant questions surrounding the many discrepancies around the sex-skewed occurrence of asthma. Another novel feature of the present study is the high impact that it would deliver in the future research options for kisspeptins in airway biology and pathologies. The discovery of kisspeptins and its receptor in the airway smooth muscle cell opens multiple new avenues for future research and therapeutics in the field of pulmonary biology. The presence of several comorbidities such as obesity can also be investigated in concurrence with kisspeptins in asthma. Moreover, it will also help explore the protective and anti-inflammatory, anti-proliferative capacities of kisspeptins in other disease conditions and help researchers to devise kisspeptin-based therapies.

5.3. Limitations

This novel study, despite advantages, also comes with its own share of limitations. The observations of a differential expression of Kp and KISS1R in males versus females although measured in post-pubertal individuals, should also be explored in an age spectrum to determine the significance of kisspeptins as an anti-asthmatic therapy across the lifespan. It would be interesting to study kisspeptins based on analyzing pubertal conditions, age and other associated factors such as menopausal and pregnancy states. Similarly, kisspeptin also has transcriptional regulatory effects which should be evaluated in the future. Moreover, sex steroid signaling is

intercalated with kisspeptin based on my observations and as observed by several other research groups in other organ systems, however, more insight is required on this feedback mechanism in the lung. Additionally, the downstream signaling mechanism of KISS1R activation is still unknown in the context of the lung, therefore further studies exploring the KISS1R -GPCR activation is warranted to shed more insight on Kp/KISS1R signaling mechanism.

Although not the primary focus of my study, the baseline differences in Kp and KISS1R between males versus females should be investigated further with regards to differential activation of the KISS1R, which may provide answers to the significant sex-skewed occurrence observed in asthma.

5.4. Future Scope

My observations provide a strong foundation to further investigate kisspeptins and the other endogenous sex steroids, namely, estrogen and testosterone crosstalk in asthma pathophysiology[101]. My preliminary studies suggested a negative impact of estrogen on Kp/KISS1R expression. On the other hand, testosterone upregulated Kp/KISS1R expression in the ASM. These sets of data provide key and novel details on the impact of sex steroids-kisspeptin crosstalk in the ASM. This may also explain the increased vulnerability of females to suffer from asthma. However, this observation warrants more investigation to better understand estrogen-Kp and testosterone-Kp crosstalk.

Moreover, kisspeptins in asthma can further be explored with relation to existence of other effectors, such as obesity. Originally heralded as an anti-obesity hormone, leptin has emerged as a marker for nutritional and metabolic status of an individual[102, 103]. Indeed, experimental work gathered in the past decade has proven the prominent function of leptin in signaling the magnitude of energy (fat) reserves to reproductive centers, thus serving as an

indispensable role for precocious puberty onset[75]. Puberty is well established to depend on satiable energy stores and fat reserve. This raises a possibility of leptin-kisspeptin crosstalk being fundamental drivers of the reproductive cascade. Here, one can easily imagine that such manipulations can be used to create exciting therapeutic possibilities. Likewise, studies have shown that absence of leptin or leptin resistance via a leptin receptor mutation leads to a phenotype of IHH and obesity[104]. Accordingly, the major question to be asked is whether leptin-induced obesity is related to the regulatory mechanisms of kisspeptins, which is yet to be determined.

My observations further indicate a strong anti-proliferative and anti-remodeling role of Kp-10. This aspect can be further exploited in other cell types or disease contexts other than asthma to further validate the potential of exogenous Kp-10 or KISS1R activation. I have also performed my studies on the chronic effects of Kp-10, including *in vitro* as well as *in vivo* studies. Future studies to investigate the acute impact of exogenous Kp-10 exposure should also be studies for an overall understanding of Kp/KISS1R signaling. KISS1R being a GPCR opens many avenues for future researchers to investigate the downstream G protein activation cascade. My studies fall short of investigating the KISS1R-based G protein activation, however, if investigated, would prove the mechanistic basis of Kp/KISS1R activation in the ASM. Relevant to GPCR, the aspects of intracellular calcium regulation and contractility aspects can also be explored in connection with kisspeptins.

Fortunately, understanding the mechanisms by which sex steroids influence structural cells of the airways has been garnering new interest with novel findings from researchers working on ASM, epithelial cells and fibroblast in the lungs (5, 46, 198). Sex steroids influence structural cells of the airways and immune cells independently. Therefore, further studies should

be done to answer the following questions: 1) Do sex steroids modulate structural cells of the airway via effects on immune cells? 2) Do direct effects of sex steroids on airway structural cells, in turn, modulate the fate of immune cells? In either question, estrogen, progesterone, and/or testosterone likely directly alter gene regulation via nuclear activation resulting in altered airway reactivity and differences in asthma. Immune cells *vis-à-vis* airway structural cells regulation by sex steroids in the pathology of asthma represents a major deficit in the field. As more and more research take place to digress kisspeptins and its role in pulmonary diseases, it would be interesting to discover kisspeptin signaling in other lung diseases. Future work should provide appropriate immunological, structural, cellular and pharmacological models to encompass the intricate pathobiological networks underlying asthma.

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