

TGF- β Signaling May Play a Role in the Development of Goblet Cell Hyperplasia in a Mouse Model of Allergic Rhinitis

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ABSTRACT

Background: Transforming growth factor- β (TGF- β) levels are elevated in the nasal mucosa in allergic rhinitis. However, because TGF- β is secreted extracellularly in latent complexes, it remains unclear whether the local TGF- β expression actually drives active signaling and affects the pathophysiology of allergic rhinitis. The objective of this study is to investigate whether TGF- β signaling is activated in allergic rhinitis and plays a role in the pathophysiology of allergic rhinitis.

Methods: An ovalbumin (OVA)-sensitized and -nasally challenged mouse model of allergic rhinitis was established and phosphorylation of Smad2 in the nasal mucosa was examined by immunohistochemistry. In addition, the effects of the pharmacological inhibition of endogenous TGF- β signaling on the allergic rhinitis model were histologically examined. Furthermore, phosphorylation of Smad2 in the nasal mucosa samples obtained from patients with allergic rhinitis was also evaluated.

Results: In the mouse model of allergic rhinitis, OVA challenge induced phosphorylation of Smad2 predominantly in epithelial cells in the nasal mucosa. In addition, the administration of an inhibitor of TGF- β type I receptor kinase activity during OVA challenge suppressed goblet cell hyperplasia in the nasal mucosa. Furthermore, phosphorylated Smad2 expression increased in nasal epithelial cells in patients with allergic rhinitis.

Conclusions: These results suggest that TGF- β signaling is activated in epithelial cells in the nasal mucosa in allergic rhinitis and may contribute to the development of goblet cell hyperplasia.

KEY WORDS

allergic rhinitis, epithelial cells, Smad, TGF- β

ABBREVIATIONS

OVA, ovalbumin.

INTRODUCTION

Allergic rhinitis (OMIM #607154) is a common chronic disease of the nasal mucosa. Over 10% of the population in developed countries suffers from allergic rhinitis, which creates societal burdens due to such factors as increased medical expenses and a loss of productivity.^{1,2} Allergic rhinitis is pathologi-

cally characterized by Th2-type allergic inflammation, including eosinophil infiltration, goblet cell hyperplasia, and mast cell accumulation in the nasal mucosa.³

TGF- β is a multifunctional cytokine that regulates cell growth, differentiation, and survival, belonging to a large family of structurally related proteins, known as the TGF- β family, to which also activins and bone morphogenetic proteins (BMPs) belong.⁴ TGF- β fam-

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ily ligands bind to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptor is activated by type II receptor upon ligand binding and transduces signals principally through the Smad family of proteins.⁵ Smad2 and Smad3 are phosphorylated by activated TGF- β and activin type I receptors whereas Smad1, Smad5, and Smad8 are phosphorylated by activated BMP type I receptors.^{5,6} Several small molecule inhibitors of TGF- β type I receptor kinase activity have been recently developed and are considered to be a promising reagent for the treatment of cancer and fibrotic diseases.⁷

In patients with allergic rhinitis, TGF- β protein expression is significantly increased in the epithelial cells in the nasal mucosa.⁸ However, because TGF- β is secreted extracellularly as latent complexes and thus requires activation to mediate its effects,⁹ the actual activity of TGF- β in the nasal mucosa of allergic rhinitis and its roles in the pathophysiology of allergic rhinitis remain uncertain.

In this study, we assessed the activation of TGF- β signaling and its roles in allergic rhinitis using a mouse model of allergic rhinitis and the nasal mucosa specimens derived from patients with allergic rhinitis by the detection of phosphorylation of Smad2 and by the pharmacological inhibition of endogenous TGF- β signaling.

METHODS

MICE

Female 4-6 wks BALB/c mice were purchased from Japan SLC (Tokyo, Japan) and were bred under specific pathogen-free conditions.

ALLERGIC RHINITIS MODEL

An allergic rhinitis model was established as previously described with some modifications.¹⁰ Briefly, the mice were actively immunized i.p. with 10 μ g of ovalbumin (OVA, Sigma Aldrich, St. Louis, MS, USA) in 4 mg of aluminum hydroxide on Day 0 and Day 7. Starting on Day 14, they were challenged intranasally with 100 μ g OVA in 10 μ l PBS twice per day for 1 week (total 14 times/week). The mice were challenged intranasally with PBS in a similar manner for the negative control. For some experiments, HTS 466284 (10 mg/kg) (Calbiochem, San Diego, CA, USA)¹¹ or a control vehicle (DMSO) was intraperitoneally administered every other day, starting on Day 14 until sacrifice. The dosage of HTS466284 (10 mg/kg) was based on previous experiments.¹² The animal experiments were approved by the Institutional Review Board of the University of Yamanashi.

HISTOLOGY

Twelve hours after the final nasal challenge, mice were killed with carbon dioxide. The heads were removed, fixed, and decalcified. Coronal nasal sections

were visualized by staining with hematoxylin and eosin (HE) or Hansel staining (to demonstrate eosinophils), or periodic acid-Schiff (PAS)/hematoxylin (to demonstrate goblet cells).

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To detect phosphorylated Smad1 and Smad2, the coronal nasal sections were deparaffinized and stained with anti-phosphorylated Smad2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-phosphorylated Smad1 antibody¹³ through the use of peroxidase-based VECTASTAIN ABC kits with DAB substrate (Vector Laboratories, Burlingame, CA, USA). Nuclei were counter-stained with hematoxylin. The sections were photographed by digital color-CCD camera (BX50, Olympus, Tokyo, Japan).

QUANTIFICATION OF HISTOLOGICAL EXAMINATION

The number of phosphorylation of Smad2-positive cells in the nasal sections was counted as previously described.¹⁴ Briefly, a minimum of 500 cells in the nasal epithelium was counted in at least 6 high power fields ($\times 400$) in each sample. The percentage of phosphorylated Smad2-positive cells in the total nasal epithelial cells was expressed (%) and the mean percentage was calculated in 6 animals or 4 human samples. The number of infiltrating eosinophils in the nasal mucosa and PAS-positive goblet cells in the nasal mucosa in the posterior portion of nasal septum was determined microscopically in a blinded manner and expressed as the number per high-power field (400 \times). Two or four specimens of the Hansel- or PAS-stained coronal sections from one mouse were selected. The mean score was counted, and then the mean scores were calculated in 6 animals.

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For phosphorylated Smad2 (pSmad2) labeling, the coronal nasal sections were blocked for 10 minutes in 3% H₂O₂, incubated with rabbit anti-pSmad2 antibody (Santa Cruz Biotechnology Inc., 1 : 200 in 1% BSA, 2 hours at room temperature) and then incubated with swine anti-rabbit antibody conjugated to RITC (red) (1 : 20 in PBS, 40min) (DAKO Cytomation, Glostrup, Denmark). The pictures were taken on an Olympus fluorescent microscope (DP30BW, Olympus, Tokyo, Japan).

BIOPSY SAMPLES

Inferior turbinate thin biopsies of 4 seasonal allergic rhinitis patients were obtained using a cup forceps device under local anesthesia. Control biopsy samples were obtained from 4 patients with idiopathic maxillary cyst during the surgical operations. The control patients had no history of allergic diseases at the operations. Informed consent for the described

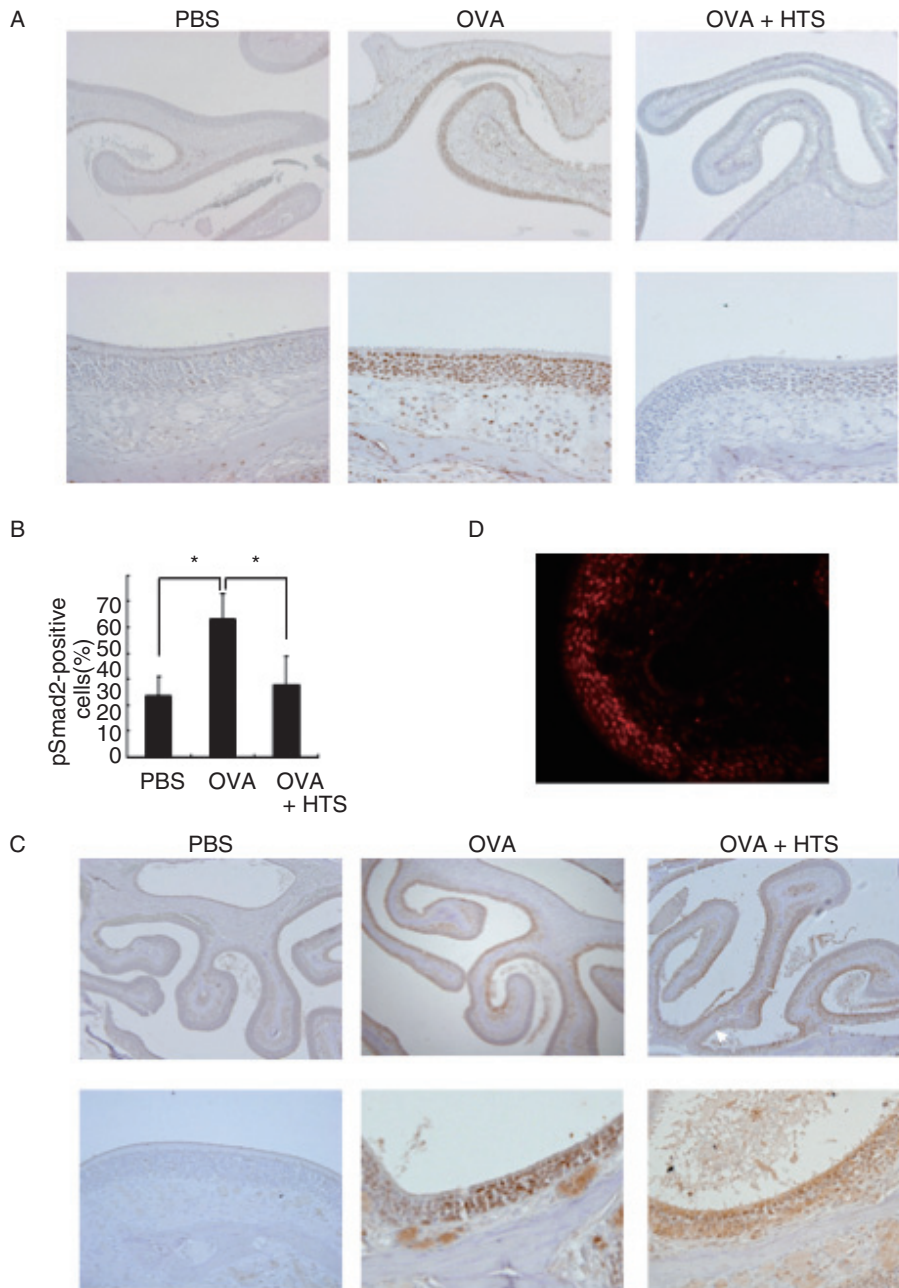


Fig. 1 Phosphorylation of Smad2 was detected predominantly in epithelial cells in the nasal mucosa in a mouse model of allergic rhinitis. The OVA-sensitized mice were intranasally challenged with OVA or PBS. During the OVA challenge, HTS466284 (OVA + HTS) or the control vehicle DMSO (OVA) was administered intraperitoneally every other day. PBS: OVA-sensitized and PBS-challenged control mice. **A-C**. Representative pictures (upper panels: $\times 40$, lower panels: $\times 400$) of immunohistochemical staining with anti-phosphorylated Smad2 antibody (**A**) or anti-phosphorylated Smad1 antibody (**C**) and a quantitative analysis of the epithelial phosphorylated Smad2 expression in the model of allergic rhinitis (**B**). Positive staining indicates as brown. **D**. Immunofluorescent analysis with anti-phosphorylated Smad2 antibody (red). Representative pictures of the nasal mucosa obtained from the OVA-sensitized and -challenged mice treated with control vehicle (DMSO) are shown. Positive staining indicates as red. Values represent the mean \pm SD of 6 mice in each group. * $p < 0.05$.

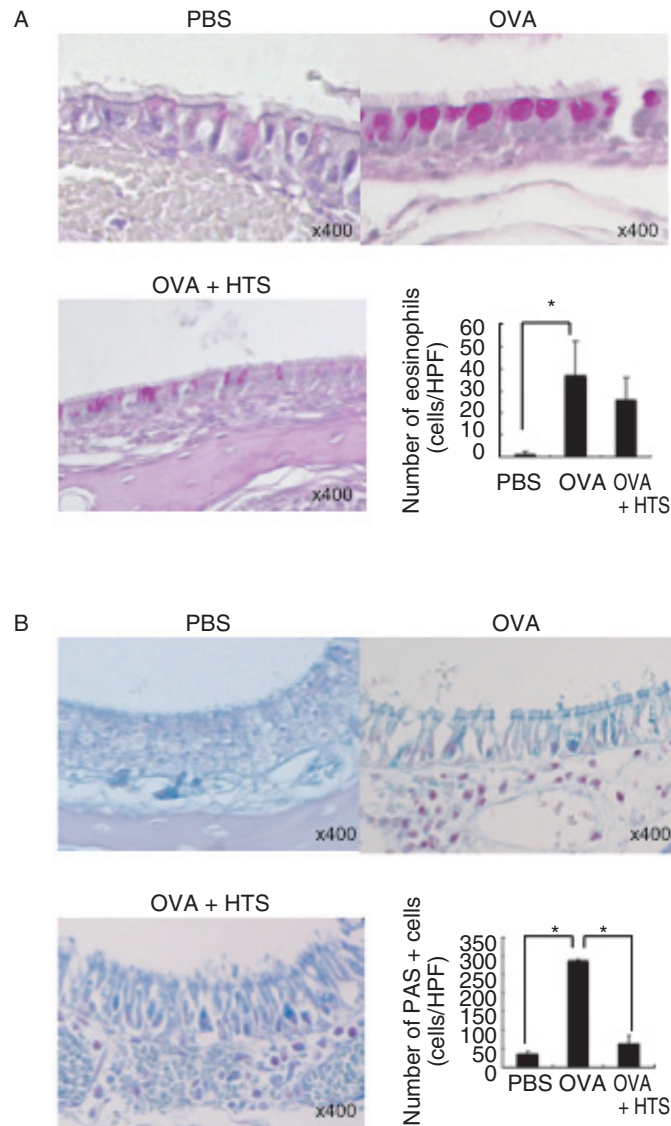


Fig. 2 TGF- β signaling may contribute to the development of goblet cell hyperplasia in the nasal mucosa in a mouse model of allergic rhinitis. The allergic rhinitis model was established as described in Figure 1 legend. **A-B.** Representative picture of Hansel (**A**) and PAS (**B**) staining of the nasal mucosa obtained from the mice treated with HTS466284 (OVA + HTS) or control vehicle DMSO (OVA) or OVA-sensitized and PBS-challenged control mice (PBS). Bar graphs show quantitative analysis of the number of eosinophils in the nasal mucosa and PAS-positive goblet cells in the nasal epithelium. Values represent the mean \pm SD of 6 mice in each group. * $p < 0.05$.

procedure was obtained from all patients. Approval for the study was given by the ethics committee of the University of Yamanashi. The specimens were fixed in 4% paraformaldehyde for 3 hours and then embedded in paraffin. The number of phosphorylated Smad2-positive cells in the nasal sections was

counted as described above.

STATISTICAL ANALYSIS

The data are summarized as the mean \pm SD. Statistical analysis was performed using the non-parametric Mann-Whitney *U* test to compare data in different two

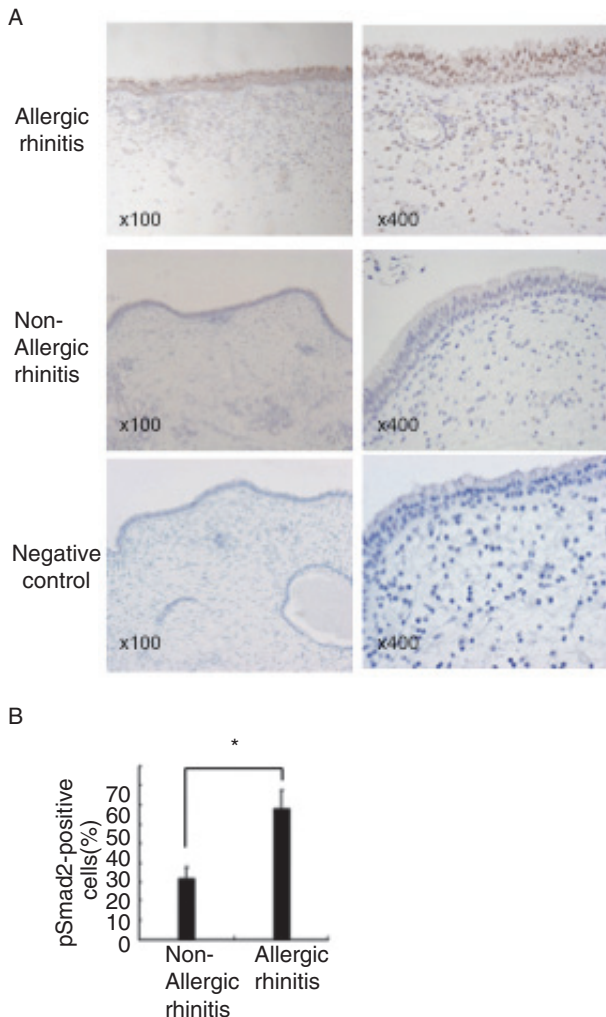


Fig. 3 Phosphorylation of Smad2 was detected predominantly in epithelial cells in the nasal mucosa in patients with allergic rhinitis. **A.** Four allergic rhinitis or 4 non-allergic rhinitis tissue specimens were immunohistochemically stained with anti-phosphorylated Smad2 antibody. Representative pictures are shown. Negative control: allergic rhinitis tissue specimens stained with control rabbit IgG antibody. **B.** A quantitative analysis of the epithelial phosphorylated Smad2 expression in allergic rhinitis and non-allergic rhinitis specimens ($n = 4$ in each group). * $p < 0.05$.

groups. A value of $P < 0.05$ was considered to be significant.

RESULTS

PHOSPHORYLATION OF SMAD2 WAS DETECTED PREDOMINANTLY IN EPITHELIAL CELLS IN THE NASAL MUCOSA IN A MOUSE MODEL OF ALLERGIC RHINITIS

To determine whether TGF- β signaling is active in allergic rhinitis, we examined the phosphorylation of Smad2 in the nasal mucosa in a mouse model of allergic rhinitis because phosphorylation of Smad2 is a

key event for initial TGF- β signal transduction.⁵

Immunohistochemical staining for phosphorylated Smad2 revealed the immunoreactivity to be increased predominantly in the nasal epithelium and in some submucosal cells after the induction of allergic rhinitis (Fig. 1A, B). Interestingly, we also found that immunoreactivity for phosphorylated Smad1, a key indicator for initial BMP signaling, also increased predominantly in the nasal epithelium (Fig. 1C).

Consistent with the immunohistochemical findings, an immunofluorescence staining also confirmed the phosphorylated Smad2-positive cells to be present predominantly in the nasal epithelium (Fig. 1D). These results suggested that epithelial cells predominantly received endogenous TGF- β activity in the nasal mucosa in a mouse model of allergic rhinitis.

ACTIVATION OF TGF- β SIGNALING MAY CONTRIBUTE TO THE DEVELOPMENT OF GOBLET CELL HYPERPLASIA IN A MOUSE MODEL OF ALLERGIC RHINITIS

Because active TGF- β signaling was present in the nasal mucosa in a mouse model of allergic rhinitis (Fig. 1), we determined whether activation of TGF- β signaling plays some roles for the development of the allergic rhinitis model. For this purpose, the effects of TGF- β type I receptor kinase inhibitor¹¹ HTS466284 on the development of allergic rhinitis were pathologically evaluated. The administration of HTS 466284 during OVA challenge significantly inhibited phosphorylation of Smad2, but not Smad1, in the nasal epithelium (Fig. 1A, C), suggesting that the inhibitor was indeed specific to TGF- β signaling.

OVA-sensitized BALB/c mice treated with control vehicle (DMSO) during OVA challenge showed massive infiltration of eosinophils into the nasal mucosa and increased number of PAS-positive goblet cells in the nasal mucosa (Fig. 2A, B). OVA-sensitized mice treated with HTS466284 during OVA challenge showed marginal reduction of the infiltration of eosinophils into the nasal mucosa (Fig. 2A). Importantly, the number of PAS-positive goblet cells in the nasal mucosa decreased to the basal levels in HTS 466284-treated mice (Fig. 2B). These results suggested that the pharmacological blockade of endogenous TGF- β signaling inhibited the development of goblet cell hyperplasia in the nasal mucosa in a mouse model of allergic rhinitis without affecting eosinophil infiltration into the nasal mucosa.

PHOSPHORYLATION OF SMAD2 WAS DETECTED PREDOMINANTLY IN EPITHELIAL CELLS IN THE NASAL MUCOSA IN PATIENTS WITH ALLERGIC RHINITIS

Finally, the relevance of the findings in mice to humans was investigated. The nasal mucosa specimens derived from 4 patients with allergic rhinitis showed an increase in the number of phosphorylated Smad2-

positive cells in nasal epithelium when compared with that in non-allergic rhinitis subjects (Fig. 3A, B). These results suggested that epithelial cells predominantly received endogenous TGF- β activity in the nasal mucosa in patients with allergic rhinitis.

DISCUSSION

A previous study using immunohistochemistry showed significantly increased immunoreactivity for TGF- β in the epithelial layer, with predominant localization to the superficial columnar epithelial cells, of the nasal mucosa obtained from patients with allergic rhinitis.⁸ However, because TGF- β is secreted extracellularly as latent complexes, it remains unclear whether the local TGF- β expression actually drives active signaling and, if any, what roles the active TGF- β signaling play in allergic rhinitis. In this study, we suggest that active TGF- β signaling is present in the nasal mucosa of allergic rhinitis and it may play a role in the development of goblet cell hyperplasia in allergic rhinitis.

Phosphorylation of Smad1 as well as that of Smad2 was detected in the nasal epithelium in the allergic rhinitis model (Fig. 1C). Because Smad1 principally mediates BMP signals,^{5,6} these results suggest that endogenous BMP signaling may be also involved in the pathophysiology of allergic rhinitis. In a mouse model of asthma and in human asthmatics, BMP signaling was reported to be activated upon allergen provocation in the airway epithelium,^{3,15} suggesting that BMP signaling may be involved in the tissue repair and control of inflammation. Thus, active BMP signaling in allergic rhinitis may also play a role in these processes. The precise roles of BMP signaling in allergic rhinitis as well as in asthma remain to be determined.

TGF- β has been implicated in the regulation of airway mucin production.¹⁶ For instance, TGF- β increased mucin MUC5AC protein expression in cultured human bronchial epithelial cells¹⁷ and neutralization of TGF- β activity using anti-TGF- β antibody or a TGF- β type I receptor kinase inhibitor suppressed antigen-induced increase in PAS-positive cells in mouse models of asthma.^{18,19} In addition, Smad3-deficient mice developed a significantly reduced percentage of airway epithelium that stained positive with PAS in a model of asthma when compared with wild type mice.²⁰ Taken together with our current *in vivo* findings, it is very likely that TGF- β signaling in allergic rhinitis contributes to the development of goblet cell hyperplasia in the nasal mucosa. Because it remains unclear whether TGF- β signaling exerts its effects either directly or indirectly on nasal epithelial cells, in particular, *in vivo* situations, future studies should focus on the effects of TGF- β signaling on the regulation of goblet cell differentiation, proliferation, and mucin production.

The pharmacological blockade of endogenous

TGF- β signaling did not affect the number of eosinophils infiltrated into the nasal mucosa in the allergic rhinitis model (Fig. 2). These results are consistent with the previous findings obtained from mouse models of asthma, showing that the blockade of endogenous TGF- β signaling did not affect airway inflammation.^{18,20,21} However, it should be noted that the roles of TGF- β in airway inflammation are still controversial, depending on the models and protocols²² and thus requires further investigations.

In summary, this study suggests that TGF- β signaling is activated in the nasal mucosa in allergic rhinitis and may contribute to the development of goblet cell hyperplasia in the nasal mucosa in allergic rhinitis. To our knowledge, this is the first report showing that active TGF- β signaling is present in allergic rhinitis and addressing possible roles of TGF- β signaling in the pathophysiology of the disease. Based on the current results, TGF- β signaling in nasal mucosa might become a potential target for the prevention of a selective pathological feature of allergic rhinitis.

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