

# Influence of Fluticasone Propionate on the Production of Periostin from Nasal Cells *In vitro* and *In vivo*

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## Abstract

**Background:** Periostin, a 90-kDa extracellular matrix protein, is well known to be an essential factor in the development and persistence of allergic rhinitis (AR). Although topical application of glucocorticoids, including beclomethasone, mometasone furoate and fluticasone propionate (FP), recommended as the first choice of agent in the treatment of AR, the influence of glucocorticoids on periostin production is not well understood. The present study was undertaken to examine the influence of FP on periostin production *in vitro* and *in vivo*.

**Methods:** Human nasal epithelial cells (HNEpC) at a concentration of  $1 \times 10^5$  cells/ml were stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of FP. After 48 h, culture supernatants were collected and assayed for periostin levels by ELISA. We also examined the influence of FP on the appearance of periostin in nasal secretions obtained from pollinosis patients treated with 27.5 µg FP twice a day for two weeks during Japanese cedar pollen seasons.

**Results:** Addition of FP into cell cultures suppressed the ability of HNEpC to produce periostin in response to IL-4 stimulation in dose-dependent manner. The minimum concentration that caused significant suppression is  $10^{-6}$ M. Treatment of patients with AR against Japanese cedar pollen with 27.5 µg FP twice a day for two weeks also suppressed the appearance of periostin in nasal secretions along with favorable modification of clinical conditions of AR.

**Conclusion:** The present results strongly suggest that glucocorticoid, especially FP, favorably modify the clinical status of AR through the suppression of periostin production from nasal cells.

## Introduction

Allergic rhinitis (AR) is well accepted to be a chronic inflammatory disease of the mucous membrane of the upper airway tract such as the nose and sinus. AR is also well known to be divided into two distinct responses, i.e. early phase response and late phase response [1, 2]. Early phase response occurs within 30 minutes after allergen exposure and lasts for approximately 3 h, which is characterized by sneezing, itching and rhinorrhea. This is followed by late phase response, which occurs after 4 to 6 h. During this phase, there is a chronic itch affecting the nose and nasal cavity with nasal congestion and conjunctivitis. Prior to the early phase, the immune system becomes sensitized to specific allergen. It is internalized and processed by antigen presenting cells such as dendritic cells and macrophages. These cells then activate and produce several types of cytokines, which are responsible for class switching of T cells to a Th2 response and activation of B cells to produce IgE. Thereafter, the allergen specific IgE attaches to receptors on the surface of mast cells and results in sensitization to specific allergen. On re-exposure to the relevant allergen, it binds to IgE molecules and mast cell degranulation occurs and releases a variety of mediators such as histamine, prostaglandins and leukotrienes [1-3], leading to what is known as the early phase response. The late phase response is characterized by intense infiltration of inflammatory cells comprising of T cells, basophils and eosinophils into the nasal mucosa. During late phase response, a wide variety of mediators are released by these cells including leukotrienes, kinins, and histamine, which result in the continuation and aggravation of the clinical symptoms [3-5].

The management of AR includes avoidance of allergens, treatment with intranasal corticosteroids, oral antihistamines and allergen immunotherapy, among others [1, 6]. Topical application of corticosteroids

such as beclomethasone, flunisolide and fluticasone propionate (FP) is reported to be more effective than other medical treatments for controlling all rhinitis symptoms [1]. The therapeutic mode of action of corticosteroids is owing, in part, to their inhibitory action of Th2 type cytokines, IL-4, IL-5 and IL-13, production from Th2 T cells and infiltrating inflammatory cells, while little effect on the production of Th1 type cytokines such as IL-12 and IFN- $\gamma$  that showing suppressive effects on the development of Th2 type immune responses [1, 7]. It is also showed that corticosteroids inhibit mediator release from mast cells and induce apoptosis of eosinophils [1].

Periostin is well accepted to be a 90-kDa matricellular protein secreted from several types of cells such as fibroblasts and airway epithelial cells after IL-4/IL-13 stimulation [8, 9]. There is much evidence that periostin is reported to play important roles in the pathological process of various diseases such as fibrosis, wound repair and inflammatory responses as well as tumorigenesis and metastasis [10, 11]. In regard to allergic diseases, periostin enhances the accumulation of eosinophils, which are an essential effector cell in the development of allergic inflammatory diseases, in airways after intranasal exposure to allergens through the increase in the ability of eosinophil to adhere fibronectin in Th2 type T cell-associated

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mucosal inflammation in both mice and human [8]. It is also reported that, in AR model mouse, periostin induces eosinophilia and type I collagen deposition in subepithelial tissues, which are important pathological feature of subepithelial fibrosis of AR [11]. Furthermore, periostin induces goblet cell metaplasia, sub-epithelial fibrosis, and basement membrane thickening in nasal mucosa [8, 13], which are called tissue remodeling and the characteristic feature of AR [14, 15]. From these reports, periostin has been attracted attention as a novel biomarker of AR, but the influence of corticosteroids on periostin production is not well understood. The present study, therefore, was undertaken to examine the influence of corticosteroids on periostin production from human nasal epithelial cells through the choice of FP *in vitro* and *in vivo*.

## Materials and Methods

### Reagents

FP was purchased from Toronto Research Chem., Inc. (North York, ON, Canada) as a preservative free pure powder. The agent was firstly dissolved in dimethyl sulfoxide and further diluted in Airway Epithelial Cell Growth Media (AECG medium; PromoCell GmbH, Heidelberg, Germany) at a concentration of  $10^{-2}$ M, sterilized by passing through 0.2  $\mu$ m filters and stored at 4°C until used. Recombinant human IL-4 purchased from R & D Systems, Inc. (Minneapolis, MN, USA) as a preservative free pure powder was also dissolved in AECG medium, sterilized and stored at 4°C until used.

### Subjects and treatment

The subjects were 10 male patients with Japanese cedar pollen-sensitized rhinitis. The patients treated with FP were recruited from the Otolaryngology Outpatient Clinic of Fujitsu Kawasaki Hospital (Kawasaki, Japan). All subjects were received a written informed consent, which was approved by the Ethics Committee of Fujitsu Co., Ltd. and of Showa University. Pollinosis was diagnosed by otorhinolaryngologist in accordance with the established criteria on the basis of patient history and rhinoscopic examination. To confirm the diagnosis and demonstrate allergen-caused pollinosis, skin prick testing (mean wheal diameter at least 4 mm greater than negative control) and a nasal provocation test was performed with commercial crude extracts used for *in vitro* and *in vivo* (Torii Pharmaceutical Co., Ltd., Tokyo, Japan). The number of eosinophils in nasal secretions obtained after the provocation test was also examined using smears stained with Wright-Giemsa solution. We also recruited 5 male healthy subjects from the member of the Showa University Fujigaoka Hospital (Yokohama, Japan) under a written informed consent approved by the Ethics Committee of Showa University. The characteristics of the subjects used in this study are shown in Table 1. Pollinosis patients were intranasally treated with 27.5  $\mu$ g FP nasal spray (GlaxoSmithKline, Tokyo, Japan) twice a day for two weeks during Japanese cedar pollen season (January 2016 to April 2016).

### Recovery of nasal secretions

Nasal secretions were obtained as previously described [16]. Briefly, filter papers (Whatman No. 42) were cut into 7 x 30 mm. A filter strip was placed on the anterior portion of the inferior turbinates of the right and left nose and left for 5 min. They were then cut into small pieces and suspended in PBS and rocking 12 h at 4°C to prepare the extract of nasal secretions. After measuring IgA concentration in

	Controls	Patients treated with FP
Number of subjects	5	10
Age, years(range)	30-51	30-66
Sex	male	male
Disease severity	nonallergic	mild
Medication	none	none
Serum IgE (U/ml)	35.6 $\pm$ 6.7	149.6 $\pm$ 13.7
IgE RAST score		
Cj	0	58.8 $\pm$ 11.2
Aa	0	0
Ap	0	0
Dg	0	0
Df	0	0
Af	0	0
Cd	0	0
Dd	0	0
Blood eosinophil count, %	2.2 $\pm$ 0.6	17.2 $\pm$ 0.7
Skin prick test	-	+++ <sup>a</sup>
Nasal provocation tes		
Symptoms	-	+++ <sup>b</sup>
Smear cytology (eosinophils, %)	< 1	> 33

Table 1: Characteristics of subjects used for treatment.

Cj = *Cryptomeria japonica*; Aa = *Ambrosia artemisiifolia*; Ap = *Artemisia princeps*; Dg = *Dactylis glomerata*; Df = *Dermatophagoides farinae*; Af = *Aspergillus fumigatus*; Cd = cat dander; Dd = dog dander.  
<sup>a</sup>Wheal reaction > 13 mm and flare reaction > 30 mm against *C. japonica* alone. <sup>b</sup>Positive for sneezing/itch, watery rhinorrhea and nasal blockage against *C. japonica* alone. FP: Fluticasone Propionate.

the extract with ELISA (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's recommendations, they were stored at -80°C until used.

### Nasal symptom scores

Nasal discharge and congestion were scored from 0 to 3 (0 = no, 1 = mild, 2 = moderate, and 3 = severe symptoms). The number of sneezes during one h were counted and transformed into a score (0 = 0 sneezes, 1 = 1-4 sneezes, 2 = 5-9 sneezes, and 3 = 10 or more sneezes). A total symptom score was calculated by adding these three scores.

### Cell culture

Human nasal epithelial cells (HNEpC), purchased from PromoCell GmbH, was suspended in AECG medium (PromoCell GmbH) at a concentration of  $1 \times 10^5$  cells/ml and used as a target cell. To examine the influence of IL-4 on periostin production from HNEpC,  $1 \times 10^5$  cells (1.0 ml) were introduced into 24-well culture plates in triplicate and stimulated with various concentrations of IL-4 in a final volume of 2.0 ml. After 24 to 96 h, culture supernatants were collected and stored at -40°C until used. To prepare culture supernatants for examining the influence of FP on periostin production from HNEpC after IL-4 stimulation,  $1 \times 10^5$  cells (1.0 ml) were introduced into 24-well culture plates in triplicate that contained various concentrations

48 h in a total volume of 2.0 ml and culture supernatants were treated in a similar manner. In all experiments, FP was added to cell cultures 2 h before stimulation.

### Assay for periostin

Periostin levels in supernatants obtained from cell culture were examined by the commercially available human periostin ELISA test kits (Phoenix Pharmaceuticals, Inc., Burlingame, Calif., USA) according to the manufacturer's recommendations, and the results were expressed as mean ng/ml ± SE. Periostin levels in nasal secretions were also measured by the commercially available human periostin ELISA test kits (Phoenix Pharmaceuticals, Inc.) in a similar manner. Simultaneously, IgA contents in nasal secretions were measured by human IgA ELISA test kits (Bethyl Laboratories, Inc.) and periostin levels in the secretions were expressed as the mean ng/ng IgA ± SE. The minimum detectable level of this ELISA kit was 0.027 ng/ml.

### Statistical analysis

Statistical significance between control and experimental groups was examined by ANOVA followed by Dunnett's multiple comparison test. Paired t-test was used to examine the statistical significance between before and after treatment with FP. Data analysis was performed by using ANOVA for Mac (SPSS Inc., Chicago, IL, USA). The level of significance was considered at a P value of less than 0.05.

## Results

### Influence of FP on periostin production from HNEpC *in vitro*

The first set of experiments was designed to examine the influence of FP on the production of periostin from HNEpC after IL-4 stimulation. To do this, we firstly examined the optimal concentration of IL-4 to induce periostin production from HNEpC. HNEpC at  $1 \times 10^5$  cells/ml was stimulated with various concentrations of IL-4 for 48 h and periostin levels in culture supernatants were examined by ELISA. As shown in Figure 1A, IL-4 stimulation increased the ability of cells to produce periostin, which was firstly observed at 5.0 ng/ml and plateaued at more than 10.0 ng/ml. The second experiments

was undertaken to examine the time course of periostin production from HNEpC after IL-4 stimulation. HNEpC ( $1 \times 10^5$  cells/ml) was stimulated with 10.0 ng/ml IL-4 for various hours and periostin levels in culture supernatants were examined by ELISA. As shown in Figure 1B, periostin contents in culture supernatants were peaked at 48 h after stimulation and plateaued thereafter. The third experiments were carried out to examine whether FP could inhibit the ability of HNEpC to produce periostin induced by IL-4 stimulation. As shown in Figure 2, addition of FP into cell cultures at more than  $10^{-6}$  M, but not less than  $10^{-7}$  M significantly suppressed periostin production from HNEpC induced by IL-4 stimulation. FP at  $10^{-3}$  M completely suppressed periostin production in response to IL-4 stimulation: periostin contents in experimental culture supernatants were nearly identical (not significant) to that detected in non-stimulated control culture supernatants.

### Influence of treatment with FP on periostin levels in nasal secretions and on clinical conditions of AR patients

The fourth experiments were undertaken to examine the influence of FP on periostin appearance in nasal secretions. Patients with Japanese cedar pollinosis were treated intranasally with 27.5 µg FP twice a day for two weeks during Japanese cedar pollen season and periostin levels in nasal secretions were examined by ELISA. As shown in Figure 3, intranasal administration of FP into patients caused significant decrease in periostin levels in nasal secretions as compared with before treatment along with favorable modification of clinical conditions (Table 2).

Symptoms	Treatment with FP	
	Before	After*
Sneezing	2.0 ± 0.4	0.5 ± 0.3
Nasal discharge	2.3 ± 0.9	0.7 ± 0.2
Congestion	2.4 ± 1.2	1.0 ± 0.3

Table 2: Changes in clinical symptom scores observed in pollinosis patients treated with Fluticasone Propionate for two weeks.

FP: Fluticasone Propionate

\*: P < 0.05 as compared with before treatment.

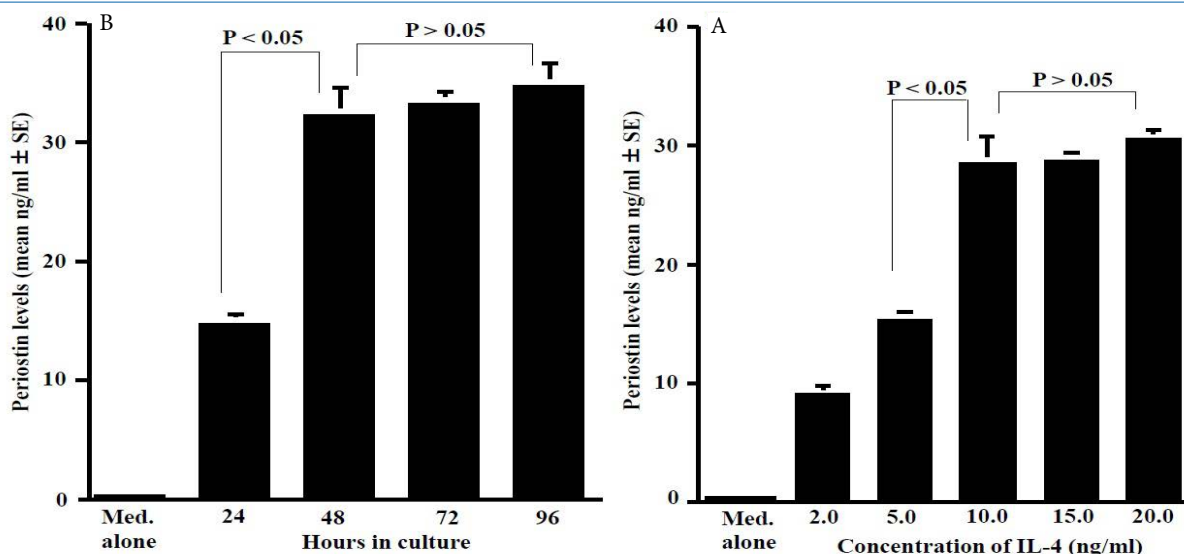


Figure 1: Influence of IL-4 stimulation on periostin production from human nasal epithelial cells (HNEpC) *in vitro*. HNEpC at a concentration of  $1 \times 10^5$  cells/ml was stimulated with various concentration of IL-4, and periostin contents in culture supernatants were examined by ELISA. A: dose response profile of IL-4 on periostin production; B: time course of IL-4-induced periostin production. Med. alone: Medium alone. The data expressed are the mean ng/ml ± SE of triplicate cultures. The experiments were repeated twice with similar results.

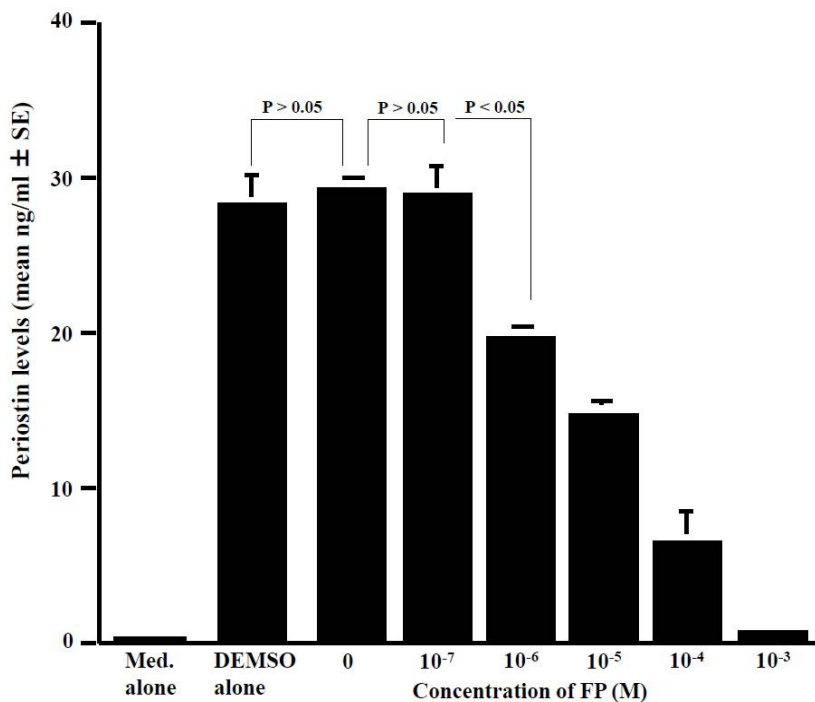


Figure 2: Influence of fluticasone propionate (FP) on periostin production from nasal epithelial cells (HNEpC) *in vitro*. HNEpC at a concentration of  $1 \times 10^5$  cells/ml was stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of FP for 48h. Periostin contents in culture supernatants were examined by ELISA. Med. alone: Medium alone. DEMSO alone: 0.01% dimethyl sulfoxide (DEMSO) alone. The data expressed are the mean ng/ml  $\pm$  SE of triplicate cultures. The experiments were repeated twice with similar results.

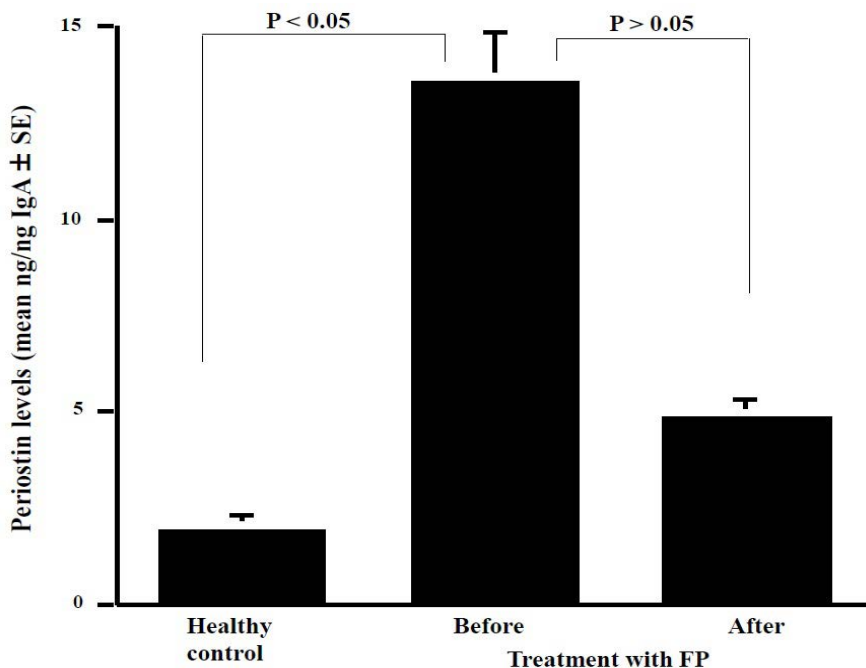


Figure 3: Influence of treatment of pollinosis patients with fluticasone propionate (FP) on the appearance of periostin in nasal secretions. Pollinosis patients were treated intranasally for two weeks during Japanese cedar pollen season with 27.5  $\mu$ g FP twice a day. Periostin levels and IgA contents in nasal secretions were measured by ELISA, respectively and the results are expressed as the mean ng/ng IgA  $\pm$  SE of 10 male patients.



## Discussion

The present results clearly show that FP inhibits the ability of nasal epithelial cells to produce periostin induced by IL-4 stimulation *in vitro*. The minimum concentration that caused significant suppression of periostin production is  $10^{-6}$ M. This suppressive effect of FP was also observed in patients with AR against Japanese cedar pollen, when they were treated intranasally with 27.5 µg FP twice a day for two weeks during pollen season.

A number of studies clearly demonstrated the histological abnormalities such as thickening of the basement membrane, sub-epithelial fibrosis, epithelial damage and torn tight junctions of the epithelial cells in nasal mucosa of patients with AR [14, 15, 17]. It is also observed goblet cell hyperplasia, mucous gland hypertrophy and angiogenesis in nasal mucosa of patients with AR [8, 15, 18]. Furthermore, immunohistochemical analysis revealed the increased expression of collagen, decorin, lumican and biglycan in both the subepithelial and submucosal layer of allergic nasal mucosa in human and murine AR model [11, 15]. There is evidence that periostin enhances fibrosis by binding to extracellular matrix proteins such as collagen I and fibronectin, and by inducing the production of chemokines to recruit inflammatory cells especially macrophages [12, 19]. It is also reported that periostin promotes proliferation and migration of both fibroblasts [20] and vascular endothelial cells [21, 22] into inflammatory sites, which are responsible for fibrosis, angiogenesis and lymphangiogenesis observed in AR [15]. The influence of periostin on the function of effector cells in allergic inflammatory responses was extensively examined by targeting eosinophils [8, 11, 23]. Periostin is reported to promote eosinophil migration [8, 24] and increases the adhesion of eosinophils from the vessel to the lamina propria *in vivo* and to the extracellular matrix component fibronectin *in vitro* [8]. Intranasal instillation of allergens [8, 11, 24] into periostin-knockout mice cannot induce eosinophil accumulation in airways as compared with wild type mice. Together with these reports, the present results strongly suggest that the suppressive activity of FP on periostin production may account, at least in part, for the clinical efficacy of FP in AR. However, before drawing the conclusion that intranasal application of FP into AR patients suppresses periostin production induced by inhalation of allergen(s) and results in favorable modification of clinical symptoms of AR, it is necessary to examine the influence of FP on periostin production *in vivo*. We, therefore, examined the influence of FP on periostin production *in vivo* using AR patients treated with FP for two weeks during Japanese cedar pollen season. The present data clearly showed that nasal secretions obtained AR patients before treatment contained higher levels of periostin as compared with those in healthy control, and that intranasal application of FP decreased periostin contents in nasal secretions along with favorable modification of clinical conditions of AR induced by inhalation of Japanese cedar pollen, suggesting that suppression of periostin production by FP may be responsible for the therapeutic mode of action of the agent on AR.

Stimulation of cells with IL-4 causes the activation of several molecules that are implicated in cellular signal transduction [25, 26]. IL-4 first binds to the IL-4 receptor alpha and the complex causes the phosphorylation of Janus kinase (Jak) 1 and 3, which leads to phosphorylation of signal transducer and activator of transcription factor-6 (STAT6) [25, 27]. STAT6 is then dimerized, migrate to the nucleus and binds to the promoter regions of various genes [25, 27]. Phosphorylation of tyrosine kinases, including Jak 1 and 3 as well as STAT6 requires intracellular free  $Ca^{2+}$  ion, which increases in cytosol

after stimulations [28, 29]. It is reported that the synthetic glucocorticoid dexamethasone (DEX) inhibits the signals via  $Ca^{2+}$ -dependent mechanisms through inhibition of  $Ca^{2+}$  elevations in T cells after anti-CD3 stimulation, which is associated with inflammatory protein production (30). From these reports, it is reasonably to speculate that FP may suppresses the increase in  $Ca^{2+}$  contents in cytosol after IL-4 stimulation and results in the inhibition of STAT6 activation responsible for periostin mRNA expression. It is reported that DEX suppress IL-4-induced transactivation of a STAT6-responsive promoter, but not IL-4-stimulated STAT6 DNA-binding (31), suggesting that FP inhibits transcriptional activity of STAT6 and results in suppression of periostin production. Anyway, further experiments are required to clarify the suppressive mechanism(s) of FP on periostin production *in vitro* and *in vivo*.

## Conclusion

The present results demonstrate that the suppressive effect of FP on periostin production by nasal cells after IL-4 and allergen stimulation constitute, at least in part, the therapeutic mode of action of the agent on allergic diseases such as AR.

## Competing Interests

The authors declare that they have no competing interests

## Author contribution

Atsuko Furuta: Contribute to study design, diagnosis and treatment of AR patients, correction of nasal secretions, contribute to cell culture and preparation of Figures and Tables.

Yoshihito Tanaka, Suguru Furukawa: Diagnosis and treatment of AR patients, correction of nasal secretions and contribute to cell culture.

Kazuhiro Asano: Assay for periostin, participate in the methodological design and contribute to the entire manuscript writing

Hitome Kobayashi: Diagnosis and treatment of AR patients, correction of nasal secretions and data analysis, including statistical analysis (for data presented in Figures 1-3 and Tables 1, 2)

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