

Expression of Semaphorin 3A and Neuropilin 1 in Asthma

Eun-Jin Shim,^{1,2} Eunyoung Chun,^{1,2}
Hae-Ryun Kang,^{1,2} Sang-Heon Cho,^{1,2}
Kyung-Up Min,^{1,2} and Heung-Woo Park^{1,2}

¹Department of Internal Medicine, and ²Institute of Allergy and Clinical Immunology, Seoul National University College of Medicine, Seoul, Korea

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Address for Correspondence:

Heung-Woo Park, MD

Department of Internal Medicine, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Korea
Tel: +82-2-2072-0699, Fax: +82-2-742-2912
E-mail: guinea71@snu.ac.kr

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Neuropilin 1 (NP1) is a part of essential receptor complexes mediating both semaphorin3A (SEMA3A) and vascular endothelial growth factor (VEGF) which is one of important mediators involved in the pathogenesis of asthma. Therefore, it is possible that SEMA3A plays a role in the pathogenesis of asthma through attenuation of VEGF-mediated effects. In the present study, we aimed to evaluate expression levels of SEMA3A and NP1 using induced sputum of asthmatics and a murine model of asthma. Firstly, SEMA3A and NP1 expressions in induced sputum of asthmatics and SEMA3A and NP1 expression on bronchoalveolar lavage (BAL) cells and lung homogenates of asthmatic mice were determined. Then we evaluated the immunolocalization of VEGF receptor 1 (VEGFR1), VEGF receptor 2 (VEGFR2), and NP1 expressions on asthmatic mice lung tissue and their subcellular distributions using fibroblast and BEAS2B cell lines. Sputum SEMA3A and NP1 expressions were significantly higher in asthmatics than controls. Similarly, SEMA3A and NP1 expressions on BAL cells and lung homogenates were significantly elevated in asthmatic mice compared to control mice. Immunohistochemical analysis showed that VEGFR1, VEGFR2, and NP1 expressions were also uniformly increased in asthmatic mice. Our observations suggest that SEMA3A and NP1 may play important roles in the pathogenesis of asthma.

Key Words: Asthma; Neuropilin; Semaphorin-3A (SEMA3A); Vascular Endothelial Growth Factor

INTRODUCTION

Asthma is characterized by allergic airway inflammation and remodeling (1). Vascular endothelial growth factor (VEGF) is one of the important mediators involved in the pathogenesis of asthma. VEGF is a multifunctional angiogenic regulator that stimulates epithelial cell proliferation, blood vessel formation, and endothelial cell survival (2, 3). Previous investigators reported that VEGF levels increased in tissues and biologic samples obtained from asthmatics (4-6). Moreover, lung-targeted VEGF transgenic mice showed the cardinal features of asthma, such as airway hyperresponsiveness (AHR), airway inflammation and remodeling (7) and VEGF levels in supernatant of induced sputum were significantly elevated in patients with severe-refractory asthma compared with patients with moderate asthma and control subjects (8). In addition to two tyrosine kinase receptors (VEGF receptor 1 [Flt-1] and VEGF receptor 2 [KDR/Flk-1]), the biological effects of VEGF are known to be mediated by neuropilin 1 (NP1) (9, 10). NP1 was initially identified in neuronal cells as receptors for class 3 semaphorins (SEMA3) (11, 12). Therefore, two seemingly disparate ligand families can bind the same receptor, which suggests that SEMA3 and VEGF possibly compete for binding to their common receptors.

Accordingly, competition between VEGF and SEMA3A for NP1 binding regulated chemotaxis of carcinoma cells (13). A combination of an anti-VEGF antibody along with an antibody targeting NP1 resulted in a greater tumor growth inhibition (14). In addition, SEMA3A dose-dependently improved skin lesions in a murine model of atopic dermatitis (15) in which an overproduction of VEGF was observed (16).

Expression of NP1, VEGF, and SEMA3 is very low in cells of normal tissues, but are highly expressed in tumor-derived cells (17). In addition, the ratio of VEGF/SEMA3A is markedly variable according to tumor cells (17), which suggests that these proteins provide an important clues for the effective therapeutic approaches for cancer patients. Along with this observation, it was reported that a decrease in SEMA3A and increase in NP1 had some relationship with disease progression in ductal breast carcinoma (18). Based on those findings, we hypothesize that SEMA3A may play an important role in the pathogenesis of asthma through the competitive binding to NP1 and resultant attenuation of VEGF-mediated effects. For the first step to test our hypothesis, we aimed to evaluate expression levels of SEMA3A and NP1 using induced sputum of asthmatics and a murine model of asthma in the present study.

MATERIALS AND METHODS

SEMA3A and NP1 expressions in induced sputum of asthmatics

To evaluate clinical relevancy of SEMA3A and NP1 in human asthma, we measured *SEMA3A* and *NP1* expressions in induced sputum of normal controls and asthmatics. Normal controls who showed negative methacholine AHR and complaint no respiratory symptoms, and asthmatics were recruited at the Seoul National University Hospital, Seoul, Korea. Asthma was diagnosed in accordance with the guideline issued by the National Institutes of Health, Bethesda, MD, USA (19). Asthma severity was determined based on lung function and the medication use index needed to obtain control, as described previously (20). All asthmatics were treatment-naïve. Sputum induction, sample processing, RNA extraction, and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) for *SEMA3A* and *NP1* were performed as previously described (21). The primers used was as follows; sense 5'-GTGCCAAGGCTGAAAT-TATCCT-3' and antisense 5'-CCCACCTTGATTCATCTCTTCT-3' for *SEMA3A* and sense 5'-ACGTGGAAGTCTTCGATGGAG-3' and antisense 5'-CACCATGTGTTTCGTAGTCAGA-3' for *NP1*.

SEMA3A and NP1 expressions in a murine model of asthma

To evaluate expressions of *SEMA3A* and *NP1*, we generated a murine model of asthma (C57BL/6) based on intraperitoneal sensitization with ovalbumin plus alum and a subsequent intranasal challenge with ovalbumin alone as described in our previous study (22). Five mice were allocated to asthma and control group respectively and one representative experiment of three replicates was presented. On the cells obtained from bronchoalveolar lavage (BAL) fluid, SEMA3A and NP1 immunofluorescence staining was performed. Cell fixation was performed in 1% paraformaldehyde for 15 min with saponin 0.1% permeabilization for 10 min and the cells were blocked with a commercial blocking solution (Super Block; ScyTek Laboratories, Logan, UT, USA) for 15 min at room temperature. The cells were then incubated with an affinity-purified polyclonal antibody to SEMA3A (Novus biologicals, Littleton, CO, USA) diluted in permeabilization solution (1:100) and NP1 (ECM bioscienc, Versailles, KY, USA) diluted in permeabilization solution (1:100) respectively overnight at 4°C, washed, and then incubated with secondary antibody (donkey anti-rabbit IgG, F[ab']₂ fragment, Cy3 conjugated, diluted 1:200; Jackson Laboratories, West Grove, PA, USA). Nuclei were counterstained with 300 nM/L 4',6-diamidino-2-phenylindole (DAPI; Sigma, Natick, MA, USA) for 1 hr at room temperature. The cells were then washed and mounted on slides. Purified non-immune rabbit IgG (SouthernBiotech, Inc., Birmingham, AL, USA) was used as an isotype control. The slides were analyzed by fluorescence mi-

croscopy (Leica Microsystems, Wetzlar, Germany). *SEMA3A*, *NP1*, and *VEGF* expressions in lung tissue were measured by real time PCR. Total RNA and cDNA were prepared as described previously (23).

Localization of VEGFR1, VEGFR2, and NP1 in a murine model of asthma

We evaluated regional distributions and cellular localizations of VEGFR1, VEGFR2, and NP1 by immunohistochemical analysis of asthmatic mice lung tissue. Immunostaining of VEGFR1, VEGFR2, and NP1 was performed on paraffin sections. The primary rabbit polyclonal antibodies used were anti-VEGFR1 (Abcam, Cambridge, UK) at a dilution of 1:100, anti-VEGFR2 at a dilution of 1:50, and anti-NP1 (Abcam) at a dilution of 1:50. After fixation in cold acetone for 10 min and blocking of non-specific binding with 2% donkey serum for 30 min, a three-stage indirect immunoperoxidase technique was used: incubation with the primary antibody at 4°C overnight, followed by the secondary biotinylated donkey anti-rabbit immunoglobulin G (Jackson, Baltimore, PA, USA) (1:1,250) then the amplification system avidin-biotin complex (Dakopatts, Glostrup, Denmark) (1:200). Negative control consisted of omission of the primary antibody and incubation with immunoglobulin of the same species at the same final concentration.

To evaluate VEGFR1, VEGFR2, and NP1 subcellular distribution, we selected the immortalized epithelial cell line (BEAS2B) and fibroblast cell line (MLg, ATCC, Rockville, MD, USA), because immunohistochemistry analysis of asthmatic mice lung tissue showed prominent expressions of VEGFR1, VEGFR2, and NP1 on the structural cells.

Statistical analysis

Results are expressed as means ± SEMs, and were assessed using paired two-tailed tests. Statistical significance was accepted for *P* values of < 0.05.

Ethics statement

All subjects enrolled in this study provided written informed consent, and the study protocol was approved by the Seoul National University Hospital institutional review board (H-1012-061-344). All experimental procedures were performed with the approval of the Seoul National University institutional animal care and use committee.

RESULTS

SEMA3A and NP1 expressions in induced sputum of asthmatics

A total of 31 asthmatics and 64 normal controls were enrolled. The characteristics of enrolled subjects are shown in Table 1. Sputum *SEMA3A* and *NP1* expressions were significantly high-

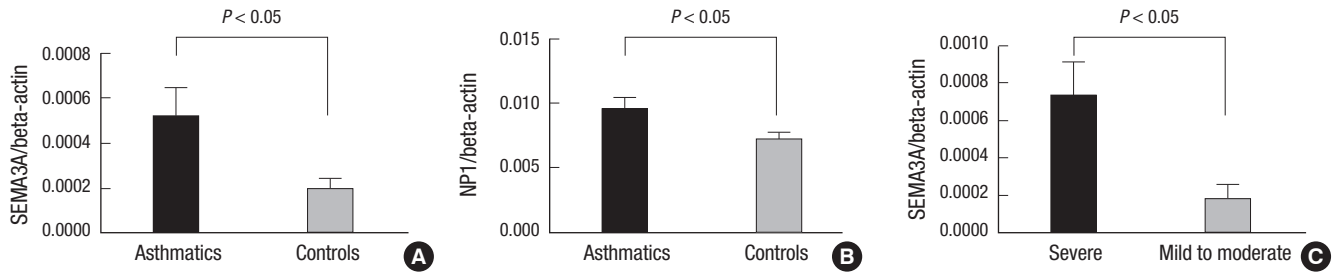


Fig. 1. Sputum *SEMA3A* and *NP1* expressions of asthmatics. *SEMA3A* and *NP1* mRNA in induced sputum was qualified using real-time quantitative polymerase chain reaction. Both values were normalized by beta-actin mRNA. Data indicate means \pm SEM. (A) *SEMA3A* expressions (asthmatics vs normal controls). (B) *NP1* expressions (asthmatics vs normal controls). (C) *SEMA3A* expressions (severe asthmatics vs mild to moderate asthmatics).

Table 1. The characteristics of subjects enrolled

Characteristics	Asthmatics n = 31	Normal control n = 64
Age (yr)	49.6 \pm 14.6	49.1 \pm 13.9
Male (%)	11 (35.5)	14 (21.8)
FEV1pred.%	79.6 \pm 18.2	102.3 \pm 14.9
Eosinophil % in induced sputum	14.5 \pm 11.6	0.8 \pm 0.7

FEV1pred. %: Predicted % of forced expiratory volume in 1 second. Data indicate means \pm SD.

er in asthmatics than controls ($P < 0.05$, respectively) (Fig. 1A, B). In asthmatics, sputum *SEMA3A* expressions were significantly higher in subjects with severe asthma ($n = 18$) compared to those with mild to moderate asthma ($n = 13$) ($P < 0.05$) (Fig. 1C).

SEMA3A and NP1 expressions in a murine model of asthma

Our immunofluorescence staining showed increased expressions of *SEMA3A* and *NP1* in BAL cells obtained from asthmatic mice compared to control mice (Fig. 2A, B). RT-PCR also showed significant increased levels of *SEMA3A* and *NP1* mRNA in lung homogenate of asthmatic mice compared to control mice ($P < 0.05$, respectively) (Fig. 3A, B). We also confirmed that *VEGF* mRNA levels significantly increased in asthmatic mice ($P < 0.05$) (Fig. 3C).

Localization of VEGFR1, VEGFR2, and NP1 in a murine model of asthma

VEGF1, *VEGF2*, and *NP1* expressions were uniformly increased in asthmatic mice compared to control mice (Fig. 4A-C). In addition to endothelium, *VEGF1* and *VEGF2* expressions were observed on bronchial cells (Fig. 4A, B). *NP1* immunostaining on bronchial cells with membrane accentuation on basal cells was shown (Fig. 4C, arrow heads). For subcellular level, *VEGF1* expressions were found predominantly in the cytoplasm of fibroblast (Fig. 5B) and BEASE2B cells (Fig. 6B) with asymmetric and crescentic nature. Meanwhile, *VEGF2* expressions were confined in the nucleus of both cell co-localized with DAPI staining (Fig. 5C and 6C). Immunostaining of fibroblast and BEASE2B cells showed a predominantly membrane

pattern of *NP1* (Fig. 5D and 6D), consistent with its role as a transmembrane receptor. Interestingly, the membrane staining of *NP1* was often brighter at intercellular contacts (Fig. 6D, arrow head), which suggests an association with cell-cell adhesion structures. In addition, abundant granular cytoplasmic staining was also associated with the membrane pattern of *NP1* in both cells.

DISCUSSION

In the present study, we observed that *SEMA3A* and *NP1* expressions were significantly elevated in induced sputum of asthmatics compared to normal controls. In addition, we found that subjects with severe asthma showed significantly increased sputum *SEMA3A* and *NP1* expressions compared to subjects with mild to moderate asthma. Likewise, we observed increased expressions of *SEMA3A* and *NP1* on BAL cells and lung homogenates of asthmatic mice. Then, we evaluated the immunolocalization of *VEGFR1*, *VEGFR2*, and *NP1* expressions on mice lung tissue and their subcellular distributions using fibroblast and BEASE2B cell lines.

Our finding that *SEMA3A* expressions significantly increased in induced sputum of asthmatics and in lung of asthmatic mice may be explained as follows. As mentioned before, *VEGF* is one of the important mediators in the pathogenesis of asthma (7) and increased *VEGF* levels were reported in asthmatics (4-6). Interestingly, it was reported that *VEGF* exposure entailed a p38 mitogen-activated protein kinase-dependent increased of *SEMA3A* production as a negative feedback mechanism in normal mesothelial cells (24). Taken together, it is plausible that *SEMA3A* is produced much in secondary to increased levels of *VEGF* in the lung of asthmatics. *SEMA3A* display highly dynamic expression patterns in the developing nervous system (25). However, recent reports demonstrated that *SEMA3A*-immunoreactive cells were found in airway and alveolar epithelial cells of mouse lungs (26, 27) and *SEMA3A* was expressed by activated human dendritic cell and T cells (28). Therefore pulmonary epithelial cells and immune cells such as dendritic cells and T cells may be the source cells for *SEMA3A* in the pathogenesis of asthma.

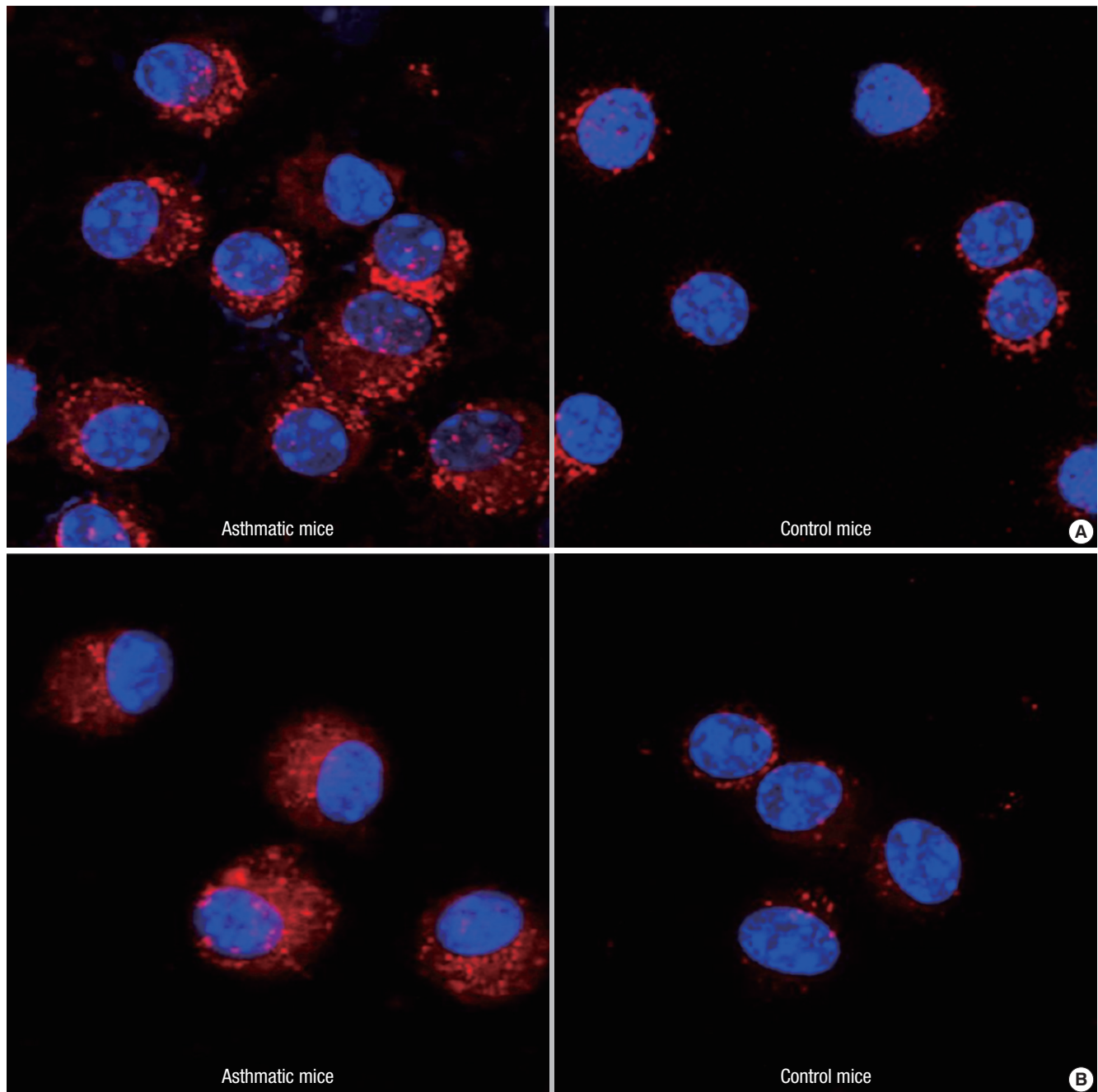


Fig. 2. SEMA3A and NP1 expressions on BAL cells of asthmatic mice. Original magnification, $\times 400$. blue (DAPI), nuclear staining. red (Cy3), SEMA3A and NP1. Representative images. (A) SEMA3. (B) NP1.

Maintenance of airway homeostasis provoked by lung injuries can be another explanation. A recent report demonstrating that cigarette smoke induced airspace enlargement and alveolar epithelial cell death was potentiated by conditional deletion of pulmonary epithelial NP1 in the lungs of adult animals led us to hypothesize that *Sema3A* might be an essential mediator of distal airspace homeostasis (29). As well known, structural damages are frequently encountered in the lung of asthmatics (30). Moreover, a recent report showed that SEMA3A may be impor-

tant determinants of plasticity following lung injury and alveolar remodeling (26). We may say that SEMA3A play a protective role in the pathogenesis of asthma either by maintaining lung homeostasis to injury or by counter-regulating VEGF signals.

Another interesting finding of our study was that NP1 expressions significantly increased in sputum of asthmatics and lung tissue of asthmatic mice. Investigators have described abundant expression of NP1 in pulmonary epithelium (27, 29, 31), and have suggested that NP1 is essential for normal lung structure

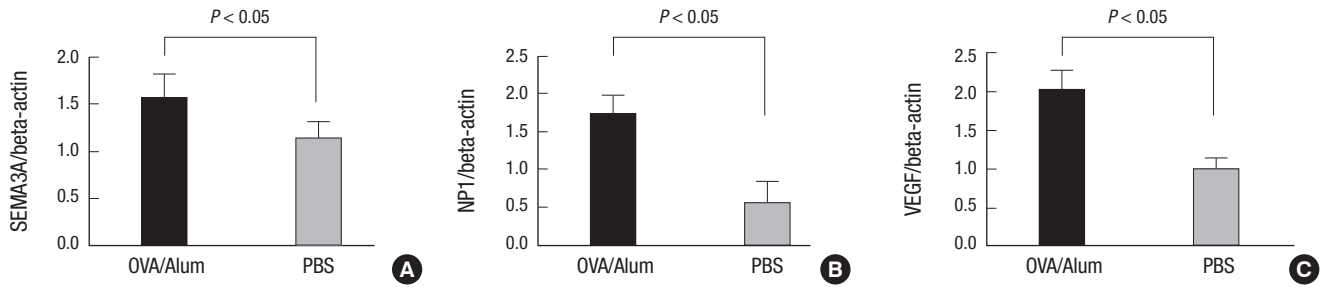


Fig. 3. *SEMA3A*, *NP1*, and *VEGF* expressions on lung homogenate of asthmatic mice. *SEMA3A* and *NP1* mRNA in lung homogenate of asthmatic mice was qualified using real-time quantitative polymerase chain reaction. Both values were normalized by beta-actin mRNA. Data indicate means ± SEM. (A) *SEMA3A* expressions (asthmatics [OVA/Alum] vs control mice [PBS]). (B) *NP1* expressions (asthmatics vs control mice). (C) *VEGF* expressions (asthmatics vs control mice).

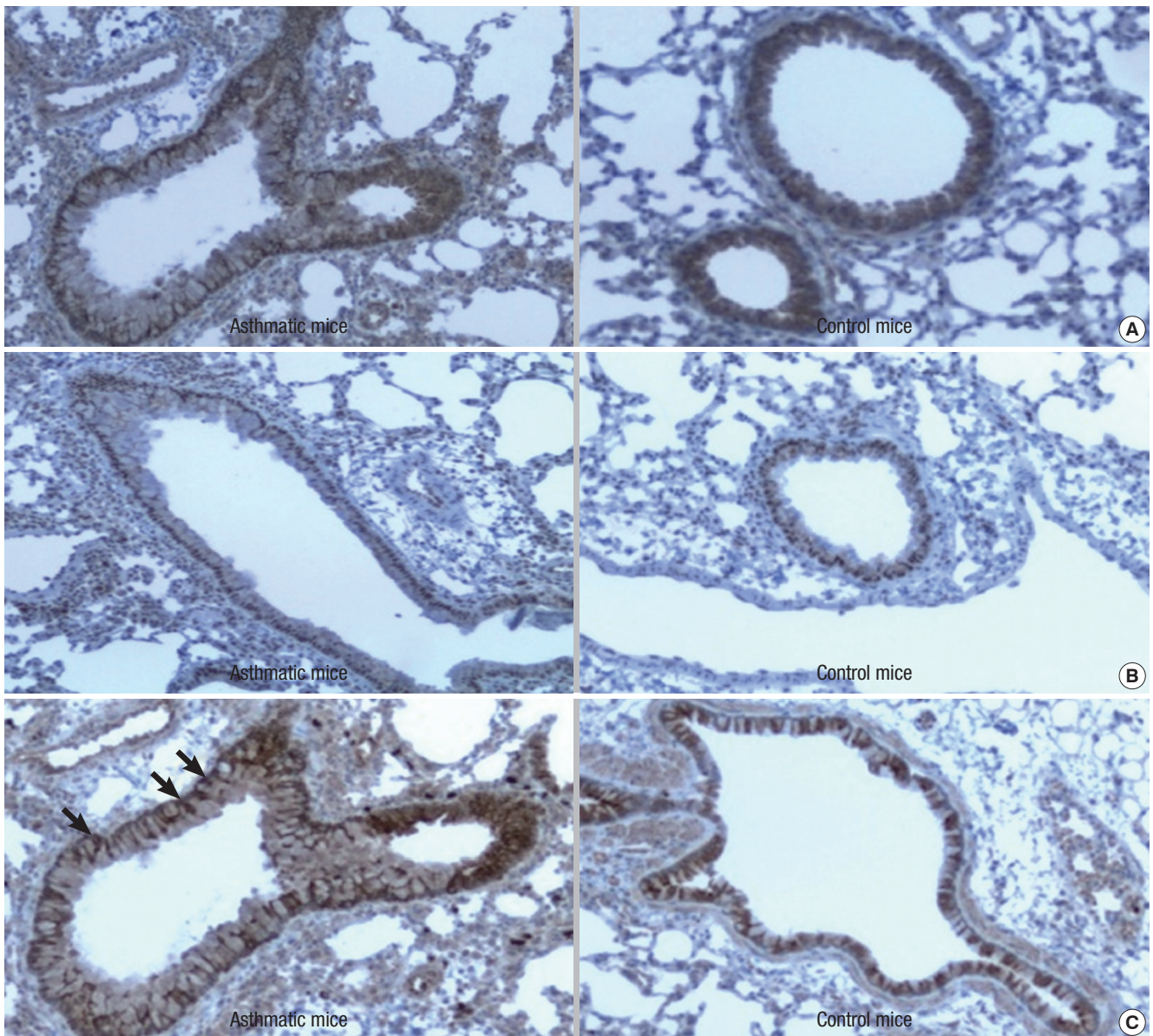


Fig. 4. Immunolocalization of VEGFR1, VEGFR2, and NP1 in the lung of asthmatic and control mice. Original magnification, × 400. Representative images. VEGFR1, VEGFR2, and NP1 expressions are uniformly increased in asthmatic mice compared to control mice. NP1 immunostaining on bronchial cells with membrane accentuation on basal cells is shown (arrow heads). (A) VEGFR1 (asthmatic vs control mice). (B) VEGFR2 (asthmatic vs control mice). (C) NP1 (asthmatic vs control mice).

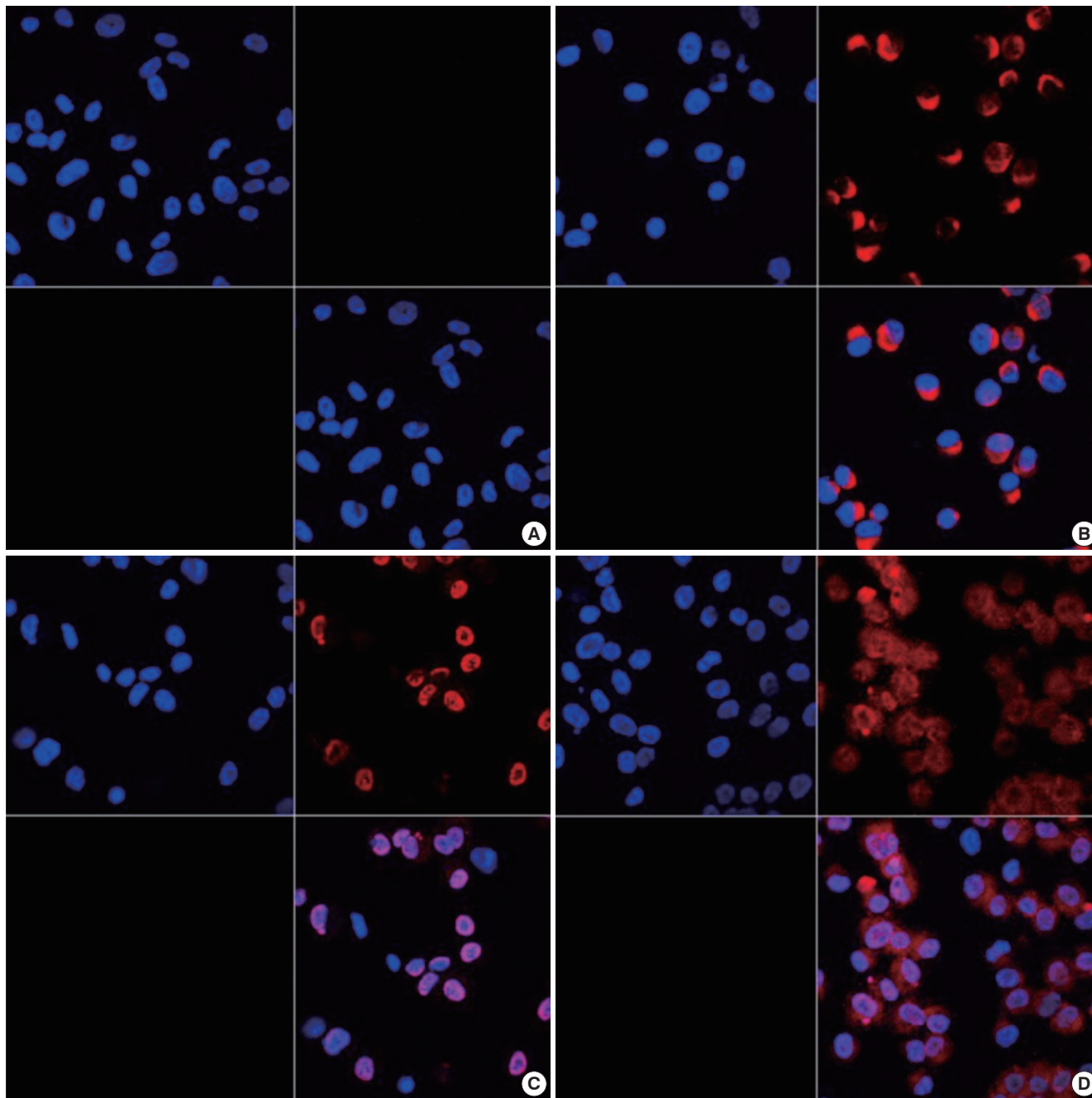


Fig. 5. VEGFR1, VEGFR2, and NP1 subcellular distributions on fibroblast cells. Original magnification, $\times 100$. blue (DAPI), nuclear staining. red (Cy3), VEGFR1, VEGFR2, and NP1. Left upper quadrant of each panel, DAPI staining image. Right upper quadrant of each panel, Cy3 staining image. Left lower quadrant of each panel, control image. Right lower quadrant of each panel, merged image. Representative images. VEGFR1 expressions are found predominantly in the cytoplasm with asymmetric and crescentic nature. VEGFR2 expressions are confined in the nucleus and co-localized with DAPI staining. NP1 expressions show predominantly membrane patterns associated with abundant granular cytoplasmic staining. (A) Control. (B) VEGFR1. (C) VEGFR2. (D) NP1.

(29). To the best of our knowledge, this is the first report showing that NP1 expression increased in asthma. However, little is known about the mechanisms underlying these observations. Nonetheless, an increase in NP1 expression in asthma raises an important issue in the treatment of asthma. It was reported that VEGF functions to autoregulate its own production in a positive feed-forward manner through NP1 in nasal epithelium of chron-

ic sinusitis patients with polyposis (32). In addition, blocking the VEGF pathway by blocking the VEGF co-receptor with anti-NP1 antibody was as effective in inhibiting VEGF-induced nasal epithelial cell growth as blocking the ligand with anti-VEGF antibody (33). Taken together, anti-NP1 treatment is needed for the complete block of VEGF pathway in the management of allergic diseases.

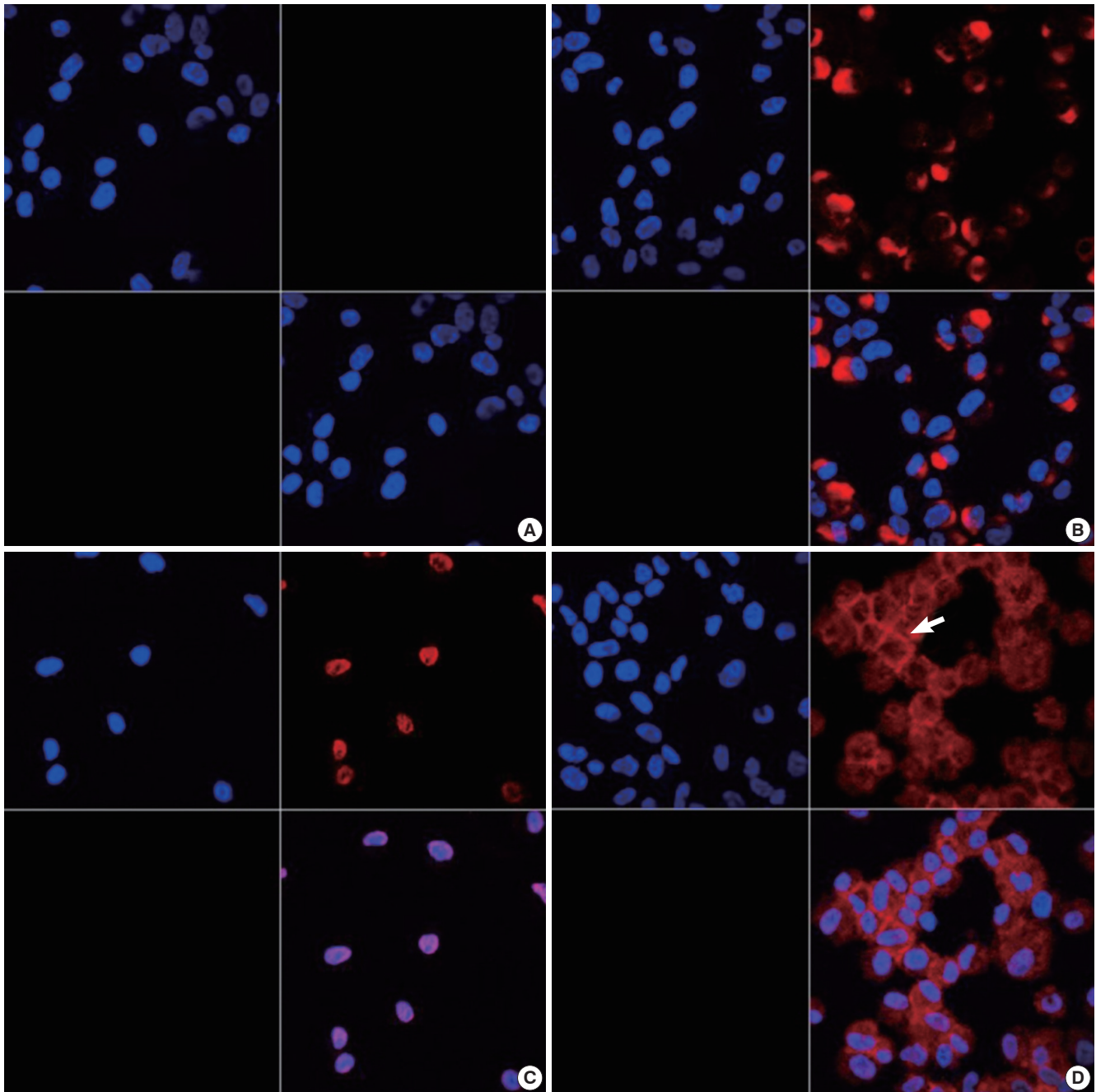


Fig. 6. VEGFR1, VEGFR2, and NP1 subcellular distributions on BEAS2B cells. Original magnification, $\times 100$. blue (DAPI), nuclear staining. red (Cy3), VEGFR1, VEGFR2, and NP1. Left upper quadrant of each panel, DAPI staining image. Right upper quadrant of each panel, Cy3 staining image. Left lower quadrant of each panel, control image. Right lower quadrant of each panel, merged image. Representative images. VEGFR2 and NP1 expressions on BEAS2B cells show similar patterns with those of fibroblast cells. The membrane staining of NP1 is often brighter at intercellular contacts (arrow head). (A) Control. (B) VEGFR1. (C) VEGFR2. (D) NP1.

The present study provided the first evidence for the possibility that SEMA3A or anti-NP1 can be a treatment option for asthma. Future studies are needed to further understand a therapeutic potential of SEMA3A.

DISCLOSURE

The authors declare that they have no proprietary, commercial,

or financial interests that could be construed to have inappropriately influenced this study.

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