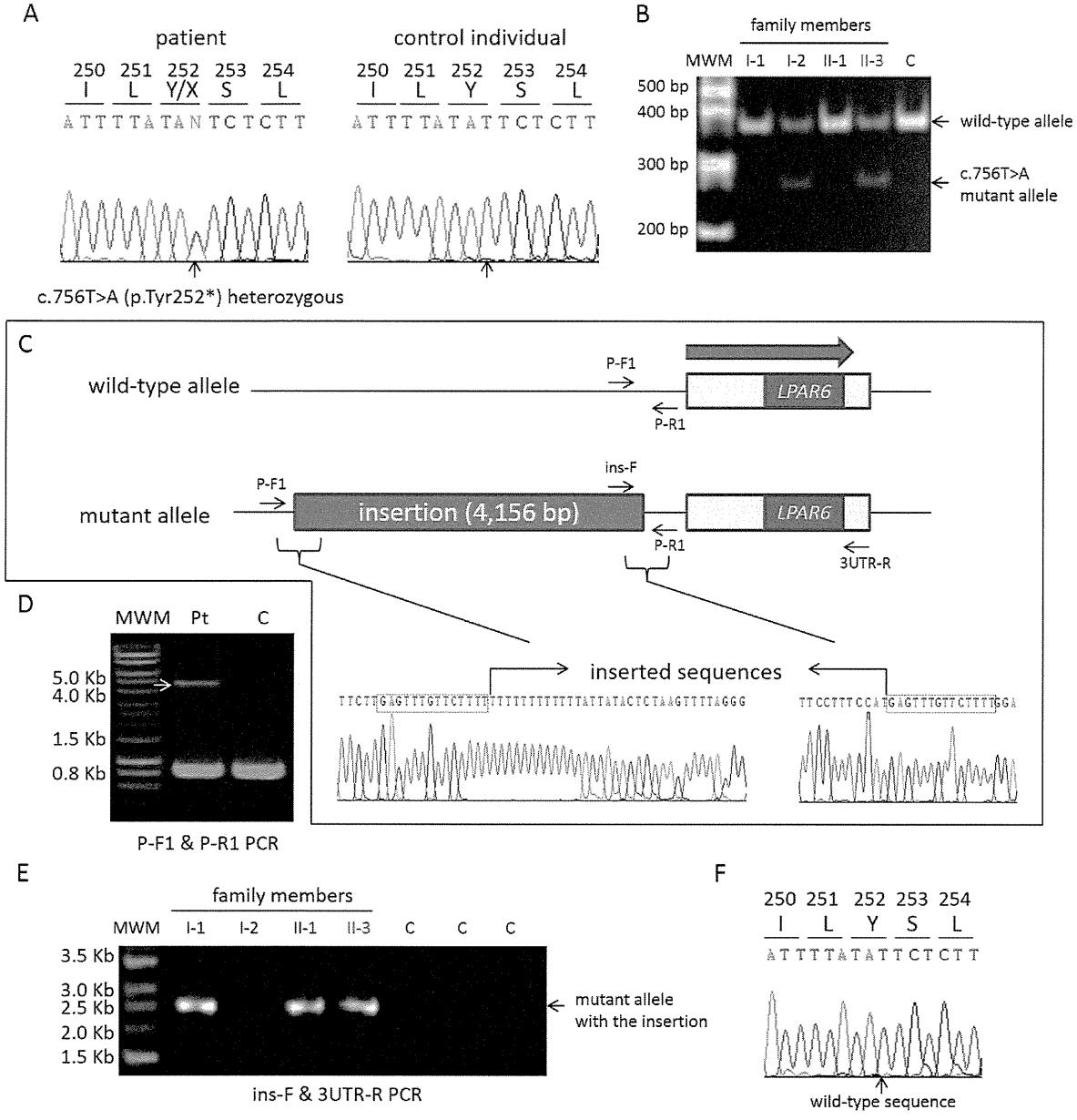

Fig. 2. *Identification of compound heterozygous LPAR6 mutations in the patient.* (A) Identification of a heterozygous nonsense *LPAR6* mutation c.756T > A (p.Tyr252*) in the affected individual. (B) Screening assay with the restriction enzyme *PsiI* revealed that the mutation was inherited from the mother. (C) Schematic representation of wild-type (upper panel) and mutant *LPAR6* allele with the insertion (lower panel). Positions of primers used in the analysis are indicated by black arrows. Direction of *LPAR6* gene transcription is indicated by a filled arrow. The non-coding and coding sequences of *LPAR6* are indicated with yellow and red boxes, respectively. The inserted sequences (4156 bp) are indicated by a blue box. Each boundary sequence of the insertion is bracketed underneath the blue box and expanded below. The duplicated sequences, 14 bp in size, are indicated by dotted boxes. (D) Results of PCR amplification with primers, P-F1 and P-R1 (C). Note that a large fragment (shown by a white arrow) was amplified only from the patient's genomic DNA (Pt). (E) PCR using primers, ins-F and 3UTR-R (C), specifically amplified a fragment derived from the mutant allele with the insertion. This assay revealed that the patient's father (I-1) and brother (II-1) also carried the mutation. (F) Direct sequencing of the PCR product from the patient (E) showed wild-type sequence at position c.756 of the *LPAR6* gene. MWM, molecular weight markers (B, D and E); C, control individuals (B, D and E). The patient (II-3) is indicated by red text (B and E).

transcription start site, and there was a 14 bp-duplication of *LPAR6* promoter sequences at both borders of the insertion (Fig. 2C). We then performed screening assay using an insertion-specific forward primer (LPAR6-ins-F) and an *LPAR6*-specific reverse primer (LPAR6-3UTR-R) and found that both his unaffected father (I-1) and brother (II-1) also had this insertion (Fig. 2E). Neither his mother (I-2) nor 100 control individuals carried this insertion (Fig. 2E; data not shown). Direct-sequencing analysis of the PCR product from the patient showed WT sequence at position c.756 in the *LPAR6* gene (Fig. 2F). Detailed analysis of the insertion using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the sequences were derived from intron 23 of the *RB1* gene (Supplementary Fig. 2A). In order to determine whether the corresponding sequences were deleted from intron

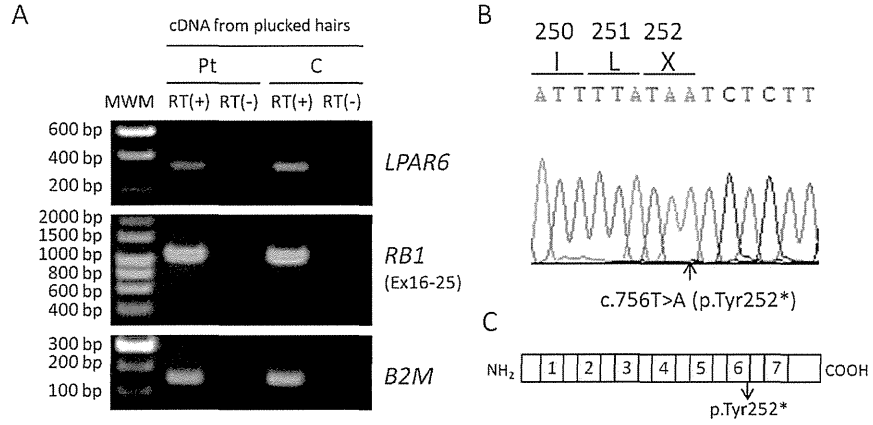

Fig. 3. Only *LPAR6*-mRNA from the c.756T > A (p.Tyr252*) mutant allele was expressed in the patient's hair follicles. (A) Reverse transcription (RT)-PCR amplification of cDNAs derived from *LPAR6* (top panel), *RB1* (middle panel), and *B2M* (bottom panel) from the follicular RNA of the patient or a control individual. The *B2M*-cDNA was amplified as a control. PCR without RT did not lead to any products, indicating that there was no genomic DNA contamination in the samples. +/− denotes reactions with or without RT. MMW, molecular weight markers; Pt, patient; C, control individual. (B) Direct sequencing of the *LPAR6* cDNA amplified from the patient's sample revealed only the c.756T > A (p.Tyr252*)-mutant *LPAR6*. (C) Schematic representation of the LPA_6 protein. Transmembrane domains are indicated by yellow boxes. Position of the mutation p.Tyr252* is indicated by an arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

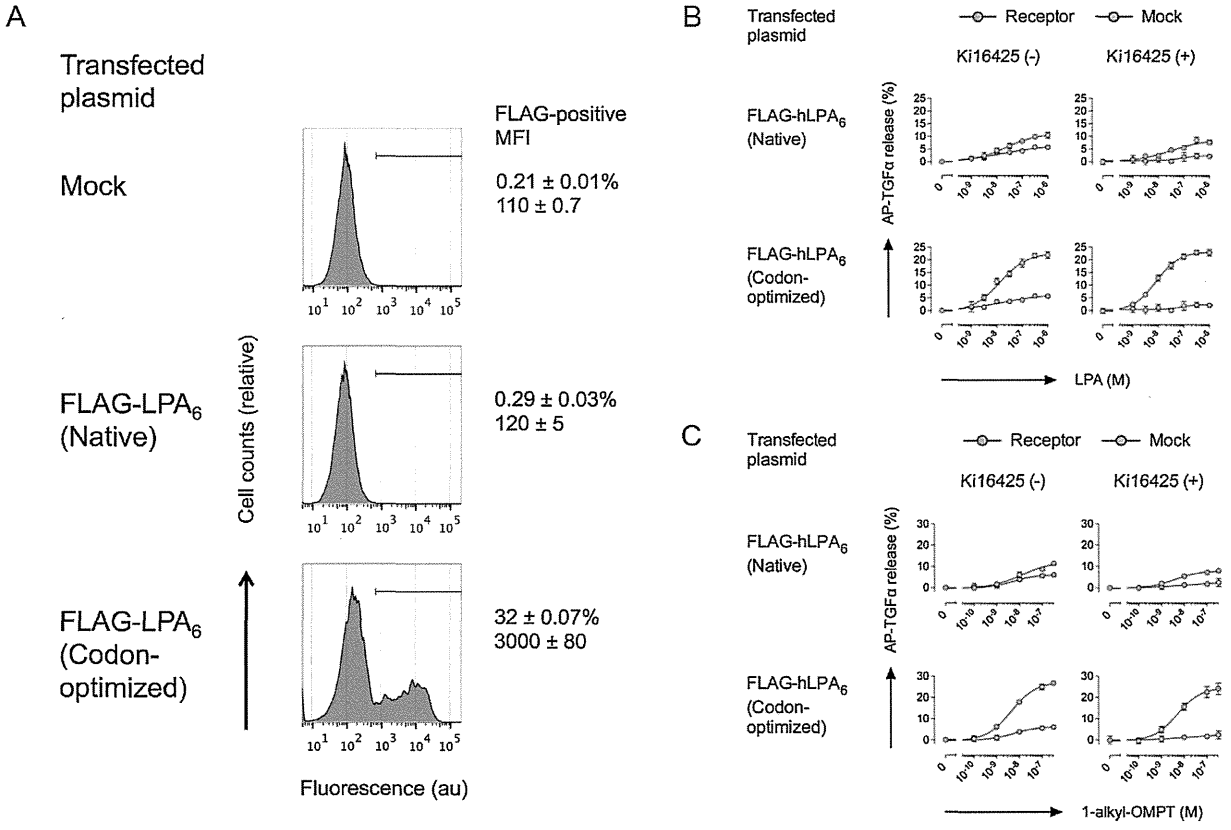

Fig. 4. *Improved expression and detection of LPA_6 by using codon optimization and Ki16425 treatment.* (A) Cells transfected with the indicated N-terminally FLAG epitope-tagged LPA_6 (native or codon-optimized sequence) or an empty vector (mock) were subject to flow cytometric analysis with an anti-FLAG antibody. FLAG-positive cells and mean fluorescent intensity are shown on the right (mean ± SD values of three culture replicates in a single assay). Data are representative of two independent experiments with similar results. (B, C) Cells transfected with AP-TGFα-encoding plasmid vector together with N-terminally FLAG epitope-tagged LPA_6 (native or codon-optimized sequence) or an empty vector (mock) were harvested and seeded in a 96-well plates; the seeded cells were treated with the indicated concentration of LPA (B) or 1-alkyl-OMPT (C) for 1 h in the presence (±) or absence (−) of 10 μM Ki16425, a LPA_{1-3} inhibitor. Release of AP-TGFα into the conditioned media was quantified by separating conditioned media from cells and measuring AP activity in both conditioned media and cells using *p*-nitrophenylphosphate as a substrate. Spontaneous AP-TGFα release in a vehicle-treated condition was set at a baseline. Note that Ki16425 treatment supressed LPA- and 1-alkyl-OMPT-induced responses in mock-transfected cells and that codon opmization enhanced LPA_6 responses. Symbols and error bars indicate mean and SD values, respectively, of three culture replicates in a single assay. Data are representative of two independent experiments with similar results.

23 of *RB1*, we amplified this region by PCR and found that intron 23 was present in both *RB1* alleles from the patient (Supplementary Fig. 2B).

3.3. Evidence for loss of transcription from the mutant LPAR6 allele with the insertion

To investigate how each mutation affected *LPAR6* expression, we used total RNA samples from hairs plucked from the patient or a control individual to perform RT-PCR analysis. *LPAR6* cDNA was amplified from each sample (Fig. 3A). However, direct sequencing of patient *LPAR6* cDNA indicated that only *LPAR6* cDNA from the c.756T > A (p.Tyr252*) was present; this finding indicated that the paternal mutant allele with the insertion in the *LPAR6* promoter was not transcribed (Fig. 3B). In PCR for the *RB1* cDNA, a clear single fragment was amplified from patient-derived or control-derived cDNA samples with similar efficiency. These findings indicated that the *LPAR6* promoter insertion did not affect splicing of *RB1* transcripts (Fig. 3A). Taken together, these results strongly indicated that only the p.Tyr252*-mutant LPA_6 protein would be stably expressed in the patient's HFs.

3.4. Establishment of a sensitive assay system for LPA_6

The c.756T > A (p.Tyr252*) mutation is located within the 6th transmembrane domain of the LPA_6 protein (Fig. 3C). To investigate how the mutation affected the function, we tried to perform a series of in vitro analysis in cultured cells. We initially transfected an N-terminally FLAG-tagged WT LPA_6 with native nucleotide sequences into HEK293FT cells and performed flow cytometry with an anti-FLAG antibody. However, to our surprise, the plasma membrane localization of the native LPA_6 was undetectable (Fig. 4A). Furthermore, the native LPA_6 poorly responded to LPA and 1-alkyl-OMPT (Fig. 4B and C). Through a serial of improvement trials, we found a codon-optimized LPA_6, which kept the amino acid sequences identical (Supplementary Fig. 1), efficiently localized to the plasma membranes (Fig. 4A) and the response to its agonists was also significantly enhanced (Fig. 4B and C). In addition, we found that pretreatment with an LPA_{1-3} antagonist Ki16425 almost completely suppressed the background response and improved the detection specificity of LPA_6-dependant activation (Fig. 4B and C). Collectively, we have established a sensitive assay system for LPA_6 and used it throughout this study.

3.5. The p.Tyr252*-mutant LPA_6 failed to localize to plasma membranes and did not function as an LPA receptor

We transiently overexpressed either WT or p.Tyr252* (Y252X)-mutant LPA_6 proteins and analyzed their plasma membrane localization. Cells that expressed WT LPA_6 on cell surfaces were detected even when the amount of transfected expression construct was as low as 1 ng per well; the cell surface expression of WT LPA_6 became more evident in a dose-dependent manner (Fig. 5). In contrast, expression of the Y252X-mutant LPA_6 on cell surfaces was not evident in any sample, even when 100 ng per well of expression construct was used for transfection (Fig. 5). The results clearly showed that the Y252X-mutant LPA_6 did not localize to plasma membranes.

We then investigated whether the p.Tyr252* (Y252X)-mutant LPA_6 responded to LPA_6 agonists. Both LPA and 1-alkyl-OMPT significantly activated the WT-LPA_6 (Fig. 6). Notably, WT LPA_6 responded to 1-alkyl-OMPT even when 0.1 ng of the expression construct was transfected per well (Fig. 6). However, the Y252X-mutant LPA_6 did not show any response to either molecule; these findings indicated that the mutant protein did not function as an LPA receptor (Fig. 6).

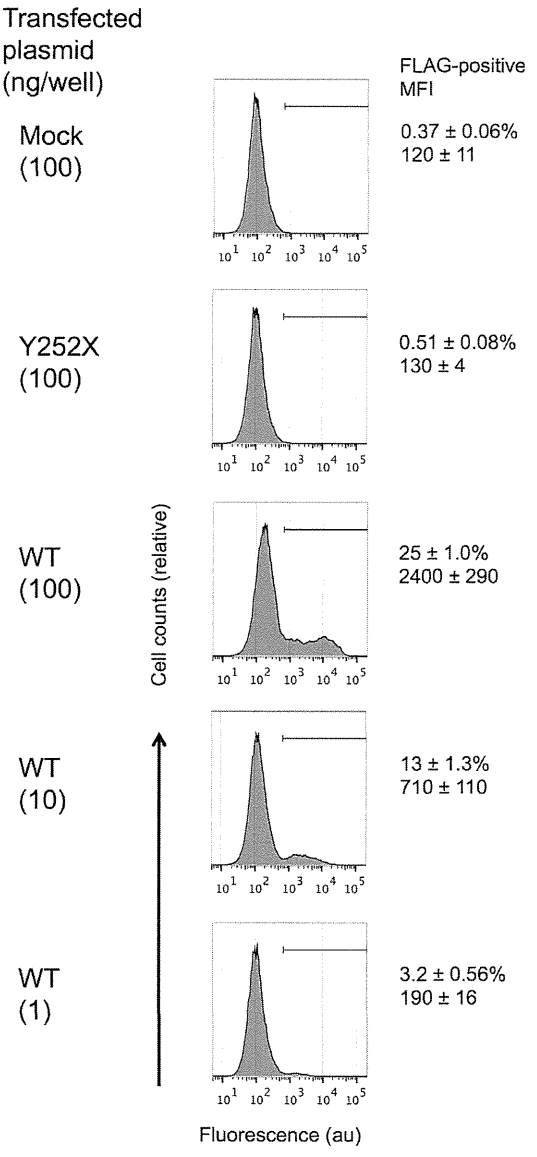

Fig. 5. *The p.Tyr252*-mutant LPA_6 protein failed to localize at plasma membranes.* Cells transfected with the indicated amount of N-terminally FLAG epitope-tagged LPA_6 (wild-type (WT) or p.Tyr252* (Y252X)-mutant) or an empty vector (Mock) were subject to flow cytometric analysis with an anti-FLAG antibody. FLAG-positive cells and mean fluorescent intensity are shown on the right (mean ± SD values of three culture replicates in a single assay). Data are representative of two independent experiments with similar results.

3.6. The p.Tyr252*-mutant LPA_6 disrupted the PA-$PLA_1\alpha$/LPA/LPA_6 axis

Finally, we used PA-$PLA_1\alpha$ to test whether the p.Tyr252* (Y252X)-mutant LPA_6 reacted to naturally occurring LPA species. As shown in previous studies [22,23,39], co-transfection of WT PA-$PLA_1\alpha$ and WT LPA_6 expression constructs markedly activated LPA_6 signaling; however, this level of activation was not evident when WT LPA_6 was co-expressed with a S154A-mutant PA-$PLA_1\alpha$; this recombinant mutant protein carried a critical amino acid substitution at a catalytic residue of PA-$PLA_1\alpha$ protein (Fig. 7). By contrast, the Y252X-mutant LPA_6 did not show any activity even when co-expressed with WT PA-$PLA_1\alpha$ (Fig. 7). Taken together, these findings indicated that Y252X-mutant LPA_6 failed to respond to endogenously produced LPA species.

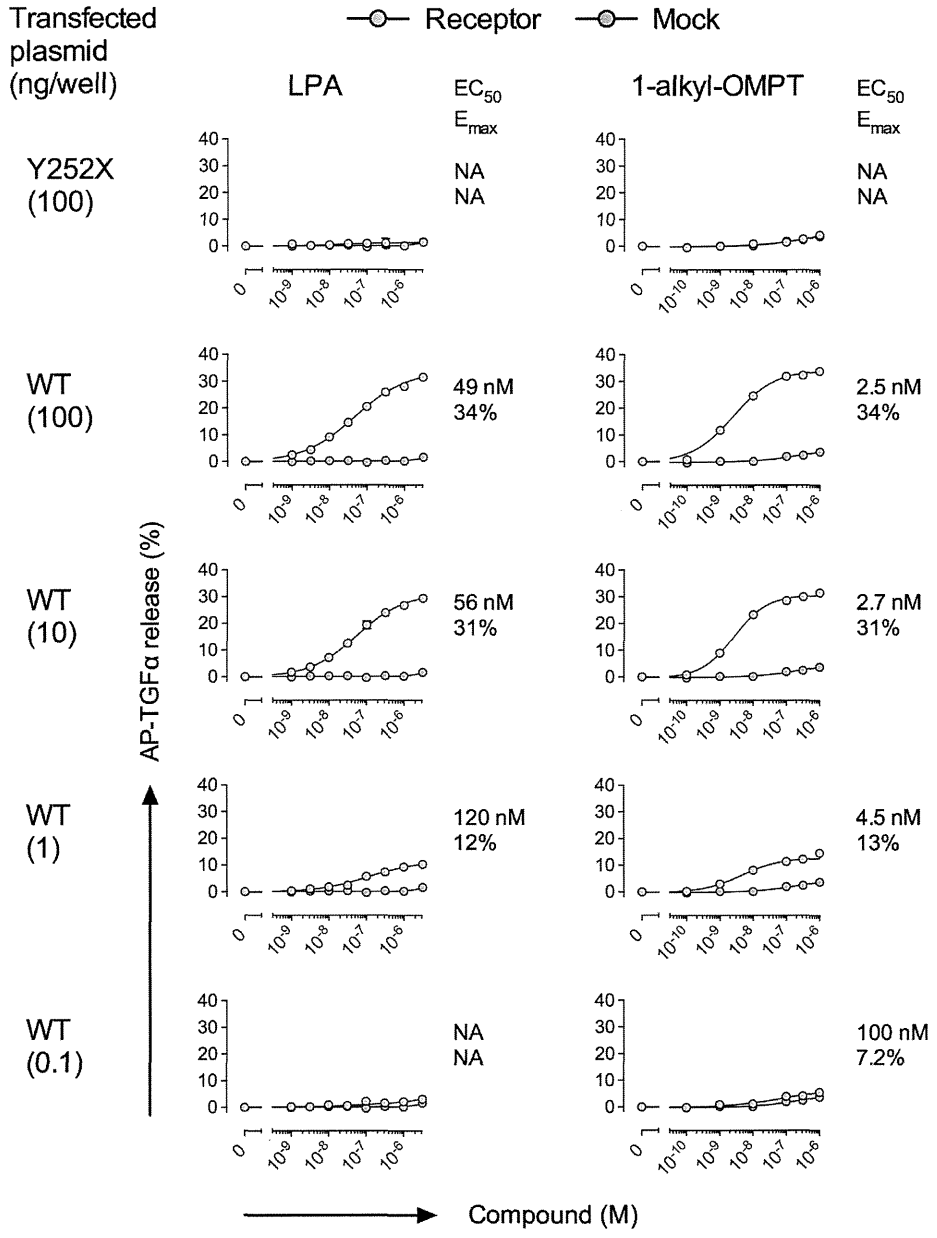

Fig. 6. *The p.Tyr252*-mutant LPA_6 protein did not respond to LPA_6 agonists.* Cells transfected with AP-TGFα-encoding plasmid vector together with indicated amount of LPA_6-encoding plasmid vector (wild-type (WT) or p.Tyr252* (Y252X)-mutant) or an empty plasmid vector (Mock) were harvested and seeded in a 96-well plates; the seeded cells were treated with the indicated concentration of 1-alkyl-OMPT or LPA for 1 h in the presence of 10 μM Ki16425. AP-TGFα release was determined as indicated in Fig. 4 legend and the method section. EC_{50} and E_{max} values were obtained by fitting data to a four-parameter logistic curve. Symbols and error bars indicate mean and SD values, respectively, of three culture replicates in a single assay. Data are representative of two independent experiments with similar results. Note that error bars are smaller than symbols in most data points. NA, not available.

4. Discussion

In this study, we analyzed a Japanese family with ARWH/hypotrichosis and identified compound heterozygous *LPAR6* mutations in the patient (Figs. 1 and 2). We initially searched for mutations in the *LIPH* gene because previously only *LIPH* mutations have been identified in Japanese patients with this disease [19,22,23,25,26]. Moreover, the clinical features of the affected individual analyzed in this study were indistinguishable from those of patients with *LIPH* mutations. To the best of our knowledge, we herein report the first Japanese case of ARWH/hypotrichosis caused by *LPAR6* mutations, and our results further indicated a close relationship in function between the *LIPH* and *LPAR6*.

Of the two *LPAR6* mutations identified in this patient, one was a large insertion mutation within the *LPAR6* promoter region; this allele was paternally inherited (Fig. 2C–F). The insertion destroyed the structure of the promoter and resulted in loss of *LPAR6* transcription from the mutant allele, as demonstrated by RT-PCR experiments (Fig. 3A and B). Interestingly, the inserted sequences were derived from intron 23 of the *RB1* gene (Supplementary Fig. 2A). Our results indicated that a part of the sequences of intron 23 of the *RB1* gene had accidentally been duplicated and inserted into the *LPAR6* promoter, which is located in intron 17 of *RB1*

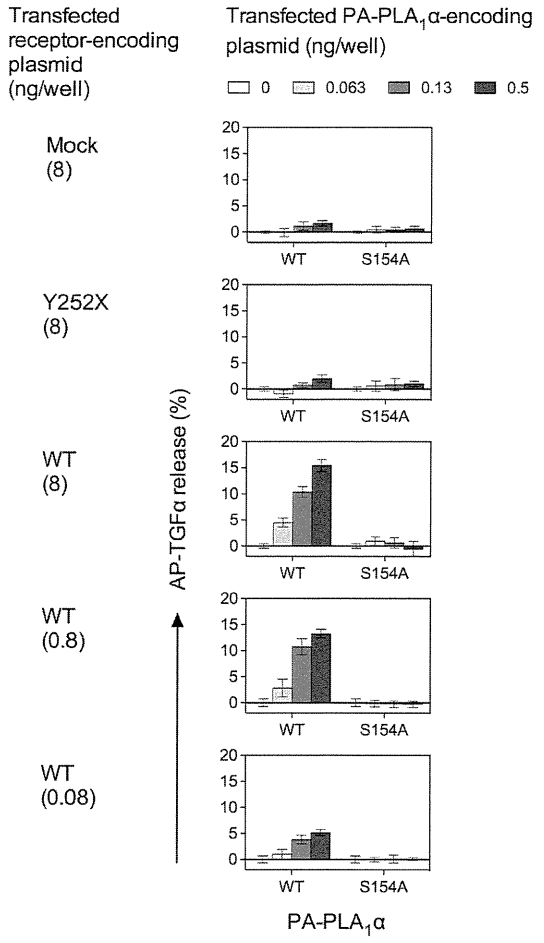

Fig. 7. *Disruption of the PA-PLA$_1$α/LPA/LPA$_6$ axis by the p.Tyr252*-mutant LPA$_6$ protein.* Cells were seeded in 96-well plates and transfected with various combinations of AP-TGFα-encoding plasmid vector, LPA$_6$-encoding plasmid vector (wild-type (WT), p.Tyr252* (Y252X)-mutant), or an empty vector (Mock) and PA-PLA$_1$α-encoding plasmid vector (WT or p.Ser154Ala (S154)-mutant). After 24 h incubation, release of AP-TGFα was quantified by separating conditioned media from cells and measuring AP activity in both conditioned media and cells using *p*-nitrophenylphosphate. Spontaneous AP-TGFα release in an enzyme plasmid-free condition was set at a baseline. Symbols and error bars indicate mean and SD values, respectively, of six culture replicates in a single assay. Data are representative of two independent experiments with similar results.

(Supplementary Fig. 2A). Such copy number variation is relatively rare, and this is the first *LPAR6* mutation detected in the *LPAR6* promoter region.

The c.756T > A (pTyr252*) nonsense mutation identified in the maternal *LPAR6* allele is predicted to generate a truncated LPA$_6$ protein that lacks the C-terminus, including the 7th transmembrane domain (Figs. 2A and B and 3C). Based on the results of RT-PCR experiment, only the p.Tyr252*-mutant LPA$_6$ was expected to be expressed stably in the patient's HFs. Previously, 24 distinct disease-causing mutations have been identified in the *LPAR6* gene [8,9,27–37]. However, expression and functional analyses for these mutations have rarely been performed. Additionally, two premature termination codon mutations, p.Gln155* and p.Lys125Asnfs*37, reportedly cause aberrant LPA$_6$ localization within the cytoplasm [8]. Moreover, LPA reportedly does not activate the p.Lys125Asnfs*37-mutant LPA$_6$ [8]. Similarly, the p.Tyr252*-mutant LPA$_6$ did not localize at plasma membranes (Fig. 5) and did not respond to any of the defined LPA$_6$ agonists tested (Fig. 6). Finally, we clearly demonstrated that the p.Tyr252*-mutant LPA$_6$ disrupted the PA-PLA$_1$α/LPA/LPA$_6$ axis, which caused ARWH/hypotrichosis in our patient (Fig. 7). We would like to emphasize that the activation of WT LPA$_6$ (Figs. 4, 6 and 7) was detected much more sensitively than that shown in previous studies [8,13,14,22,23,39]. In this study, we have added two critical modifications into the *in vitro* assay system that we (A.I. and J.A.) have recently reported [13,14]: (1) we used codon-optimized LPA$_6$ to significantly increase the efficiency of plasma membrane localization of recombinant LPA$_6$ (Fig. 4A, Supplementary Fig. 1); (2) we treated the cells with an LPA$_{1-3}$ antagonist Ki16425 to inhibit the effect of endogenous LPA receptors expressed in HEK293FT cells [13] (Fig. 4B and C). As a result, we have finally established an assay system to sensitively detect LPA$_6$ activation (Fig. 4). We believe that our assay system will become a useful tool to search for ideal agonists of LPA$_6$ which can be used to produce a medicine for the treatment of ARWH/hypotrichosis in the future.

Our results provide important information regarding the molecular basis for ARWH/hypotrichosis in the Japanese population; they also further highlight the crucial roles of the PA-PLA$_1$α/LPA/LPA$_6$ signaling in HF development and hair growth in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.03.006.

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Article

Variation in the Effect of Particulate Matter on Pulmonary Function in Schoolchildren in Western Japan and Its Relation with Interleukin-8

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Abstract: This study aimed to investigate the effects of particulate matter (PM) on pulmonary function in schoolchildren, as well as the relationships of these effects with interleukin-8. Morning peak expiratory flow (PEF) was measured daily in 399 children during April–May 2012, and in 384 of these children during March–May 2013. PEF's

association with the daily levels of suspended particulate matter (SPM) and PM < 2.5 μm ($PM_{2.5}$) was estimated using a linear mixed model. Interleukin-8 promoter activity was assessed in THP-G8 cells stimulated by fallen PM collected at Tottori University Hospital during four periods (two in 2012 and two in 2013). An increase of 14.0 μg/m^3 in SPM led to PEF changes of −2.16 L/min in 2012 and −0.81 L/min in 2013, respectively. An increment of 10.7 μg/m^3 in $PM_{2.5}$ was associated with PEF changes of −2.58 L/min in 2012 and −0.55 L/min in 2013, respectively. These associations were only significant in 2012. Interleukin-8 promoter activity was significantly higher in both periods of 2012 than in 2013. There was a significant association between pulmonary function in schoolchildren and daily levels of SPM and $PM_{2.5}$, but this association may differ depending on the PM's ability to elicit interleukin-8 production.

Keywords: interlukin-8; peak expiratory flow; $PM_{2.5}$; schoolchildren; suspended particle matter

1. Introduction

Particulate matter (PM) is an important component of ambient air pollution. It is categorized based on particle size as PM_{10}, $PM_{2.5}$, or $PM_{0.5}$, which represent median aerodynamic diameters of less than 10, 2.5, and 0.5 μm, respectively. Numerous epidemiological research studies have demonstrated that exposure to PM correlates with human health risks [1,2]. For example, the specialized cancer agency of the World Health Organization, the International Agency for Research on Cancer, has reported that exposure to PM increases risk of lung cancer [3]. Globally, ambient pollutants are now the third leading contributor to disability-adjusted life years associated with chronic respiratory disease [4].

Similarly, many epidemiological studies have demonstrated significant associations between PM and pulmonary function in children [5]. Short-term exposures to PM can reduce pulmonary function and increase respiratory symptoms, especially among children with respiratory diseases [6,7]. However, several studies have been unable to find an association between PM and pulmonary function in children with asthma [8,9]. A European multicenter study of asthmatic children failed to detect any consistent relationship between PM and short-term health effects, despite the wide ranges of climatic conditions and pollutant mixtures that were encountered across the sites [10].

PM is continuously affected by both stationary sources (e.g., power plants, industries, incinerators, and residential heating) and mobile sources (e.g., road traffic) [11–13]. It can also change size, morphology, phase state, and chemical composition via coagulation, condensation, and chemical reactions [14]. In one study, the inflammatory potential of ambient PM exhibited heterogeneity based on city and season [15]. Neutrophils migrate to the lung during acute inflammation induced by exposure to air pollutants [16]. One study found that exposure to air pollutants also increased both the concentration of interleukin (IL)-8 in bronchial lavage fluid and IL-8 mRNA expression in bronchial biopsy tissue from healthy participants [17]. Considering this previous evidence, the various adverse respiratory effects that are induced by exposure to PM may be related to the production of pro-inflammatory cytokines, and the presence or absence of cytokine production may depend on the particular composition of the PM.

In 2012, a study investigated the influence of air pollutants on pulmonary function (as assessed via peak expiratory flow [PEF]) in schoolchildren in western Japan. In 2013, we also conducted an extended survey to investigate the different influences of Asian dust storms on pulmonary function in children [18]. In the current study, we used the same cohort of schoolchildren who were surveyed in 2012 and 2013 to assess the effects of different PM on the pulmonary function of children who did and did not have asthma. For each year of data, we also used an IL-8 luciferase assay to investigate IL-8 promoter activity and its relationship with the detrimental effects of PM on pulmonary function.

2. Experimental Section

2.1. Participants

The study was performed in Matsue, which is the capital city of Shimane Prefecture and is located in Southwest Japan. The population of Matsue is about 200,000 and the area is 530.2 km^2. In March 2012, all fourth-grade students aged 8–9 years from four of the 35 elementary schools in Matsue were enrolled in the study. The four elementary schools were within 10 km of each other and all participants lived within 1 km of the schools. The children's morning PEF values were monitored daily from March to May of 2012 and 2013. March 2012 was used as trial period to allow the children to familiarize themselves with the monitoring process.

The recruitment process for 2012 and 2013 is shown in Figure 1. A total of 401 children were recruited into the study in March 2012. Two were subsequently excluded because of failures to keep the daily PEF records. Thus, records of daily PEF were analyzed for 399 children in 2012. In March 2013, we re-recruited the same 401 children, although one child was excluded because of Moyamoya disease. Sixteen children were subsequently excluded because of failures to keep the daily records of PEF value. Thus, records of daily PEF values were analyzed for 384 children in 2013.

In both March 2012 and March 2013, each participant was asked to record his or her age, sex, height, and weight, as well as the presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies. Participants were defined as having asthma if they met any of the following criteria in the past year: (1) diagnosis of asthma by a pediatrician; (2) wheezing; (3) use of asthma medication; or (4) regular visits to a hospital for asthma. Similarly, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy were judged to be present if the participants met any of the following criteria in the past year: (1) diagnosis by a pediatrician; (2) use of relevant medication for the disease; or (3) regular visits to a hospital for the disease.

The study was approved by the institutional ethics committee of Tottori University (Ethics Committee of Tottori University, Approval Number 1764). The Matsue City Board of Education approved and submitted the study proposal to the schools. The study was also approved by the Parent Teacher Association (PTA) of each participating elementary school. The children and their parents were informed by teachers and provided written consent.

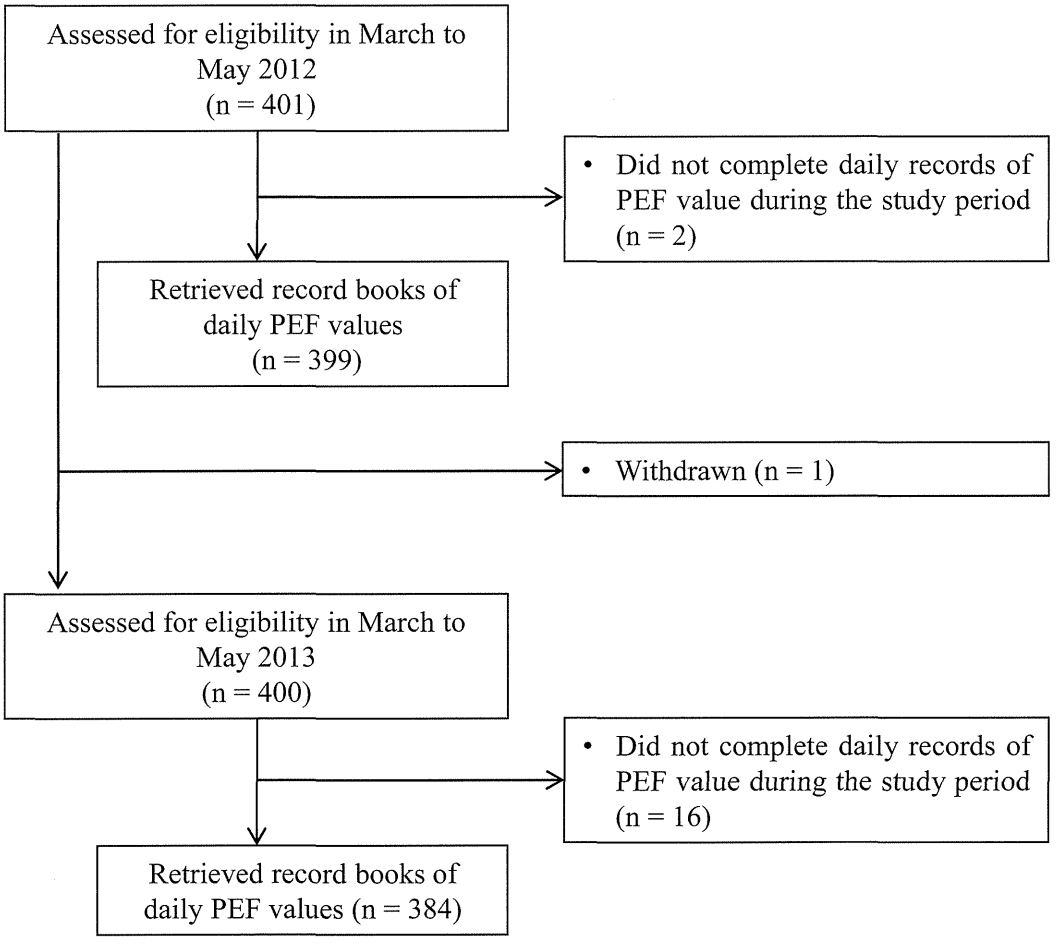

Figure 1. Flow chart showing the recruitment of the children in the study.

2.2. Monitoring of PEF

Before the study, the children and their teachers were taught how to measure PEF values. All children then measured their morning PEF values daily using peak flow meters (Mini-Wright, Harlow, UK; American Thoracic Society scale) from March to May of 2012 and 2013, excepting weekends and public holidays. Each child was asked to record his or her greatest PEF value based on three attempts after arriving at school between 8 AM and 9 AM.

2.3. Measurement of Air Pollutant Levels

PM is classified into several categories according to size. PM_{10} is defined as any particle measuring less than l0 μm in diameter with a 50% cut-off, while $PM_{2.5}$ as any particle measuring less than 2.5 μm in diameter with the same cut-off as PM_{10} [19]. In Japan, suspended particulate matter (SPM) is defined under the National Air Quality Standard as any particle with a diameter of less than 10 μm with a 100% cut-off [20]. The theoretical 50% cut-off diameter for SPM is assumed to be approximately 7 μm [20]. The particle diameter of SPM measurement in Japan is intermediate to the diameters that are used in the evaluation of $PM_{2.5}$ and PM_{10}. Although the daily fluctuations of SPM are similar to those of $PM_{2.5}$ [19,20], the constituents of PM may differ across countries. The Japanese Ministry of the Environment monitors the levels of SPM instead of PM_{10}. In Matsue City, the concentrations of

SPM, $PM_{2.5}$, sulfur dioxide (SO_2), nitrogen dioxide (NO_2), and ozone are also monitored by the Japanese Ministry of the Environment. These data were used to examine the relationships between changes in PEF values and air pollutant levels. Data on the daily temperature, humidity, and atmospheric pressure were obtained from the Japan Meteorology Agency in Matsue City. The observatories of the Japanese Ministry of the Environment and the Japan Meteorology Agency are both located within 10 km of the four elementary schools.

2.4. Preparation of the PM

Samples of fallen PM were collected four times at Tottori University Hospital, Yonago, which is located 30 km east of Matsue: (1) during April 7–April 20, 2012; (2) during April 26–May 10, 2012; (3) during April 8–22, 2013; and (4) during April 30–May 13, 2013. The collections were conducted with a large acrylic basin that had a collection area of 5,000 cm^2 and a depth of 30 cm [21]. The collected samples of fallen PM were sterilized at 121 °C for 30 min in an autoclave (Tomy SX-300; Tomy Co., Tokyo, Japan) and stored in a freezer at −20 °C to prevent the growth of bacteria and fungi. For stimulation of the THP-G8 cells, the fallen PM was diluted to various concentrations with distilled deionized water.

2.5. IL-8 Promoter-Luciferase Gene Reporter Assay

THP-G8 cells are a THP-1-derived reporter cell line that expresses stable luciferase orange (SLO) and stable luciferase red (SLR) genes under the control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively [22]. The THP-G8 cell line was cultured as described previously [22]. Luciferase activity (LA) was determined using a microplate luminometer with a Phelios multicolor detection system (Atto Corp., Tokyo, Japan) using Tripluc luciferase assay reagent (Toyobo Co., Osaka, Japan). IL-8 promoter activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA, and the fold induction of nSLO-LA was calculated as the nSLO-LA level of treated cells divided by that of untreated cells [22]. The induction of IL-8 promoter activity was measured by comparing THP-G8 cells (5 × 104 cells/100 μL/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) stimulated for 5 h with (a) solvent only (negative control); (b) 100 ng/mL lipopolysaccharide (LPS; positive control); or (c) 1 mg/mL PM collected in 2012 and 2013.

2.6. Statistical Analyses

To estimate the effect of exposures to SPM and $PM_{2.5}$ on the children's daily PEF values, we used linear mixed models that accounted for correlations among repeated measurements within the same participant [23,24]. The linear mixed models included a random intercept for the participants in the analysis. Additionally, the participants' individual characteristics (sex, height, weight, asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy), gaseous air pollutants (SO_2, NO_2, and ozone), and meteorological variables (daily temperature, humidity, and atmospheric pressure) were modeled as potential confounding factors. In our results, estimates are presented as the absolute difference in PEF values per interquartile range (IQR) change in exposure, and are accompanied by 95%

confidence intervals (CIs). Linear mixed model analyses were performed using R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Differences in the nSLO-LA of THP-G8 cells were evaluated using analysis of variance (ANOVA) in SPSS statistical software (Japanese ver. 21.0 for Windows; IBM Japan, Tokyo, Japan). All quoted p values are two-sided and the significance levels of all tests were set to 0.05.

3. Results

3.1. Characteristics of the Children

The characteristics of the children in the 2012 and 2013 studies are shown in Table 1.

Table 1. Characteristics of the schoolchildren included in the study.

	2012	**2013**
Number	399	384
Boy/Girl	205/194	194/190
Height (cm)	132.3 ± 5.9	137.7 ± 7.0
Boy	132.2 ± 5.5	136.9 ± 6.3
Girl	132.4 ± 6.4	138.5 ± 7.7
Weight (kg)	29.5 ± 5.8	32.4 ± 6.6
Boy	29.6 ± 6.2	32.3 ± 6.8
Girl	29.3 ± 5.4	32.6 ± 6.4
Allergic disease (number)		
Asthma	38	45
Allergic rhinitis	78	74
Allergic conjunctivitis	8	15
Atopic dermatitis	44	36
Food allergy	19	20

Data are shown as the mean ± standard deviation.

3.2. PEF

Results of the estimated changes in PEF values for interquartile range (IQR) increases in exposure to SPM and $PM_{2.5}$ are presented in Table 2. In the 2012 survey, SPM was significantly associated with PEF in both children who did and did not have asthma, with an increase of 14.0 μg/m^3 in SPM reducing the PEF value by 2.16 L/min (−2.06 L/min in children without asthma and −3.11 L/min in children with asthma). Similarly, $PM_{2.5}$ was negatively associated with the PEF value, with a 10.7 μg/m^3 increase in $PM_{2.5}$ decreasing the PEF value by 2.58 L/min in the total study cohort (−2.46 L/min in children without asthma and -3.69 L/min in children with asthma). In the 2013 survey, increases of 14.0 μg/m^3 in SPM and 10.7 μg/m^3 in $PM_{2.5}$ led to changes in the PEF value of −3.41 L/min and −2.42 L/min in children with asthma, respectively. However, in 2013, there was no significant association between the PEF value and either SPM or $PM_{2.5}$ for the total study cohort or for children without asthma. Figure 2 shows the relationship between the daily average PEF value and the daily levels of SPM and $PM_{2.5}$.

Table 2. Associations of PEF values with interquartile increases in SPM and $PM_{2.5}$ in linear mixed-effects models for the 2012 and 2013 surveys.

Year	Exposure Metric	IQR	All Children		
			Change in PEF Value (L/min)	95%CI	*p* Value
2012	SPM	14.0 μg/m³	−2.16	−2.88, −1.43	<0.0001
	$PM_{2.5}$	10.7 μg/m³	−2.58	−3.59, −1.57	<0.0001
2013	SPM	14.0 μg/m³	−0.81	−1.68, 0.06	0.068
	$PM_{2.5}$	10.7 μg/m³	−0.55	−1.30, 0.19	0.146
Year	**Exposure Metric**	**IQR**	**Children without Asthma**		
			Change in PEF Value (L/min)	**95%CI**	***p* Value**
2012	SPM	14.0 μg/m³	−2.06	−2.81, −1.30	<0.0001
	$PM_{2.5}$	10.7 μg/m³	−2.46	−3.51, −1.41	<0.0001
2013	SPM	14.0 μg/m³	−0.44	−1.37, 0.47	0.337
	$PM_{2.5}$	10.7 μg/m³	−0.29	−1.07, 0.49	0.464
Year	**Exposure Metric**	**IQR**	**Children with Asthma**		
			Change in PEF Value (L/min)	**95%CI**	***p* Value**
2012	SPM	14.0 μg/m³	−3.11	−5.70, −0.54	0.018
	$PM_{2.5}$	10.7 μg/m³	−3.69	−7.28, −0.10	0.044
2013	SPM	14.0 μg/m³	−3.41	−6.11, −0.70	0.014
	$PM_{2.5}$	10.7 μg/m³	−2.42	−4.70, −0.13	0.039

IQR: interquartile range; CI: confidence interval; PEF: peak expiratory flow; SPM: suspended particulate matter; $PM_{2.5}$: particulate matter smaller than 2.5 μm in diameter.

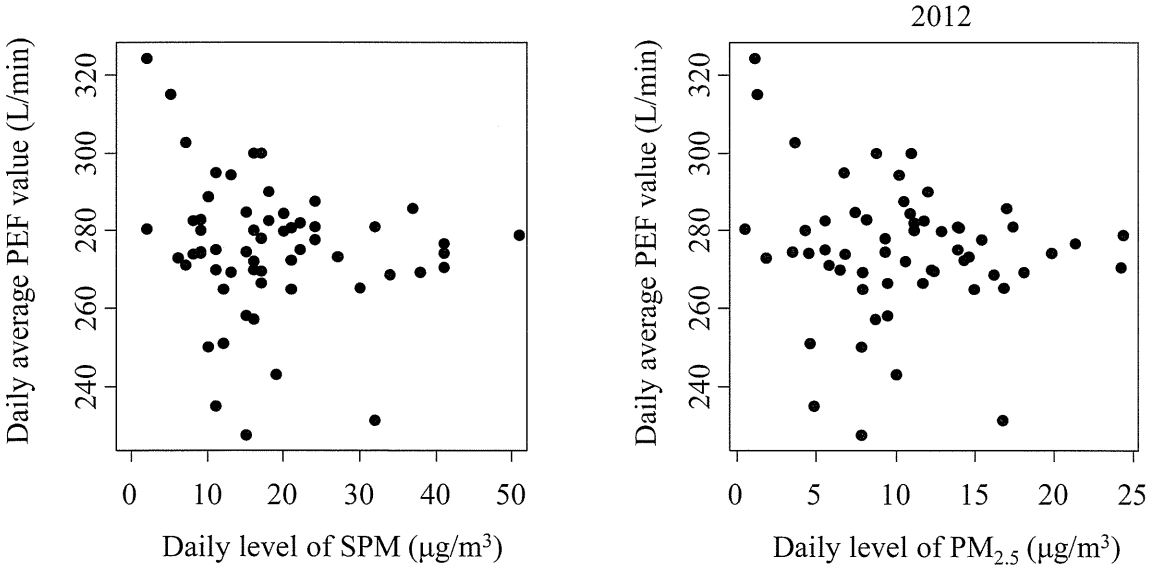

Figure 2. *Cont.*

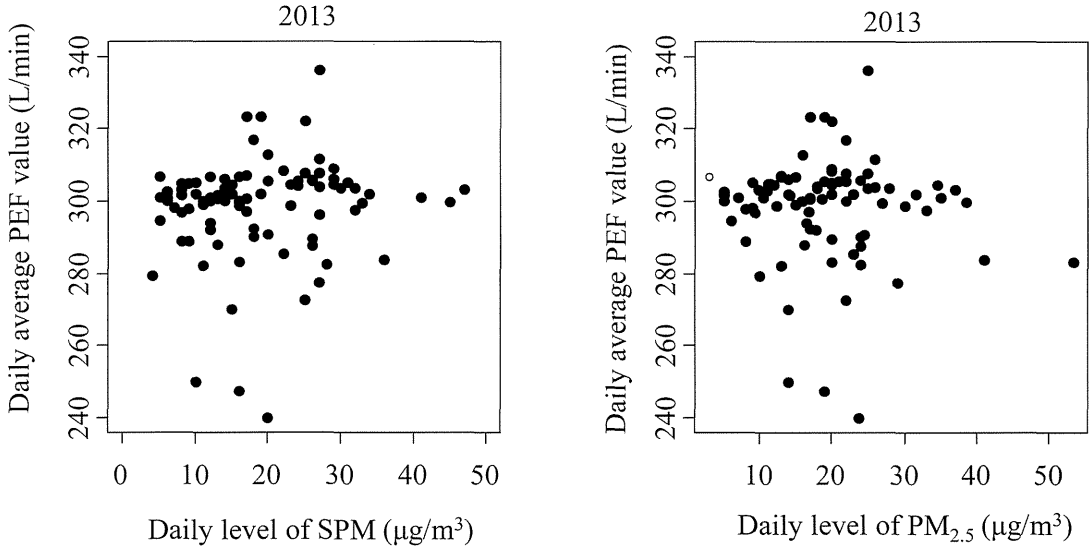

Figure 2. The associations of daily average peak expiratory flow (PEF) values and daily levels of suspended particulate matter (SPM) and particulate matter smaller than 2.5 μm in diameter ($PM_{2.5}$) in the 2012 and 2013 surveys.

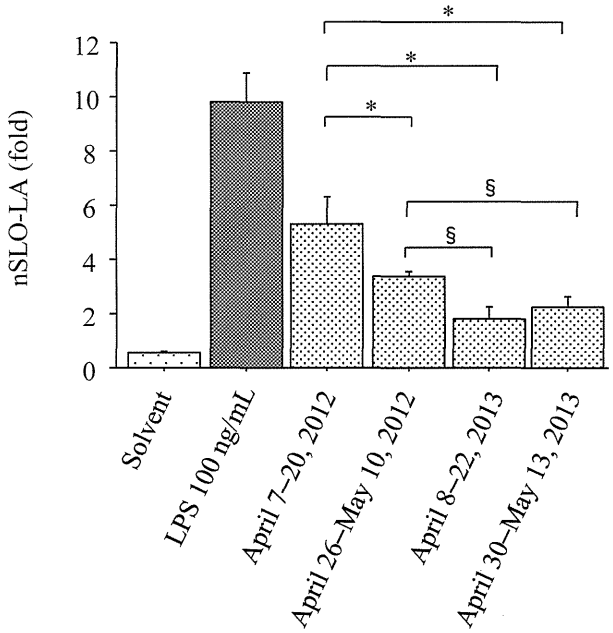

Figure 3. Interleukin-8 transcriptional activity measured using an interleukin -8 luciferase assay in a stable THP-1-derived interleukin-8 reporter cell line. Interleukin-8 transcriptional activity is based on normalized stable luciferase orange luciferase activity (nSLO-LA), which was calculated as stable luciferase orange luciferase activity divided by stable luciferase red luciferase activity. Cells were treated with solvent only (n = 6, negative control), lipopolysaccharide (LPS) (n = 6, 100 ng/mL, positive control), or particulate matter collected on: April 7–20, 2012 (n = 6, 1 mg/mL); April 26–May 10, 2012 (n = 6, 1 mg/mL); April 8–22, 2013 (n = 6, 1 mg/mL); and April 30–May 13, 2013 (n = 6, 1 mg/mL). * $p < 0.0001$ for the comparison with particulate matter collected during April 7–20, 2012. § $p < 0.005$ for the comparison with PM collected during April 26–May 10, 2012.

3.3. IL-8 Promoter Activity in THP-G8 Cells

The nSLO-LA values (IL-8 promoter activity) of THP-G8 cells (Figure 3) changed by 0.53 ± 0.05 fold (solvent, n = 6), 9.79 ± 1.10 fold (LPS, n = 6, 100 ng/mL), 5.32 ± 1.01 fold (April 7–20, 2012, n = 6, 1 mg/mL), 3.35 ± 0.22 fold (April 26–May 10, 2012, n = 6, 1 mg/mL), 1.82 ± 0.40 fold (April 8–22, 2013, n = 6, 1 mg/mL), and 2.23 ± 0.38 fold (April 30–May 13, 2013, n = 6, 1 mg/mL). Both of the collected PM samples from 2012 differed from the two 2013 samples in terms of the nSLO-LA values in THP-G8 cells (Figure 3). After stimulation by the fallen PM, the viability of the THP-G8 cells exceeded 95% in all four samples, as assessed using a trypan blue-exclusion test.

3.4. Supplementary Results

In addition to the analyses described above, we evaluated the sex-specific relationships of PEF with SPM and $PM_{2.5}$; however, our results showed no interaction effect between the changes in PEF values and the participants' sexes (Supplementary File, Table S1). In a second supplementary analysis, we evaluated IL-8 promoter activity in THP-G8 cells stimulated with 0.2 mg/mL, 0.4 mg/mL, and 1 mg/mL of collected PM. As expected, we found that nSLO-LA increased in a concentration-dependent manner (Supplementary File, Figure S1).

4. Discussion

Although many studies have clearly shown that exposure to PM is significantly associated with pulmonary function [5–7], several other studies have failed to find a consistent relationship [8–10]. In Japan, only been a limited number of studies have investigated the association between PM and pulmonary function. We conducted an extended survey to reassess the relationship between PM and pulmonary function in schoolchildren in Japan. In both 2012 and 2013, there were significant associations between pulmonary function and both SPM and $PM_{2.5}$ in children with asthma. However, we were unable to find the same relationship between pulmonary function and SPM or $PM_{2.5}$ in children without asthma, even though these children belonged to the schools and grades as the children with asthma. The effects of PM on the production of pro-inflammatory cytokines differed according to the period of collection. The fallen PM that had been collected in 2012 induced significantly greater IL-8 promoter activity than did the PM that had been collected in 2013. These results suggest that the effect of PM on pulmonary function in children may be mediated by the extent of airway inflammation.

Previous studies of PM and pulmonary function have shown heterogeneous results [6–10]. Although some of these differences may be attributable to differences in study design, methods, or data analysis, such factors are thought to account for only a small portion of the variation between study results [7,10,25,26]. The present study was conducted using methods that are similar to those employed in other studies, and the differences in study design, methods, and data analysis cannot account for the difference between the year 2012 and year 2013 effects of PM in children without asthma. Therefore, other, more substantial factors are likely to explain these discordant results.

A large proportion of fine and ultrafine PM has anthropogenic origins (e.g., emissions from combustion and motor vehicles) [27]. Ambient coarse PM and sand dust can also include components of geological origin [27,28]. The acute effects that are triggered by short-term exposure to particles can

exacerbate inflammatory responses, and inflammation is associated with the long-term development of lung disease [29–31]. Dusts of geological origin are known to induce the production of pro-inflammatory cytokines [32]. Kumar *et al.* showed that ambient PM was more important than traffic-derived PM as a cause of injury to airway epithelial cells leading to the production of pro-inflammatory cytokines [33]. Therefore, in this study, we assessed and compared the potential production of IL-8 by fallen PM that had been collected in 2012 and 2013. Both PM samples collected during 2012 increased the levels of IL-8 significantly more than did the two samples from 2013. This could explain why the pulmonary function of children without asthma was not associated with SPM or $PM_{2.5}$ in 2013. These results suggest that the association between PM and reduced pulmonary function depends on the ability of the PM to cause the production of pro-inflammatory cytokines.

The heavy dust emissions that originate from the deserts in East Asia have produced the second largest dust emissions worldwide. These dust storms have been referred to as Asian dust storms (ADS) [34]. ADS include high counts of aerosolized air pollutants in addition to mineral dust particles [35]. These sand emissions are known to induce the production of pro-inflammatory cytokines [32]. Therefore, in order to avoid the effects of ADS airborne particles on the secretion of IL-8, we collected PM during study periods that did not include days of ADS exposure. Additionally, the collection periods differed between 2012 and 2013.

Asthma is usually characterized by chronic airway inflammation [36]. It is defined by a history of respiratory symptoms such as wheezing, shortness of breath, chest tightness, and cough that vary over time and in intensity, along with variable expiratory airway limitations. Additionally, asthma is usually associated with airway hyperresponsiveness. Airway hyperresponsiveness itself usually persists, even when symptoms are absent and lung function is normal. Both airway limitation and respiratory symptoms are often triggered by various factors. Irritant exposure is one common trigger for impaired pulmonary function and respiratory symptoms in patients with asthma. Therefore, in this study, we performed subgroup analyses for the associations of pulmonary function with SPM and $PM_{2.5}$, stratifying the children according to asthma status. The children with asthma exhibited associations between pulmonary function and SPM and $PM_{2.5}$ in both 2012 and 2013.

There is a relationship between atopic disposition and airway hyperresponsiveness in children [37]. The influences of pollutants on one's airway may differ depending on whether allergic diseases are present. Therefore, we also adjusted for allergic diseases in our analysis of the associations between air pollutants and PEF. In addition, the participants' sex may influence the risk of wheezing and the prevalence of asthma throughout childhood [38]. We evaluated the sex-specific relationships of asthma with PEF; however, our results showed no interaction effect between the changes in PEF values and the participants' sexes (Supplementary File).

To investigate the potential of PM to elicit production of IL-8, this study used samples of fallen PM. Experimental studies have repeatedly found that coarse PM is the most injurious to cells, even when other size fractions are delivered at the same mass or concentration [15,39]. However, several studies have reported contrasting findings—specifically that the associations between chemical compositions and particle toxicity tend to be stronger for fine and ultrafine PM [40,41]. Collectively, these findings show that the effects of PM on the production of pro-inflammatory cytokines can vary considerably, and that the composition of PM affects the observed differences. Future studies should measure the potentials of SPM and $PM_{2.5}$ to elicit pro-inflammatory cytokines.

In a previous study, we investigated IL-8 transcriptional activity using an IL-8 promoter luciferase assay in THP-G8 cells (the so-called IL-8 Luc assay). We found that IL-8 transcriptional activity correlated significantly with the secretion of IL-8 [18]. At present, it is extremely difficult to collect sufficient quantities of PM and to separate it according to defined aerodynamic diameters. Smaller PM is especially difficult to collect. We used the IL-8 Luc assay in this study because it has high sensitivity for the evaluation of IL-8 levels using a small amount of material.

There are several limitations to the present study. First, we defined asthma based on the parents' reports and were unable to diagnose asthma on the basis of airway hyperresponsiveness to methacholine and reversible airflow limitation. Therefore, the number of children with asthma differed between 2012 and 2013. Several children may have been incorrectly diagnosed with asthma. An over-diagnosis bias would have resulted in underestimations of the associations between PEF values and SPM and $PM_{2.5}$. Second, missing PEF values due to school absences were excluded from the data analysis. However, this intermittent missing data was statistically independent and did not cause any serious bias in the results. Third, the study periods were limited. In particular, we were unable to assess any seasonal variation in the association of pulmonary function with SPM and $PM_{2.5}$. Fourth, this study was unable to analyze the components and size distribution of collected PM. Therefore, it remains unclear which specific substance have substantial effects on decreases in pulmonary function and induction of IL-8. Fifth, we were unable to measure the individual amounts and durations of exposure to SPM and $PM_{2.5}$. To the extent that we were able to confirm the relevant information from teachers, the educational curricula of 2012 and 2013 did no lead to differences in outdoor activities. The differences in the individual amounts and durations of exposure to SPM and $PM_{2.5}$ may not been substantial, at least on school days. Finally, in the course of our investigation of the effects of PM on pulmonary function and induction of IL-8, we were unable to estimate the IL-8 concentrations in sputum and blood. Therefore, further study is needed to investigate these effects.

5. Conclusions

We found a significant association between pulmonary function in schoolchildren and daily levels of SPM and $PM_{2.5}$. However, the effects of SPM and $PM_{2.5}$ on pulmonary function may differ depending on the production of pro-inflammatory cytokines induced by PM.

Acknowledgments

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Author Contributions

Masanari Watanabe, Jun Kurai, Hiroyuki Sano, and Eiji Shimizu designed the study. Masanari Watanabe and Hisashi Noma wrote the manuscript. Masanari Watanabe and Jun Kurai contributed to the data collection. Masanari Watanabe, Jun Kurai, Rumiko Saito, Yutaka Kimura, Setsuya Aiba, and Mitsuo Oshimura contributed to the experiments. Masanari Watanabe and Hisashi Noma performed

the statistical analysis and interpreted the results. Masanari Watanabe, Hisashi Noma, and Eiji Shimizu contributed to critical revision of important intellectual content. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Abbreviations

ADS	Asian dust storm
ANOVA	analysis of variance
CI	confidence intervals
IL	interleukin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IQR	interquartile range
LPS	lipopolysaccharide
nSLO-LA	normalized stable luciferase orange luciferase activity
PEF	peak expiratory flow
PM	particulate matter
PM_{10}	particulate matter smaller than 10 μm
$PM_{2.5}$	particulate matter smaller than 2.5 μm
$PM_{0.5}$	particulate matter smaller than 0.5 μm
PTA	Parent Teacher Association
SD	standard deviation
SLO	stable luciferase orange
SLR	stable luciferase red
SPM	suspended particle matter

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