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Signaling pathway of isophorone diisocyanate-responsive IL-8 in airway smooth muscle cells

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Running title: The molecular mechanism of IPDI-induced asthma

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ABSTRACT

This study is first to analyze the soluble factors, secreted by the bronchial epithelium after exposure to isophorone diisocyanate (IPDI), responsible for increasing migration and proliferation of primary normal human bronchial smooth muscle cells (BSMC).

We treated immortalized non-tumorigenic human bronchial epithelial cells (BEAS-2B) and primary normal human bronchial epithelial cells (HBEC) with IPDI, and then collected the condition medium (IPDI-BEAS-2B-CM and IPDI-HBEC-CM), which was added to BSMC. Exposure of BEAS-2B and HBEC to IPDI increased IL-8 production. Culture of BSMC with IPDI-BEAS-2B-CM and IPDI-HBEC-CM increased BSMC proliferation and migration, which are major features in asthma remodeling. Induction of BSMC proliferation and migration by IPDI-BEAS-2B-CM and IPDI-HBEC-CM was associated with increased FAK, Src, ERK1/2 and AKT activation. Blocking FAK by a specific inhibitor significantly decreased BSMC migration and proliferation by inhibiting ERK1/2 activation. FAK and ERK1/2 inhibitor also decreased IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8-mediated BSMC proliferation and migration, whereas blocking Rnd3 by siRNA failed to affect BSMC proliferation, suggesting that Rnd3 was only involved in the regulation of BSMC migration.

Our study suggests that inhibition of IL-8 or IL-8-mediated FAK/ERK/Rnd3 signaling is an attractive therapeutic target for IPDI-mediated asthma.

KEYWORDS: isophorone diisocyanate, occupational asthma, IL-8, Rnd3, proliferation, migration

INTRODUCTION

Exposure to isocyanates and other low molecular weight chemicals causes 5-15% of all occupational asthma, which is a major health problem [1]. Isophorone diisocyanate (IPDI), an aliphatic diisocyanate used to manufacture polyurethane plastics, has been reported to cause occupational adult asthma [2, 3]. Characteristics of bronchial and lymph node biopsies from diisocyanate asthma patients or laboratory animals show pathological features similar to those seen in atopic asthma, including increase of inflammatory and T helper cell 2 response and enhancement of airway remodeling [1, 4]. The major features of airway remodeling include: loss of epithelial integrity, subepithelial fibrosis, goblet cell and submucosal gland enlargement, increased bronchial smooth muscle mass and increased angiogenesis [5]. Human bronchial smooth muscle cells (BSMC) play a key role in the modulation of airway tone. In an asthmatic airway, BSMC not only increase secretory and proliferative ability, but also migrate to the subepithelial area [5, 6]. In addition, BSMC also release proinflammatory cytokines, which are responsible for the progression of asthma pathogenesis. The degree of change in bronchial smooth muscle mass has been correlated to asthma severity [6].

Focal adhesion kinase (FAK), a nonreceptor protein tyrosine kinase, is involved in mediation of signaling cascade and plays an important role in cell proliferation, survival, adhesion and movement [7, 8]. The activation of FAK is regulated by

phosphorylation at the sixth tyrosine residue. Autophosphorylation of FAK at tyrosine 397 forms a binding site for Src, which in turn phosphorylates FAK at tyrosine 576/577, which is essential for optimum FAK kinase activity. FAK/Src complex also phosphorylates FAK at tyrosine 925, which forms an adaptor Grb2 docking site and activates the MAP kinase pathway [9]. Rnd3/RhoE, a subgroup of Rho GTPases, is activated by Raf/MEK/ERK [10], and has been reported to be involved in many cellular biologic functions, including cell migration, proliferation, cell cycle progression, apoptosis and differentiation [11-13]. Overexpression of Rnd3/RhoE disrupts actin cytoskeleton organization and focal adhesions in fibroblast and epithelial cells [14]. For example, height expression of Rnd3 has been found to increase the invasive ability of melanoma [11], but the function of Rnd3 has been less well studied in BSMC.

We hypothesize that IPDI may cause epithelial cells to produce the soluble factor(s) which in turn increase proliferation and migration of BSMC. We therefore treated BSMC with IPDI-treated bronchial epithelial cells condition medium, and assessed the mechanism of BSMC proliferation and migration. This model provided evidence of the interaction between bronchial epithelial cells and BSMC, and the mechanism of BSMC migration after IPDI exposure.

METHODS

Cell culture and condition medium

Immortalized non-tumorigenic human bronchial epithelial cells (BEAS-2B; ATCC CRL-9609) were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Primary normal human bronchial smooth muscle cells (BSMC) and primary normal human bronchial epithelial cells (HBEC) were obtained from Lonza (Walkersville, MD). BSMC were cultured in SmGM-2 smooth muscle medium and HBEC were cultured in BEGM (bronchial epithelial cell growth medium) (Lonza).

To obtain IPDI-treated BEAS-2B and HBEC condition medium (IPDI-BEAS-2B-CM and IPDI-HBEC-CM), BEAS-2B and HBEC (2×10^6 cells/100 mm dish) were treated with vehicle control and various concentrations of IPID for 6 h. After treatment, the medium was replaced and the supernatants harvested after 24 h of incubation. IL-8 depletion from IPDI-BEAS-2B-CM and IPDI-HBEC-CM was performed using anti-IL-8 antibodies (2 μg/mL) and Sepharose A/G beads following regular immunoprecipitation techniques. Cytokine depletion was confirmed by IL-8 ELISA assay kit.

Enzyme-linked immunosorbent assay (ELISA) and cytokine array

The levels of IL-8, CXCL5 and IL-1β were determined by ELISA-based kits (R&D Systems Europe, Abingdon, UK). ELISAs were performed according to the

manufacturer's instructions. The profile of cytokine expressed on IPDI-treated BEAS-2B cells was also assessed by RayBio® Human Inflammation Antibody Array (RayBiotech, Inc., Norcross, Ga, USA) according to the manufacturer's instructions.

Cell proliferation

Cells (4×10^3/well) were plated in 96-well culture plates. After 24 h incubation, the cells were treated with vehicle control-CM, IPDI-BEAS-2B-CM or IPDI-HBEC-CM for 72 h. The proliferation of BSMC was determined by Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions.

Cell migration assay

Cell migration was carried out using the QCM chemotaxis 8 μm cell migration assay system (Chemicon, Temecula, CA) (Millipore Corp, Bedford, MA, USA) according to the manufacturer's instructions. Cells were seeded into the migration chamber, and IPDI-BEAS-2B-CM, IPDI-HBEC-CM, vehicle control medium, IL-8 depletion IPDI-BEAS-2B-CM, IL-8 depletion IPDI-HBEC-CM or medium containing 20 ng/ml rhIL-8 was placed in the lower chamber. After allowing cell migration for 24 h, cells that had migrated through the membrane were stained, lysed, and quantified on a microplate at 520 nm.

Immunoblot/immunoprecipitation

Cells were lysed on ice for 15 min by M-PER lysis reagent (Pierce, USA). Cell lysate was centrifuged at 14,000 × *g* for 15 min, and the supernatant fraction collected for immunoblot. Equivalent amounts (20 μg/ml) of protein were resolved by SDS-PAGE (8-12%) and transferred to PVDF membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1-16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins detected using an enhanced chemiluminescence kit (Millipore) according to the manufacturer's instructions.

Real-time RT-PCR and microarray

RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo (dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed by using SYBR Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contained 200 nM of each primer, 10 μL of 2xSYBR Green PCR Master Mix (Applied Biosystems), 5 μl cDNA and RNase-free water in a total volume of 20 μl. The PCR reaction was carried out with a denaturation step at 95°C for 10 min, and then for 40 cycles at 95°C for 15 s and 60°C for 1 min. All PCRs were performed in triplicate and normalized to internal control glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) mRNA. Relative expression was presented using the $2^{-\Delta\Delta CT}$ method.

Microarray experiment procedures were carried out following the manufacturer's protocols. Total RNA (1 μg) was amplified by an Agilent Quick Amp Labeling Kit (Agilent Technologies, USA). IPDI-BEAS-2B-CM and IPDI-HBEC-CM-treated BSMC RNA was labeled by Cy5, and contro-CM-treated BSMC RNA was labeled by Cy3 in an *in vitro* transcription process. 0.825 μg of Cy-labled cRNA was cleavaged to an average size of about 50-100 nucleotides by incubation with fragmentation buffer (Agilent Technologies) at 60^{o}C for 30 minutes. An equal amount of Cy-labeled cRNA was pooled and hybridized to Agilent Whole Human Genome 4x44k oligo microarray (Agilent Technologies, USA) at 65°C for 17 h. After washing and drying by nitrogen gun blowing, microarrays were scanned by an Agilent microarray scanner (Agilent Technologies, USA), at 535 nm for Cy3 and 625 nm for Cy5. Scanned images were analyzed by Feature extraction software 10.5 (Agilent Technologies, USA), an image analysis and normalization software used to quantify signal and background intensity for each feature, which substantially normalized the data using the rank-consistency-filtering LOWESS method.

siRNA knockdown

BSMC were transfected with 1 μmol/L Non-target or Rnd3 Accell siRNAs pool (Dharmacon) in Accell delivery media (B-005000), according to the manufacturer's instructions. Positive controls, Accell GAPDH siRNA and Non-target Accell siRNA pool, were used in the experiments. After 72 h transfection, the medium was changed to whole medium, and the cells treated with a mixture of cytokine and pterostilbene for an additional 15 h. The changes of Rnd3 were measured by real-time PCR as described above.

Statistical analysis

Data were expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by Dunnett's test.

RESULTS

The condition medium of IPDI-treated BEAS-2B and HBEC increased proliferation and migration of bronchial smooth muscle cells

Increase in the mass of bronchial smooth muscle cells (BSMC) and decreased distance between BSMC and bronchial epithelia cells are important features of the remodeled wall in asthmatic airways [5]. These changes in BSMC can be induced by many factors produced by epithelial cells [5]. We collected the IPDI-treated BEAS-2B and HBEC-condition medium (IPDI-BEAS-2B-CM and IPDI-HBEC-CM), then

assessed the effects of two condition media on the proliferation and migration of BSMC. As shown in Figure 1A, IPDI-BEAS-2B-CM and IPDI-HBEC-CM increased the proliferation of BSMC in a dose-dependent manner after 72 h. Furthermore, IPDI-BEAS-2B-CM and IPDI-HBEC-CM increased the migration of BSMC in a concentration-dependent manner (Figure 1B).

IPDI-BEAS-2B-CM and IPDI-HBEC-CM **increased the expression of inflammatory, adherence and chemotaxis factors**

Increased inflammatory response of BSMC is a cardinal feature in the development of airway remodeling [15]. We assessed whether IPDI increased the inflammatory response through cross-talk of the epithelium and BSMC. As shown in Figure 2A, IPDI-BEAS-2B-CM increased the transcription of inflammatory cytokines, including IL-1β, IL-6, IL-8, CXCL2, CXCL3, CXCL5, CCL5 and ICAM, as assessed by microarray. The data was also confirmed by real-time PCR in both IPDI-BEAS-2B-CM and IPDI-HBEC-CM-treated BSMC (Figure 2B). Similarly, IPDI-BEAS-2B-CM and IPDI-HBEC-CM also increased the amount of IL-8, IL-1β, and CXCL5 on the protein level (Figure 2C, D, and E).

Involvement of IL-8 on IPDI-BEAS-2B-CM and IPDI-HBEC-CM-mediated proliferation and migration

Increase of inflammatory response in the epithelium plays an important role in the development of asthma [16, 17]. We consequently assessed whether IPDI increased the production of inflammatory factor in epithelial cells, which in turn enhances the proliferation and migration of BSMC. We first determined the cytokine profile of BEAS-2B after IPDI treatment by cytokine array. The data showed that IPDI only increased the amount of IL-8 (Figure 3A), which was confirmed by ELISA kit. As shown in Figure 3B, IPDI increased the amount of IL-8 in BEAS-2B and HBEC in a dose-dependent manner. In contrast, IPDI failed to affect IL-6, CCL5/RANTES and CXCL5 levels in BEAS-2B and HBEC (data not shown). We also assessed the effect of recombinant human interleukin-8 (rhIL-8) on the proliferation and migration of BSMC. The results showed that rhIL-8 not only increased BSMC proliferation (Figure 3C), but also enhanced BSMC migration (Figure 3D).

Next, we removed IL-8 from IPDI-BEAS-2B-CM and IPDI-HBEC-CM to ascertain the role of IL-8 on BSMC proliferation and migration. The successful depletion of IL-8 from IPDI-BEAS-2B-CM and IPDI-HBEC-CM was confirmed by IL-8 ELISA kit (data not shown). As shown in Figure 3E, IL-8 depleted IPDI-BEAS-2B-CM and IPDI-HBEC-CM were added to BSMC, effectively reversing the proliferation of BSMC caused by IPDI-BEAS-2B-CM and IPDI-HBEC-CM. Similarly, increase of

BSMC migration by IPDI-BEAS-2B-CM and IPDI-HBEC-CM was terminated upon IL-8 depletion (Figure 3F).

The effects of IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 on FAK/Rnd3 signaling pathway

FAK is postulated to integrate growth factor, cytokine and integrin signals, and be involved in cell migration. We assessed whether IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 induces BSMC migration by FAK. As shown in Figure 4A, IPDI-BEAS-2B-CM**,** IPDI-HBEC-CM and rhIL-8 increased the phosphorylation of FAK at Tyr397, 576/577 and 925 sites in BSMC. However, IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 treatment did not cause any change in the protein levels of total FAK. Exposure of BSMC to IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 increased active form Src (Tyr416 phosphorylation) and decreased inactive form Src (Tyr527). In addition, the association of FAK and Src increased in a time-dependent manner in IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8-treated BSMC, as determined by immunoprecipitation assay (Figure 4B). Similar responses were observed for the phosphorylated forms of two other FAK downstream targets, ERK1/2 and AKT (Figure 4A).

Because Rnd3/RhoE is a target of MEK-ERK1/2 signaling [12], we next checked the expression of Rnd3 in BSMC. As shown in Figure 4C and D, IPDI-BEAS-2B-CM,

IPDI-HBEC-CM and rhIL-8 increased the amount of Rnd3 in both mRNA and protein levels in BSMC.

The role of FAK and ERK on BSMC proliferation and migration

To understand the upstream of Rnd3, we assessed the roles of FAK and ERK1/2 on the expression of Rnd3 by specific chemical inhibitors. BSMC were pretreated with FAK Inhibitor 14 (inhibitor for FAK) and PD98059 (inhibitor for ERK1/2), after which the cells were exposed to IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8. The effect of FAK and ERK inhibitors on the expression of Rnd3, cell migration and proliferation was then examined. Pretreatment of BSMC with FAK inhibitors reduced phosphorylation of FAK induced by IPDI-BEAS-2B-CM, IPDI-HBEC-CM or rhIL-8 (Figure 5A). The specific inhibitor of FAK halted ERK1/2 phosphorylation and Rnd3 upregulation in BSMC after IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 treatment (Figure 5A). Cell proliferation and migration also ceased in BSMC after IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 treatment (Figure 5B to 5E).

Specific inhibition of ERK1/2 activation by PD98059 decreased IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8-induced Rnd3 upregulation in BSMC (Figure 6A). Similar to FAK inhibition, PD98059 also decreased the effect of IPDI-BEAS-2B-CM**,** IPDI-HBEC-CM and rhIL-8 on BSMC proliferation and migration (Figure 6B to 6E)

The role of Rnd3 on bronchial smooth muscle cell migration induced by IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8

To confirm the central role of Rnd3 on BSMC migration induced by IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8, we transfected BSMC with Rnd3 siRNA. As shown in Figure 7A, Rnd3 siRNA reduced Rnd3 expression by approximately 90%, in comparison with control siRNA transfection. Specific knockdown of Rnd3 expression decreased the effect of IPDI-BEAS-2B-CM**,** IPDI-HBEC-CM and rhIL-8 on migration in BSMC, but failed to affect BSMC proliferation (Figure 7B to 7E). These results suggest that Rnd3 may play a key role in IPDI mediated BSMC migration.

DISCUSSION

This study is the first to investigate the interaction of airway epithelium and smooth muscle after exposure to the environmental chemical agent isophorone diisocyanate (IPDI). IPDI caused immortalized non-tumorigenic human bronchial epithelial cells (BEAS-2B) and primary normal human bronchial epithelial cells (HBEC) to produce IL-8, which increased the proliferation and migration of primary normal human bronchial smooth muscle cells (BSMC). In addition, IPDI-treated BEAS-2B and HBEC condition medium (IPDI-BEAS-2B-CM and IPDI-HBEC-CM) and recombinant human interleukin-8 (rhIL-8) increased FAK activation, subsequently

increasing ERK1/2 and AKT activation in BSMC. In short, blocking the upstream molecule FAK or downstream factor ERK1/2 activation by specific inhibitors effectively reverses their induction effect on BSMC proliferation and migration. Furthermore, inhibition of Rnd3 by siRNA also decreased IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8-mediated BSMC migration. These data suggest that FAK/ERK1/2/Rnd3 plays a critical role in IPDI-mediated asthma (Figure 8).

CXCL8/IL-8, a proinflammatory CXC chemokine, is secreted by a variety of human bronchial epithelial cells or BSMC exposed to pro-inflammatory cytokines such as IL-1 and TNF-α, and promotes an inflammatory response [17, 18]. IL-8 has been reported to trigger calcium release, contraction, and migration in BSMC through functional CXCR1 and CXCR2 receptors [18, 19]. Another isocynate, toluene diisocyanate (TDI), has been found to increase bronchial epithelial cells' production of IL-8, which in turn recruits neutrophils, resulting in inflammatory response [20, 21]. However, the role of IL-8 on IPDI-related asthma remains unknown. We found that IPDI increases BEAS-2B and HBEC secretion of IL-8, which in turn increases the proliferation and migration of BSMC. However, depleting IL-8 from IPDI-BEAS-2B-CM and IPDI-HBEC-CM reversed that. Moreover, treatment of BSMC with IPDI also increased their proliferation and migration. This is an important

correlation to our finding on the clinical significance of elevated IL-8 levels in IPDI-mediated asthma.

FAK, a nonreceptor tyrosin kinase, has been shown to be a critical mediator of cell proliferation, survival and migration in a variety of cell types [22]. Many studies have reported that activation of FAK is strongly associated with vascular smooth muscle cell growth and migration, whereas loss of FAK activation inhibited smooth muscle cell proliferation and migration [22, 23]. Activated FAK can associate with Src to form a complex, which relieves inactivated phosphorylation at Tyr527 and promotes activated autophosphorylation at Tyr416, resulting in Src activation. Activated Src has been shown to phosphorylate FAK at multiple tyrosine residues, subsequently activating AKT and ERK1/2 and promoting cell proliferation and migration. Although Lin et al. reported that inhibition of FAK by antisense oligodeoxynucleotides inhibits human pulmonary artery smooth muscle cells' proliferation, resulting in cell apoptosis, the effect of FAK on bronchial smooth muscle remained unknown. [22]. In our study, we found that IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 increased the phosphorylation of FAK at three different tyrosine sites and enhanced the interaction of FAK and Src. In addition, exposure of BSMC to IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 also activated AKT and ERK1/2. Inhibition of FAK activation decreased ERK1/2 activation, suggesting that FAK is the upstream molecule

of ERK1/2. In addition, selective inhibition of FAK and ERK1/2 by chemical inhibitors also decreased the effects of IPDI-BEAS-2B-CM, IPDI-HBEC-CM or rhIL-8 on BSMC proliferation and migration, suggesting that FAK/ERK1/2 plays a crucial role in IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8-mediated BSMC change.

Rnd3/RhoE, an atypical Rho family protein, exhibits a regulatory role in many cellular biological processes, such as cytoskeleton formation, cell survival, apoptosis, cell cycle progression and differentiation [23, 24]. Unlike typical Rho proteins, with activity dependent on the GTP or GDP-binding states, the activation of RhoE is controlled by transcription or post-transcription modification [25, 26]. However, the function of endogenous Rnd3 in bronchial smooth muscle cells remains unknown. Recent studies indicate that endogenous Rnd3 is associated with ROCK-mediated apoptosis and myoblast alignment [14]. In this study, we have shown that treatment of BSMC with IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 resulted in increased Rnd3 expression at both transcriptional and translation levels. Inhibition of FAK and ERK1/2 decreased the upregulation of Rnd3 by IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8, suggesting that FAK/ERK1/2 is the upstream event of Rnd3. In addition, knockdown Rnd3 by siRNA decreased IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8-mediated BSMC migration, indicating that Rnd3 participates in the migration of

BSMC. The regulatory role of Rnd3 on BSMC migration provides a critical new function for Rnds involved in airway remodeling.

Taken together, our findings indicate that conditioned media from IPDI-treated epithelial cells stimulate bronchial epithelial cells' proliferation and migration. IL-8 in the conditioned medium results in an enhanced effect on BSMC growth and movement, while FAK/ERK/Rnd3 has been found responsible for BSMC migration. In light of this finding, inhibition of IL-8 signaling is an attractive therapeutic target for IPDI-caused occupational asthma.

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Figure legends

Figure 1. The effect of IPDI-BEAS-2B-CM and IPDI-HBEC-CM on the proliferation and migration of BSMC. (A) IPDI-BEAS-2B-CM and IPDI-HBEC-CM increased BSMC proliferation. (B) IPDI-BEAS-2B-CM and IPDI-HBEC-CM enhances the migratory ability of BSMC. BEAS-2B and HBEC (2×10^6 cells/100 mm dish) were treated with vehicle control and various concentrations of IPDI for 6 h. The medium was replaced with fresh medium, then cells were harvested after 24 h incubation. The harvested medium was defined as IPDI-BEAS-2B-CM and IPDI-HBEC-CM. The effect of IPDI-BEAS-2B-CM and IPDI-HBEC-CM on BSMC proliferation was assessed by WST-1 after 72 h incubation. The BSMC migration was assessed by QCM Chemotaxis cell migration assay system. The asterisk indicates a significant difference between control and test groups, * $p < 0.05$.

Figure 2. IPDI-BEAS-2B-CM and IPDI-HBEC-CM increased inflammatory and chemotaxis response in BSMC. IPDI-BEAS-2B-CM and IPDI-HBEC-CM increased the expression of inflammatory factors and chemotaxis in mRNA levels, assessed by microarray (A) and real-time PCR (B). IPDI (50 μM)-BEAS-2B-CM and IPDI (50 μM)-HBEC-CM increased the amount of IL-8 (C), IL-1β (D) and CXCL5 (E) on protein levels. BSMC were treated with IPDI (50 μM)-BEAS-2B-CM and IPDI (50 μM)-HBEC-CM for 6 h and the expressions of mRNA were assessed by microarray and real-time PCR. For (C) to (E), BSMC were treated with IPDI (50 μM)-BEAS-2B-CM and IPDI (50 μM)-HBEC-CM for the indicated times, and the amounts of various proteins were detected by ELISA kit. The asterisk indicates a significant difference between control and test groups, * $p < 0.05$.

Figure 3. IL-8 is involved in BSMC proliferation and migration induced by IPDI-BEAS-2B-CM and IPDI-HBEC-CM. IPDI increased the amount of IL-8 in BEAS-2B assed by cytokine array (A) and ELISA kit (B). The addition of rhIL-8 increased BSMC proliferation (C) and migration (D). Depletion of IL-8 from IPDI-BEAS-2B-CM and IPDI-HBEC-CM decreased BSMC proliferation (E) and migration (F) induced by IPDI-BEAS-2B-CM and IPDI-HBEC-CM. BEAS-2B and HBEC were treated with IPDI (50 μM) for 6h. The supernatants were collected and the IL-8 levels of IPDI-BEAS-2B-CM and IPDI-HBEC-CM assessed by cytokine array

and ELISA kit. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($p < 0.05$).

Figure 4. IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 activates FAK signaling. (A) IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 increased the phosphorylation of FAK, Src, ERK1/2 and AKT in BSMC. (B) IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 enhanced the interaction of FAK and Src. IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 increased the amount of Rnd3 at both mRNA (C) and protein levels (D). BSMC were treated with IPDI (50 μM)-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 (20 ng/ml) for the indicated times. Phosphorylated and unphosphorylated proteins were assessed by immunoblot, and the interaction of FAK and Src detected by immunoprecipitation. The amount of Rnd3 mRNA was assayed by microarray and real-time PCR.

Figure 5. The role of FAK on BSMC proliferation and migration. (A) The effects of FAK inhibitor on the phosphorylation of FAK and ERK1/2 and Rnd3 expression. The effect of FAK inhibitor on BSMC proliferation caused by IPDI-BEAS-2B-CM and IPDI-HBEC-CM (B), as well as rhIL-8 (C). The effect of FAK inhibitor on BSMC migration induced by IPDI-BEAS-2B-CM and IPDI-HBEC-CM (D), as well as rhIL-8 (E). BSMC were pre-treated with FAK inhibitor for 1 h then exposed to IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 (45 min for ERK phosphorylation, 3

h for Rnd3 expression, 72 h for proliferation assay and 24 h for migration assay). The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($p < 0.05$).

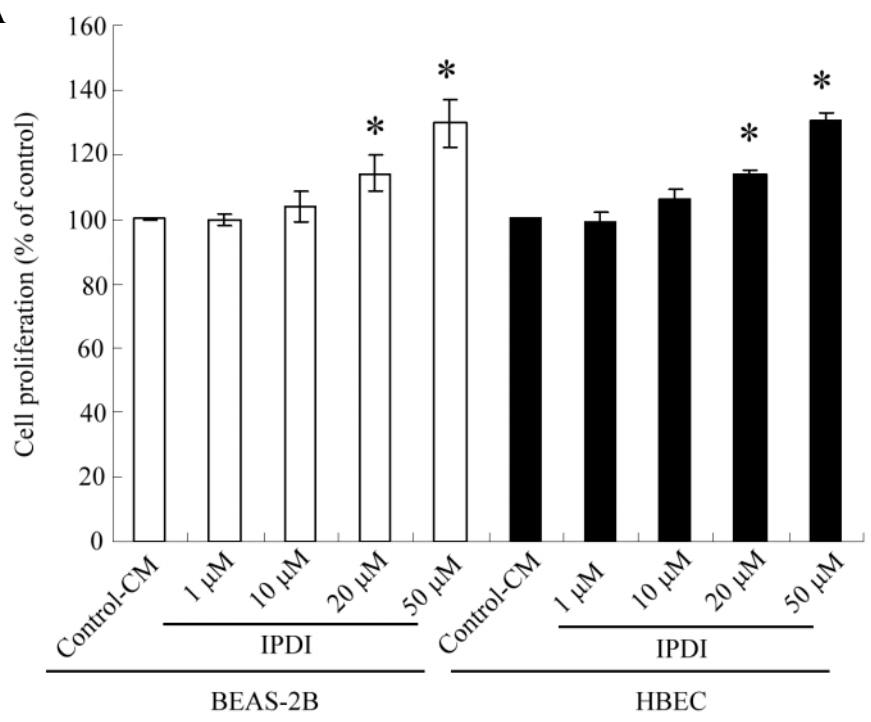
Figure 6. The role of ERK1/2 on BSMC proliferation and migration. (A) The effects of MEK inhibitor on the phosphorylation of ERK1/2 and Rnd3 expression. The effect of ERK inhibitor on BSMC proliferation induced by IPDI-BEAS-2B-CM and IPDI-HBEC-CM (B) as well as rhIL-8 (C). The effect of ERK inhibitor on BSMC migration caused by IPDI-BEAS-2B-CM and IPDI-HBEC-CM (D), as well as rhIL-8 (E). BSMC were pre-treated with PD98059 (20 μM) for 1 h then exposed to IPDI-BEAS-2B-CM, IPDI-HBEC-CM and rhIL-8 (45 min for ERK phosphorylation, 3 h for Rnd3 expression, 72 h for proliferation assay and 24 h for migration assay). The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($p < 0.05$).

Figure 7. The role of Rnd3 on BSMC proliferation and migration. (A) Rnd3 was knockdown by siRNA. Rnd3 inhibition did not affect either the inductive effect of IPDI (50 μM)-BEAS-2B-CM and IPDI (50 μM)-HBEC-CM (B), or the effect of rhIL-8 (20 ng/ml) (C) on BSMC proliferation. Rnd3 inhibition decreased IPDI-BEAS-2B-CM and IPDI-HBEC-CM (D), as well as rhIL-8 (E)-mediated BSMC migration. The asterisk

indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($p < 0.05$).

Figure 8. The molecular mechanism of IPDI-induced asthma. IPDI caused bronchial epithelial cells to produce IL-8, which increased the proliferation and migration of bronchial smooth muscle cells (BSMC). IL-8 in the condition medium results in an enhanced effect on BSMC growth and movement, while FAK/ERK/Rnd3 has been found to be responsible for BSMC migration.

A



B

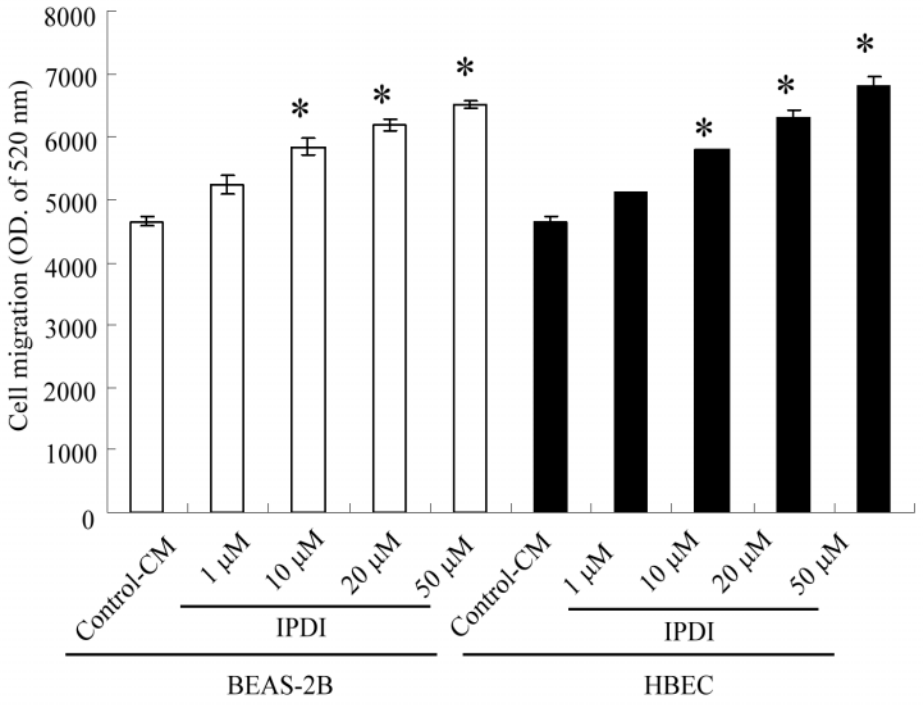
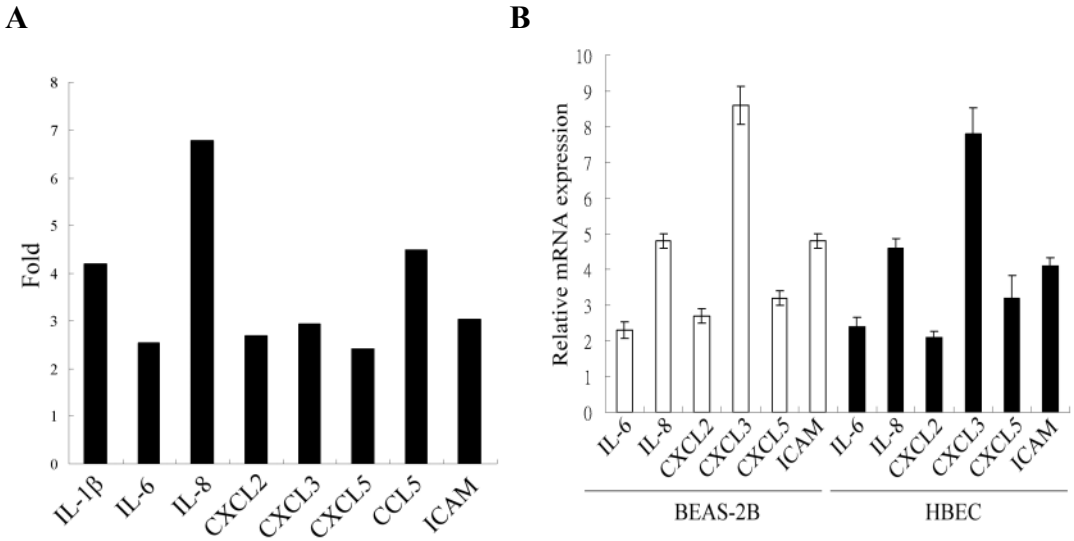

Cell migration (OD. of 520 nm)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
Control-CM
1 μM
10 μM
20 μM
50 μM
IPDI
BEAS-2B
HBEC

Figure 1


A
B
Fold
IL-1β
IL-6
IL-8
CXCL2
CXCL3
CXCL5
CCL5
ICAM
Relative mRNA expression
BEAS-2B
HBEC

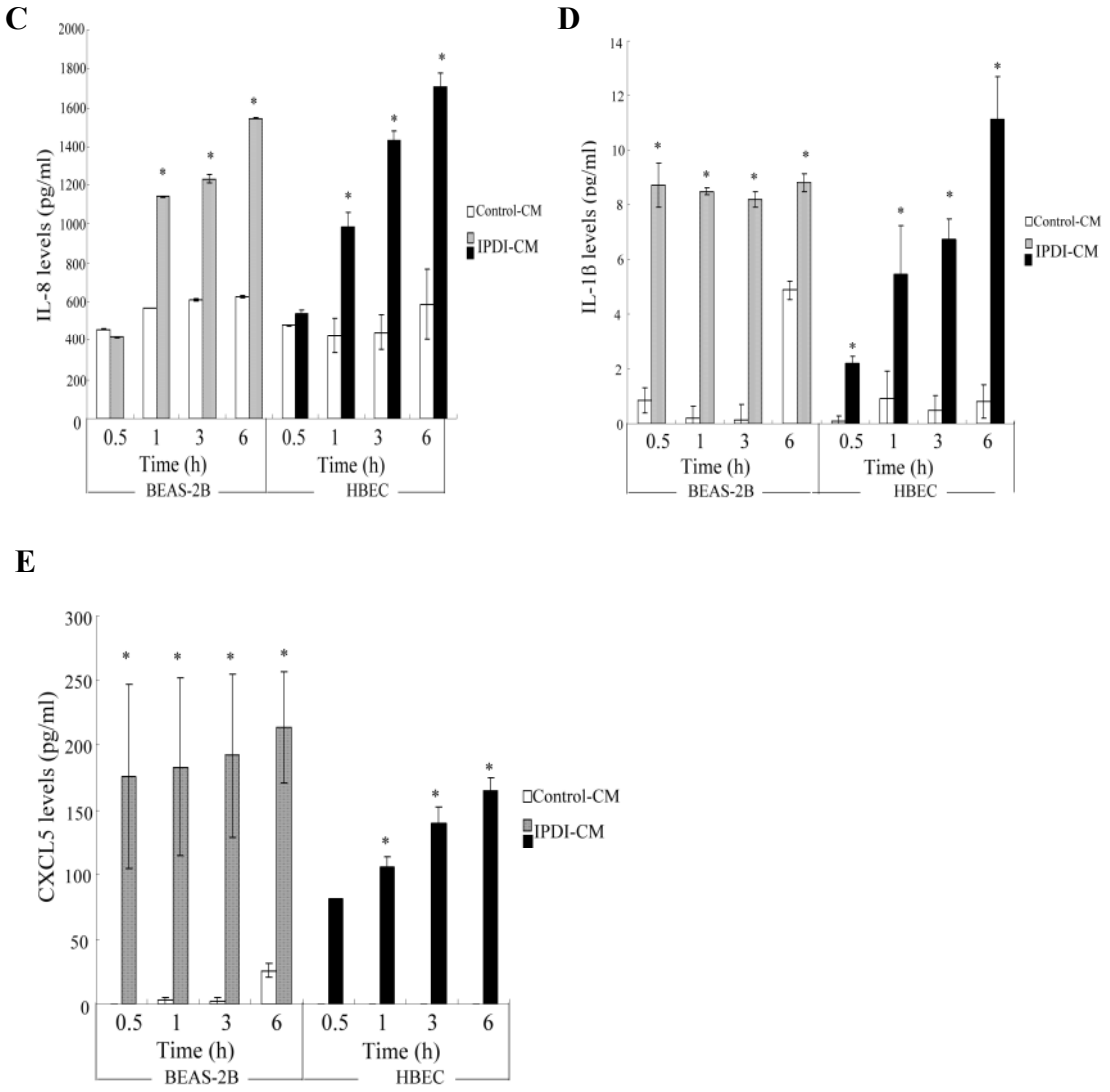
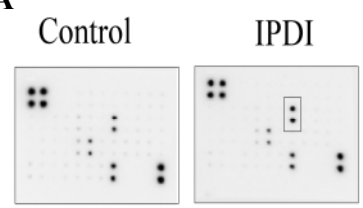


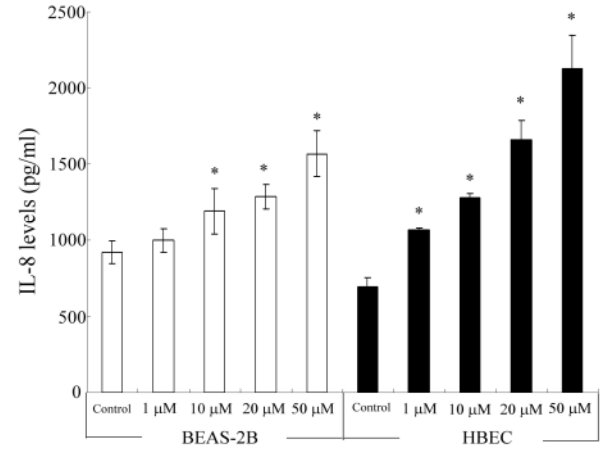
Figure 2

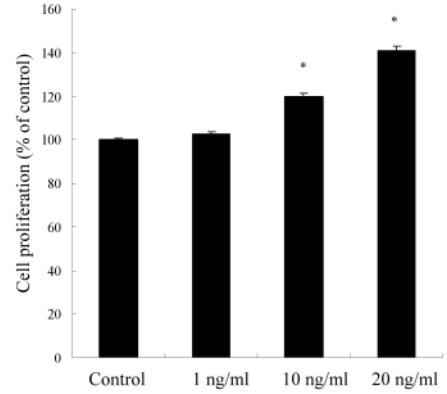
A

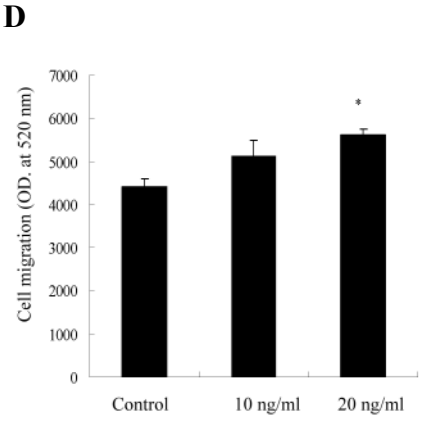


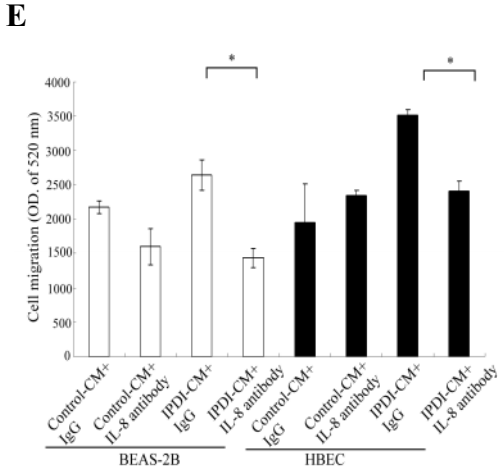
A	B	C	D	E	F	G	H	I	J	K	L
POS	POS	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
POS	POS	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
RANTES	TGF-β1	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
RANTES	TGF-β1	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

B


IL-8 levels (pg/ml)
2500
2000
1500
1000
500
0
Control
1 μM
10 μM
20 μM
50 μM
BEAS-2B
HBEC


C
Cell proliferation (% of control)
160
140
120
100
80
60
40
20
0
Control
1 ng/ml
10 ng/ml
20 ng/ml


D
Cell migration (OD. at 520 nm)
7000
6000
5000
4000
3000
2000
1000
0
Control
10 ng/ml
20 ng/ml


E
Cell migration (OD. of 520 nm)
4000
3500
3000
2500
2000
1500
1000
500
0
Control-CM+ IgG
Control-CM+ IL-8 antibody
IPDL-CM+ IgG
IPDL-CM+ IL-8 antibody
BEAS-2B
HBEC

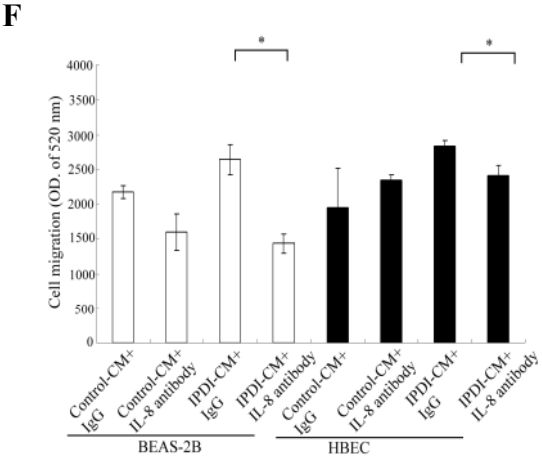

F
Cell migration (OD. of 520 nm)
4000
3500
3000
2500
2000
1500
1000
500
0
Control-CM+ IgG
Control-CM+ IL-8 antibody
IPDL-CM+ IgG
IPDL-CM+ IL-8 antibody
BEAS-2B
HBEC

Figure 3

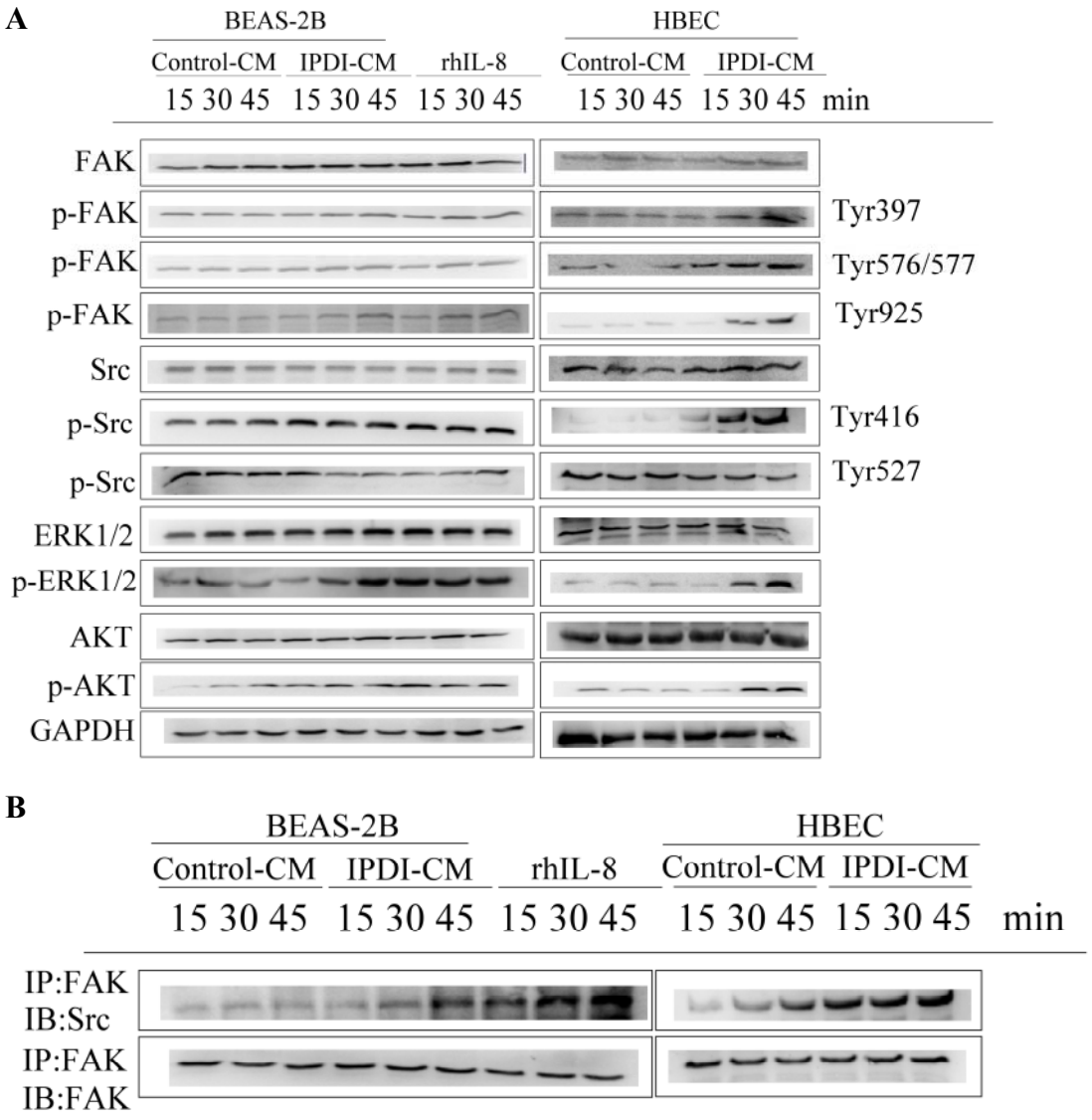

A
BEAS-2B
HBEC
Control-CM
IPDI-CM
rhIL-8
Control-CM
IPDI-CM
15 30 45 15 30 45 15 30 45 15 30 45 15 30 45 min
FAK
p-FAK
Tyr397
p-FAK
Tyr576/577
p-FAK
Tyr925
Src
p-Src
Tyr416
p-Src
Tyr527
ERK1/2
p-ERK1/2
AKT
p-AKT
GAPDH
B
BEAS-2B
HBEC
Control-CM
IPDI-CM
rhIL-8
Control-CM
IPDI-CM
15 30 45 15 30 45 15 30 45 15 30 45 15 30 45 min
IP:FAK
IB:Src
IP:FAK
IB:FAK
C
Relative mRNA expression (fold)
3
2.5
2
1.5
1
0.5
0
Rnd3
Microarray
Real-time PCR

Figure 4

D

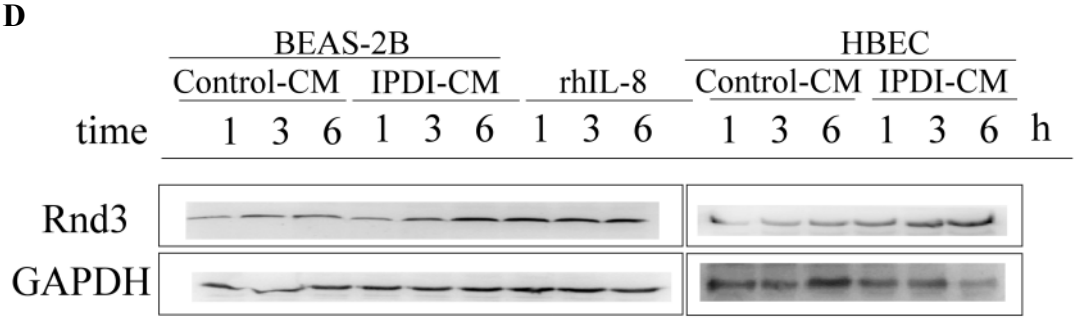

BEAS-2B
HBEC
Control-CM
IPDI-CM
rhIL-8
Control-CM
IPDI-CM
time
1 3 6 1 3 6 1 3 6 1 3 6 1 3 6 h
Rnd3
GAPDH

Figure 4

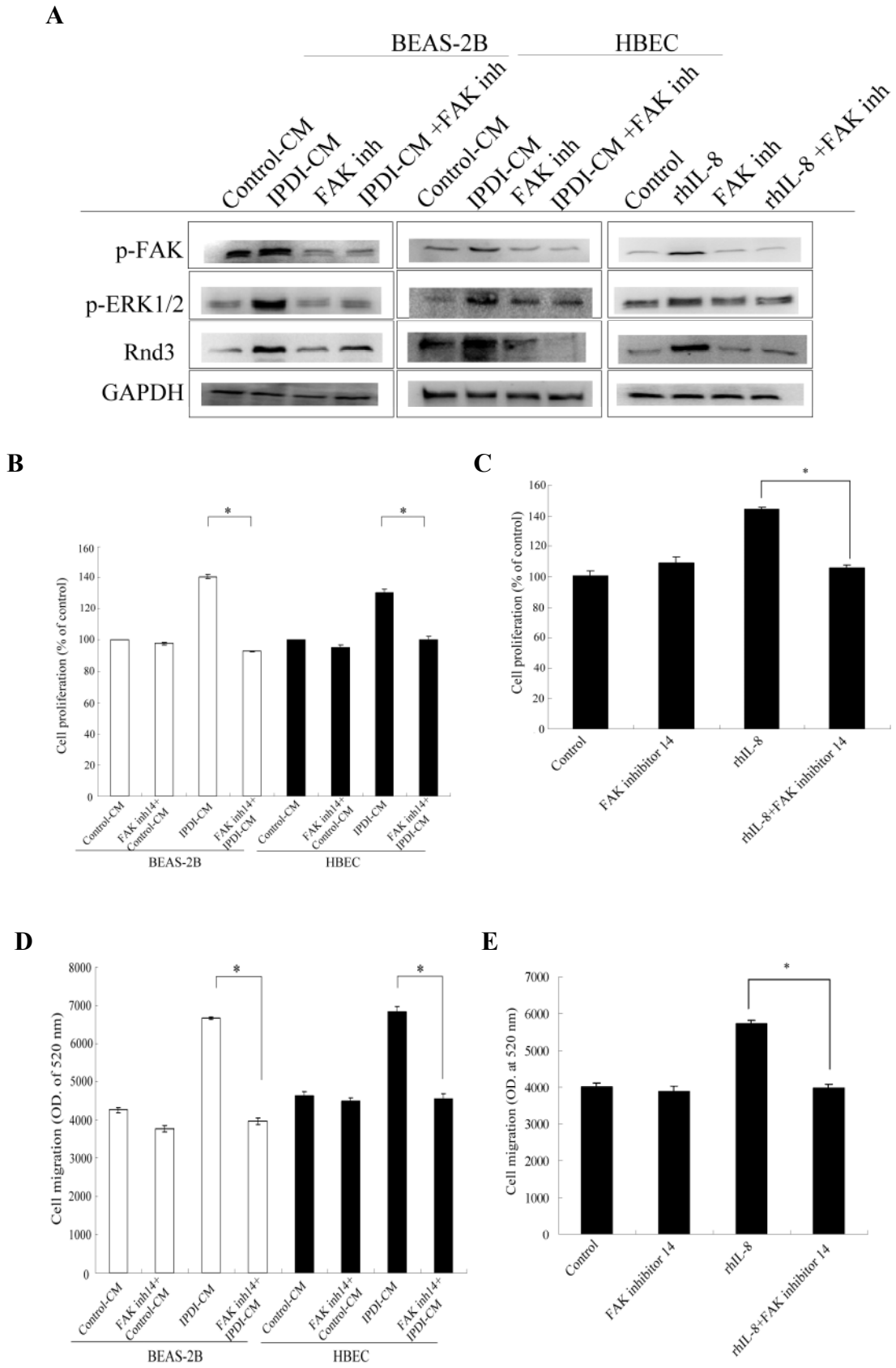

A
BEAS-2B
HBEC
Control-CM
IPDI-CM
FAK inh
IPDI-CM +FAK inh
Control-CM
IPDI-CM
FAK inh
IPDI-CM +FAK inh
Control
rhIL-8
FAK inh
rhIL-8 +FAK inh
p-FAK
p-ERK1/2
Rnd3
GAPDH
B
Cell proliferation (% of control)
Control-CM
FAK inh14+ Control-CM
IPDI-CM
FAK inh14+ IPDI-CM
BEAS-2B
HBEC
C
Cell proliferation (% of control)
Control
FAK inhibitor 14
rhIL-8
rhIL-8+FAK inhibitor 14
D
Cell migration (OD. of 520 nm)
BEAS-2B
HBEC
E
Cell migration (OD. at 520 nm)
Control
FAK inhibitor 14
rhIL-8
rhIL-8+FAK inhibitor 14

Figure 5

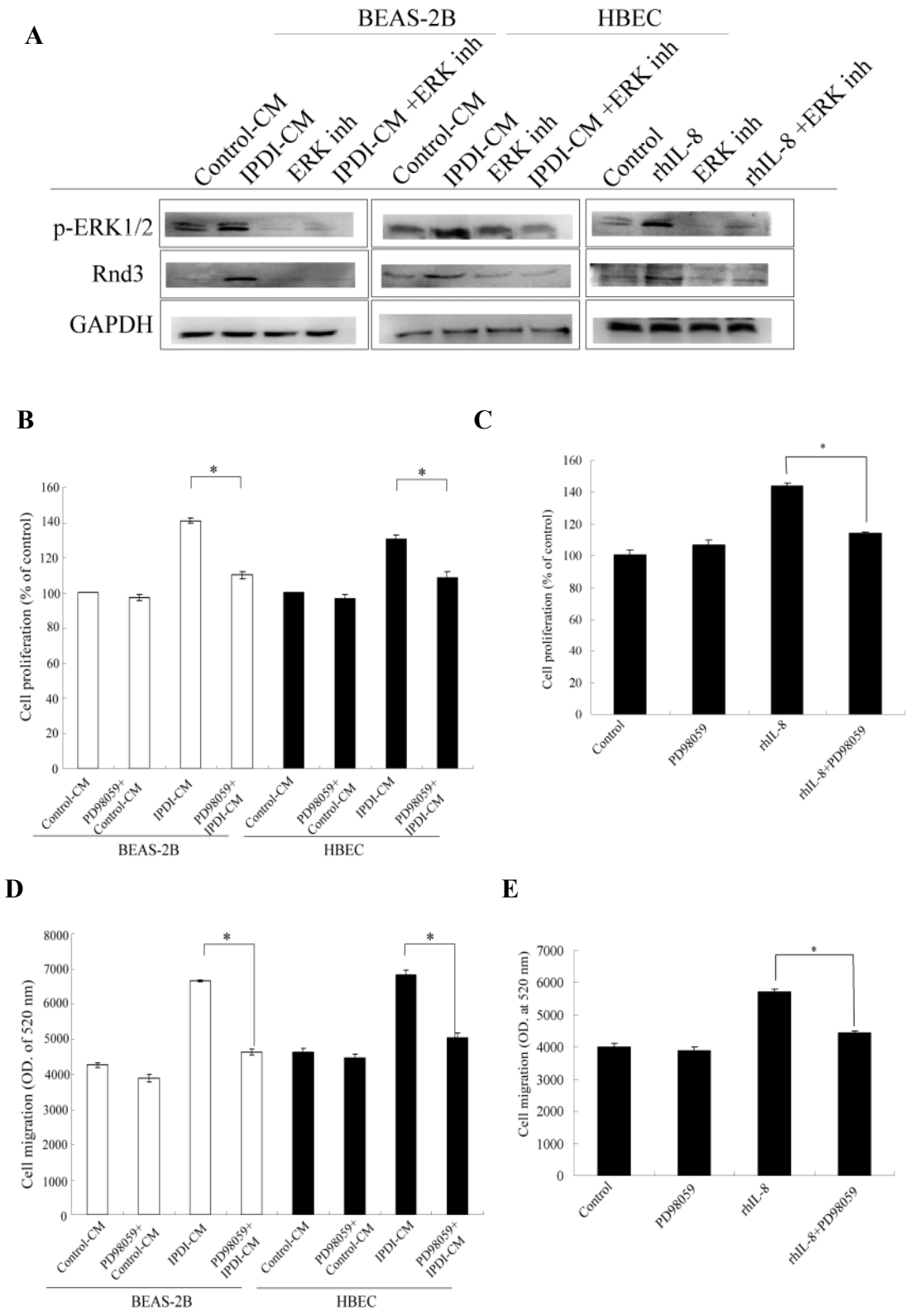
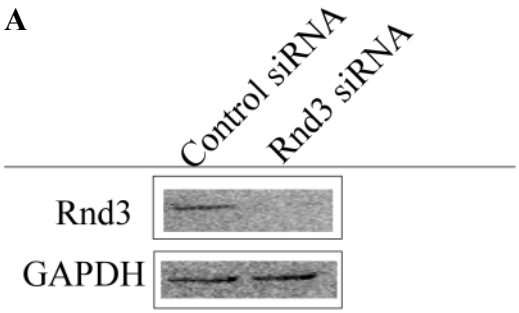
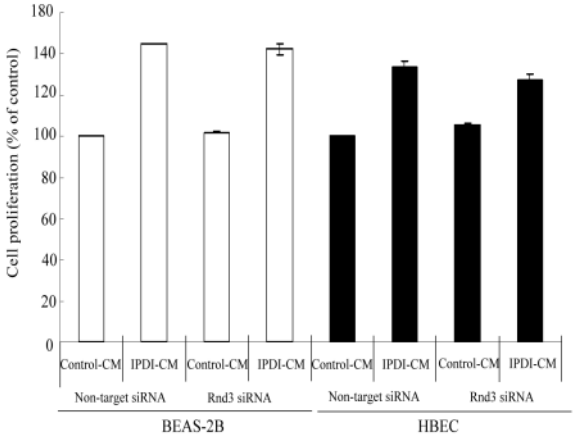
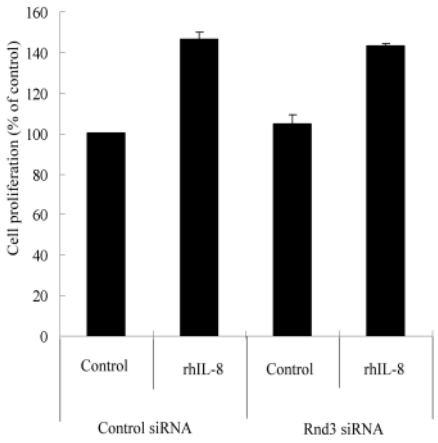
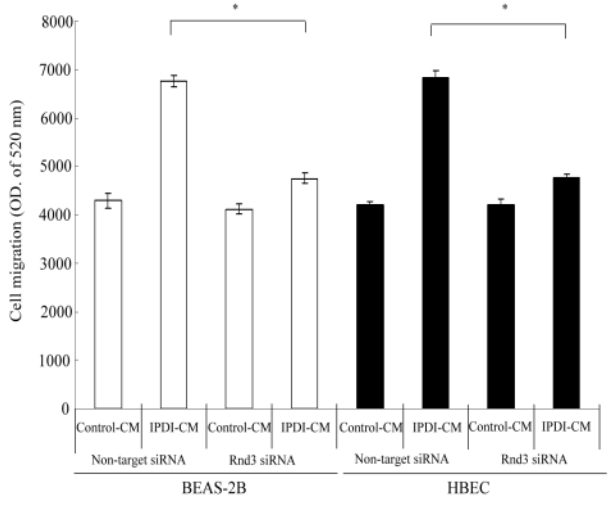

A
BEAS-2B
HBEC
Control-CM
IPDI-CM
ERK inh
IPDI-CM +ERK inh
Control-CM
IPDI-CM
ERK inh
IPDI-CM +ERK inh
Control
rhIL-8
ERK inh
rhIL-8 +ERK inh
p-ERK1/2
Rnd3
GAPDH
B
Cell proliferation (% of control)
Control-CM
PD98059+ Control-CM
IPDI-CM
PD98059+ IPDI-CM
BEAS-2B
HBEC
C
Cell proliferation (% of control)
Control
PD98059
rhIL-8
rhIL-8+PD98059
D
Cell migration (OD. of 520 nm)
E
Cell migration (OD. at 520 nm)

Figure 6


A
Control siRNA
Rnd3 siRNA
Rnd3
GAPDH


B
Cell proliferation (% of control)
180
140
120
100
80
60
40
20
0
Control-CM
IPDI-CM
Control-CM
IPDI-CM
Control-CM
IPDI-CM
Control-CM
IPDI-CM
Non-target siRNA
Rnd3 siRNA
Non-target siRNA
Rnd3 siRNA
BEAS-2B
HBEC


C
Cell proliferation (% of control)
160
140
120
100
80
60
40
20
0
Control
rhIL-8
Control
rhIL-8
Control siRNA
Rnd3 siRNA


D
Cell migration (OD. of 520 nm)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
Control-CM
IPDI-CM
Control-CM
IPDI-CM
Control-CM
IPDI-CM
Control-CM
IPDI-CM
Non-target siRNA
Rnd3 siRNA
Non-target siRNA
Rnd3 siRNA
BEAS-2B
HBEC

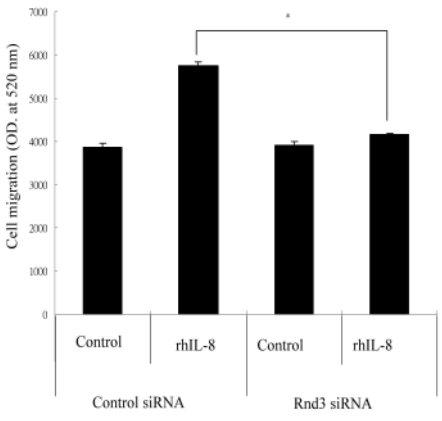

E
Cell migration (OD. at 520 nm)
7000
6000
5000
4000
3000
2000
1000
0
*
Control
rhIL-8
Control
rhIL-8
Control siRNA
Rnd3 siRNA

Figure 7

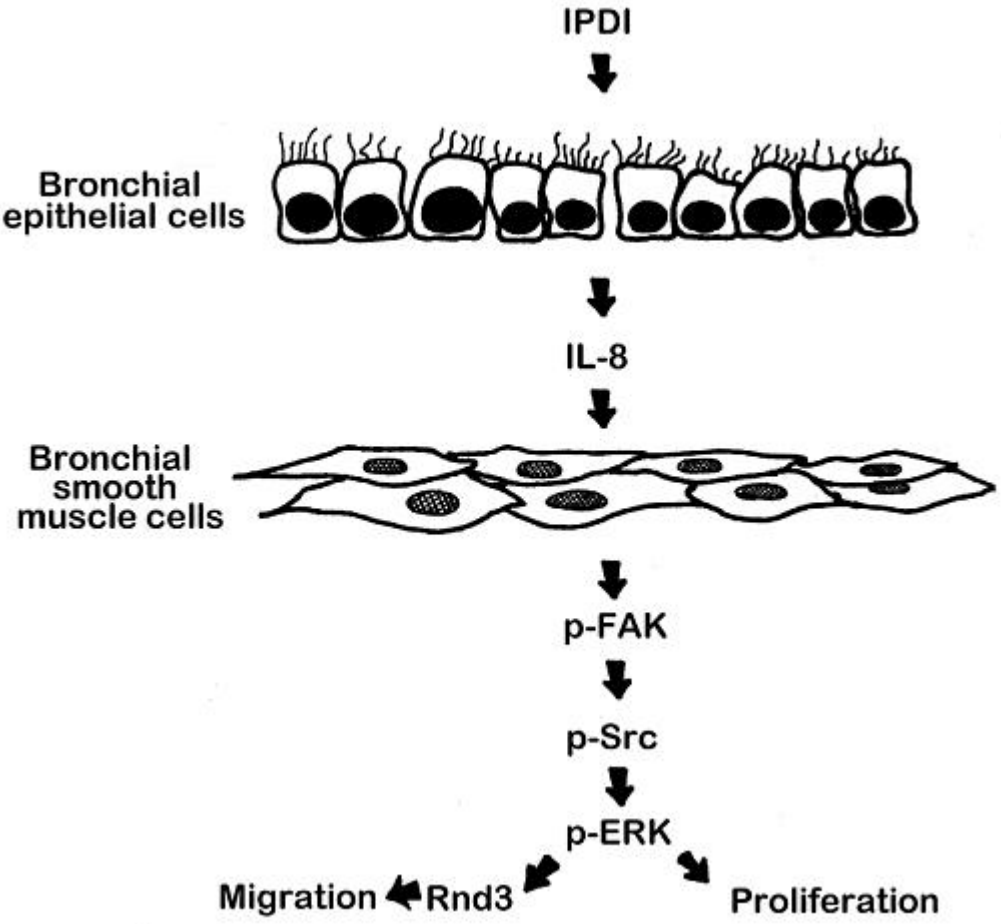

IPDI
Bronchial epithelial cells
IL-8
Bronchial smooth muscle cells
p-FAK
p-Src
p-ERK
Migration
Rnd3
Proliferation

Figure 8