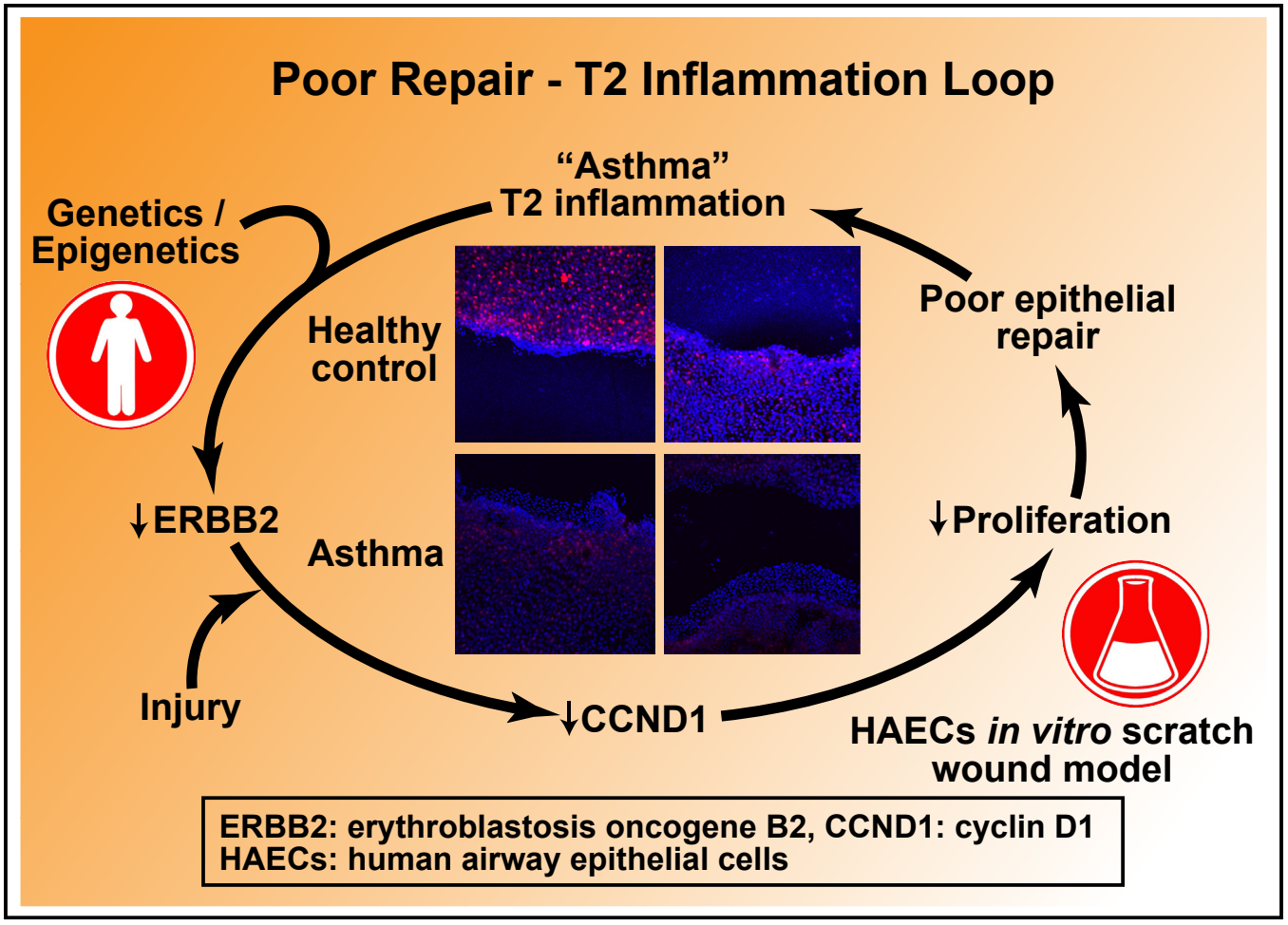
Dysfunctional ErbB2, an EGF receptor family member, hinders repair of airway epithelial cells from asthmatic patients

Hideki Inoue, MD, PhD,[a,b] Takeshi Hattori, MD, PhD,[a] Xiuxia Zhou, PhD,[a] Emily B. Etling, BS,[a] Brian D. Modena, MD,[c,d] John B. Trudeau, BA,[a] Fernando Holguin, MD, MPH,[e] and Sally E. Wenzel, MD[a] *Pittsburgh, Pa, Tokyo, Japan, La Jolla and San Diego, Calif, and Aurora, Colo*

GRAPHICAL ABSTRACT



From [a]the Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Pittsburgh; [b]the Division of Pulmonary and Allergy Medicine, Department of Medicine, Showa University School of Medicine, Tokyo; [c]Molecular and Experimental Medicine, Scripps Research Institute, La Jolla; [d]the Department of Allergy, Asthma and Immunology, Scripps Health, San Diego; and [e]the Division of Medicine-Pulmonary Sciences & Critical Care, University of Colorado School of Medicine, Aurora.

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Corresponding author: Sally E. Wenzel, MD, 130 Desoto St, 4127 Public Health, Pittsburgh, PA 15213. E-mail: SWENZEL@pitt.edu.

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Background: Genetic and genomic data increasingly point to the airway epithelium as critical to asthma pathogenesis. Epithelial growth factor (EGF) family members play a fundamental role in epithelial differentiation, proliferation, and repair. Although expression of erythroblastosis oncogene B2 (ErbB2) mRNA, an EGF family receptor, was reported to be lower in asthmatic patients, little is understood about its functional role.
Objective: We sought to determine whether decreased ErbB2 activation in freshly isolated human airway epithelial cells (HAECs) from asthmatic patients associated with impaired wound closure *in vitro*.
Methods: An *in vitro* scratch-wound model of air-liquid interface cultured and freshly isolated HAECs were compared between HAECs from healthy control subjects (HCs) and asthmatic patients in relation to ErbB2.
Results: Freshly brushed HAECs from asthmatic patients had impaired ErbB2 activation compared with those from HCs. In an *in vitro* scratch-wound model, HAECs from asthmatic patients showed delayed wound closure compared with HAECs from HCs. Cell proliferation, as assessed based on [^{3}H] thymidine incorporation after wounding, and expression or activation of ErbB2 and cyclin D1 at the leading edge of the wound were lower in HAECs from asthmatic patients and HCs. A selective ErbB2 tyrosine kinase inhibitor, mubritinib, impaired wound closure and decreased cyclin D1 expression in healthy HAECs, with less effect on cells from asthmatic patients, supporting diminished activity in asthmatic patients.
Conclusion: These results implicate a primary defect in the ErbB2 pathway as constraining epithelial repair processes in asthmatic patients. Restoration of homeostatic ErbB2 function should be considered a novel asthma therapeutic target. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: *Asthma, epidermal growth factor receptor, erythroblastosis oncogene B2, epithelial cell, wound repair, airway inflammation, cell proliferation, cyclin D1, air-liquid interface culture*

Abbreviations used
ALI: Air-liquid interface
CCND1: Cyclin D1
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ErbB2: Erythroblastosis oncogene B2
FENO: Fraction of exhaled nitric oxide
HAEC: Human airway epithelial cell
HC: Healthy control subject
IQR: Interquartile range
pErbB2: Phosphorylated ErbB2
qRT-PCR: Quantitative RT-PCR
ROI: Region of interest
tErbB2: Total ErbB2
%WC: Percentage of wound closure

Airway epithelial cells critically maintain barrier function, trigger innate immune pathways, and intersect with adaptive immunity.[1,2] Epithelial abnormalities in asthmatic patients are increasingly observed,[3-6] with recent genomic studies of fresh human airway epithelial cells (HAECs) reporting vast abnormalities in "epithelial growth and repair"–related gene expression in association with worsening disease.[7,8] Some studies have suggested that epithelial repair abnormalities persist even after long culture duration, supporting genetic (or epigenetic) influences.[9] In fact, the most consistently observed genetic locus in relation to asthma, 17q21, is highly enriched for epithelium-related genes,[10-12] with a recent genome-wide methylation study further linking this locus with molecular pathways and asthma risk.[13] However, the precise mechanisms by which these genetic-epigenetic abnormalities affect asthma pathogenesis, including specific alterations in proliferation, migration, apoptosis, tight junction formation, or mucociliary differentiation (and the molecular pathways behind them), remain unclear.

The epidermal growth factor (EGF) receptor (EGFR) family plays a central role in maintaining epithelial cell homeostasis, and EGF itself is necessary for *in vitro* cell propagation and differentiation.[14] The pathway consists of 4 known receptors: erythroblastosis oncogene B1 (ErbB1) to erythroblastosis oncogene B4 (ErbB4). These receptors, which function as homodimers or heterodimers, are activated by a range of EGF family ligands, from EGF to various "-regulin" family members to the mucin MUC4β. Phosphorylation of these receptors increases proliferation, migration, and tight junction formation; affects HAEC differentiation; and augments mucus production.[15,16] EGF pathway involvement in airway epithelial dysfunction in asthmatic patients is unclear, with some studies suggesting overactivity and others suggesting underactivity of the pathway.[17-20] One of the tyrosine kinase receptors (ErbB2; also known as HER2, Neu, or CD340) is located within 200 kbp of the 17q12-21 hotspot and has specifically been identified on genome-wide association study pathway analysis, methylation, and gene expression studies in relation to asthma and severe asthma.[7,13,21] Although ErbB2 overactivity is strongly linked to some adenocarcinomas, ErbB2 is also essential for normal HAEC differentiation and integrity. Knockouts are embryonically lethal, confirming their crucial role in survival,[22-24] with microarray data reporting reduced *ERBB2* mRNA expression in severe asthma clusters.[7] However, its role in epithelial cell repair in asthmatic patients has not been investigated.

We hypothesized that the impaired wound repair response observed in HAECs from asthmatic patients both *in vivo* and *in vitro* would be caused by a fundamental reduction in proliferative responses to wounding, stemming from decreased activation of ErbB2 pathways. To address this hypothesis, HAECs were analyzed for ErbB2 activation by using phosphorylated ErbB2 (pErbB2) expression *ex vivo* and cultured in an air-liquid interface (ALI) with an *in vitro* scratch-wound model to compare repair and proliferative responses between groups.

METHODS

Subjects

Healthy control subjects (HCs) and asthmatic patients were recruited as part of several clinical studies, as fully described in the Methods section in this article's Online Repository at www.jacionline.org. Because of the length of the study, several subgroups of subjects were recruited (see the Results section). This study was approved by the Institutional Review Board of the University of Pittsburgh, and written informed consent was obtained from all study subjects before enrollment.

Bronchoscopy

Bronchoscopy was performed, and epithelial brushings were obtained from fourth- to fifth-generation bronchi, as previously described.[25,26]

TABLE I. Subjects' demographics

	In vivo (freshly brushed HAECs)		*In vitro* (ALI culture)			
Experiment	**WB**	**IF**	**WC/mRNA/WB**	**[^{3}H] thymidine**	**ErbB2 inhibitor (WC/WB/IF)**	***P* value**
No.	26	13	34	13	12	
HC/MMA/SA	6/8/12	4/5/4	12/11/11	5/4/4	6/6/0	
Age (y)	38.7 ± 14.1	38.5 ± 12.2	42.1 ± 11.2	46.8 ± 8.2	33.9 ± 13.3	.08
Sex, male/female	9/17	5/8	13/21	5/8	6/6	.93
Race, C/AA/O	20/3/3	11/1/1	25/8/1	10/3/0	10/2/0	.62
Inhaled corticosteroids, yes/no	16/10	7/6	16/18	7/6	3/9	.32
FEV_1 (% predicted)	71.5 ± 23.1	73.2 ± 31.2	79.6 ± 26.2	84.6 ± 24.9	91.0 ± 17.0	.18
FENO (ppb)	26.5 (19.7–44.3)	16 (14.5–31.5)	24 (17–64)	23 (17.3–60.3)	16 (10.5–35.7)	.27

Data are represented as means ± SDs or medians (IQRs). *P* values were analyzed by means of ANOVA.
AA, African American; *C*, Caucasian; *[^{3}H] thymidine*, [^{3}H] thymidine incorporation assay; *IF*, immunofluorescent staining; *MMA*, patients with mild-to-moderate asthma; *O*, others; *SA*, patients with severe asthma; *WB*, Western blotting; *WC*, wound closure.

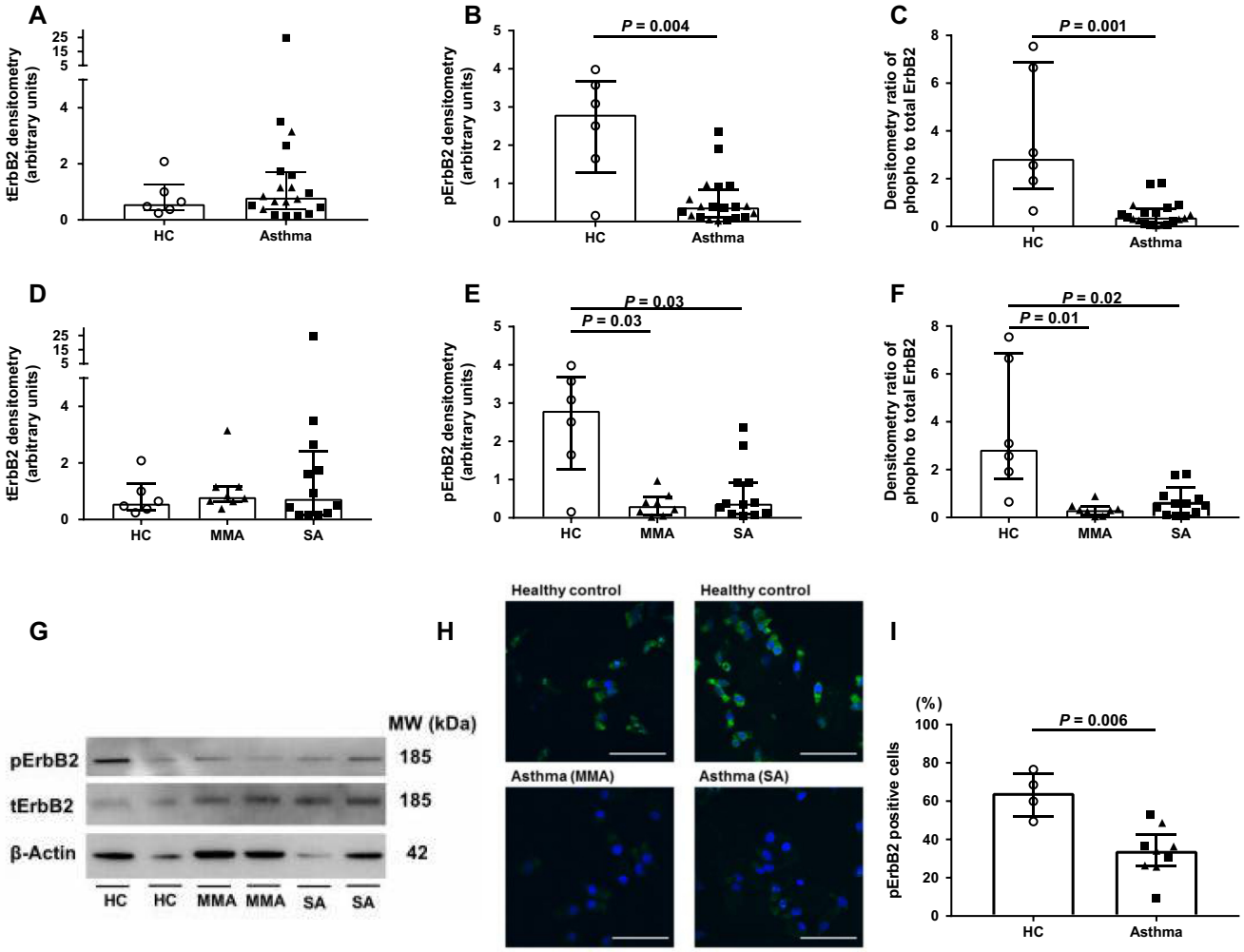

FIG 1. *In vivo* pErbB2 protein expression in freshly isolated HAECs is lower in asthmatic patients than in healthy subjects. tErbB2 and pErbB2 protein expression in freshly isolated HAECs was analyzed by means of Western blotting **(A-G)** and immunofluorescent staining of cytospin preparations (**H** and **I**). Fig 1, *A* and *D*, There were no differences in tErbB2 protein expression across the groups. Fig 1, *B*, *C*, *E*, and *F*, pErbB2 protein (Fig 1, *B* and *E*) and the ratio of pErbB2 to tErbB2 (p/t ErbB2; Fig 1, *C* and *F*) was lower in HAECs from asthmatic patients but without difference by severity. Fig 1, *G*, Representative Western blots for ErbB2 among freshly isolated HAECs. Fig 1, *H*, Representative immunofluorescent pictures for pErbB2 among cytospin HAEC samples. Green, pErbB2; blue, nucleus. The *white bar* represents 100 μm. Fig 1, *I*, pErbB2-positive cells were less in HAECs from asthmatic patients than in those from HCs. Each *bar* represents medians with IQRs. Statistical analysis was performed by using the Wilcoxon rank sum test. ○, HCs; ▲, patients with mild-to-moderate asthma; ■, patients with severe asthma. *MMA*, Mild-to-moderate asthma; *MW*, molecular weight; *SA*, severe asthma.

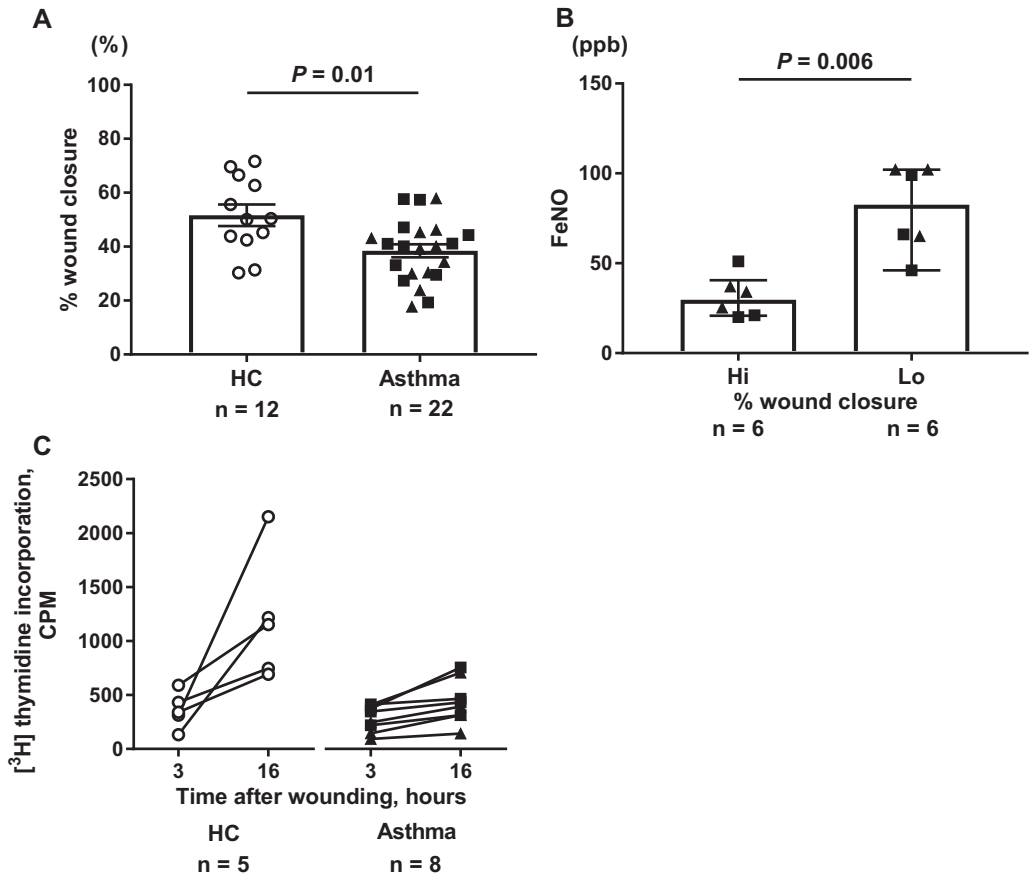

FIG 2. Wound closure and cell proliferation after wounding are impaired in HAECs from asthmatic patients. **A,** Wound closure was decreased in HAECs from asthmatic patients compared with that in HAECs from HCs in ALI culture. HAECs were cultured in ALI for 7 days. Wound area was measured at baseline and 7 hours after wounding, and %WC at 7 hours after wounding was calculated. Each *bar* represents means ± SEMs. Statistical analysis was performed with the Student *t* test. **B,** Asthmatic patients with the worst HAEC wound closure *(bottom quartile)* had greater FENO levels than those whose wound closure overlapped with normal wound repair *(top quartile)*. High %WC was defined as a %WC of greater than 45%, and low %WC was defined as a %WC of less than 30%. **C,** Cell proliferation after wounding was impaired in HAECs from asthmatic patients compared with HAECs from HCs. [^{3}H] thymidine incorporation at 16 hours after wounding was greater in HAECs from HCs than in HAECs from asthmatic patients. The fold change of [^{3}H] thymidine incorporation between 3 and 16 hours after wounding was significantly greater in HAECs from HCs than in those from asthmatic patients. Statistical analyses were performed by using the Student *t* test and Wilcoxon rank sum test, respectively. ○, HCs; ▲, patients with mild-to-moderate asthma; ■, patients with severe asthma.

Primary HAEC culture at the ALI

Primary HAECs from airway brushings were cultured by using an ALI (see the Methods section in this article's Online Repository).[25,26] After 7 days of ALI culture, when full differentiation, including ciliated and mucus cells, was achieved,[27,28] HAECs were harvested, as described below. For ErbB2 inhibitor experiments, cultured cells were treated with a selective tyrosine kinase inhibitor of ErbB2 (mubritinib, 100 nmol/L; Selleck Chemicals, Houston, Tex) or dimethyl sulfoxide 16 hours before wounding until the time of harvest.[29]

Epithelial wound model

A scratch-wound model for ALI culture was developed to evaluate the wound repair response of airway epithelial cells. On day 7 of ALI, 3 straight lines were manually etched on cell-culture surfaces by using a 200-μL pipette tip, and the wound area was measured by using live cell imaging microscopy (see the Methods section in this article's Online Repository). The percentage of wound closure (%WC) at 7 hours was reported. Greater %WC indicates better wound healing.

[^{3}H] thymidine incorporation assay

Cell proliferation was measured by using the [^{3}H] thymidine incorporation assay on day 7 of ALI, as shown in the Methods section in this article's Online Repository. The time point of 3 hours after wounding was used for baseline [^{3}H] incorporation, and that of 16 hours after wounding was used for measurement of cell proliferation based on the DNA synthesis cycle. The fold change of [^{3}H] thymidine incorporation between 3 and 16 hours after wounding was calculated to compare cell proliferation between HAECs from HCs and asthmatic patients.

Quantitative RT-PCR and Western blot analysis

On day 7 of ALI and 7 hours after scrape wounding, HAECs were harvested and processed for quantitative RT-PCR (qRT-PCR) and Western blot analysis of targeted cyclin D1 (CCND1; as a cell proliferation marker regulated by activation of EGF and other pathways),[30,31] as well as ErbB2. Freshly brushed HAECs were also processed for total ErbB2 (tErbB2) and pErbB2 protein expression. Detailed methods are described in the Methods section in this article's Online Repository.

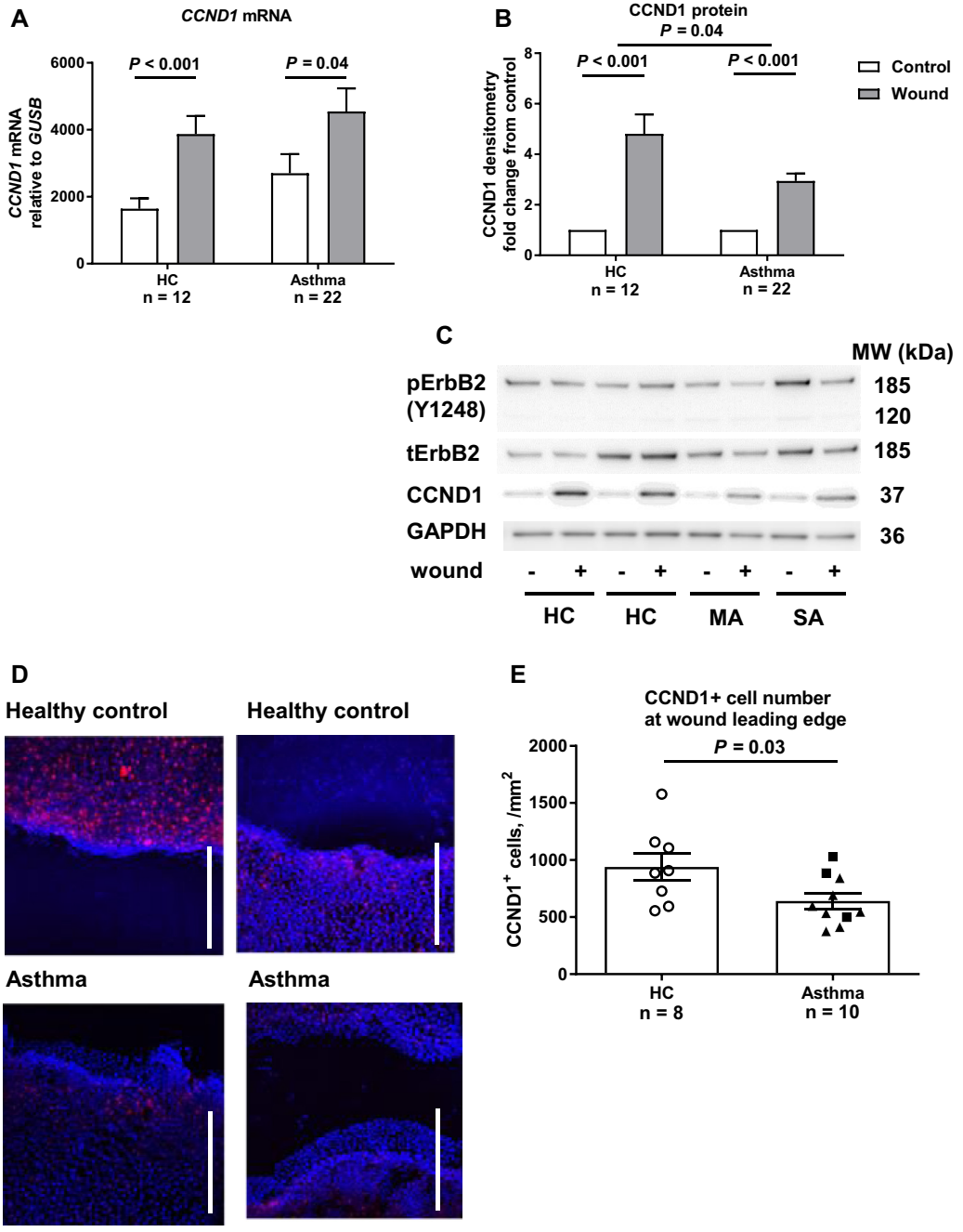

FIG 3. Wounding-induced CCND1 expression is reduced in HAECs from asthmatic patients. **A,** *CCND1* mRNA expression. **B,** CCND1 protein expression. **C,** Representative Western blot for ErbB2 and CCND1 protein expression with or without wounding. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *MA*, mild-to-moderate asthma; *MW*, molecular weight; *SA*, severe asthma. **D,** Representative immunofluorescent staining images for CCND1 at the wound's leading edge. Red, CCND1; blue, nucleus. The *white bar* represents 300 μm. **E,** CCND1-positive cells along the wound's leading edge were greater in HAECs from HCs compared with those form asthmatic patients. Each *bar* represents means ± SEMs. Statistical analysis was performed with the Student *t* test. ○, HCs; ▲, patients with mild-to-moderate asthma; ■, patients with severe asthma.

Immunofluorescent staining

At 7 hours after scraping, the entire Transwell membrane was processed for immunofluorescent staining for CCND1 and pErbB2. Cytospin samples of freshly brushed HAECs were also stained for pErbB2. For Transwell membrane samples, regions of interest (ROIs) were randomly and intermittently obtained along the wound's leading edge on each wounded culture. Numbers of CCND1-positive cells and fold change of fluorescent intensity of pErbB2 between the wound's leading edge and the nonwounded area were measured. For cytospin samples, percentages of pErbB2-positive cells were analyzed (see the Methods section in this article's Online Repository).

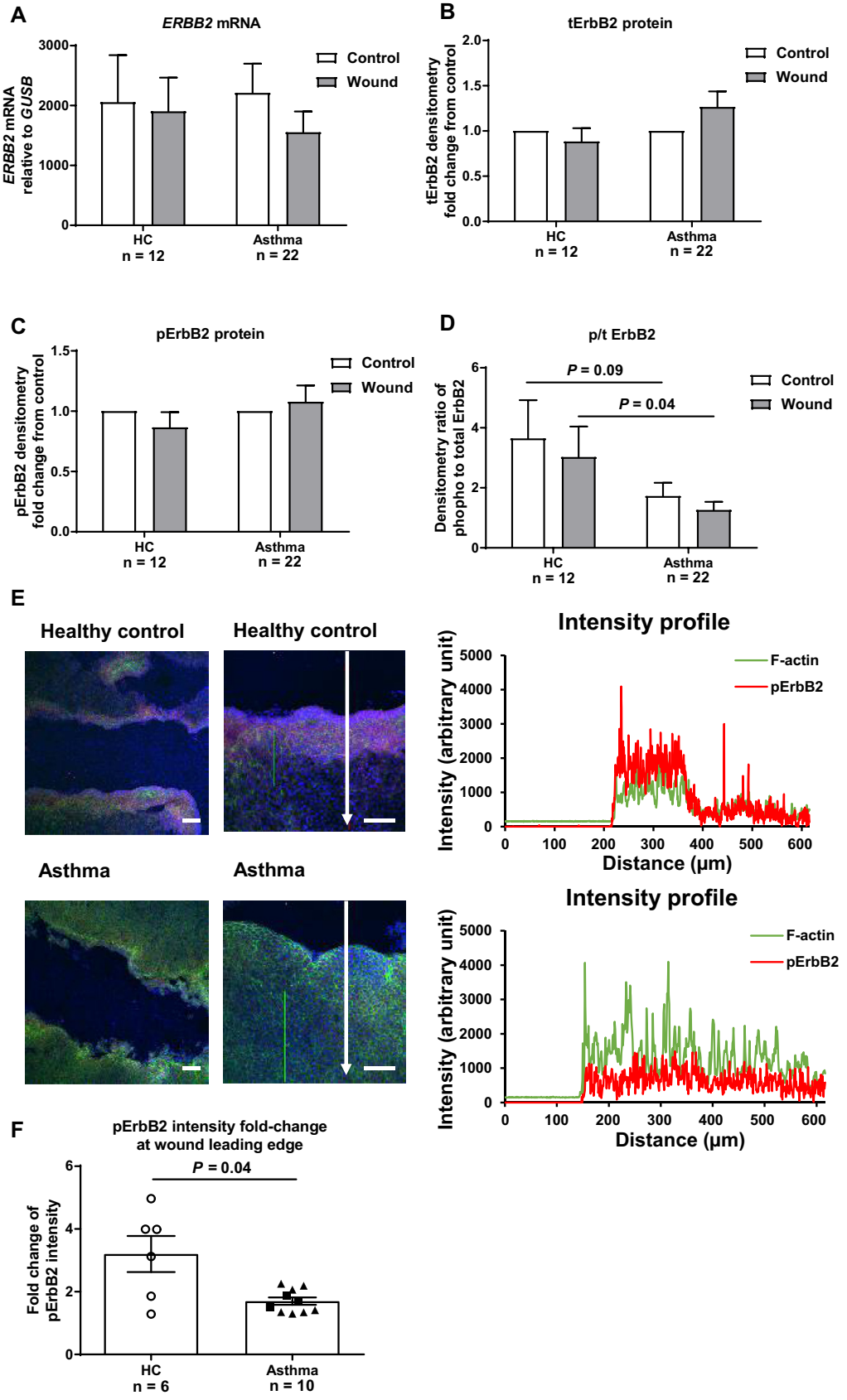

FIG 4. Phosphorylation of ErbB2 after wounding is impaired in HAECs from asthmatic patients. **A,** *ERBB2* mRNA expression. **B,** tErbB2 protein expression. **C,** pErbB2 protein expression. **D,** Ratio of phosphorylated to tErbB2 protein ($P = .09$ and .04 for difference in ratio between HCs and asthmatic patients before and after

Statistics

Summary statistics are presented as means ± SEMs or medians (interquartile ranges [IQRs]) for fraction of exhaled nitric oxide (FENO), *in vivo* pErbB2 expression analysis, and fold change of [^{3}H] thymidine incorporation between 3 and 16 hours caused by nonlinear data distribution. *In vitro* comparisons across subject type or condition are expressed as fold change compared with control values, primarily because of varying baseline values. Differences in fold changes are then analyzed by means of ANOVA, followed by multiple comparison testing with Bonferroni correction ($P < .0125$). All statistical analysis was performed with JMP Pro software (version 12; SAS Institute, Cary, NC). P values of less than .05 were considered statistically significant.

RESULTS

Study subjects

Because of the complexity of the study and sample availability, each experiment used subgroups of the overall study population (Table I and see Tables E1-E5 in this article's Online Repository at www.jacionline.org). There were no demographic differences between subgroups except for the ErbB2 inhibitor experiment, which included no patients with severe asthma because of ease of availability and the observed lack of effect of severity on wound closure.

pErbB2 expression is lower in freshly isolated HAECs from asthmatic patients than in HAECs from HCs

tErbB2 and pErbB2 protein expression was analyzed in freshly isolated HAECs (Table I and see Table E1).[7] Although there were no differences in tErbB2 expression, pErbB2 expression (and its ratio to tErbB2 [p/t ErbB2]) was lower in HAECs from asthmatic patients than in HAECs from HCs (Fig 1, *A-C* and *G*). There were no differences by asthma severity (Fig 1, *D-F*). pErbB2 expression was evaluated by means of immunofluorescent staining on freshly brushed HAEC cytospin preparations to confirm these results (see Table E2). HAECs from asthmatic patients had fewer pErbB2-positive cells than those from HCs (Fig 1, *H-I*), without a difference by severity.

HAECs from asthmatic patients exhibit abnormal wound closure in a scratch-wound model

Among the subgroup of *in vitro* wound-closure experiments, there were lower percent predicted values of FEV_1 and higher FENO levels in asthmatic patients compared with HCs, whereas age, sex, and race were not different (see Table E3). Seven hours after scraping (day 7 of ALI culture), %WC was 51.6% ± 4.0% in HAECs from HCs compared with 38.5% ± 2.4% in HAECs from asthmatic patients (mean ± SEM; $P = .01$), as measured by using live cell microscopy (Fig 2, *A*). There were no differences in %WC between cells from patients with mild-to-moderate asthma and those from patients with severe asthma (37.1% ± 3.4% and 39.8% ± 3.6%, $P = .59$). However, asthmatic patients in the lowest quartile for %WC (%WC < 30%) had greater FENO levels than those in the greatest %WC quartile (%WC > 45%; 80.0 ± 24.1 vs 31.4 ± 11.8 ppb; $P = .006$; Fig 2, *B*). No other clinical characteristics (including inhaled corticosteroid use, atopic status, exacerbation history, and physiologic or inflammatory patterns) differentiated %WC values in asthmatic patients or HCs.

Cell proliferation is impaired in HAECs from asthmatic patients after wounding

[^{3}H] thymidine incorporation was measured to evaluate cell proliferation after wounding. Although there were no differences in proliferation as measured by counts per minute at 3 hours after wounding, by 16 hours, the [^{3}H] thymidine counts per minute were significantly greater in HAECs from HCs than those from asthmatic patients (Fig 2, *C*). The fold change of [^{3}H] thymidine incorporation between 3 and 16 hours was also less in HCs from asthmatic patients compared with those form HCs (median, 1.56 [IQR, 1.29-1.95] and 2.04 [IQR, 1.84-8.05]; $P = .03$; Fig 2, *C*). Asthma severity did not affect [^{3}H] thymidine incorporation ($P = .15$). No significant increase was observed at 7 hours after wounding (see Fig E1 in this article's Online Repository at www.jacionline.org). Despite similar trends in wound repair and proliferation, [^{3}H] thymidine incorporation and %WC did not correlate.

CCND1 expression after wounding is decreased in HAECs from asthmatic patients

CCND1 expression in total cells from the Transwell membrane increased compared with control conditions at both the mRNA and protein levels in all cultures after wounding (Fig 3, *A* and *B*, and see Fig E2 in this article's Online Repository at www.jacionline.org). Wound-related fold induction of CCND1 protein (but not mRNA) was greater in HAECs from HCs than in those from asthmatic patients (Fig 3, *B* and *C*). Similarly, expression of CCND1 at the wound edge, evaluated by using immunofluorescent staining, revealed greater numbers of CCND1-positive cells at the leading edge of the wound in HAECs from HCs compared with those from asthmatic patients (Fig 3, *D* and *E*). CCND1 expression values did not correlate with %WC values.

Phosphorylation of ErbB2 along the leading edge of the wound is decreased in HAECs from asthmatic patients

To determine whether ErbB2 pathway activation could be contributing to the abnormal wound repair, we evaluated *ERBB2* mRNA expression in HAECs at 7 hours. *ERBB2* expression did not change after wounding, and there were no differences

wounding, respectively). **E,** Representative immunofluorescent staining pictures and their intensity profile for pErbB2 and F-actin at the wound's leading edge. Red, pErbB2; green, F-actin; blue, nucleus. The *white bar* represents 100 μm. **F,** pErbB2 expression along the wound's leading edge was greater in HAECs from HCs compared with that in HAECs from asthmatic patients. Each *bar* represents means ± SEMs. Statistical analysis was performed with the Student *t* test. ○, HCs; ▲, patients with mild-to-moderate asthma; ■, patients with severe asthma.

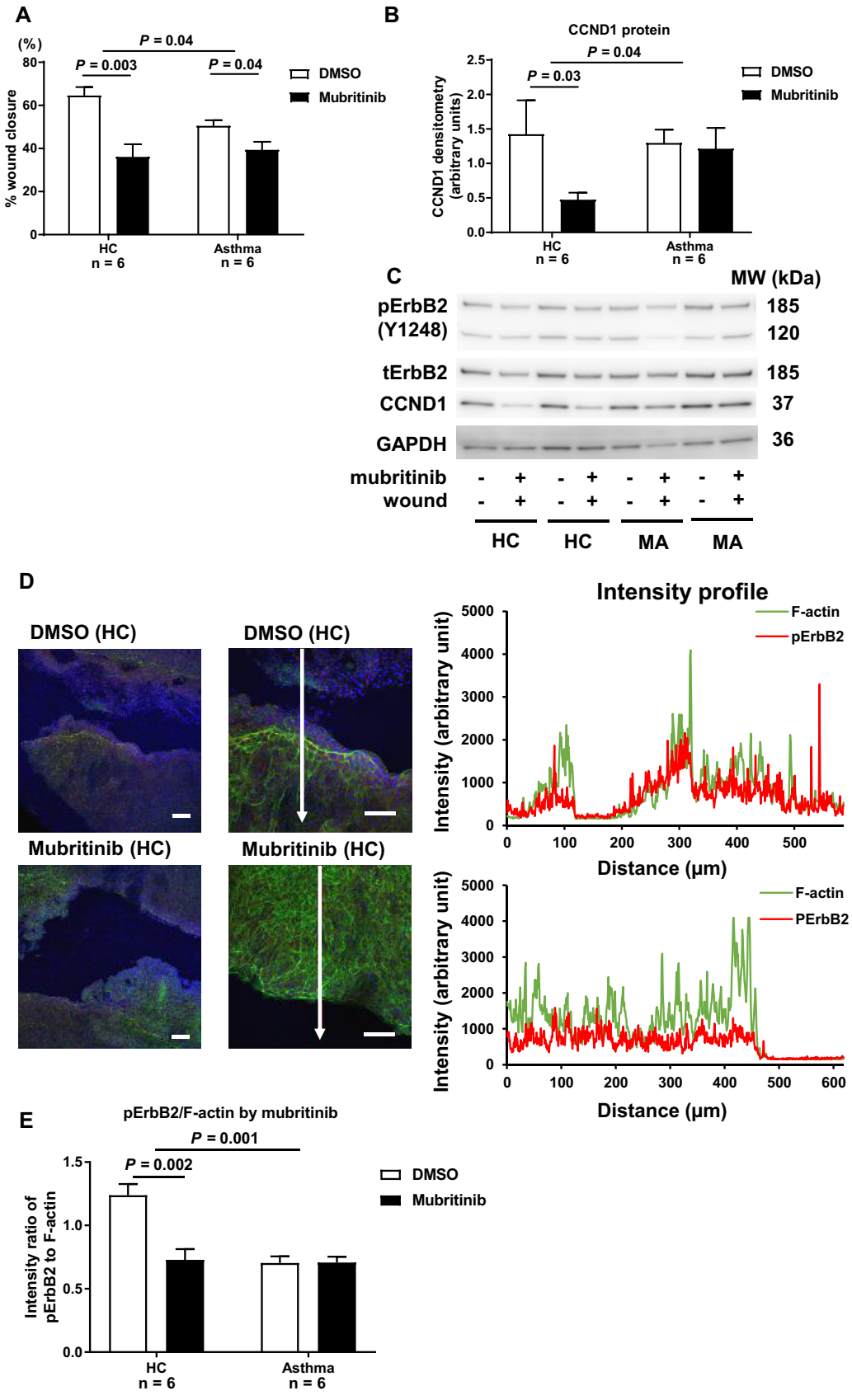

FIG 5. Inhibition of ErbB2 phosphorylation impairs wound closure, and the effect is greater in HAECs from HCs. **A,** Mubritinib (100 nmol/L) treatment decreased wound closure after 7 hours of wounding, with a greater effect in HAECs from HCs compared with HAECs from asthmatic patients ($P = .04$ for difference

between subject groups when evaluating the entire Transwells (Fig 4, *A*). However, despite the lack of significant changes, *ERBB2* mRNA expression correlated strongly with *CCND1* mRNA expression after wounding ($\rho = 0.82$, $P < .0001$). In Western blots, 2 bands were observed for pErbB2, a full-length (transmembrane form, 185 kDa) and a truncated (nuclear translocated form, 120 kDa) form, as shown in Figs 3, *C*, and 5, *C*. The former was used for analyses. Although there were no consistent changes in pErbB2 or tErbB2 protein levels after wounding from either subject group, as determined by means of Western blotting (Fig 4, *B* and *C*, and see Fig E3 in this article's Online Repository at www.jacionline.org), the ratio of pErbB2 to tErbB2 protein (p/t ErbB2) was less in HAEC wounds from asthmatic patients compared with those from HCs (Fig 4, *D*). In small numbers there were no other correlations between ErbB2 expression and any clinical parameters, including FEV_1 or dose of inhaled corticosteroid. Local ErbB2 expression at the leading edge of the wound was also analyzed by using immunofluorescence 7 hours after wounding. pErbB2 was upregulated along the leading edge of the wound compared with the nonwounded area in the intensity profile analysis (Fig 4, *E*). The fold change in the intensity of pErbB2 from the "no wound/control" area to the leading edge of the wound was less in HAECs from asthmatic patients than in those from HCs (Fig 4, *F*), supporting lower wound-associated activation of ErbB2 in asthmatic patients. ErbB2 expression did not directly correlate with %WC.

Inhibition of ErbB2 phosphorylation differentially affects wound closure in HAECs from HCs compared with those from asthmatic patients

Inhibition of ErbB2 phosphorylation by mubritinib significantly decreased wound closure in HAECs from both HCs and asthmatic patients (Fig 5, *A*). However, the decrease in wound closure after mubritinib treatment was greater in HAECs from HCs than in those from asthmatic patients (Fig 5, *A*). Similarly, CCND1 protein expression decreased with mubritinib treatment to a greater degree in HAECs from HCs than in those from asthmatic patients (Fig 5, *B* and *C*). These results were verified by using immunofluorescent staining because mubritinib treatment suppressed ErbB2 phosphorylation along the leading edge to a greater degree in HAECs from HCs than in those from asthmatic patients, likely because of lower baseline ErbB2 activation in the cells from asthmatic patients (Fig 5, *D* and *E*).

DISCUSSION

In this study lower ErbB2 activation was observed in freshly brushed HAECs from asthmatic patients compared with those from HCs. These *ex vivo* findings were then mechanistically explored in primary ALI cultures, in which HAECs from asthmatic patients exhibited diminished wound closure, a reduction in proliferation, changes in CCND1 expression, and ErbB2 activation compared with cells from HCs. Combined with previous genetic and genomic studies, these results strongly support abnormalities of the ErbB2 pathway in human airway epithelial cells from asthmatic patients on wound repair.

The delayed airway epithelial repair in asthmatic patients observed here could allow easier access of pathogens to the submucosa and the rich array of immune cells located there, promoting airway inflammation or even airway remodeling. The reasons for the observed low activation of the ErbB2 pathway in HAECs from asthmatic patients are not well known. Although genetic factors controlling both expression and possibly activity of the ErbB2 pathway are likely, given the gene's strong linkage disequilibrium with the 17q12-21 "hotspot," epigenetic factors could also be involved.[10-13]

Our findings that the worst wound repair was seen in cells from patients with higher FENO levels *(in vivo)* suggest that T2 inflammation might have epigenetically altered cells in such a way as to promote lower ErbB2 activation responses, even after nearly a month in culture. Alternatively, genetic or epigenetic changes in HAECs could promote generation of the T2 immune processes observed *in vivo*. In the previous microarray study, reduced ErbB2 expression was seen in patients with the highest levels of FENO, a marker of T2 inflammation.[7] IL-13 has been reported to have epigenetic effects through inhibition of microRNA expression, which might regulate cell differentiation, at least in part through effects on the NOTCH1 pathway. However, its ability to affect ErbB2 expression/activation is unknown.[32] Finally, loss of EGF receptor family ligands, including MUC4β, the only known endogenous ligand for ErbB2 homodimers, could also prolong epithelial repair through decreased activation of heterodimers of ErbB2 with other EGFR family members.[33,34]

Although many pathways are considered to be involved with epithelial repair, including migration, apoptosis, and barrier function, our studies suggest that reduced proliferation is playing an important role in HAECs from asthmatic patients. ErbB2 is widely considered critical to wound healing and epithelial differentiation, but the vast majority of published studies address ErbB2 in the context of cancer, including breast and gastric tumors, in which its overexpression is believed to contribute to its dimerization with EGFR or ErbB3, resulting in aberrant cell survival and proliferation.[35-37] However, ErbB2 has also been studied in relation to epithelial repair and differentiation.

Vermeer et al[38] identified ErbB2 phosphorylation along the leading edge of a physical wound using an ALI model of HAECs. They reported that treatment with an anti-ErbB2 antibody decreased ciliated and increased metaplastic cells in differentiated HAEC cultures.[22] The findings reported here of very low expression (and activity) in freshly brushed cells (by mRNA and protein) and low activity in cultured asthmatic cells after

in decrease in wound closure between HCs and asthmatic patients). **B,** CCND1 protein expression was inhibited by mubritinib treatment in HAECs from HCs but not in HAECs from asthmatic patients ($P = .04$ for difference between HCs and asthmatic patients). **C,** Representative Western blot for ErbB2 and CCND1 protein expression with or without mubritinib treatment. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *MA*, mild-to-moderate asthma; *MW*, molecular weight. **D,** Phosphorylation of ErbB2 along the wound's leading edge was impaired by mubritinib primarily in HAECs from HCs. Representative immunofluorescent staining for pErbB2 and F-actin at the wound's leading edge. Red, pErbB2; green, F-actin; blue, nucleus. The *white bar* represents 100 μm. **E,** Intensity ratio of pErbB2 to F-actin at the wound's leading edge was decreased by mubritinib in HAECs from HCs but not in HAECs from asthmatic patients. Each *bar* represents means ± SEMs. Statistical analysis was performed with the Student *t* test.

wounding support the polar opposite situation in which low levels of ErbB2 activity and expression prevent normal epithelial repair and might explain why *Erbb2* knockout mice are embryonically lethal.[24]

To confirm the relationship between wound closure, CCND1, and ErbB2, we inhibited the pathway using mubritinib, a selective ErbB2 tyrosine kinase inhibitor. Mubritinib is a selective ErbB2 inhibitor compared with several other tyrosine kinases that nonselectively also target EGFR, fibroblast growth factor receptor, and platelet-derived growth factor receptor.[29] Mubritinib's antiproliferative effect was only seen in an ErbB2-overexpressing breast cancer cell line (inhibitory concentration of 50% = 5 nmol/L), without inhibitory effects in EGFR/ErbB1-overexpressing cell lines (inhibitory concentration of 50% > 25 μmol/L). As anticipated, inhibition of ErbB2 by mubritinib decreased ErbB2 phosphorylation, most notably at the leading edge of the wound, whereas it profoundly decreased CCND1 mRNA and protein levels, supporting the link between this specific EGF family receptor and cell proliferation. Importantly, these effects were significantly greater in HCs, in whom the expression and activation of ErbB2 was highest, having minimal effect in cells from asthmatic patients (ErbB2 suppressed).

ERBB2 and *CCND1* mRNA expression was highly correlated in cultured ALI cells, suggesting interactions between ErbB2 and CCND1 in cell proliferation. This correlation is consistent with our previously published gene expression study.[7] Although other factors could also stimulate proliferation in epithelial cells, the EGF pathway is widely considered the most relevant.[39] Importantly, our published gene array data did not show differences in expression of any other EGF family receptors between HAECs from HCs and asthmatic patients. These findings contrast with a recent RNA sequencing profiling study of sputum-derived cells, which reported concomitant upregulation of type 2 and EGFR/ERBB1 and ERBB2 in house dust mite–sensitive wheezers than nonatopic control subjects. However, they did not control for cell type, with these differences potentially caused by more epithelial cells in asthmatic sputum.[40] However, given the complexities of dimerization patterns among the EGFRs and its effect on ligand selectivity, it is conceivable other EGFRs are involved to some degree as well.

Our studies have both similarities and differences with previous studies. Stevens et al[41] also reported a defect in wound repair but in airway epithelial cells in a monolayer system from children. This system is very different from the differentiated/polarized cells studied here. An additional study showed that overexpression of plasminogen activator inhibitor 1, which is induced by EGF, contributed to the abnormal repair seen in HAECs from asthmatic patients.[42] Their findings contrast with ours, perhaps because of differences in culture systems.

There are some limitations in this study. First, the sample size for the *in vitro* studies is small. However, the sample size of this study was much larger than that for any similar study, and the diagnosis of asthma was rigorously confirmed.

Second, tErbB2 protein levels determined by means of Western blotting did not differ between cells from asthmatic patients and those from HCs *in vitro*. However, phosphorylation is more critical to evaluate ErbB2 pathway activation than tErbB2 expression because their homodimerization or heterodimerization with other EGFR family members is necessary for pathway activation,[43] and importantly, we observed markedly lower ErbB2 phosphorylation both *in vitro* in response to wounding and *in vivo* in freshly brushed, perhaps already "wounded," asthmatic epithelial cells.

Third, there was discordance in the time points between [^{3}H] thymidine levels, which were measured at between 3 and 16 hours and %WC after 7 hours. %WC was evaluated at 7 hours because most wounds were completely closed at 16 hours. Proliferation signals could continue beyond the simple closure, with the 3- to 16-hour window capturing the total amount. However, it is almost certain that other wound-healing processes, such as migration, also contribute to wound closure, particularly at the earlier timepoints.

Fourth, there was no difference by asthma severity in the wound-closure experiments. This could certainly be due to the prolonged (approximately 4 weeks) dedifferentiation and redifferentiation of the cultured cells and differences in the overall environment of the cultured cells versus those in the asthmatic epithelium.

Finally, current technical limitations of primary HAECs prevent us from adding or generating specific stimuli to phosphorylate ErbB2 in HAECs from asthmatic patients, preventing us from directly confirming the importance of the deficiency of this ErbB2 pathway in asthmatic cells.

In conclusion, decreased activation of ErbB2 impedes the proliferative response to wound repair in both *in vitro* HAEC models and in freshly brushed HAECs *in vivo*. The relationship between the *in vitro* and *in vivo* systems and the effect of ErbB2 on the CCND1 pathway and wound repair, combined with the previous genetic data, suggest that studies that focus on understanding the reasons for the downregulation of this pathway can lead to effective and even preventive asthma therapies, reversing a fundamental underlying "defect" of the disease.

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Key messages

- **Airway epithelial cells from asthmatic patients have impaired wound closure with decreased cell proliferation after wounding compared with those from HCs.**
- **Dysregulated activation of ErbB2 was observed both in *in vivo* and *in vitro* among airway epithelial cells from asthmatic patients, suggesting a novel therapeutic target for asthma treatment.**

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METHODS

Subjects

HCs and asthmatic patients (<5 pack years and no smoking in the previous year) were recruited as part of the Severe Asthma Research Program III, Immune Airway-Epithelial Interactions in Steroid-Refractory Severe Asthma, or the Electrophilic Fatty Acid Derivatives in Asthma study.[E1] All patients with asthma met the American Thoracic Society definition of asthma and included patients with mild-to-severe asthma, as defined by the European Respiratory Society–American Thoracic Society guidelines.[E2,E3] HCs had no history of respiratory disease and a negative methacholine challenge result. All participants were required to have no history of cancer of any kind. Percent predicted values of FEV_1 on spirometry were calculated by using the Global Lung Function Initiative 2012 equations.[E4]

Primary HAEC culture at the ALI

Primary HAECs from airway brushings were expanded under submerged conditions by using BronchiaLife growth medium (Lifeline Cell Technology, Frederick, Md). Once confluent, first-passage cells were cultured in Transwell plates, as previously described, supplemented with basic epithelial growth medium mixed with Dulbecco modified eagle medium at 1:1 (EGF concentration, 0.5 ng/mL).[E5,E6] Once confluent, the apical media was reduced to generate an ALI and achieve full differentiation of HAECs, including ciliated and mucus cells, after 7 days of ALI culture.[E7,E8] Cells were harvested on day 7 of ALI, as described below.

Epithelial wound model

On day 7 of ALI, 3 straight lines were manually etched on cell-culture surfaces with a 200-μL pipette tip, and the wound area was measured by using live cell imaging microscopy with differential interference contrast (Nikon Eclipse Ti inverted microscope; Nikon, Tokyo, Japan) and then again 7 hours later. Each field was automatically integrated into a complete field encompassing the entire Transwell insert. The wound area was calculated with a Nikon NIS-Elements viewer (Nikon Instruments, Melville, NY). Wound-closure areas were calculated by subtracting the post–7-hour wound area from the baseline wound area. %WC at 7 hours was then reported.

[^{3}H] thymidine incorporation assay

Cell proliferation was measured by using the [^{3}H] thymidine incorporation assay on day 7 of ALI. Cells were starved of EGF for 24 hours and incubated with 1 μCi/mL [^{3}H] thymidine (specific activity, 20 Ci/mmol) immediately after scraping the 6-line wounds. At 3, 7, and 16 hours, media were removed, cells were washed with ice-cold PBS, the entire Transwell membrane was removed and put into a scintillation vial with scintillation fluid (Ready Safe; Beckman Coulter, Brea, Calif), and radioactivity was counted on a liquid scintillation counter (Beckman LS6500). Samples were harvested in duplicates in 6 of 13 samples, whereas 7 subject's samples were analyzed as single wells because of low cell numbers. The time point of 3 hours after wounding was used as baseline [^{3}H] incorporation, and that of 16 hours after wounding was used for measurement of cell proliferation based on the DNA synthesis cycle. The fold change of [^{3}H] thymidine incorporation between 3 and 16 hours after wounding was calculated to compare cell proliferation between HAECs from HCs and those from asthmatic patients.

qRT-PCR and Western blot analysis

On day 7 of ALI and 7 hours after scrape wounding, epithelial cells were harvested in QIAzol, and mRNA was extracted with the RNeasy Mini Kit (Qiagen, Germantown, MD). Expression of *CCND1* as a cell proliferation marker and *ERBB2* mRNA expression were analyzed by using qRT-PCR on the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, Calif). Primers and probes were purchased from Applied Biosystems, as follows: *CCND1*, Hs00765553_m1; *ERBB2*, Hs01001599_m1; and glucuronidase β (*GUSB*), Hs99999908_m1, as a housekeeping gene.[E9] Relative expression to *GUSB* was calculated by using the Δ cycle threshold method.

Cultured and freshly brushed HAECs were also harvested in cell lysis buffer plus protein inhibitors for protein analysis by using Western blotting. Samples were run on a 4-12% SDS-PAGE gel and blotted on a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, Calif). Each protein was detected by using polyclonal antibodies for CCND1 (ab134175, rabbit, 1:1,000; Abcam, Cambridge, Mass), pErbB2 (Y1248, sc-293110, rabbit, 1:100; Santa Cruz Biotechnology, Santa Cruz, Calif), tErbB2 (29D8, rabbit, 1:1,000; Cell Signaling, Beverly, Mass), β-actin (A5441, mouse, 1:10,000; Sigma-Aldrich, St Louis, Mo), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NB300-320, goat, 1:20,000; Novus Biologicals, Littleton, Colo).

Chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific, Waltham, Mass) was used to detect the antibodies and read on an Amersham Imager 600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Each targeted protein was indexed to β-actin to determine a densitometric value, and the fold change of these values from control to wounding were calculated. Phosphorylation of ErbB2 was evaluated based on the ratio of pErbB2 to tErbB2.

Immunofluorescent staining

At 7 hours after scraping, the entire Transwell membrane was fixed with 3% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 in PBS. Cytospin samples of freshly brushed HAECs were fixed with 2% paraformaldehyde according to the same permeabilization method. Cell samples were blocked with 5% goat serum in 0.5% BSA for 45 minutes and incubated overnight with primary antibody against CCND1 or pErbB2 (same antibodies for Western blotting both at 1:100) at 4°C. After washing, samples were incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG, A11037, 1:400; Invitrogen, Carlsbad, Calif) and phalloidin for F-actin staining (Phalloidin CruzFluor, 1:2000; Santa Cruz Biotechnology) for 1 hour at room temperature. After nuclear counterstaining with 4′-6-diamidino-2-phenylindole dihydrochloride (D9542; Sigma-Aldrich), images were taken on an Olympus FluoView 1000 confocal microscope (Olympus, Tokyo, Japan). For Transwell membrane samples, ROIs were randomly and intermittently obtained (each of 300 × 630 μm) along the wound's leading edge on each wounded culture. Numbers of CCND1-positive cells per square millimeter were calculated at 5 randomly selected ROIs. pErbB2 expression was calculated as the fold change of fluorescent intensity of pErbB2 between the wound's leading edge and the nonwounded area at 10 randomly selected ROIs. The intensity ratio of pErbB2 to F-actin was also calculated. For cytospin samples, cells were blocked with 5% donkey serum in 0.5% BSA, incubated overnight with primary antibody against pErbB2, and then incubated with secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG, A21206, 1:400; Invitrogen). Five ROIs (each of 300 × 300 μm) were randomly and intermittently obtained and the percentage of pErbB2-positive cells analyzed in each field using ImageJ software (National Institutes of Health, Bethesda, Md). The threshold of pErbB2 positivity was set by using the "Maxentropy" algorithm of the software.

Statistics

Summary statistics are presented as means ± SEMs or medians (IQRs) for FENO, *in vivo* pErbB2 expression analysis, and the fold change of [^{3}H] thymidine incorporation between 3 and 16 hours because of nonlinear distribution of the data. *In vitro* comparisons across subject type or condition are expressed as the fold change compared with control, primarily because of varying baseline values. Differences in fold change are then analyzed by means of ANOVA, followed by multiple comparison testing with Bonferroni correction ($P < .0125$). All Statistical analysis was performed with JMP Pro software (version 12; SAS Institute). P values of less than .05 were considered statistically significant.

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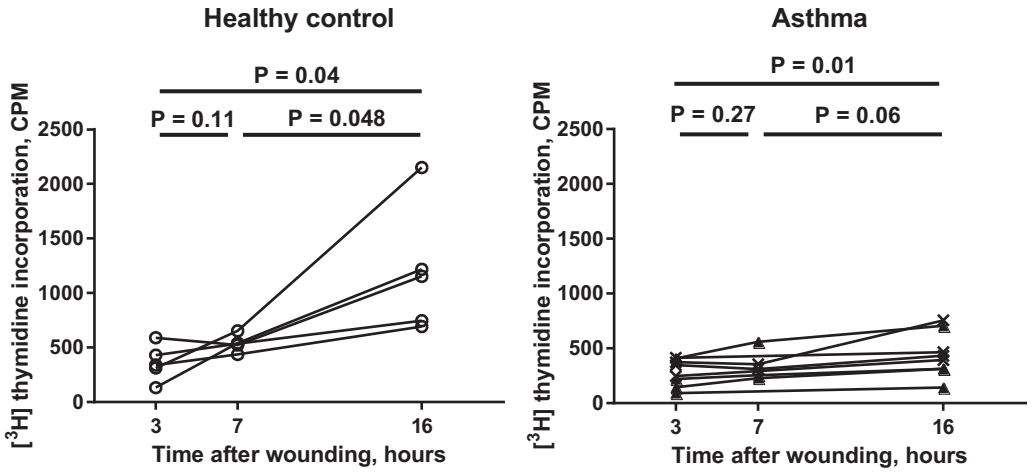

FIG E1. [^{3}H] thymidine incorporation was increased at 16 hours after wounding but not at the 7-hour time point in both healthy subjects and asthmatic patients.

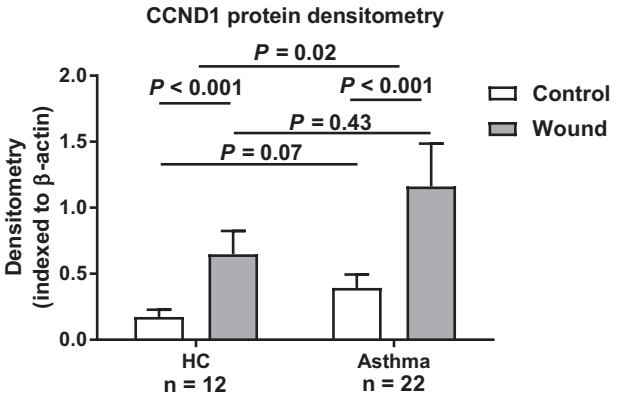

FIG E2. HAECs from asthmatic patients tended to have higher absolute levels of CCND1 protein, as determined by using Western blotting, than those from HCs both in the control and wounded conditions but without a significant difference ($P = .07$ for HC vs asthmatic patient baseline values and $P = .43$ for differences between HCs and asthmatic patients after wounding). However, wounding induced a greater fold change in CCND1 protein expression in HAECs from HCs than in those from asthmatic patients (medians and 25th to 75th percentiles: fold change, 4.0 [2.2-11.8] vs 2.9 [0.4-6.0]; $P = .02$).

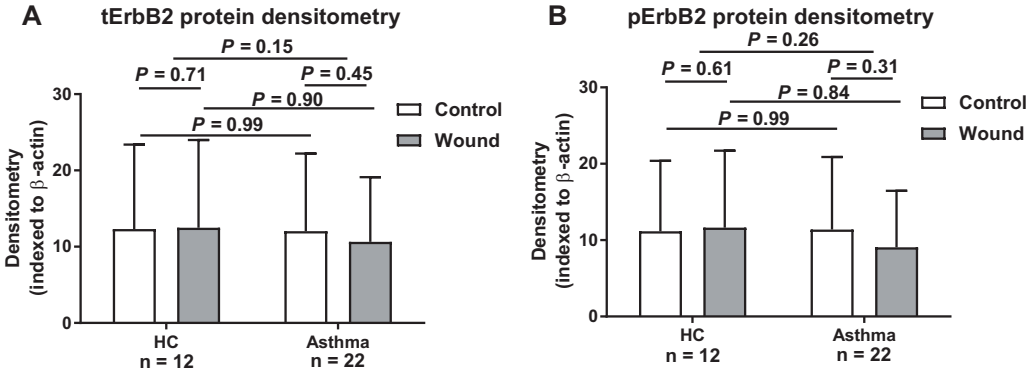

FIG E3. tErbB2 **(A)** and pErbB2 **(B)** protein expression using densitometric absolute values. No difference was observed between HAECs from HCs and asthmatic patients both in control and wounded conditions.

TABLE E1. Subjects' demographics: Western blot studies of freshly brushed HAECs

	HCs	Patients with mild-to-moderate asthma	Patients with severe asthma	*P* value
No.	6	8	12	
Age (y)	28.7 ± 5.1	29.1 ± 11.7	50.1 ± 9.3	.001
Sex, male/female	3/3	3/5	3/9	.56
Race, C/AA/O	4/1/1	5/2/1	11/0/1	.68
Inhaled corticosteroids, yes/no	NA	4/4	12/0	<.001
FEV_1 (% predicted)	92.8 ± 6.4	84.1 ± 15.8	52.3 ± 16.3	<.001
F_{ENO} (ppb)	18 (12-41)	24 (21-35.5)	43 (23-45.6)	.10

Data are represented as means ± SDs or medians (IQRs). *P* values were analyzed by means of ANOVA.
AA, African American; *C*, Caucasian; *NA*, not applicable; *O*, others.

TABLE E2. Subjects' demographics: Immunofluorescent staining studies of freshly brushed HAECs

	HCs	Patients with mild-to-moderate asthma	Patients with severe asthma	*P* value
No.	4	5	4	
Age (y)	37.3 ± 14.7	37.2 ± 15.9	41.5 ± 5.2	.87
Sex, male/female	2/2	1/4	2/2	.56
Race, C/AA/O	4/0/0	5/0/0	2/1/1	.68
Inhaled corticosteroids, yes/no	NA	3/2	4/0	.02
FEV_1 (% predicted)	100.5 ± 14.1	82.8 ± 16.7	34.0 ± 11.0	<.001
F_{ENO} (ppb)	14.5 (9.5-15.8)	16 (15-38)	24.5 (21.8-46)	.05

Data are represented as means ± SDs or medians (IQRs). *P* values were analyzed by means of ANOVA.
AA, African American; *C*, Caucasian; *NA*, not applicable; *O*, others.

TABLE E3. Subjects' demographics: *In vitro* wound closure/mRNA/western blot studies

	HCs	Patients with mild-to-moderate asthma	Patients with severe asthma	*P* value
No.	12	11	11	
Age (y)	42.3 ± 10.7	40.3 ± 13.3	43.7 ± 10.2	.78
Sex, male/female	6/6	5/6	2/9	.24
Race, C/AA/O	9/3/0	8/3/0	8/2/1	.68
Inhaled corticosteroids, yes/no	NA	5/6	11/0	<.001
FEV_1 (% predicted)	98.5 ± 16.8	86.2 ± 18.6	52.4 ± 17.8	<.001
F_{ENO} (ppb)	18 (11.5-23)	37 (18.3-66)	46 (16-66)	.03

Data are represented as means ± SDs or medians (IQRs). *P* values were analyzed by means of ANOVA.
AA, African American; *C*, Caucasian; *NA*, not applicable; *O*, others.

TABLE E4. Subjects' demographics: *In vitro* [^{3}H] thymidine incorporation studies

	HCs	Patients with mild-to-moderate asthma	Patients with severe asthma	*P* value
No.	5	4	4	
Age (y)	50.6 ± 2.3	47.8 ± 8.4	41.3 ± 11.3	.25
Sex, male/female	2/3	1/3	2/2	.76
Race, C/AA/O	3/2/0	3/1/0	4/0/0	.37
Inhaled corticosteroids, yes/no	NA	3/1	4/0	.007
FEV_1 (% predicted)	105.7 ± 22.6	80.3 ± 17.5	62.6 ± 9.4	.02
FENO (ppb)	20 (10.5-23.5)	26 (17.3-57.3)	56 (22.4-67.1)	.14

Data are represented as means ± SDs or medians (IQRs). *P* values were analyzed by means of ANOVA.
AA, African American; *C*, Caucasian; *NA*, not applicable; *O*, others.

TABLE E5. Subjects' demographics: *In vitro* ErbB2 inhibitor studies

	HCs	Patients with mild-to-moderate asthma	*P* value
No.	6	6	
Age (y)	31.4 ± 14.4	36.3 ± 12.8	.78
Sex, male/female	3/3	3/3	1.00
Race, C/AA/O	6/0/0	4/2/0	.12
Inhaled corticosteroids, yes/no	NA	3/3	NA
FEV_1 (% predicted)	99.2 ± 4.6	82.8 ± 21.3	.10
F_{ENO} (ppb)	12.5 (9.5-21.9)	23 (14-58)	.15

Data are represented as means ± SDs or medians (IQRs). *P* values were analyzed by means of ANOVA.
AA, African American; *C*, Caucasian; *NA*, not applicable; *O*, others.