

Characterization of protocadherin-1 expression in primary bronchial epithelial cells: association with epithelial cell differentiation

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ABSTRACT *Protocadherin-1 (PCDH1)* is a novel susceptibility gene for asthma that is expressed in airway epithelium. We aimed to characterize *PCDH1* mRNA transcripts and protein expression in primary bronchial epithelial cells and to determine regulation of *PCDH1* during mucociliary differentiation. Total RNA and protein were isolated from human primary bronchial epithelial cells. *PCDH1* transcripts were characterized by rapid amplification of cDNA ends in bronchial epithelial cells of 4 subjects. *PCDH1* expression was quantified by quantitative RT-PCR and Western blotting in bronchial epithelial cells directly *ex vivo* and after air liquid interface (ALI) or submerged culture. We identified 5 novel exons on the 5' end and 1 exon on the 3' end of *PCDH1*. Novel transcripts showed major variation in expression of intracellular conserved motifs. Expression levels of *PCDH1* transcripts encoding exon 1–2 were 4-fold higher, and transcripts encoding exon 3–4 were 15-fold higher in freshly isolated bronchial epithelial cells than in submerged cultures. *PCDH1* mRNA (3- to 8-fold) and protein levels (2- to 3-fold) were strongly up-regulated during mucociliary differentiation of primary bronchial epithelial cells in ALI cultures. In summary, *PCDH1* transcripts display remarkable variability in expression of conserved intracellular signaling domains. Enhanced *PCDH1* expression levels strongly correlate with differentiation of bronchial epithelial cells.—Koning, H., Sayers, I., Stewart, C. E., de Jong, D., ten Hacken, N. H. T., Postma, D. S., van Oosterhout, A. J. M., Nawijn, M. C., Koppelman, G. H. Characterization of protocadherin-1 expression in primary bronchial epithelial cells: association with epithelial cell differentiation. *FASEB J.* 26, 439–448 (2012). www.fasebj.org

Key Words: asthma • brush • rapid amplification of cDNA ends • air-liquid interface • splice variants

PROTODADHERINS ARE THE largest subfamily of the cadherin superfamily of adhesion molecules (1). Protocadherins can be subdivided into clustered protocadherins (α -, β -, and γ -protocadherins), flamingo (CELSR) cadherins, the large (Fat- and Dachshous-related) protocadherins, and nonclustered protocadherins (ϵ - and δ -protocadherins; refs. 2, 3). The δ -protocadherin family consists of $\delta 1$ -protocadherins (PCDH1, PCDH7, PCDH9, and PCDH11X/Y) and $\delta 2$ -protocadherins (PCDH8, PCDH10, PCDH17, PCDH18, and PCDH19; ref. 4). δ -Protocadherins are characterized by the presence of 7 ($\delta 1$) or 6 ($\delta 2$) extracellular cadherin repeats and variable intracellular signaling domains with no similarity to classic cadherins (5). They are generally highly conserved, with a remarkable conservation of 2 intracellular signaling domains across evolution (6). The expression of clustered protocadherins is mainly restricted to the nervous system, but interestingly the nonclustered $\delta 1$ -protocadherins are additionally expressed in a wide range of different tissues (7, 8). $\delta 1$ -Protocadherins are implicated in several diseases, such as non-small-cell lung cancer (PCDH7; ref. 9), autism spectrum disorder (PCDH9; ref. 10), and Alzheimer's disease (PCDH11; ref. 11).

Recently, we identified *protocadherin-1 (PCDH1)* as a novel susceptibility gene for asthma (12). *PCDH1*, as well as other members of the $\delta 1$ -protocadherin subfamily, is characterized by the presence of 3 conserved motifs (CM1, -2, and -3) in the intracellular cytoplasmic tail (3). The CM1 and CM2 motifs are present in both $\delta 1$ - and $\delta 2$ -protocadherins, while the CM3 motif is characteristic

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for δ 1-protocadherins only. CM3 has been shown to bind to the catalytic subunit of protein phosphatase 1 α (13), a protein implicated in lung morphogenesis (14). PCDH1 also contains a PDZ-domain binding site (PDZ-BS) at the C-terminal end of the cytoplasmic tail (15). The extracellular cadherin repeats of PCDH1 have been shown to display homotypic adhesion activity but weaker than classical cadherins (16, 17). Clearly, the function of PCDH1 is largely unknown and hence its putative role in the pathogenesis of asthma.

PCDH1 mRNA is expressed in brain (6), skin (18), nose (19), and lung (12), as well as in a range of endothelial and epithelial cell lineages throughout mouse embryonic development (19). *PCDH1* mRNA expression is up-regulated in a skin keratinocyte wounding model, suggesting a possible role of *PCDH1* in epithelial repair (18). We have shown expression of *PCDH1* in human peripheral blood mononuclear cells, fibroblasts, and primary bronchial epithelial cells (PBECs) and confirmed the presence of 2 *PCDH1* mRNA isoforms in the airway epithelium based on published annotated short 3-exon and long 5-exon isoforms (12).

The expression of δ 1-protocadherins in the nervous system is well characterized, but apart from their homotypic adhesion function, the exact function, expression, and regulation of these genes are unknown. As δ 1-protocadherins are highly conserved across evolution, analysis of the expression of PCDH1 in epithelial cells will contribute to the understanding of the function of the δ 1-protocadherin family in general. Therefore, we aimed to characterize *PCDH1* mRNA and protein expression levels in freshly isolated and cultured PBECs. Moreover, we investigated whether mucociliary differentiation of bronchial epithelial cells has an effect on *PCDH1* mRNA and protein expression levels.

MATERIALS AND METHODS

Cell culture

PBECs were obtained by bronchial brushings of 19 patients with asthma, using endoscopic cytology brushes (1.9 mm, Cellebri; Boston-Scientific-International, Nanterre, France). All patients had a doctor's diagnosis of asthma, showed reversibility, and were hyperresponsive to histamine and/or adenosine 5'-monophosphate (AMP). Lung function measurements, histamine and AMP provocation tests, and atopy measurements have been described previously (20). All subjects gave written informed consent. Two bronchial brushings were collected from each subject. One brush was directly stored in RLT buffer (Qiagen Benelux, Venlo, The Netherlands) for RNA extraction, while the second brush was collected in HBSS on ice for cell culture. PBECs were cultured as described previously (21) for 3 passages before RNA, and protein was extracted. The human bronchial epithelial cell-line 16HBE14o⁻ (16HBE) was kindly provided by D. C. Gruenert (Department of Medicine, University of Vermont, Burlington, VT, USA, and University of California, San Francisco, CA, USA) and cultured as described previously (22).

To investigate the potential role of PCDH1 in airway epithelial cell differentiation, a time course series of air-liquid interface cultured cells (ALIs) and submerged cultured cells was purchased (MucilAir; Epithelix Sàrl, Geneva, Switzer-

land). These cells originated from bronchial airways of a healthy female donor (60 yr). Bronchial epithelial cells were cultured under ALI and submerged conditions at Epithelix according to the following protocol: bronchial airways were digested enzymatically for 2 d, and epithelial cells were isolated. Then, 250,000 cells/well were seeded in 24-well Transwell inserts and cultured submerged for 2 d. Next, cells were cultured at ALI or under submerged culture conditions for another 45 d (7 wk). At time points 1, 7, 21, and 45 d after the start of ALI culture, cells were harvested both for RNA and protein in duplicate. Differentiation status was confirmed by FoxJ1 and zona occludens 1 (ZO-1) protein expression levels, both markers of epithelial differentiation (23–25).

RNA and protein isolation

RNA was extracted using RNeasy Mini or Micro Elute kits (Qiagen Benelux) according to the manufacturer's protocol. Extensive in-solution *DNaseI* treatment (Qiagen Benelux) was performed to remove gDNA traces. For isolation of total protein, T25 culture dishes and Transwell inserts were treated with Triton-X lysis buffer (1% Triton-X 100, 150 mM NaCl, 5 mM MgCl₂, and 10 mM HEPES), and 2 \times Leammli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8), immediately boiled for 5 min, and stored until further usage.

Rapid amplification of cDNA ends (RACE)

RACE-ready cDNA was generated using RNA isolated from 4 submerged cultured PBECs and 16HBE cells using RNA-ligase-mediated RACE (GeneRacer-RLM-RACE kit; Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The 5'- and 3' (nested)-RACE reactions were performed using specific primer sets (Table 1), using PCR cycling conditions as described by manufacturer's protocol. The resulting PCR products were cloned into TOPO pCR2.1 plasmids using TOPO TA cloning kit for Sequencing (Invitrogen). Twenty-five colonies (detection of transcripts with >4% abundance) were picked for each RACE reaction (5' and 3') for 4 patients and 16HBE cells. Plasmids were isolated using Qiagen Miniprep kit (Qiagen Benelux), and cloned PCR products were identified by nucleotide sequencing (M13-reverse or T7-forward primers; Baseclear, Leiden, The Netherlands).

For confirmation of the 5'-PCDH1 RACE results, long-range PCR reactions were performed from the newly discovered 5' exons toward either exon 2 untranslated region (UTR; primer 3656-Rev; corresponding to isoform 1) or toward exon 3 (primer 3327-Rev; corresponding to isoform 2), using the Expand High Fidelity PCR System (Roche Diagnostics, Almere, The Netherlands; see Table 1 for primer sequences). The 3'-PCDH1 RACE results were confirmed by a PCR reaction spanning exon 3–5 (see Table 1 for primer sequences). The DNA sequence of the PCR products was confirmed by nucleotide sequencing (Baseclear).

Determination of transcript quantities by quantitative (q)RT-PCR

To determine the relative expression levels of individual PCDH1 transcripts, qRT-PCR assays were designed across exon-exon boundaries. qRT-PCR was performed using inventoried or in-house designed Taqman assays (Applied Biosystems Europe, Nieuwekerk A/D IJssel, The Netherlands). qRT-PCRs were performed on the ABI7900HT cycler in 384-well format. For *PCDH1* 2 inventoried assays (assay exon 1–2, Hs00170174_m1; assay exon 3–4, Hs00260937_m1), and 2 On-

TABLE 1. *GeneRacer and PCDH1 primers and combinations for 5'- and 3'-RACE reactions*

No.	Primer	Sequence, 5'-3'
RACE reaction primers		
1	GeneRacer 5'-primer	CGACTGGAGCACGAGGACACTGA
2	GeneRacer 5'-nested primer	GGACACTGACATGGACTGAAGGAGTA
3	GeneRacer 3'-primer	GCTGTCAACGATACGCTACGTAACG
4	GeneRacer 3'-nested primer	CGCTACGTAACGGCATGACAGTG
5	5'-RACE 684	GAGTGATGACTGGTGAGGCGAAG
6	5'-RACE nested 437	TAGCTTGTACAGGTGCCCCACAT
7	3'-RACE 2973	AAGTTCAACCTGATGAGCGATGC
8	3'-RACE nested 3213	TCCGACTACAGCTACCGCACCA
9	3'-RACE Ex3	CGCCGTCCAGCAAGTCATCCTCA
10	3'-RACE nested Ex3	CTGATGGCAGCATAGGAGAGATGGA
PCR exon 3-5 primers		
11	Ex3-FW3b	GCCTGAGGATCACTATGAGCGCACCA
12	Ex5-Rev2b	ACACGGGTCTTTTCATTGACAGCTCAGCA
Long-range PCR reaction primers		
13	Ex1B-FW1	CTGGGGACAGGTGTTTTGACT
14	Ex1C-FW1	CCAGATTCAGGCAGGAGAAGC
15	Ex1F-FW3A	TACACCCAGCTGTGGATGAGATTCA
16	Ex1D-FW1	TCGGCGTCTCTGGGAG
17	Ex1E-FW1	AGCAAGGAGGAAAAGAAGGAAAGAG
18	3327-Rev	GGACGGCGTCTCAGACTCCT
19	3656-Rev	TCAGTTATCCACAGGACCCCG
qRT-PCR assay primers and probes		
20	Ex1B-FW	ACTTCCCTTGCTCGACTTC
21	Ex1B-Rev	GGGAGGCCCCAGAATCAG
22	Ex1B-Probe	FAM-ACAGGTGTTTTGAGTAGGCC-NFQ
23	Ex1C-FW	CGCCCCGCCACGTTA
24	Ex1C-Rev	GGGAGGCCCCAGAATCAG
25	Ex1C-Probe	FAM-TCGGGACATTATTATTCC-NFQ
26	Ex1F-FW	TACACCCAGCTGTGGATGAGATTCA
27	Ex1F-Rev	CCCAGAATCAGGAGGGCCTCTC
28	Ex1F-Probe	FAM-TGTGCTGGGACTGACTGCTTCTGT-BHQ
RACE PCR reactions		
1A	GeneRacer 5'-primer + 5'-RACE 684	
1B	GeneRacer 5'-nested primer + 5'-RACE nested 437	
2A	GeneRacer 3'-primer + 3'-RACE 2973	
2B	GeneRacer 3'-nested primer + 3'-RACE nested 3213	
3A	GeneRacer 3'-primer + 3'-RACE Ex3	
3B	GeneRacer 3'-nested primer + 3'-RACE nested Ex3	

Demand assays were purchased (assay exon 1B-1 and assay 1C-1; Applied Biosystems Europe). In addition, predesigned assays for 4 housekeeping genes were used: *GAPDH* (Hs99999905_m1), β -*actin* (Hs99999903_m1), *RPLPO* (Hs99999902_m1), and β -2-*microglobulin* (Hs99999907_m1). Assay exon 1F-1 was designed in house; FAM-labeled probe was ordered at Eurogentec (Liege, Belgium; see Table 1 for primer sequences).

cDNA was produced using Omniscript reverse transcriptase (Qiagen Benelux). A total of 1.75 μ g of RNA was reverse transcribed into cDNA using Oligo-dT (12-18) primers in a 35- μ l reaction volume, including RNase inhibitor, at 37°C for 1 h. Each qRT-PCR reaction contained 50 ng of cDNA, 250 nM of probe, 900 nM of forward and reverse primers, and 5 μ l TaqMan Universal PCR Master Mix (Applied Biosystems Europe) in a final volume of 10 μ l. All samples were measured in triplicate using recommended cycling conditions. Data were analyzed using SDS2.3 software by applying the $\Delta\Delta C_t$ method (Applied Biosystems User Bulletin 2). As 4 housekeeping genes were used, the best combination of

housekeeping genes for normalization was determined by using the Normfinder applet (26). Efficiencies of all assays were determined by a 2- or 10-fold dilution series of pooled brush or cultured cells cDNA (Supplemental Table E1).

Detection of CpG islands *in silico*

CpG islands were detected using the Emboss CpGPlot program (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/>) by submitting a sequence of 10 kb upstream of exon 1, derived from Genbank template AC094107.

Statistics

To test the differences between brushed PBECs and submerged cultured PBEC qRT-PCR results, a nonparametric paired Wilcoxon test was performed using SPSS 16.0 (IBM Netherlands, Nieuwegein, The Netherlands).

Detection of PCDH1 isoform 1, isoform 2, FoxJ1, and ZO-1 by Western blotting

Both for cultured PBECs and ALI cultures, protein samples were separated on 8% acrylamide SDS-page gels (Bio-Rad Laboratories, Veenendaal, The Netherlands) or precast NuView 4–20% gradient gels (Generon, Maidenhead, UK). Blotting procedures were performed as described previously (12), using affinity-purified PCDH1 isoform-specific polyclonal antibodies, FoxJ1 antibody (AF3619; R&D Systems Europe, Abingdon, UK), ZO-1 antibody (33–9100; Zymed Laboratories; Invitrogen, Carlsbad, CA, USA), and β -actin antibody (sc-47778; Santa-Cruz Biotechnology, Heidelberg, Germany). Polyclonal PCDH1 antibodies were generated from immunized rabbits against 15-aa peptide QPFQLSTPQ PLPHYPH, ep78, for isoform 1 and 15-aa peptide SPSPE DRNTKTAPV, ep76, for isoform 2 (Fig. 1A). Antibodies were affinity column purified against immunizing peptides (Eurogentec). Validation of antibodies is described in Supplemental Data. Protein levels were quantified by densitometric analysis using Quantity One 4.6.2 software (Bio-Rad), relative to the β -actin loading control.

RESULTS

Identification of novel PCDH1 transcripts in PBECs

The *PCDH1* gene encodes 2 main isoforms through alternative splicing in brain (6): a short 3-exon isoform (Genbank NM_002587) and a long 5-exon isoform (Gen-

bank NM_032420). The short isoform contains a specific sequence at exon 2, which is spliced out in the long isoform (Fig. 1A). RACE was performed to identify the presence of annotated and possible novel *PCDH1* transcripts in PBECs that had been cultured under submerged conditions for 3 passages. We revealed expression of 5 novel exons located 5' to annotated exon 1 of *PCDH1* (NM_002587) and 1 novel exon located 3' to annotated exon 4 of *PCDH1* (NM_032420) in cultured PBECs. In contrast, we did not detect expression of the annotated exon 1A previously observed in brain (6) (Fig. 1B). Expression of newly identified exons 1B–1F was confirmed by sequence determination of transcript fragments PCR amplified from cDNA samples of PBECs (see Table 1 for primer sequences, Fig. 1B for transcripts). We detected expression of novel 5' exons 1D, 1E, and 1F in transcripts encoding both long and short *PCDH1* isoforms, whereas exons 1B and 1C were only detected in transcripts encoding the short isoform. All novel 5' exons were found in separate transcripts spliced directly to exon 1, with the exception of exon 1E, for which we also detected a transcript in which it was spliced to exon 1F and then to exon 1 (Fig. 1B). In addition to novel 5' and 3' exons, we observed the presence of a gap within exon 4 or even full skipping of exon 4 in *PCDH1* transcripts of the long isoform (Fig. 1C). Since exon 4 contains the highly conserved CM2 and PDZ-BS domains, these variants will affect the expression of these intracellular signaling domains (Fig. 1A, C).

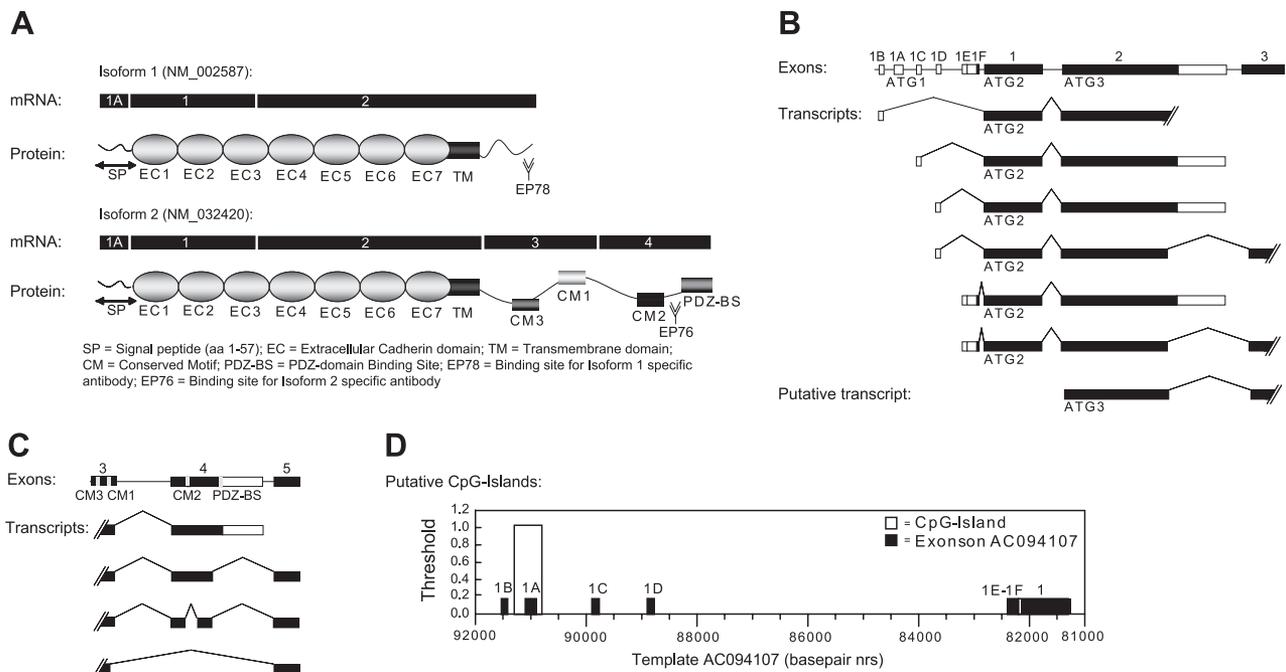


Figure 1. Protocadherin-1 gene, RNA, and protein structure. *A*) RNA and protein structure of PCDH1 isoforms 1 and 2. Note that exon numbering follows the annotation of mouse *Pcdh1* by Vanhalst *et al.* (6). Ep76 and Ep78 indicate the presence of the polypeptide sequence that was used to generate isoform specific antibodies. *B*) Variation on 5' end of PCDH1 in primary bronchial epithelium of patients with asthma. Novel exons were connected to isoform 1 or isoform 2 or both. Exons 1E and 1F exist both separately and connected to each other, both with isoform 1 and 2. *C*) Variation on 3' end of PCDH1, which can result in alternative usage of conserved domains (CM2 and PDZ-BS). *D*) A CpG island was detected surrounding exon 1A. Genbank accession numbers and prevalence of exons are provided in Supplemental Table E2.

Culture of PBECs influences *PCDH1* expression levels and detected isoforms

We designed quantitative RT-PCR assays spanning all novel exon-exon boundaries and successfully validated these for transcripts containing the exon boundaries 1B-1, 1C-1, and 1F-1. As expression levels were found to be low for these transcripts, only presence or absence of these transcripts in cultured *vs.* freshly isolated brush PBECs was determined. For submerged cultured PBECs, expression of transcripts encoding both exon 1C-1 (17/19 subjects) and exon 1F-1 (18/19 subjects) was detected, while transcripts encoding exon 1B-1 were not detected. In a preliminary study of 3 healthy subjects, we also detected expression of transcripts encoding exon 1C-1 and 1F-1 in PBECs but no expression of transcripts encoding exon 1B-1 (results not shown). In freshly isolated PBEC samples, expression of only the transcript encoding exon 1F-1 was detected in a subset of subjects (6/19). These results suggest that submerged culturing of cells induces alternative 5' exon usage in *PCDH1* transcripts. Epigenetic mechanisms can be an explanation for the low expression levels of these novel exons. Therefore, we investigated by *in silico* analysis whether the exons were contained within a CpG island. We detected a CpG island of 565 bp containing exon 1A, which was not detected by our RACE experiments, but no CpG islands were detected around the other exons (Fig. 1D).

In addition to exons 1B-1, 1C-1, and 1F-1, we quantified expression of transcripts containing exon 1–2 and exons 3–4 boundaries in PBECs. According to the annotated gene structure of *PCDH1*, qRT-PCR assay for exon 1–2 can detect both short and long *PCDH1* isoforms, while the exon 3–4 assay is specific for the long isoform. *PCDH1* expression levels in submerged cultured PBECs and freshly isolated brush samples were quantified relative to the average of 3 housekeeping genes (*β-actin*, *GAPDH*, and *RPLP0*) using prevalidated qRT-PCR assays. We detected similar expression levels of *PCDH1* transcripts with the exon 1–2 and exon 3–4 assays in cultured PBECs, indicating that almost all of *PCDH1* transcripts encode *PCDH1* isoform 2 (Fig. 2A). Surprisingly, and in strong contrast to cultured PBECs, higher expression levels of *PCDH1* transcripts were detected with the exon 3–4 assay compared with the

exon 1–2 assay in freshly isolated PBECs of all 19 subjects (Fig. 2B). *PCDH1* expression levels in PBECs from 3 healthy controls displayed a very similar level and regulation between culture conditions as those in PBECs of patients with asthma (results not shown). The expression difference between exon 1–2 and 3–4 indicates that a *PCDH1* mRNA transcript exists in freshly isolated PBECs that contains exon 3 and 4, but not exon 1 and/or 2, encoding the extracellular and transmembrane domains. This novel *PCDH1* transcript has not been reported before and was not identified by our RACE approach since we used primers located in exon 1 to characterize the 5' sequences of the *PCDH1* transcripts and performed our analysis in submerged cultured PBECs that may not contain this novel transcript.

Therefore, we performed additional 5'-RACE experiments from exon 3 in differentiated ALI cultured PBECs to identify a putative novel transcript encoding exons 3–4 but lacking exons 1 and/or 2. Indeed, we identified a *PCDH1* transcript that does not encode exon 1 and presumably starts translation from within exon 2, where an in-frame start codon is present in the context of a strong Kozak sequence (TCAGTGATG-GAG, ATG3; Fig. 1B), allowing the expression of a *PCDH1* protein encoding the extracellular EC6 and EC7 domains and the transmembrane and intracellular domains but missing the first 5 extracellular cadherin repeats. In addition to this novel *PCDH1* transcript, we also observed significantly lower *PCDH1* mRNA expression levels in submerged cultured cells compared with freshly isolated PBECs ($P < 0.0001$), both with the exon 1–2 assay (Fig. 2C) and the exon 3–4 assay (Fig. 2D). Specifically, exon 1–2 expression levels were 4-fold higher in freshly isolated PBECs, while exon 3–4 expression levels were 15-fold higher in freshly isolated than in cultured PBECs. These data show that higher *PCDH1* mRNA expression levels are observed in freshly *ex vivo* isolated brush cells compared with submerged cultured PBECs.

We found evidence for 3 *PCDH1* transcripts: the short annotated isoform encoding exon 1 and exon 2 and displaying alternative upstream exon usage; the long annotated isoform encoding exons 1, 2, 3, and 4 and using alternative upstream exons; and a novel *PCDH1* transcript encoding exon 3–4 but not exon 1

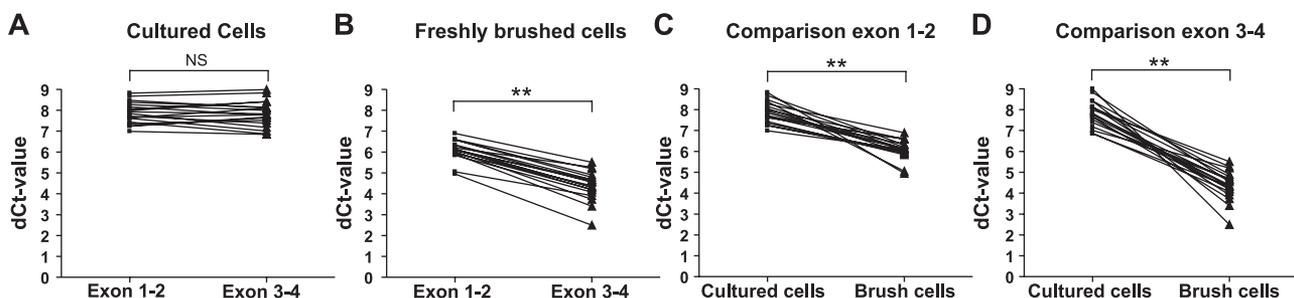


Figure 2. Expression of exon 1–2 and 3–4 in submerged cultured cells and freshly brushed cells. *A, B* Quantification of transcripts encoding *PCDH1* exon 1–2 and exon 3–4 in submerged cultured (*A*) and freshly brushed cells (*B*). *C, D* Comparison of exon 1–2 (*C*) and exon 3–4 (*D*) expression between submerged cultured and brush cells. dCt, ΔC_t ; NS, nonsignificant. $**P < 0.0001$.

and only part of the coding sequences of exon 2, identified by RACE in differentiated PBECs. We next aimed to identify the protein products encoded by these different transcripts. To this end, we first determined which PCDH1 protein products were expressed in submerged cultured PBECs and 16HBE cells using novel antibodies that were raised to peptides specific to the cytoplasmic tail of the 2 annotated PCDH1 isoforms (Fig. 1A and Supplemental Fig. E1 for validation). The antibody specific to the exon-2 encoded intracellular sequences (annotated isoform 1) detected the full-length protein only, whereas the antibody specific to the exon-4 encoded intracellular sequences (annotated isoform 2) detected both the full-length protein and a shorter protein isoform of ~50 kDa (Supplemental Fig. E1A). The size of the latter 50-kDa protein is consistent with a PCDH1 protein product encoded by the newly identified transcript. In cultured PBECs, we detected hardly any expression of the annotated PCDH1 protein isoform 1, in agreement with our qRT-PCR data (Supplemental Fig. E2A). The antibody specific to annotated isoform 2 detected two PCDH1 protein products: 1 corresponding to the full-length protein (170 kDa) that was detected in cultured PBECs and 16HBE cells, and the short protein product of ~50 kDa in 16HBE cells, but not in cultured PBECs, which is consistent with our qRT-PCR data (Supplemental Fig. E2B).

PCDH1 expression levels increase during ALI culture conditions

We observed a strong reduction of *PCDH1* mRNA expression levels during the 3 passages of submerged culture used to expand the PBECs, compared with freshly brushed cells of the same subject (Fig. 2). This expression difference between cultured and freshly brushed PBECs was not restricted to PBECs of patients with asthma, as we observed the same expression pattern after preliminary analysis of *PCDH1* expression levels in PBECs of healthy subjects (results not shown).

We hypothesized that the loss of mucociliary differentiation of PBECs due to submerged culture conditions (27) reduced *PCDH1* expression levels in PBECs. We therefore compared *PCDH1* mRNA expression lev-

els between PBECs of healthy subjects grown under submerged and those grown under ALI culture conditions, as ALI cultures have been described to induce mucociliary differentiation in PBECs (28) (Fig. 3). We confirmed the kinetics of this differentiation in our ALI cultures using the ciliated-cell-specific protein FoxJ1 (23) and the ratio of the isoforms of ZO-1 associated with differentiation (24, 25) (Fig. 4A). We observed a strong increase (3- to 8-fold) in *PCDH1* mRNA expression levels with both exon 1–2 and exon 3–4 assays after 1 wk of ALI culture, compared with d 1 of ALI culture. *PCDH1* mRNA expression levels gradually increased to reach similar levels at wk 7 in ALI and submerged cultures when detected with the exon 1–2 assay but remained much higher in ALI than submerged cultures at all times when detected with exon 3–4 assay (Fig. 3A).

As seen before (Fig. 2), equal expression levels of transcripts encoding exon 1–2 and transcripts encoding exon 3–4 were observed at d 1, when cells were transferred to ALI culture conditions from submerged cultures. In contrast, at d 45 of ALI culture, a 2.8-fold higher expression of *PCDH1* transcripts was detected with the exon 3–4 assay compared with the exon 1–2 assay. For submerged cultures, this difference was less pronounced (Fig. 3B).

To determine whether the up-regulation of *PCDH1* mRNA levels in differentiated ALI cultures is also observed at the protein level, we analyzed protein levels of PCDH1 isoforms in the 7-wk PBEC cultures, maintained either under ALI or submerged culture conditions. Both PCDH1 full-length protein isoforms (corresponding to the extracellular domains, the transmembrane domain and the intracellular domains for both annotated isoforms) were strongly (2- to 3-fold) increased after 7 wk of ALI culture compared with submerged culture conditions (Fig. 4B). In addition, we detected a 50-kDa protein product, previously detected in 16HBE cells (Supplemental Figs. E1 and E2B), at the latest time points (d 21 and 45) of ALI culture, with higher expression in ALI cultured PBECs compared with submerged cultured PBECs (Fig. 4B). These data indicate that PCDH1 protein expression levels during PBEC culture by and large follow the kinetics of the qRT-PCR data, with elevated PCDH1 expression

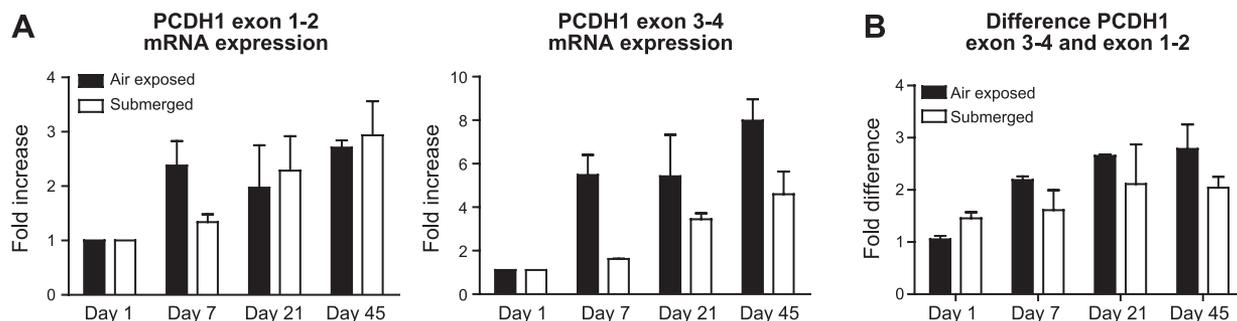


Figure 3. PCDH1 mRNA expression in ALI cultures of epithelial cells over time. *A*) Quantification of transcripts encoding PCDH1 exon 1–2 and exon 3–4 in ALI cultured cells. *B*) Difference of exon 3–4 and 1–2 mRNA expression levels in ALI and submerged cultured cells. There is a larger difference between exon 3–4 and 1–2 expression levels in ALI than in submerged cultured cells. Error bars show SD of duplicates of one experiment.

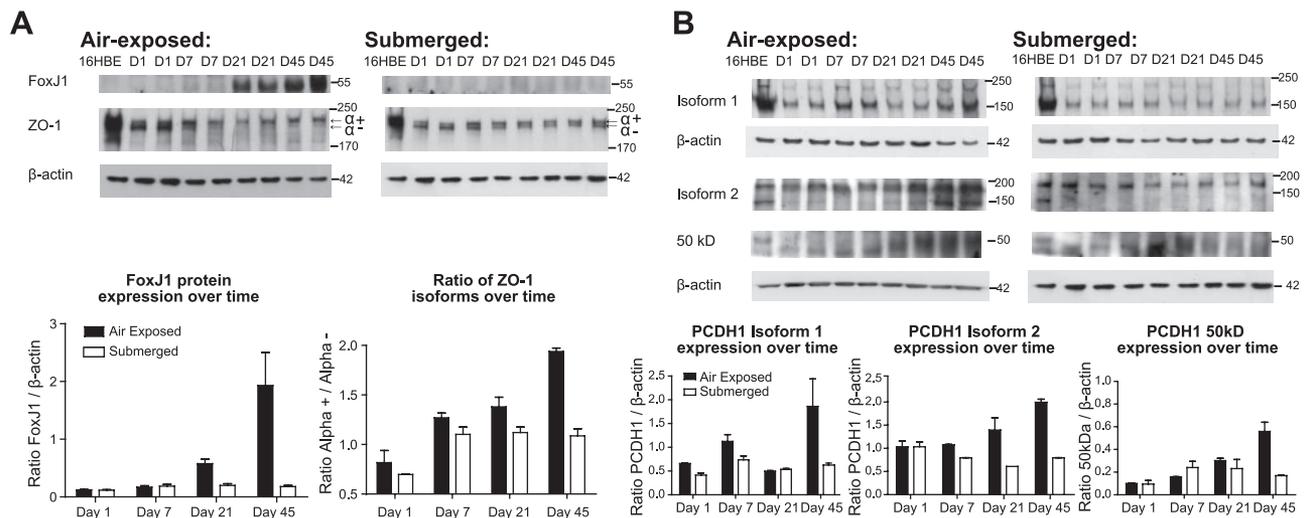


Figure 4. PCDH1 isoform 1 and isoform 2 protein expression in ALI cultures of epithelial cells over time. A) Mucociliary differentiation is indicated by FOXJ1 expression and the ratio between ZO-1 α^+ and ZO-1 α^- isoforms, which are both markedly increased in ALI cultures, but not in submerged cultures. B) An increase of PCDH1 isoform 1 and 2 expression is observed, with the highest expression at day (D) 45. Submerged cultures show no clear increase in PCDH1 expression. Graphs show densitometric quantification of PCDH1 bands on Western blots, relative to β -actin. A representative example of 3 independent experiments with comparable results is shown. Error bars represent sd of duplicates.

under ALI culture conditions, both for the long PCDH1 isoforms and the 50-kDa protein.

DISCUSSION

We recently identified *PCDH1* as a novel gene for asthma that is expressed in airway epithelial cells. PCDH1 is a member of the δ 1-protocadherins, which encode transmembrane proteins with 7 extracellular cadherin repeats and in some isoforms intracellular conserved protein-protein interaction domains that are involved in signal transduction. δ 1-Protocadherins have been shown to engage in homotypic interactions, but otherwise the functional role of these proteins is unknown. To explore a possible functional role for PCDH1 in bronchial epithelial cells, we first characterized the *PCDH1* transcripts present in PBECs and the regulation of *PCDH1* isoform expression during PBEC mucociliary differentiation in the ALI culture model.

Identification of novel PCDH1 mRNA isoforms

The starting points of our analysis were the annotated *PCDH1* mRNA isoforms, originally identified in brain tissue: a 3-exon (isoform 1; Genbank NM_002587) and a 5-exon isoform (isoform 2; Genbank NM_032420; Fig. 1A). Both isoforms share an extracellular and a transmembrane domain but differ in their intracellular domains: isoform 1 carries a short unique cytoplasmic region encoded by exon 2, whereas isoform 2 has a long cytoplasmic region encoding the conserved signaling domains encoded by exons 3 and 4 (6). In our RACE experiments on cultured PBECs, we identified 5 novel *PCDH1* 5' exons that are mainly expressed in undifferentiated cells during submerged cell culture (Fig. 1B).

By long-range PCR, exons 1B and 1C were shown to be present only in transcripts encoding the short PCDH1 isoform, while exons 1D, 1E, and 1F were present in transcripts encoding both PCDH1 isoforms. The alternative usage of 5' exons between the transcripts terminating at the exon 2 UTR (the annotated short isoform) and those terminating at the exon 4 UTR (the annotated long isoform), indicates that promoter usage might be different between these 2 main isoforms. Remarkably, we did not detect the annotated exon 1A. PCR amplification of exon 1A may, however, have been hampered by the very high GC content (81.5%) of this exon, precluding identification by RACE as well as by RT-PCR. In fact, exon 1A is located within a CpG island, indicating that epigenetic mechanisms may contribute to regulation of *PCDH1* gene expression (Fig. 1D). The annotated *PCDH1* isoforms contain 2 inframe start codons, 1 in exon 1A (ATG1) and 1 in exon 1 (ATG2). In addition, we identified a putative additional in-frame start codon in exon 2 (ATG3) in the context of a strong KOZAK sequence. Of the 5 novel 5' exons, only exon 1F contains an in-frame ATG sequence but no conserved Kozak sequence; hence, it is a less likely translation initiation site. The lack of conserved translational start sites and relatively low expression of the novel 5' exons suggest that these 5' exons may contribute to mRNA translation regulation as part of the 5'-UTR sequences. Vanhalst *et al.* (6) suggested that translation initiation of *PCDH1* employs the first start-codon of exon 1 (ATG2), since the corresponding Kozak sequence of ATG2 was best conserved between species.

We investigated the expression levels of *PCDH1* in 3 healthy controls. *PCDH1* exon 1–2 and 3–4 expression levels were by and large similar between healthy controls and subjects with asthma, but a thorough comparison between healthy and asthma groups will be the

focus of a future study. Furthermore, we investigated whether *PCDH1* exon 1–2 and 3–4 expression levels were influenced by 3 *PCDH1* gene variants (IVS3–116, rs14359, and rs3797054), previously associated with bronchial hyperresponsiveness and asthma (12). With expression levels of 19 subjects, we unfortunately were underpowered for this analysis. One of our most remarkable findings was the detection of higher *PCDH1* mRNA expression levels with an exon 3–4 specific assay than an exon 1–2 specific assay, both in freshly brushed epithelial cells and at later time points in the ALI cultured PBECs but not in submerged cultured PBECs. These data were in contrast to our expectations based on the published annotated isoforms of *PCDH1*. We expected *PCDH1* expression detected by the exon 1–2 specific assay to reflect expression of both isoforms, thereby constituting the total *PCDH1* mRNA pool, whereas *PCDH1* expression detected by the exon 3–4 specific assay was expected to reflect the level of the long 5-exon isoform only. Based on our results, we here hypothesize the presence of a novel putative *PCDH1* transcript lacking exon 1 and/or 2. Notably, the expression difference between exon 1–2 and exon 3–4 was not due to lower efficiency of the exon 1–2 qRT-PCR assay (Supplemental Table E1). For this putative variant, translation may start from within exon 2, where an in-frame start codon is present within a Kozak sequence (ATG3). In RACE experiments, we confirmed the presence of a transcript variant carrying ATG3 but not ATG2 or ATG1 (Fig. 1B). Translational initiation from the putative ATG3 start codon located in exon 2 would result in a truncated protein product, lacking 5 of the extracellular domains, but retaining the conserved intracellular signaling domains. As adhesion typically forms by engagement of the EC1 domain, this isoform would not be able to participate in cell-cell adhesion but could still participate in signal transduction. A truncated variant was previously described for PCDH15, where PCDH15 isoform B has a deletion of 10 of 11 cadherin domains but still retains the transmembrane domain and intracellular domains (29). Interestingly, we did identify a PCDH1 protein product of 50 kDa using the antibody directed against the intracellular part of isoform 2. This 50-kDa product could be the protein product of the putative novel isoform, although we can not exclude the possibility that this product is the result of a full-length isoform modified by post-translational cleavage or shedding. Such a shedding process was previously identified for the clustered γ -protocadherins and the nonclustered protocadherin-12 (30, 31) but is yet unknown for δ 1-protocadherins. Future studies will be directed at the characterization of this novel PCDH1 protein.

δ 1-Protocadherins are characterized by 3 conserved motifs in their intracellular tail (CM1–CM3), of which the function of CM1 and CM2 is unknown. At the 3' end of *PCDH1* mRNA, we observed substantial variability in the transcription of exon 4: we identified transcripts with an intraexon gap (in-frame deletion of 96 bp) as well as transcripts with exon skipping of exon 4.

This gap in exon 4 has also been described to be present in mouse *Pcdh1* (Genbank AY861418) but has no consequence for the expression of conserved domains (6). In contrast, deletion of the entire exon 4 would result in the translation of a PCDH1 protein lacking CM2 and a putative PDZ-domain binding site. The CM2 motif is strongly conserved during metazoan evolution and is therefore considered an important motif for downstream functions of many protocadherins (32). However, function and putative binding partners for CM2 have not been identified to date. PDZ-containing proteins can provide linkage to the cytoskeleton, or initiate signaling functions either directly or indirectly by acting as scaffolds for large protein complexes. Deletion of the PDZ-BS might therefore potentially influence coupling of PCDH1 to the cytoskeleton or downstream signaling functions (15, 33). Besides PCDH1, the δ 1-protocadherin PCDH11 has also been shown to be subject to complex splicing patterns, resulting in several transcripts with alternative expression of conserved motifs (34). Thus, δ 1-protocadherins seem to have multiple protein isoforms with different use of the 3 intracellular CM-domains, allowing the specific protein isoforms to selectively address certain, as of yet unknown, signaling pathways depending on the presence or absence of the different CM domains. Future research will be needed to identify the relevant signaling pathways regulated by the δ 1-protocadherins, as well as the regulation of splicing events leading to inclusion or exclusion of specific CM domains. We here thus describe evidence for an intriguing diversity of *PCDH1* transcripts with strongly variable 5'- and 3'-exon usage, likely affecting protein function, and additionally for a novel transcript isoform encoding the intracellular and transmembrane domains, but only containing 2 of the extracellular cadherin repeats. Given the strong conservation between δ 1-protocadherin family members, these data are likely to be of relevance to the other family members as well.

Interestingly, we observed higher *PCDH1* mRNA expression levels in freshly isolated cells than in PBECs of the same subject cultured for 3 passages under submerged conditions, both with the exon 1–2 and exon 3–4 specific assays. The cells obtained by bronchial brushing are ~85–90% epithelial cells (35, 36), and we consider these brushed PBECs to be terminally differentiated. In contrast, submerged tissue culture conditions are likely to render undifferentiated cells, especially after multiple passages (27, 28). We therefore hypothesized that *PCDH1* expression levels are regulated by the differentiation status of bronchial epithelial cells. In agreement with this, we observed increased *PCDH1* mRNA and protein expression levels in PBECs cultured under ALI conditions, compared with submerged cultured cells. Since ALI cultures have been shown to induce terminal mucociliary differentiation of PBECs (28, 37) and we also observed the same differences in PCDH1 expression levels between freshly isolated (terminally differentiated) PBECs from bronchial brushes and cultured (relatively undifferentiated) cells, we conclude that high PCDH1 expression levels

are associated with terminal differentiation of bronchial epithelial cells.

We are the first to show an increase of protocadherin-1 expression at the mRNA and protein level over time in differentiated ALI cultures. Together with a proposed role of PCDH1 in cell-cell adhesion, and its identification as an asthma susceptibility gene, this suggests that there may be a role for PCDH1 in epithelial differentiation. Previously protocadherin of the liver, kidney, and colon (PCDH-LKC) was shown to be regulated during Madin-Darby canine kidney (MDCK) epithelial cell differentiation (38). Furthermore, RNA expression of protocadherin-7, a closely related $\delta 1$ -protocadherin gene, was shown to be up-regulated during bronchial epithelial cell differentiation in a microarray experiment (39). Therefore, the increase of protocadherin expression levels during epithelial differentiation might be a common regulatory mechanism for the nonclustered $\delta 1$ -protocadherins. Epithelial differentiation encompasses several processes, including up-regulation of adhesion molecules, establishment of apical/basolateral cell polarity and planar cell polarity, and development of cilia (40). Asthma is characterized by a decreased epithelial barrier function (41) and abnormal repair of epithelial cells (42–44). One aspect of epithelial repair is the regrowth of columnar epithelial cells or differentiation and proliferation of basal epithelial cells (45). We propose that dysregulation of PCDH1 expression results in slower or incomplete differentiation of the epithelial layer, thereby contributing to an altered response to injury and a weaker epithelial barrier function. FJ

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