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RESEARCH ARTICLE

Proprotein convertase inhibition promotes ciliated cell differentiation – a potential mechanism for the inhibition of Notch1 signalling by decanoyl-RVKR-chloromethylketone

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Abstract

Chronic repetitive rounds of injury and repair in the airway lead to airway remodelling, including ciliated cell loss and mucous cell hyperplasia. Airway remodelling is mediated by many growth and differentiation factors including Notch1, which are proteolytically processed by proprotein convertases (PCs). The present study evaluated a novel approach for controlling basal cell-type determination based on the inhibition of PCs. It was found that decanoyl-RVKR-chloromethylketone (CMK), a PC inhibitor, promotes ciliated cell differentiation and has no effect on the ciliary beat frequency in air–liquid interface (ALI) cultures of human nasal epithelial cells (HNECs). Comparative microarray analysis revealed that CMK considerably increases ciliogenesis-related gene expression. Use of cell-permeable and cell-impermeable PC inhibitors suggests that intracellular PCs regulate basal cell-type determination in ALI culture. Furthermore, CMK effect on ciliated cell differentiation was reversed by a Notch inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT). CMK inhibited the processing of Notch1, a key regulator of basal cell differentiation toward secretory cell lineages in the airway epithelium, and down-regulated the expression of Notch1 target genes together with furin, a PC. Specific lentiviral shRNA-mediated knockdown of *furin* resulted in reduced Notch1 processing and increased numbers of ciliated cells in HNECs. Moreover, CMK inhibited Notch1 processing and promoted regeneration and ciliogenesis of the mouse nasal respiratory epithelium after $ZnSO_4$ injury. These observations suggest that PC inhibition promotes airway ciliated cell differentiation, possibly through suppression of furin-mediated Notch1 processing.

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Keywords **airway remodelling; basal progenitor cells; ciliated cell differentiation; cultured human nasal epithelial cells; proprotein convertases; notch1**

1. Introduction

The human airway epithelium is a complex pseudostratified multicellular layer that contains basal, secretory and ciliated cells, and provides the first line of defence against inhaled pathogens and allergens (Puchelle *et al*., 2006; Rock *et al*., 2010). In normal turnover and injury repair, basal cells function as epithelial progenitor/stem cells that differentiate into other specialized cell lineages, including secretory and ciliated cells, to maintain the epithelial barrier function (Puchelle *et al*., 2006; Rock *et al*., 2010). However, rounds of repeated tissue injury and repair by chronic environmental stimuli alter the basal cell differentiation process to other cell lineages, leading to aberrant tissue remodelling including basal cell and mucus cell hyperplasia, and dysfunction or loss of ciliated cells (Puchelle *et al*., 2006; Fahy and Dickey, 2010; Rock *et al*., 2010). Finally, deterioration of mucociliary clearance results in chronic respiratory infections and inflammatory damage (Rock *et al*., 2010). Thus, understanding the molecular mechanism that regulates the selection of secretory vs. ciliated cell fate in basal cells is important for therapeutic approaches for chronic airway diseases manifested by increased secretory cell numbers and decreased ciliated cell numbers.

Proprotein convertases (PCs) are involved in the regulation of cell growth and differentiation via the proteolytic activation of many secretory proteins, including growth/differentiation factors and their receptors, adhesion molecules, enzymes, neuropeptides and peptide hormones (Thomas, 2002). Indeed, several growth factors and receptors relevant to airway remodelling, such as epidermal growth factor (EGF), transforming growth

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factor-β (TGF-β), insulin-like growth factor 1 (IGF-1) and its receptor IGF-1R, and Notch, have been shown to be substrates for PCs (Masui *et al*., 1986; Boland *et al*., 1996; Khatib *et al*., 2002; Rock *et al*., 2011; Seidah and Prat, 2012; Shaykhiev *et al.*, 2013; Shiryaev *et al.*, 2013). Both EGF and TGF-β induce migratory and squamous phenotypes in airway basal cells (Masui *et al*., 1986; Boland *et al*., 1996; Shaykhiev *et al*., 2013). Insulin-like growth factor 1 signalling is involved in airway remodelling, including epithelial cell hyperplasia, mucus overproduction, and extracellular matrix deposition (Krein *et al*., 2003; Chand *et al*., 2012). The Notch signalling pathway plays critical roles in the lineage selection of epithelial basal progenitor/stem cells for differentiation into either secretory or ciliated cells in many adult and embryonic tissues (Chiba, 2006; Danahay *et al*., 2015; Liu *et al*., 2010; Rock *et al*., 2011; Mori *et al*., 2015; Pardo-Saganta *et al*., 2015a,b). Mammals express four Notch receptors (Notch1–4) that are single-pass, heterodimeric, transmembrane proteins (Kopan and Ilagan, 2009). Interestingly, sustained activation of the intracellular domain of Notch1 and Notch3 receptors (NICD1 and 3) promotes the differentiation of basal cells into secretory cell lineages in both human and mouse airways (Rock *et al*., 2011; Gomi *et al*., 2015). In mouse tracheobronchial epithelium, basal cells are significantly enriched for *Notch1* transcripts, while there is no differential expression of *Notch2* and *Notch3* in basal and luminal cells and *Notch4* transcripts are not detected in either population (Rock *et al*., 2011). It has recently been reported that human bronchial epithelial cells cultured under submerged or hypoxic conditions inhibit ciliated cell differentiation by maintaining the Notch1 signalling pathway (Gerovac *et al*., 2014). Importantly, the Notch1 precursor is cleaved by PCs (S1 cleavage) in the *trans*-Golgi network (TGN) to generate a mature form and then is transported to the cell surface (Logeat *et al*., 1998; Rand *et al*., 2000).

To date, seven different PCs [furin, paired basic amino acid-cleaving enzyme 4 (PACE4), PC1/3, PC2, PC4, PC5/6, and PC7] have been identified in mammals (Thomas, 2002). Recently, it was reported that furin and PACE4 are expressed in human nasal epithelial cells (HNECs) (Lee *et al*., 2015). Based on this knowledge, it is hypothesized that PCs control the differentiation of

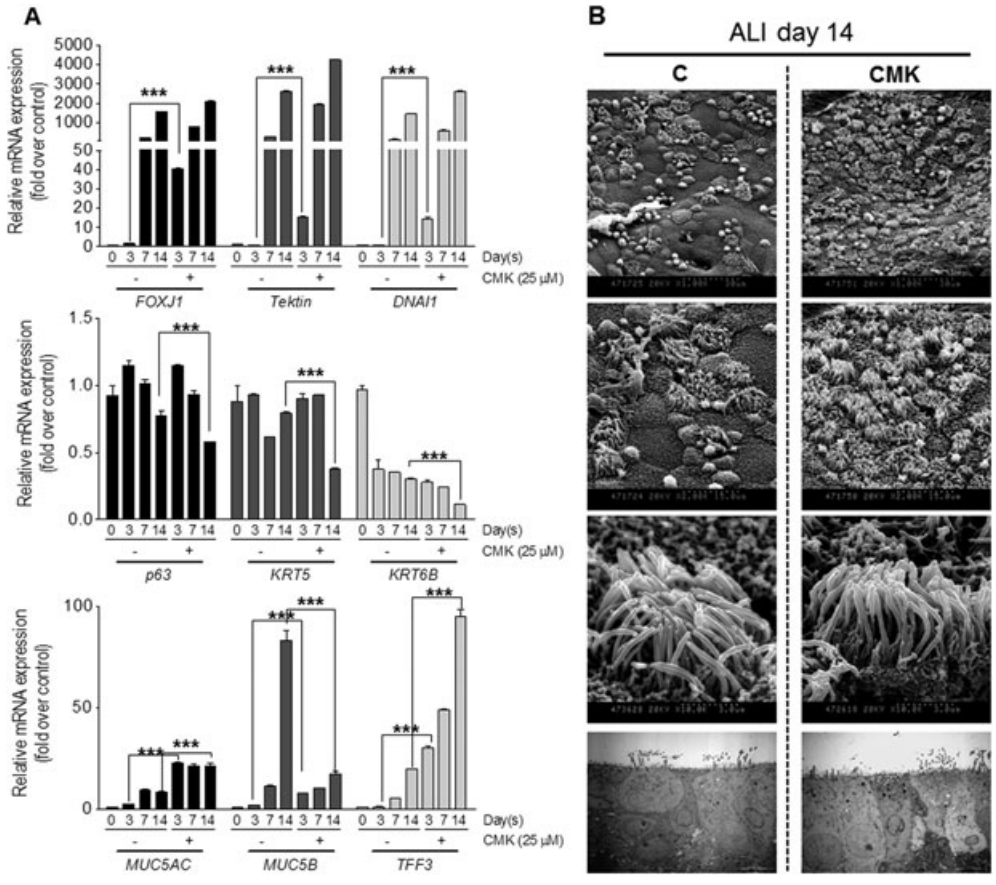

Figure 1. Decanoyl-RVKR-chloromethylketone (CMK) promotes basal cell differentiation in an air–liquid interface (ALI) culture. (a) Cells were differentiated on an ALI for 14 days in the presence and absence of 25 μM CMK from the basolateral side. The numbers indicate the days after the addition of CMK at ALI day 1. Cells were harvested on days 0, 3, 7 and 14 days and assessed for relative mRNA amounts of ciliated cell markers (*FOXJ1*, *tektin* and *DNAI1*), basal cell markers (*p63*, *KRT5* and *KRT6B*) and secretory cell markers (*MUC5AC*, *MUC5B* and *TFF3*) using quantitative real-time polymerase chain reaction. Values for each mRNA were normalized to 18S rRNA and the relative quantity was calculated relative to day 0 of culture. Results are expressed as mean ± SD of four individual experiments, each measured in triplicate (one-way ANOVA $***p < 0.0001$). (b) On day 14, cells were evaluated for morphological changes by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Bars: 30 μm (top row), 15 μm (middle row) and 3 μm (bottom row) for SEM; 10 μm for TEM. (c) On day 14, cells were immunostained for acetylated α-tubulin (green) and MUC5AC (red), respectively. (d) For each membrane, the numbers of secretory (left) and ciliated cells (middle) from 10 different fields were counted and the relative quantity was calculated relative to the untreated control. The results were analysed by Student's *t*-test ($*p < 0.01$, $***p < 0.0001$). Ciliary beat frequency Analyses were performed on at least five different ciliated cells per ALI culture (right). All experiments were conducted on ALI cultures obtained from three different donors. [Colour figure can be viewed at wileyonlinelibrary.com]

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basal cells by regulating Notch1 activation in HNECs. Using an *in vitro* air–liquid interface (ALI) primary HNEC culture model to mimic the epithelial repair process after injury (Puchelle *et al*., 2006; Whitcutt *et al*., 1988), it was shown that the PC inhibition using a PC-specific inhibitor, decanoyl-RVKR-chloromethylketone (CMK, a cell-permeable and irreversible inhibitor) (Hallenberger *et al*., 1992), and molecular silencing methods promotes ciliated cell differentiation, possibly by suppressing furin-mediated Notch1 processing. Furthermore, the results demonstrates that CMK reduces Notch1 processing and promotes regeneration and ciliogenesis of mouse nasal respiratory epithelium after nasal irrigation with a $ZnSO_4$ treatment (Matulionis, 1975).

2. Materials and methods

See the Materials and Methods section in the Supplementary material online, for additional information. The sequences of polymerase chain reaction (PCR) primer sets used in this study are shown in the Supplementary material online, Table S1. The Institutional Review Board of Yonsei University College of Medicine approved the study protocols (IRB# 4-2014-0737 and IACUC# 2012-0330).

3. Results

3.1. Proprotein convertase inhibition stimulates differentiation of basal cells in an ALI culture

To study the effect of PC inhibition on cell differentiation in an ALI culture, CMK (25 μM) was added to the basolateral compartment for 14 days starting on day 1 of ALI culture and the expression of ciliated cell markers (*FOXJ1*, *tektin* and *DNAI1*), basal cell markers (*p63*, *KRT5* and *KRT6B*) and secretory cell markers (*MUC5AC*, *MUC5B* and *TFF3*) (Ross *et al*., 2007; Hackett *et al*., 2011) on days 0, 3, 7 and 14 after treatment were monitored by qPCR (Figure 1a). In untreated control cells, the expression levels of both ciliated and secretory cell markers were upregulated in a differentiation-dependent manner, with simultaneously decreasing expression of basal cell markers. Upon CMK treatment, the expression levels of ciliated cell markers were dramatically increased, indicating that CMK has a strong effect on the acquisition

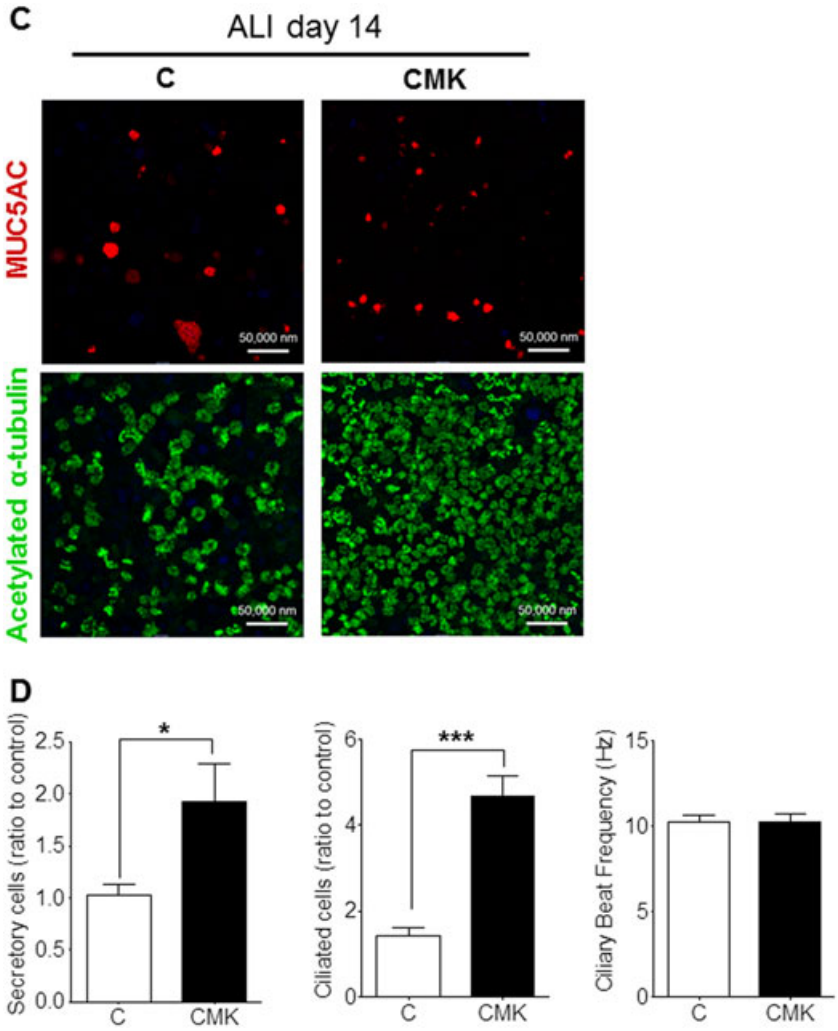

Figure 1. (Continued)

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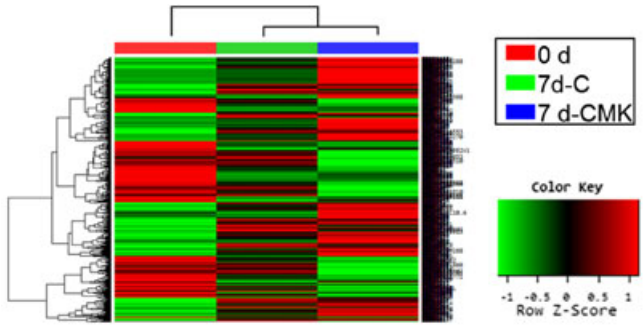

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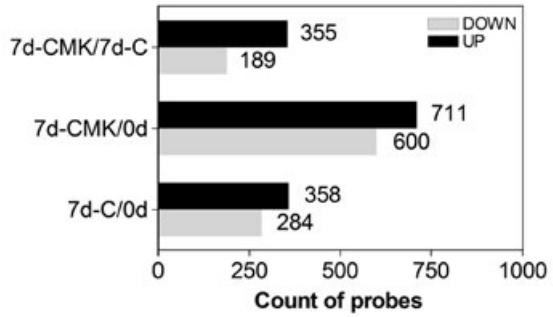

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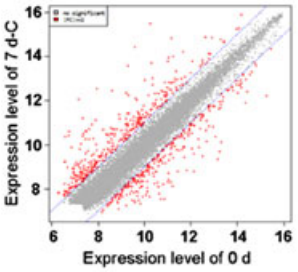
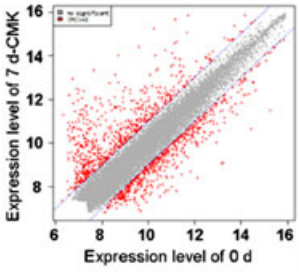
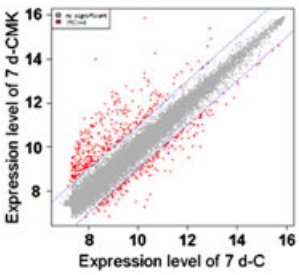


Biological Process	Up	Down	p value
Amino acid metabolism	6	6	1.20E-02
Coenzyme and prosthetic group metabolism	6	4	1.43E-02
Signal transduction	62	45	3.70E-02
Transport	26	21	2.25E-03
Homeostasis	10	6	1.43E-05
Other metabolism	16	12	2.24E-05
Cell structure and motility	20	27	8.01E-05
Developmental processes	56	30	3.89E-06
Electron transport	15	5	5.26E-07
Lipid, fatty acid and steroid metabolism	30	19	1.95E-09
Carbohydrate metabolism	24	15	3.22E-09
Immunity and defense	44	52	2.91E-19



Biological Process	Up	Down	p value
Intracellular protein traffic	44	17	1.32E-02
Homeostasis	7	9	1.60E-02
Non-vertebrate process	4	0	4.24E-02
Phosphate metabolism	8	2	4.97E-02
Signal transduction	102	122	1.18E-03
Coenzyme and prosthetic group metabolism	8	10	2.47E-03
Electron transport	19	3	9.42E-04
Other metabolism	25	17	2.10E-04
Transport	37	53	2.34E-04
Cell cycle	53	28	2.96E-05
Carbohydrate metabolism	24	29	2.38E-06
Developmental processes	83	76	2.82E-07
Cell adhesion	16	42	5.57E-07
Immunity and defense	56	64	1.21E-08
Lipid, fatty acid and steroid metabolism	40	38	3.73E-09
Cell structure and motility	54	60	3.09E-14



Biological Process	Up	Down	p value
Intracellular protein traffic	16	12	3.20E-02
Non-vertebrate process	3	0	4.00E-02
Phosphate metabolism	5	1	4.70E-02
Developmental processes	41	27	3.02E-04
Cell cycle	31	12	1.33E-05
Cell structure and motility	41	17	3.52E-11

Figure 2. Global analysis of differentially expressed transcripts in decanoyl-RVKR-chloromethylketone (CMK)-treated human nasal epithelial cells (HNECs). Cells were cultured in the presence and absence of 25 μM CMK for 7 days. RNA was isolated from air–liquid interface (ALI) cultures on day 0, day 7 without CMK, and day 7 with CMK, and then analysed using microarrays. (a) Cluster analysis of Z-score data. Heat map represents colour-coded expression levels for each sample in selected functional categories with respect to day 0 of culture. Results show 1568 transcripts that are differentially expressed in at least one of the three conditions ($p < 0.05$). (b) Array data export processing and analysis was performed using Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0). Results were filtered according to the magnitude of the change in expression, and only genes that were at least twofold upregulated or downregulated were considered ($p < 0.05$). (c) Scatter plot demonstrates the differential expression of the illustrated genes: grey dots represent genes that did not achieve significant changes in expression, red dots above the grey indicate genes with significantly downregulated expression, and red dots below indicate genes with significantly upregulated expression (left panel). Enriched classification of the biological processes of differentially expressed genes was determined using Gene Ontology analysis (right panel). The number of probe sets for each term is given together with the *p*-value for each term. The experiments were performed with ALI cultures obtained from two different donors. [Colour figure can be viewed at wileyonlinelibrary.com]

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of the ciliated cell phenotype. Expression of basal cell markers displayed a gradual decrease followed by a large decrease at day 14 in CMK-treated cells relative to untreated control cells. *MUC5AC* expression showed a sharp increase at day 3 with a steady increase from day 3 to day 14 in CMK-treated cells. In contrast, *MUC5B* expression exhibited an initial increase at day 3 followed by a drastic decrease at day 14 in CMK-treated cells relative to untreated control cells. Interestingly, *TFF3* expression was upregulated during the culture period of both CMK-treated and untreated control cells, although its expression level in CMK-treated cells was considerably higher than that in untreated control cells, suggesting that the upregulation of *TFF3* may be relevant to ciliated cell differentiation of airway epithelial cells (LeSimple *et al*., 2007). In addition, the effect of CMK on the expression of the cell-type specific markers was concentration dependent and the most effective dose for the expression of ciliated cell markers was 25 μM of CMK (see the Supplementary material online, Figure S1).

To further demonstrate the morphological changes of ALI culture by CMK treatment, both CMK-treated and untreated control cells were analysed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). As shown in Figure 1B, CMK-treated cells displayed similar morphology to normally differentiated epithelial cells containing mature ciliated cells. On day 14, the numbers of ciliated and secretory cells was quantified by immunofluorescent staining for acetylated α-tubulin (a component of cilia axonemes) and MUC5AC, respectively (Figure 1c,d). Consistent with the gene expression data (Figure 1a), CMK treatment increased the numbers of both secretory and ciliated cells relative to untreated control cells. However, the numbers of MUC5AC-positive cells was much lower than the numbers of acetylated α-tubulin-positive cells in both CMK-treated and untreated control cells (Figure 1c). Furthermore, there was no difference in ciliary beat frequency (CBF) between CMK-treated and untreated control cells (Figure 1d), indicating that CMK did not affect ciliary function. Thus, PC inhibition by CMK treatment promotes mucociliary differentiation primarily toward ciliated cell differentiation during an ALI culture of HNECs.

3.2. Enhancement of ciliogenesis-related gene expression by CMK

To examine the gene expression profiles associated with ciliogenesis in HNECs treated with or without CMK, a comparative microarray analysis was performed using RNA samples isolated from ALI cultures on day 0, day 7 with CMK, and day 7 without CMK (Figure 2). The data were filtered to identify probe sets that displayed at least a twofold change either in expression relative to: day 0 of culture for day 7 without CMK of culture (7 d-C/0 d);

Table 1. List of ciliated, basal, and secretory cell-related genes that are differentially expressed in decanoyl-RVKR-chloromethylketone (CMK)-treated human nasal epithelial cells

		Fold change			
RefSeq_NM	Gene	7 d-C/0 d	7 d-CMK/0 d	7 d-CMK/7 d-C	Gene description
Ciliated cell-related genes					
NM_004344.1	*CETN2*	1.10	2.22	2.02	Centrin, EF-hand protein, 2
NM_012144.2	*DNAI1*	2.27	16.85	7.42	Dynein, axonemal, intermediate chain 1
NM_025184.3	*EFHC2*	1.67	7.28	4.37	EF-hand domaincontaining2
NM_001454.2	*FOXJ1*	12.84	74.46	5.80	Forkhead box J1
NM_018010.2	*IFT57*	1.65	4.79	2.91	Intraflagellar transport 57 homologue
NM_018365.1	*MNS1*	2.63	16.76	6.36	Meiosis-specific nuclear structural 1
NM_031916.2	*ROPN1L*	3.79	35.72	9.44	Ropporin 1-like
NM_172242.1	*SPAG6*	2.87	19.22	6.71	Sperm-associated antigen 6
NM_053285.1	*TEKT1*	2.73	21.43	7.85	Tektin 1 (testicular)
Basal cell-related genes					
NM_033157.2	*CALD1*	−2.37	−3.98	−1.67	Caldesmon 1
NM_001753.3	*CAV1*	−1.73	−2.28	−1.32	Caveolin 1
NM_133493.1	*CD109*	−4.67	−4.20	1.11	CD109 molecule
NM_001001392.1	*CD44*	−1.45	−2.21	−1.53	CD44 molecule
NM_000210.2	*ITGA6*	−1.33	−2.23	−1.68	Integrin, alpha 6
NM_139277.1	*KLK7*	−4.90	−5.22	−1.07	Kallikrein-related peptidase 7
NM_000424.2	*KRT5*	−1.24	−1.29	−1.04	Keratin 5
NM_005554.3	*KRT6A*	−1.47	−1.64	−1.12	Keratin 6A
NM_058242.1	*KRT6B*	−1.92	−4.66	−2.42	Keratin 6B
NM_005557.2	*KRT16*	−1.88	−3.24	−1.72	Keratin 16
NM_006216.2	*SERPINE2*	1.04	−2.38	−2.49	Serpin peptidase inhibitor, clade E, member 2
NM_001114979.1	*TP63*	−1.06	−1.34	−1.26	Tumour protein p63
NM_032525.1	*TUBB6*	−1.43	−1.82	−1.27	Tubulin, beta 6
Secretory cell-related genes					
NM_001018021.1	*MUC1*	1.06	1.00	−1.06	Mucin 1, cell surface associated
XM_495860.2	*MUC5AC*	2.49	3.63	1.46	Mucin 5AC, oligomeric mucus/gel-forming
XM_039877.12	*MUC5B*	1.00	−1.10	−1.10	Mucin 5B, oligomeric mucus/gel-forming
NM_024690.2	*MUC16*	10.71	13.09	1.22	Mucin 16, cell surface associated
NM_183234.1	*RAB27A*	1.06	−1.04	−1.11	RAB27A, member RAS oncogene family
NM_052863.2	*SCGB3A1*	1.65	1.98	1.20	Secretoglobin, family 3A, member 1
NM_012391.1	*SPDEF*	1.24	1.19	−1.04	SAM pointed domain containing ETS transcription factor
NM_003226.2	*TFF3*	2.32	21.81	9.40	Trefoil factor 3 (intestinal)

Data from microarray analysis.

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day 0 of culture for day 7 with CMK of culture (7 d-CMK/0 d); or day 7 without CMK of culture for day 7 with CMK (7 d-CMK/7 d-C). Expression microarrays identified 1568 genes that were differentially expressed in at least one comparison of the three conditions ($p < 0.05$; Figure 2a). In the transition from day 0 to day 7 without CMK, 358 induced genes and 284 repressed genes that reached statistical significance were identified, whereas in the transition from day 0 to day 7 with CMK, 711 induced genes and 600 repressed genes were identified ($p < 0.05$; Figure 2b). A comparison of microarray data from 7 d-CMK relative to 7 d-C showed that 355 genes were upregulated and 189 genes were downregulated in CMK-treated cells ($p < 0.05$; Figure 2b). To obtain biological information about the predominant biological processes of the genes that are differentially expressed in the ALI culture treated with vs. without CMK, genes were grouped into different Gene Ontology (GO) terms for biological processes using the DAVID (http://david.abcc.ncifcrf.gov/) and PANTHER (http://www.pantherdb.org/) tools (Figure 2c, Supplementary material online, Tables S2 and S3). Figure 2c shows the top statistically significant terms belonging to a specific biological process for genes with twofold changes either for 7 d-C/0 d (top panel), for 7 d-CMK/0 d (middle panel), or for 7 d-CMK/7 d-C (bottom panel). The highest-scoring biological process category for differentially expressed genes for 7 d-C/0 d was immunity and defence genes, which are related to the protective function of the airway epithelium. Interestingly, the highest scoring category for differentially expressed genes both for 7 d-CMK/0 d and for 7 d-CMK/7 d-C was cell structure and motility, including genes relevant to ciliogenesis, cytoskeletal organization, and cell adhesion (see the Supplementary material online, Tables S2 and S3). Table 1 shows a list of representative ciliated, basal, and secretory cell-related genes that are differentially expressed in at least one comparison of the three conditions (7 d-C/0 d, 7 d-CMK/0 d, and 7 d-CMK/7 d-C). Consistent with quantitative PCR (qPCR) gene expression data on day 7 (Figure 1a), CMK treatment exhibited drastically increased expression of ciliated cell markers, decreased expression of basal cell makers and increased expression of *MUC5AC* and *TFF3*, but there was change in *MUC5B* expression. A list of ciliogenesis-related genes showing increases of twofold or greater in at least one comparison of the three conditions is shown in the Supplementary material online, Table S4 (Ivliev *et al*., 2012). Among these, 10 ciliogenesis-related genes (*CETN2*, *DNAI1*, *DNAH7*, *EFHC2*, *FOXJ1*, *IFT57*, *MNS1*, *ROPN1L*, *SPAG6* and *TEKT1*) were selected and used to validate microarray data

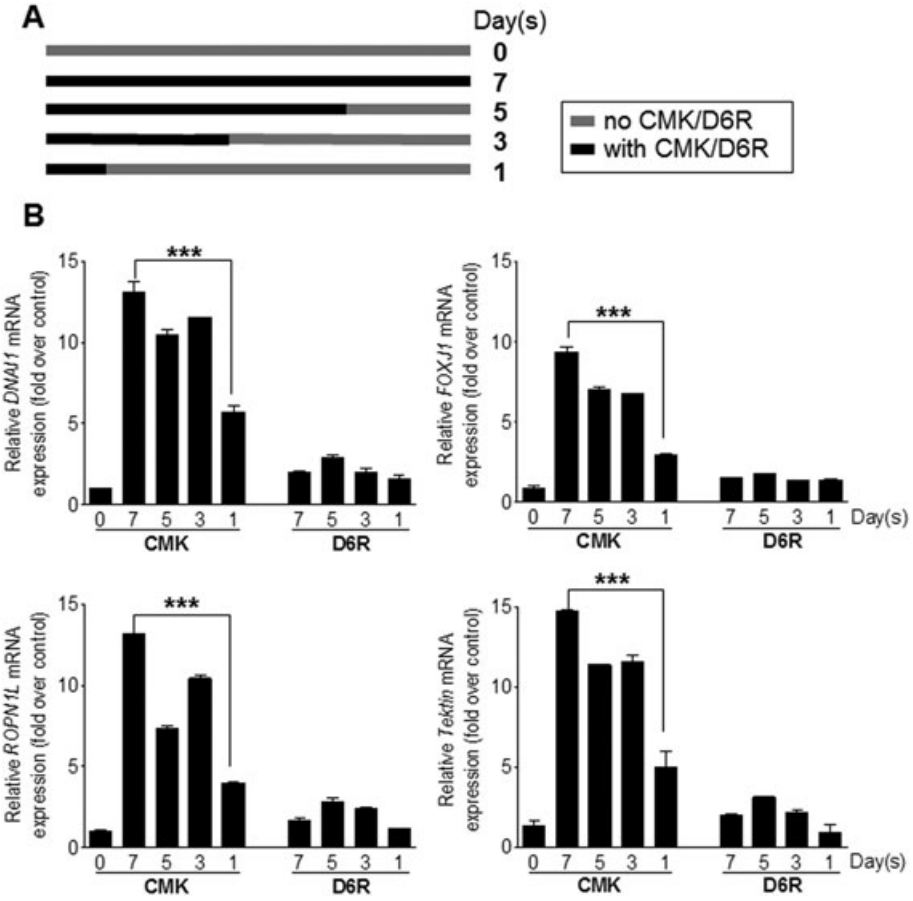

Figure 3. Intracellular PCs regulate cell lineage specification during basal cell differentiation in an air–liquid interface (ALI) culture. (a) Protocols for the treatment of human nasal epithelial cells (HNECs) with PC inhibitors. The HNECs were treated with 25 μM decanoyl-RVKR-chloromethylketone (CMK) or 10 μM hexapeptide D-arginine (D6R) for different periods (i.e. for 1 day from day 0–1, for 3 days from days 0–3, for 5 days from days 0–5 and for 7 days from days 0–7). Cells were then cultured without CMK or D6R until day 7. The numbers represent the days of treatment from day 0. (b) The relative mRNA amount of *DNAI1*, *FOXJ1*, *RPON1L* and *Tektin* was evaluated by quantitative polymerase chain reaction. Values for each mRNA were normalized to 18S rRNA, and the relative quantity was calculated relative to the untreated control. Results are expressed as mean ± SD of three individual experiments, each measured in triplicate (one-way ANOVA ***$p < 0.0005$)

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with qPCR (see the Supplementary material online, Figure S2). Consistent with the microarray data, the expression levels of ciliogenesis-related genes were considerably increased in 7 d-CMK compared with either 0 d or 7 d-C. These results suggest that CMK induces upregulation of ciliogenesis-related genes in HNECs.

3.3. Intracellular PCs regulate cell fate determination during basal cell differentiation in an ALI culture

Next, whether continuous treatment of CMK is necessary for its maximal relative effect on ciliated cell differentiation during ALI culture of HNECs was examined. For this, HNECs cells were treated with CMK from the basolateral side of an ALI culture for various periods starting on day 0, and then cultured in fresh medium without CMK until day 7 (Figure 3a). Analysis of qPCR data revealed that the maximal induction of the expression of ciliogenesis-related genes by CMK treatment is observed as early as within 3 days of ALI culture, when undifferentiated basal cells are mostly present (Figure 3b), indicating a role of PCs in regulating basal cell-type determination in ALI culture. In addition, it was tested whether the effect of a PC inhibitor on ciliated cell differentiation of HNECs is mediated by inhibition of either intracellular PCs or extracellular PCs. Here, the ALI cultures were treated with the cell-impermeable PC inhibitor hexapeptide D-arginine (D6R) (Cameron *et al*., 2000) with the same protocol as for CMK treatment (Figure 3a), and the expression levels of ciliogenesis-related genes by qPCR were analysed. Compared with CMK, D6R increased the expression of ciliogenesis-related genes with much less effect on ciliogenesis-related gene expression (Figure 3b).

Together, these data suggest that intracellular PCs play a major role in controlling cell-type determination during basal cell differentiation in an ALI culture through the processing of secreted substrates for PCs in TGN/endosomal compartments within basal cells.

3.4. CMK promotes ciliated cell differentiation by inhibiting furin-mediated Notch1 processing

Notch signalling is known to be essential for inducing the differentiation of basal cells into secretory cell lineages during airway epithelial repair (Rock *et al*., 2011; Gomi *et al*., 2015). Microarray data revealed substantial differential expression of Notch target genes containing *HEY1*, *HEY2*, *HES5*, *HES6*, *ID1*, *ID3*, *etc.* in CMK-treated cells relative to untreated control cells (Table 2 and Supplementary material online, Table S5) (Meier-Stiegen *et al*., 2010). In particular among them, the expression levels of *HEY1*, *ID1* and *ID* transcripts were significantly decreased ($p < 0.02$), while the expression level of *HES6* transcripts, an inhibitor of *Hes1* (Bae *et al*., 2000), was considerably increased ($p < 0.02$) in CMK-treated cells relative to untreated control cells. However, the differential expression of *HES1* transcript, a Notch downstream effector (Meier-Stiegen *et al*., 2010), was not detected by the microarray.

To assess a role of Notch signalling in the promotion of ciliated cell differentiation by CMK, 5 μM DAPT {*N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester}, a Notch inhibitor, in the presence and absence of 25 μM CMK was added to the basolateral compartment for 14 days starting on day 1 of ALI culture, and differentiation of basal cells into ciliated cells was quantified by immunofluorescent staining for acetylated α-tubulin (Figure 4a). Compared with dimethylsulphoxide (DMSO) vehicle treated controls, DAPT alone slightly reduced the numbers of ciliated cells. In contrast, CMK treatment dramatically increased the numbers of ciliated cells. When DAPT and CMK treatments were combined, the numbers of ciliated cells was similar to that in DMSO-treated control cells, but much lower than that in CMK-treated cells. The effects of DAPT on the expression of basal and secretory cell markers on ALI day 7 by qPCR (Figure 4b) were also examined. It was found that DAPT alone led to increased expression of *p63* and decreased expression of *MUC5AC*. Addition of DAPT and CMK completely reversed the upregulated *MUC5AC* expression observed in CMK-treated cells, while slightly increasing expression of *p63*. These results suggest that Notch signalling might be relevant to basal cell differentiation modulated by CMK treatment.

To further investigate the role of PCs in the processing of Notch receptors in HNECs, the relative expression levels of four *NOTCH* receptor transcripts (*NOTCH1*, *2*, *3* and *4*) on ALI day 1 were first verified by qPCR. As shown in Figure 4c, NOTCH1, 2 and 3 were well expressed by HNECs, while *NOTCH4* was barely expressed at levels about 1000-fold less than *NOTCH1* ($p < 0.0001$). The expression level of *NOTCH1* was down-regulated over the differentiation time course in both CMK-treated and untreated control cells, indicating the preferential

Table 2. List of Notch target genes that are differentially expressed in decanoyl-RVKR-chloromethylketone (CMK) treated human nasal epithelial cells

		Fold change			
RefSeq_NM	Gene	7 d-C/0 d	7 d-CMK/0 d	7 d-CMK/7 d-C	Gene description
NM_001040708.1	*HEY1*	1.15	−1.60	−1.84	Hairy/enhancer-of-split related with YRPW motif 1
NM_012259.1	*HEY2*	−1.34	−1.56	−1.17	Hairy/enhancer-of-split related With YRPW motif 2
NM_001010926.2	*HES5*	1.13	−1.71	−1.94	Hairy and enhancer of split 5
NM_018645.3	*HES6*	3.28	17.42	5.31	Hairy and enhancer of split 6
NM_181353.1	*ID1*	−1.51	−4.54	−3.01	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
NM_002167.2	*ID3*	−2.32	−7.46	−3.21	Inhibitor of DNA binding 3, dominant negative helix–loop–helix protein

Data from microarray analysis.

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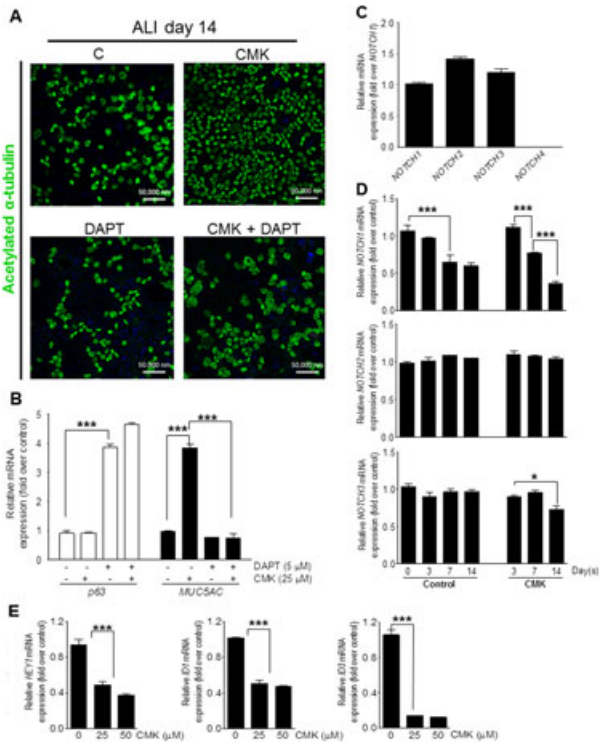

Figure 4. Decanoyl-RVKR-chloromethylketone (CMK) promotes ciliated cell differentiation by inhibition of Notch signalling pathway. (a,b) Cells were cultured on ALI cultures in the presence of either CMK (25 μM), DAPT (5 μM), or *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine t-butyl ester (DAPT) and CMK. Treatment with dimethylsulfoxide (DMSO) was used as the drug vehicle control. (a) On day 14, cells were immunostained for acetylated α-tubulin (green) and 4 ,6-diamidino-2-phenylindole (DAPI; nuclei, blue). (b) Quantitative polymerase chain reaction (qPCR) was used to assess expression of basal cell markers (*p63* and *KRT5*) and secretory cell markers (*MUC5AC* and *TFF3*). Values for each mRNA were normalized to 18S rRNA, and the relative quantity was calculated relative to the untreated control. Results are expressed as mean ± SD of three individual experiments, each measured in triplicate (one-way ANOVA $***p < 0.0005$). (c) The relative mRNA amount of *NOTCH1*, *NOTCH2*, *NOTCH3* and *NOTCH4* was evaluated by qPCR on air–liquid interface (ALI) day 1. The relative quantity was calculated relative to *NOTCH1*. (b) Cells were cultured on ALI for 14 days in the presence and absence of 25 μM CMK, harvested on days 0, 3, 7, and 14, and assessed for relative mRNA amounts of *NOTCH1*, *NOTCH2*, and *NOTCH3* using qPCR. The relative quantity was calculated relative to day 0 of culture ($*p < 0.01$, $***p < 0.0005$). (e,f) Cells were treated with different concentration of CMK (0–50 μM) for 2 days starting on day 1 of ALI culture and subjected to qPCR analysis to determine the relative mRNA levels of *HEY1*, *ID1*, and *ID3* (e, $***p < 0.0005$), and to western blot analysis to determine the protein levels of Notch1, Notch3, furin, and PACE4 (f). Beta-actin was used as a loading control. (g) Immunofluorescence double staining of cytospin preparations on ALI day 1 using anti-furin (red) and anti-Notch1 (green) antibodies. Furin was co-localized with Notch1 in HNECs. Bar: 20 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

expression of *NOTCH1* in the basal cells: its expression, however, displayed a large decrease at day 14 in CMK-treated cells relative to untreated control cells (Figure 4d). In contrast, *NOTCH2* did not show any change in expression level during the time-course in both CMK-treated and untreated control cells. *NOTCH3* expression did not change over time in untreated control cells, while in CMK-treated cells, its expression exhibited a slight decrease at day 14 compared with that in untreated control cells. It was next tested whether CMK modulates gene expression of the Notch signalling pathway components, RNA and protein lysates isolated from HNECs treated with different concentrations of CMK (0–50 μM) for 2 days starting on day 1 of ALI culture were subjected to qPCR and western blot analyses, respectively. Figure 4e shows that the addition of CMK significantly reduced the expression level of Notch downstream molecules (HEY1, ID1 and ID3).

The full-length precursor of Notch (approximately 300 kDa) is cleaved by three subsequent proteolytic cleavages by PCs, ADAM-type metalloproteases and γ-secretase, resulting in the release of the Notch intracellular domain (NICD) (Logeat *et al*., 1998; Rand *et al*., 2000; Kopan and Ilagan, 2009). As sustained activation of NICD1 or NICD3, but not NICD2 and NICD4, promotes the differentiation of basal cells into secretory cell lineages (Gomi *et al*., 2015), it was investigated whether CMK can modulate the processing of Notch1 and Notch3. Interestingly, CMK treatment considerably reduced the cleavage of full-length Notch1 (FL-Notch1), but not FL-Notch3, in a dose-dependent manner, indicating that Notch1, but not Notch3, is a substrate for PCs (Figure 4 f). Next the effect of CMK on the expression of two PCs, furin and PACE4, which are expressed in HNECs (Lee *et al*., 2015), was examined. It was found that CMK dramatically reduced the expression of furin, but not PACE4 (Figure 4f). Further, furin co-localization with Notch1 was seen by immunofluorescence double staining of cytospin preparations on ALI day 1 using anti-furin (red) and anti-Notch1 (green) antibodies (Figure 4g), indicating the involvement of furin in Notch1 processing. To confirm these results, HNECs were transduced with lentivirus expressing either a scrambled shRNA or shRNAs targeting either *furin* or *PACE4*. Scrambled and PACE4 shRNAs were used as negative controls. At 2 days post-transduction, the expression of endogenous *furin* and *PACE4* was assessed by quantitative reverse-transcription PCR (qRT-PCR) and western blotting (Figure 5a). As expected, HNECs infected with either scrambled or *PACE4* shRNA did not affect the expression at both the mRNA and protein levels of furin, while *furin*-shRNA-infected HNECs exhibited dramatically decreased expression of furin. *Furin* knockdown, but not the *PACE4* knockdown,

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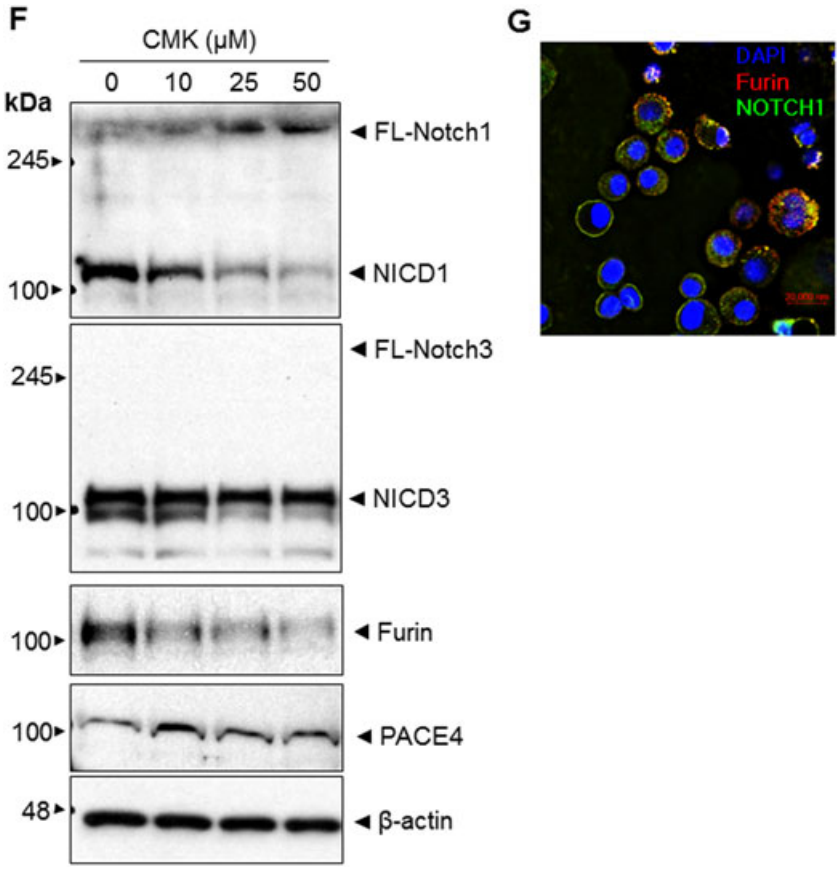

Figure 4. (Continued)

impaired the processing of Notch1, indicating the involvement of furin in Notch1 processing. To further investigate the functional correlation of the observed knockdown on differentiation of HNECs, at 14 days post transduction, basal cell differentiation into ciliated and secretory cells was assessed by either haematoxylin and eosin (H&E) or Alcian Blue (AB) staining, or immunofluorescent staining for acetylated α-tubulin. The H&E and AB staining demonstrated that *Furin* knockdown resulted in a significant increase in ciliated cell numbers, with no significant change in secretory cell numbers (Figure 5b). Furthermore, immunofluorescent staining displayed increased numbers of ciliated cells in *sh-furin*-infected cells compared with *sh-Scramble*-infected control cells (Figure 5c). Therefore, these results suggest that CMK promotes the differentiation of basal cells toward ciliated cells, possibly by suppressing furin-mediated Notch1 processing in HNECs.

3.5. CMK promotes regeneration and ciliogenesis of the mouse nasal respiratory epithelium during repair

An injury/repair model of mouse nasal respiratory epithelium (NRE) was used to study the effect of CMK on regeneration and ciliogenesis in the NRE *in vivo*. As nasal irrigation with $ZnSO_4$ is known to induce necrosis of ciliated cells from the apical surface in the NRE of C57BL/6 mice (Matulionis, 1975), a 1% $ZnSO_4$ solution was applied to the NRE of the mice. The experimental design is depicted schematically in Figure 6a. One day after $ZnSO_4$ treatment, mice were treated with either distilled water (sham-treated mice) or 100 μ CMK (CMK-treated mice) three times every other day. Control mice were exposed to distilled water (control mice). The animals were then using SEM 1 day after the final treatment to observe any morphological changes in the nasal septum (Figure 6b; see the Supplementary material online, Figure S3a). To minimize sampling errors, the posterior portion of the nasal septum, which is in close proximity to olfactory epithelium, was used. Compared with the NRE of control mice, the NRE of sham-treated mice exhibited shortened ciliary precursor structures, which are a ciliated phenotype at an early stage of ciliogenesis. In contrast, the NRE of CMK-treated mice were fully reconstituted to a normal form.

Notch1 processing in the NRE obtained from control mice on day 0, and from sham- and CMK-treated mice on days 0, 2, 4, 6 and 8 after injury were assessed by western blotting. As shown in Figure 6c, Notch1 processing was inhibited in CMK-treated NRE on day 2 post-injury, compared with sham-treated NRE. In addition, sham-treated NRE showed a gradual increase in the expression of acetylated α-tubulin from day 6 post-injury, while CMK-treated NRE exhibited considerably increased expression from day 4, suggesting that CMK-treated NRE regenerate faster than sham-treated NRE. This was confirmed by whole-mount immunofluorescence staining for acetylated α-tubulin in untreated-, sham- and CMK-treated NRE over the time-course (see the Supplementary material online, Figure S3b). Together, these results suggest that CMK promotes regeneration and ciliogenesis of

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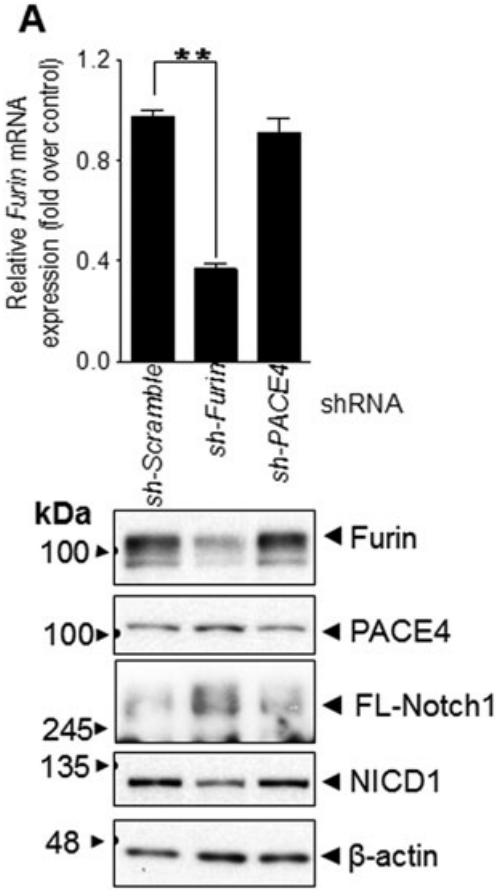

Figure 5. Furin knockdown induces expression of a ciliated cell phenotype in human nasal epithelial cells (HNECs) by suppressing Notch1 processing. (a) On air–liquid interface (ALI) day 0, HNECs were transduced with lentivirus expressing either a scrambled shRNA control (*scramble*) or shRNAs targeting either *furin* or *PACE4*. Two days post-transduction, cells were analysed for *furin* expression by quantitative polymerase chain reaction (qPCR). The relative quantity was calculated relative to the *sh-Scramble* control. Results are expressed as mean ± SD of three individual experiments, each measured in triplicate (one-way ANOVA $^{*}p < 0.01$). Protein levels of furin, PACE4, and Notch1 were determined by western blotting. Beta-actin was used as a loading control. (b,c) Fourteen days post-transduction, epithelial sheets on filters were stained with either haematoxylin and eosin (H&E, top panels) or Alcian Blue (AB, bottom panels) reagent (b), or immunostained with anti-acetylated α-tubulin antibodies (c). (c) For each membrane, the numbers of ciliated cells from 10 different fields were counted and the relative quantity was calculated relative to the *sh-scramble*-infected cells. Scale bar: 50 μm. The results were analysed by Student's *t*-test ($^{***}p < 0.0005$). All experiments were conducted on ALI cultures obtained from three different donors. [Colour figure can be viewed at wileyonlinelibrary.com]

the NRE after $ZnSO_4$ injury, possibly by suppressing the Notch1 signalling pathway.

4. Discussion

Chronic airway epithelial damage and repair lead to aberrant tissue remodelling, including basal cell and mucus cell hyperplasia, mucus overproduction and dysfunction or loss of ciliated cells (Puchelle *et al*., 2006; Fahy and Dickey, 2010; Rock *et al*., 2010). It is, therefore, important to understand the molecular mechanisms and the signalling pathways that regulate the proliferation and differentiation of basal cells during the airway epithelial repair. The results of the present study demonstrate that PC inhibition promotes ciliated cell differentiation in HNECs and the regeneration and ciliogenesis of mouse nasal respiratory epithelium after $ZnSO_4$ injury, possibly by suppressing furin-mediated Notch1 signalling pathway.

Using the PC-specific pharmacological inhibitor CMK (Hallenberger *et al*., 1992) in an ALI model (Whitcutt *et al*., 1988; Puchelle *et al*., 2006), it was found that CMK treatment exhibits drastically increased expression of ciliated cell markers with normal ciliary function and significantly decreased expression of basal cell makers (Ross *et al*., 2007; Hackett *et al*., 2011; Ivliev *et al*., 2012), suggesting that CMK promotes basal cell differentiation toward ciliated cell lineages during an ALI culture of HNECs. Basal cells function as epithelial progenitor cells that differentiate into secretory and ciliated cells to maintain normal epithelial barrier function during normal turnover and injury repair (Puchelle *et al*., 2006; Rock *et al*., 2010). Interestingly, our analyses revealed that acquisition of the ciliated cell phenotype by CMK treatment is accompanied by the altered expression of Notch signalling pathway components (Meier-Stiegen *et al*., 2010; Gomi *et al*., 2015), including key lineage-specific transcription factors and their repressors (*ID1*, *ID3*, *HEY1*, *HEY2*, *HES5* and *HES6*, etc.) (Table 2; see the Supplementary material online, Table S5). The Notch signalling pathway plays an essential role in regulating the fate of adult epithelial basal cells (Chiba, 2006; Liu *et al*., 2010; Rock *et al*., 2011). Notch signalling is not active under steady-state conditions when cellular turnover of the airway epithelium is low, whereas, during the repair process, it is considerably enhanced in epithelial progenitor cells (Kauffman, 1980; Rock *et al*., 2010). Many pharmacological and genetic gain- and loss-of-function studies performed using *in vivo* and *in vitro* models have demonstrated that activation of the Notch signalling increases secretory cell differentiation, while inhibition of Notch enhances ciliated cell differentiation (Rock *et al*., 2011; Gerovac *et al*., 2014; Gomi *et al*., 2015). Full-length Notch is cleaved by PCs in the TGN to generate a mature form and then is transported to the cell surface (Logeat *et al*., 1998; Rand *et al*., 2000), followed by two subsequent proteolytic cleavages by ADAM-type metalloproteases and γ-secretase after ligand binding, resulting in the release of the activated NICD (Kopan and Ilagan, 2009). In the present study, inhibition of Notch signalling with a γ-secretase inhibitor DAPT resulted in a largely increased expression of a basal cell marker and slightly decreased expression of secretory and ciliated cell markers in HNECs, indicating that Notch signalling is essential for the luminal differentiation of basal cells. In addition, the effect of CMK on ciliated cell differentiation in ALI culture was reversed by DAPT addition, suggesting that Notch signalling might be relevant to basal cell differentiation modulated by CMK treatment.

Proteolytic cleavage of PC-specific substrates occurs in TGN/endosomal compartments, at the cell surface, and/or in the extracellular space (Bassi *et al*., 2005; Seidah *et al*., 2008; Seidah and Prat, 2012). Recently, it was reported that cultured HNECs express two PC family members: furin and PACE4 (Lee *et al*., 2015). These two PCs have functional redundancy for the processing of numerous substrates *in vitro* and *ex vivo* (Seidah *et al*., 2008; Seidah and Prat, 2012). Furin is primarily localized

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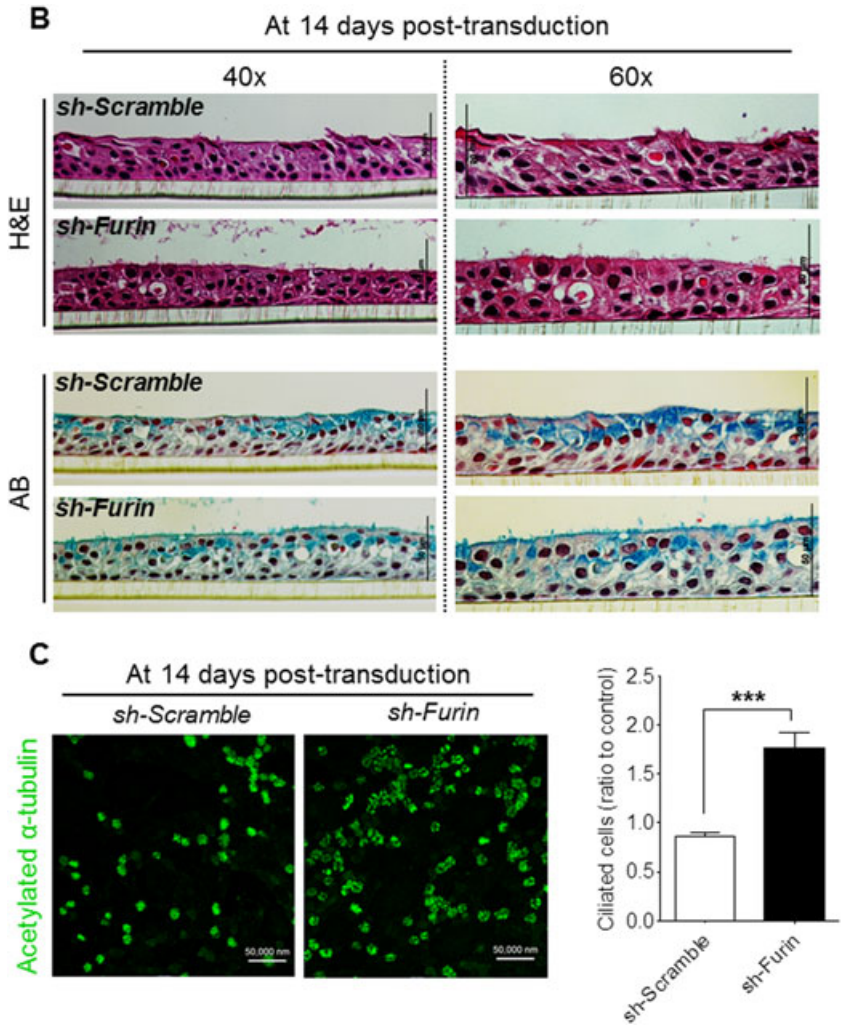

Figure 5. (Continued)

to the TGN, cycles from the TGN to the cell surface and is retrieved through the endosomal pathway, while PACE4 is localized at the cell surface or in the extracellular matrix by binding to heparan sulfate proteoglycans (HSPGs) (Thomas, 2002; Seidah and Prat, 2012). The results of the present study, based on PC inhibition using either the cell-permeable PC inhibitor CMK or the cell-impermeable PC inhibitor D6R during ALI culture of HNECs (Figure 4), suggest that basal cell differentiation is mostly regulated by intracellular PCs. It was also observed that the maximal induction of ciliogenesis-related gene expression by CMK treatment occurs as early as within 3 days of an ALI culture, when undifferentiated basal cells are mostly present, indicating a role for PCs in controlling basal cell-type determination in an ALI culture. Previous studies have shown that components of the Notch signalling pathway including Notch ligands (*Dll* and *Jag*), receptors (*Notch1*, *Notch2* and *Notch3*) and downstream effectors (*Id* and *Hes/Hey* proteins), are expressed in human airway basal cells (Tilley *et al*., 2009; Gomi *et al*., 2015). Here, it is shown that HNECs express *NOTCH1*, *NOTCH2* and *NOTCH3* with barely detectable levels of *NOTCH4*. Interestingly, *NOTCH1* expression was down-regulated over the differentiation time course in HNECs, indicating that basal cells may preferentially express NOTCH1. However, *NOTCH2* and *NOTCH3* did not show any change in expression levels during the time-course in HNECs, suggesting that all cell populations, including basal, ciliated and secretory cells, express both genes. These results are consistent with the findings of a previous study which showed that basal cells are significantly enriched for *Notch1* transcripts in mouse tracheobronchial epithelium, while there is no differential expression of *Notch2* and *Notch3* in basal and luminal cells and *Notch4* transcripts are not detected in either population (Rock *et al*., 2011).

It has been reported that sustained activation of NICD1 or NICD3, but not NICD2 and NICD4, promote the differentiation of human airway basal cells into secretory cell lineages (Gomi *et al*., 2015), *miR-449* microRNA promotes multiciliogenesis by directly repressing endogenous *Notch1* and *Dll1* transcripts in both human airway epithelium and *Xenopus laevis* embryonic epidermis (Marcet *et al*., 2011), and the expression of Notch1 is increased in areas of goblet cell metaplasia and hyperplasia observed from chronic obstructive pulmonary disease (COPD) patients (Boucherat *et al*., 2012). Interestingly, *in silico* analysis of the human proteome for the identification of potential furin substrates has demonstrated that Notch1, Notch2 and Notch4, but not Notch3, are targets of furin (Shiryaev *et al*., 2013). Indeed, CMK treatment

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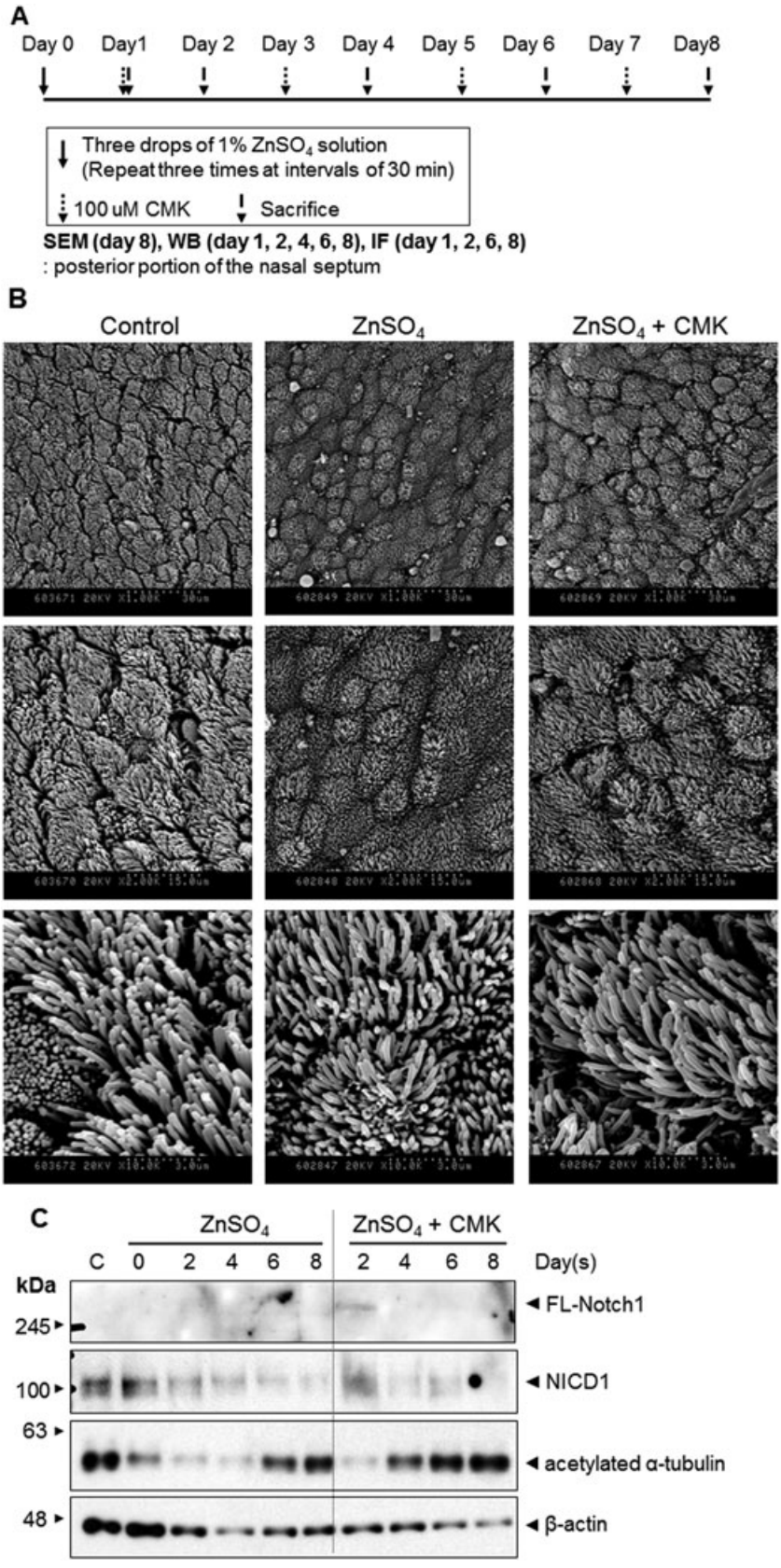

Figure 6. Decanoyl-RVKR-chloromethylketone (CMK) enhances regeneration and ciliogenesis of mouse nasal respiratory epithelium following nasal irrigation in response to $ZnSO_4$. (a) Protocol for treatment of mice with $ZnSO_4$ and CMK. Nasal respiratory epithelia of C57BL/6 mice were irritated with a 1% aqueous $ZnSO_4$ solution. One day after $ZnSO_4$ treatment, mice were treated with either distilled water (sham-treated mice) or 100 μM CMK (CMK-treated mice) three times every other day. Control mice were exposed to distilled water. Mice were killed 1 day after the final treatment, fixed with 1% paraformaldehyde, and processed for scanning electron microscopy. (b) The nasal respiratory epithelia completely regenerated in CMK-treated mice, but not in sham-treated mice. Four control, four sham-treated and four CMK-treated mice were analysed and the results were similar. Scale bars: 30 μm (top row), 10 μm (middle row), 3 μm (bottom row). (c) The nasal respiratory epithelia obtained from untreated control mice on day 0, and from sham- and CMK-treated mice on days 0, 2, 4, 6, and 8 after injury were subjected to western blotting to determine the protein levels of Notch1 and acetylated α-tubulin. Beta-actin was used as a loading control

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considerably decreased Notch1 cleavage, but had no effect on Notch3 cleavage, suggesting that Notch1, a substrate for PCs, controls the balance of secretory and ciliated cell fates in HNECs. We also found that furin knockdown, but not PACE4 knockdown, reduces Notch1 processing and increases the numbers of ciliated cells. In addition, CMK treatment dramatically reduced expression of furin, but not PACE4, speculating that the decreased furin expression by CMK treatment is not due to direct inhibitory effect of CMK on the expression of furin, but rather to the acquisition of the ciliated cell phenotype in HNECs by CMK treatment. It has recently been reported that the positive-feedback loop between Notch1 and furin amplifies the Notch1 signalling (Logeat *et al*., 1998; Qiu *et al*., 2015). Taken together, our data suggest that CMK treatment promotes airway ciliated cell differentiation through suppression of furin-mediated Notch1 processing in HNECs.

We note that CMK also alters the expression of secretory cell marker (*MUC5AC, MUC5B*, and *TFF3*), with a different expression pattern. CMK treatment resulted in significantly increased expression of *MUC5AC* in the early stage of an ALI culture, followed by a steady increase of *MUC5AC* expression. As DAPT treatment reverses upregulation of *MUC5AC* expression observed in CMK-treated cells and Notch3 can be cleaved in a furin-independent manner in HNECs, the CMK-induced increase in *MUC5AC* expression might be mediated by Notch3 in HNECs. However, it cannot be ruled out that CMK modulates other mechanisms that require proteolytic events mediated by PCs: PCs activate various growth factors that affect epithelial cell proliferation and differentiation (Seidah *et al*., 2008; Seidah and Prat, 2012). In the future, as other as-yet unknown substrates of PCs are identified, it will be important to readdress this issue. Notably, although CMK increases the expression of *MUC5AC* in the early stage of an ALI culture with a tendency toward an increasing numbers of MUC5AC-positive cells, the overall numbers of secretory cells is much lower than the numbers of ciliated cells in ALI culture. Interestingly, expression of *MUC5B*, unlike *MUC5AC*, was significantly increased in the early stage of an ALI culture by CMK treatment, followed by a drastic decrease of *MUC5B* expression, indicating that the expression of these genes is differentially regulated by transcriptional and post-transcriptional mechanisms (Gosalia *et al*., 2013). It was also observed that *TFF3* expression is upregulated over the differentiation time-course in both CMK-treated and untreated control cells. LeSimple *et al.* (2007) reported that TFF3 promotes airway epithelial ciliated cell differentiation, indicating a cross-talk between TFF3 and ciliogenesis signalling pathways.

The present study also reports that CMK promotes regeneration and ciliogenesis in mouse NRE following necrosis and sloughing of ciliated cells, but not basal cells, in response to $ZnSO_4$ (Matulionis, 1975). The data revealed that CMK treatment after $ZnSO_4$ injury strongly reduced Notch1 processing, while simultaneously increasing expression of acetylated α-tubulin in the mouse NRE, leading to the faster restoration of a fully functional airway epithelium in CMK-treated mice compared with sham-treated mice. It has been reported that basal stem cells isolated from steady-state mouse tracheobronchial epithelium express *Notch1*, *Dll1*, *Jag1* and *Jag2* (Rock *et al*., 2011). Rock *et al*. also showed in an injury/repair model of mouse tracheobronchial epithelium with SO_2 treatment to induce the death and sloughing of most luminal cells, that Notch signalling is active in early progenitor cells derived from basal stem cells and the luminal differentiation of basal cells depends on Notch signalling (Rock *et al*., 2011). Moreover, the constitutive expression of NICD1 in adult mouse basal cells promotes secretory cell differentiation (Rock *et al*., 2011). However, additional experiments are required to determine whether furin can process the Notch1 receptor and control the differentiation of mouse airway basal cells *in vivo*.

In summary, the findings demonstrate that PC inhibition by CMK promotes the differentiation of functional ciliated cells from basal cells during ALI culture of HNECs, possibly by inhibiting furin-mediated Notch1 processing. As CMK is not furin-specific and also inhibits other furin-like PCs (Becker *et al*., 2012), identification of more efficient, selective and non-toxic inhibitors may be a useful strategy for preventing the development of pathologies associated with the dysfunction or loss of ciliated cells in human nasal epithelium.

Conflict of interest

The authors have declared that there is no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. CMK alters the expression of cell-type specific markers in a dose-dependent manner in an ALI culture.
Figure S2. CMK increases the expression of ciliogenesis-related genes in an ALI culture.
Figure S3. CMK enhances regeneration and ciliogenesis of mouse nasal respiratory epithelium following nasal irrigation in response to $ZnSO_4$.
Method S1. Supplementary methods and materials.
Table S1. List of primer sequences used for qPCR.
Table S2. Gene ontology analysis of upregulated genes in CMK-treated HNE cells.
Table S3. Gene ontology analysis of downregulated genes in CMK-treated HNE cells.
Table S4. List of ciliogenesis-related genes whose expression is up-regulated by CMK treatment in HNE cells.
Table S5. List of genes involved in mRNA transcription whose expression is affected by CMK in HNECs.

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