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# **Muco-ciliary differentiation of nasal epithelial cells is decreased after wound healing in vitro**

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## **Abstract**

**Epithelial damage and modifications of cell differentiation are frequent in airway diseases with chronic inflammation, in which Transforming Growth Factor- $\beta$ 1 (TGF- $\beta$ 1) plays an important role. The aim of this study was to evaluate the differentiation of human nasal epithelial cells (HNEC) after wound healing and the potential effects of TGF- $\beta$ 1.**

**Basal, mucous and ciliated cells were characterized by cytokeratin-14, MUC5AC and  $\beta$ IVtubulin immunodetection, respectively. Their expression was evaluated in situ in nasal polyps and in an in vitro model of wound healing in primary cultures of HNEC after wound closure, under basal conditions and after TGF- $\beta$ 1 supplementation. Using RT-PCR, the effects of TGF- $\beta$ 1 on MUC5AC and DNAI1 genes, specifically transcribed in mucous and ciliated cells, were evaluated.**

**In situ , high TGF- $\beta$ 1 expression was associated with low MUC5AC and  $\beta$ IVtubulin expression. In vitro , under basal conditions, MUC5AC expression remained stable, cytokeratin-14 expression was strong and decreased with time, while  $\beta$ IV tubulin expression increased. TGF- $\beta$ 1 supplementation down-regulated MUC5AC and  $\beta$ IV tubulin expression as well as MUC5AC and DNAI1 transcripts.**

**After a wound, differentiation into mucous and ciliated cells was possible and partially inhibited in vitro by TGF- $\beta$ 1, a cytokine that may be involved in epithelial remodeling observed in chronic airway diseases.**

**MESH Keywords** Axonemal Dyneins ; Cell Differentiation ; drug effects ; Cells, Cultured ; Cilia ; metabolism ; Down-Regulation ; Dyneins ; metabolism ; Epithelial Cells ; cytology ; metabolism ; Humans ; Keratin-14 ; metabolism ; Mucin 5AC ; genetics ; metabolism ; Mucins ; metabolism ; Nasal Mucosa ; cytology ; metabolism ; Nasal Polyps ; metabolism ; pathology ; Transforming Growth Factor beta1 ; metabolism ; pharmacology ; Tubulin ; metabolism ; Wound Healing

**Author Keywords** ciliated cells ; human nasal epithelial differentiation ; mucous cells ; TGF-beta 1 ; wound healing

## **INTRODUCTION**

Epithelial damage of the airways seems to be an important event in the pathophysiology of chronic inflammatory diseases, such as asthma, chronic obstructive pulmonary disease or nasal polyposis (1, 2). The differentiation in mucous and ciliated cells participates in creating a protective barrier of the airways (3) which may be damaged after injury. Wound healing may restore the epithelium ad integrum or induce remodeling of the airways, resulting in chronic inflammation and altered differentiation (4).

The first step of the repair process requires loss of differentiation, spreading and migration of airway epithelial cells from the edges of the wound (5). The second step, epithelial "redifferentiation" which has been much less clearly defined is a major issue.

Epithelial cells, activated during the injury and repair process, interact with fibroblasts and inflammatory cells, secreting growth factors and cytokines including transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (6). TGF- $\beta$ 1 is known to regulate inflammatory cell activation (7), enhance fibrosis formation (8), and accelerate in vitro cell migration (4, 9). The action of TGF- $\beta$ 1 on epithelial cell differentiation, especially in the airways, has been poorly investigated.

We wondered whether a damaged epithelium could restore mucous and ciliated differentiation and, if so, how TGF- $\beta$ 1 could influence this restoration. We therefore investigated the in vitro epithelial cell differentiation after wound closure in a model of primary culture of human nasal epithelial cells (HNEC) and studied the effects of TGF- $\beta$ 1 supplementation on this differentiation.

## **MATERIALS AND METHODS**

Nasal polyps were collected from 20 patients with nasal polyposis during ethmoidectomy. The patients did not mention any allergic background and were negatively tested for multirast. The study was performed after obtaining informed consent from all patients and complied with all criteria defined by the hospital ethics committee (CCPPRB-Henri Mondor).

Reagents are listed in Annex 1 .

### **TGF- $\beta$ 1 expression and modification of epithelial differentiation in polyps in situ**

A relationship between TGF- $\beta$ 1 expression and modification of epithelial differentiation was investigated by using immunolabeling for TGF- $\beta$ 1, MUC5AC and  $\beta$ IV tubulin in nasal polyps (n=8). Nasal polyps were surgically removed, immediately fixed in 4% formalin, embedded in paraffin, and cut into 5- $\mu$ m sections. After deparaffinization, rehydration and blocking of non-specific binding sites (2% BSA), tissue sections were incubated with primary antibody, anti-TGF- $\beta$ 1, anti-MUC5AC or anti- $\beta$ IV tubulin monoclonal antibodies (dilution 1/500, room temperature, one hour). After incubation with biotinylated horse anti-mouse antibody (dilution 1/200, room temperature, one hour), immunolabeling was visualized according to the avidin-biotin-peroxidase complex method (Elite ABC kit). Two negative controls were performed by omitting primary antibody and using non-immune mouse serum.

TGF- $\beta$ 1, MUC5AC and  $\beta$ IV tubulin expression was evaluated in various epithelial areas of each polyp by conventional light microscopy.

### **In vitro model of epithelial wound healing modulated by TGF- $\beta$ 1**

An in vitro model of epithelial wound healing (9 ) was used to study the modifications of epithelial differentiation related to TGF- $\beta$ 1 overexpression.

#### **Primary culture of human nasal epithelial cells**

Epithelial cell differentiation was studied in a model of primary culture of HNEC, which contains only epithelial cells and allows the formation of a differentiated pseudo-stratified epithelium composed of three major cell types (i.e. basal cells, mucous cells and ciliated cells). HNEC were isolated from polyps from nine different patients and cultured at air-liquid interface as previously described (for details see in Papon and all. (10 )).

#### **In vitro wound healing model**

The in vitro wound repair assay was carried out using a model of mechanical injury adapted from a previously described method (9 ). A 12 mm linear wound was made by scraping the HNEC with a pipette tip, followed by extensive washing to remove cellular debris (9 ). The site of the wound was indicated by a mark performed on the structure of the well. Wounding was performed on day 12, when cells were differentiated. Wounds closed in about 24 hours, as previously shown (9 ). The experiments of the present study were started when wound closure was achieved (T0). These experiments were performed 2 days after closure (T1), 5 days after closure (T2) and 12 days after closure (T3).

Epithelial differentiation was studied under basal conditions (i.e . DMEM/F-12-antibiotics with 2% Ultrosor G) and after TGF- $\beta$ 1 supplementation.

#### **TGF- $\beta$ 1 supplementation**

The dose of 5 ng/ml was previously selected for TGF- $\beta$ 1 supplementation in our culture model and the absence of toxicity was verified by electrophysiological and viability assays (Trypan blue) (9 –12 ). TGF- $\beta$ 1 was added to the culture medium of wounded HNEC cultures daily from T0 to the end of experiments (T3). For each culture, cell differentiation was compared between TGF- $\beta$ 1-supplemented and non-supplemented wounded wells (i.e . grown under basal conditions) at T1, T2, T3.

#### **Evaluation of epithelial differentiation after immunolabeling**

Basal, mucous and ciliated cells were characterized by immunolabeling with specific mouse monoclonal antibodies (see annex 1 ). After rinsing, inserts with cell layers were fixed (4% paraformaldehyde, 30 minutes, 4°C). After blocking non-specific binding sites (1% BSA), inserts were incubated (room temperature, one hour) with either anti-cytokeratin 14, anti-MUC5AC or anti- $\beta$ IV tubulin monoclonal antibodies (1/20, 1/500 and 1/500 antibody dilutions, respectively). Inserts were then incubated with the Alexa 488-conjugated monoclonal

goat anti-mouse IgG (1/1000 dilution, one hour). Filters supporting the cell layers were cut away from the inserts and mounted on glass slides with Vectashield mounting medium with DAPI (Abcys Paris, France). Two negative controls were performed by omitting primary antibody and using non-immune mouse serum. The cells were observed under a Zeiss microscope equipped with epifluorescence illumination.

Even after closure, the wounded area was easily differentiated from the non-wounded area under light microscopy (c.f. wounding site marked on the structure of the well). The control area was defined as the cells located midway between the wounded area and the edges of the well. Five different cultures were first examined to verify that the wounding and repair processes did not modify cell differentiation of the control area.

The study then consisted of comparing cell differentiation in the wounded area to that of the control area at T1, T2, T3.

A single blinded observer evaluated, in random order, immunolabeling preparations coded (for date and TGF- $\beta$ 1 supplementation) by an independent investigator. Quantitative results were obtained by calculating the ratio between the number of immunopositive cells for each antibody and the total number of cells, determined by counting the nuclei revealed by DAPI staining in one microscopic field (final magnification,  $\times$  200). This quantification was repeated in 10 random fields each including about 1,500 cells.

For each immunolabeling, we first compared the positivity in wounded and control area from T1 to T3, in cultures grown under basal conditions. The time-course of positive labeling was then compared for each immunolabeling in the wounded area between TGF- $\beta$ 1-supplemented cultures and cultures grown under basal conditions. Similarly, the time-course of positive labeling was compared for each immunolabeling in the control area between TGF- $\beta$ 1-supplemented cultures and cultures grown under basal conditions. Results of evaluation of cell positivity were compared using a Hotelling's trace multivariate analysis (SPSS Version 13.0). Data are expressed as means  $\pm$  SEM. A p value less than 0.05 was considered statistically significant.

#### **Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) quantification of DNAI1 and MUC5AC transcripts under basal conditions and after TGF- $\beta$ 1 supplementation**

Due to the prominent effects of TGF- $\beta$ 1 on in vitro epithelial cell differentiation, we chose to investigate whether TGF- $\beta$ 1 could modulate the levels of DNAI1 and MUC5AC transcripts expressed during differentiation of ciliated (13) and mucous cells (14, 15), respectively.

#### **RNA isolation and Reverse Transcription**

Total RNA was isolated from three primary cultures of HNEC, two days after confluence, using the commercially available reagent RNeasy (Qiagen, Carlsbad, CA). This culture time corresponded to the beginning of cell differentiation. TGF- $\beta$ 1 was added to culture medium for two days after confluence, using the same protocols as those previously described (9–11). First-strand cDNA synthesis was performed in a total volume of 20  $\mu$ L, 5  $\mu$ g of total RNA were reverse transcribed using 200 units of Superscript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen), 50 pmol of random primer (Promega), 60 units of RNaseOUT ribonuclease inhibitor (Invitrogen) and 1X reaction buffer. A 10-minute pre-incubation period at 25°C ensured improved annealing of the primers with the RNA matrix. The mix was then incubated for 50 minutes at 42°C, followed by a denaturation step consisting of 15 minutes at 65°C. Integrity and quantity of RNAs were previously determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The cDNA obtained were used for qRT-PCR with no step of storage.

#### **qRT-PCR quantification of cDNA**

qRT-PCR assays were performed using an ABI PRISM 7000 Sequence detection system instrument and the corresponding software (PE Applied Biosystems, Inc.). Quantitative data of relative gene expression were obtained using the comparative C<sub>T</sub> method ( $\Delta\Delta$ C<sub>T</sub> method), as described by the manufacturer. Assays-on-Demand, consisting of a mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye-labeled) for the target genes DNAI1 (assay Hs00201755\_m1), MUC5AC (assay Hs00873638\_m1) and 18S (assay Hs9999901\_s1), were purchased from PE Applied Biosystems. As we have previously demonstrated that expression of 18S is stable in our culture system (12), we used this gene as endogenous control.

PCR was performed with 12.5  $\mu$ L TaqMan Universal PCR Master Mix (PE Applied Biosystems), 1.25  $\mu$ L probe/primer mix and two dilutions of cDNA (1X and 0.1X, data not shown) in a 25- $\mu$ L final reaction mixture. After a 2-minute incubation at 50°C to allow for uracil-N-glycosylase (UNG) cleavage, AmpliTaq Gold was activated by incubation for 10 minutes at 95°C. Each of the 45 PCR cycles consisted of 15 seconds of denaturation at 95°C, and hybridization of the probe and the primers for 1 minute at 60°C. All reactions were carried out in triplicate and the threshold cycle (C<sub>T</sub>) values obtained were plotted against the base 10 log of the quantity of cDNA. A slope close to -3.32, defining a reaction with 100% efficiency, was verified for each qRT-PCR before performing the assays. For evaluation of PCR efficiencies, serial dilutions of cDNA obtained from culture cells were used to construct a standard curve. To confirm the specificity of the PCR reaction, PCR products were electrophoresed on a 2% agarose gel.

The relative difference in changes in expression of transcripts in untreated samples compared to changes in stimulated samples ( $\Delta\Delta C_T$ ) was determined.

## RESULTS

### TGF- $\beta$ 1 expression and modification of epithelial differentiation in polyps in situ

MUC5AC,  $\beta$ IV tubulin and TGF- $\beta$ 1 were expressed in all polyp samples. Depending on the area, well differentiated epithelium showed strong positivity for MUC5AC and  $\beta$ IV tubulin and weak positivity of surrounding TGF- $\beta$ 1 (Figure 1A). In contrast, in the case of loss of epithelial differentiation, MUC5AC and  $\beta$ IV tubulin were weakly positive and TGF- $\beta$ 1 was strongly positive (Figure 1B). High TGF- $\beta$ 1 expression levels were therefore associated with a trend towards loss of mucous and ciliated cell differentiation.

### Epithelial cell differentiation in wounded and control area, from T1 (2 days after wound closure) to T3 (12 days after wound closure), in cultures grown under basal conditions

Basal cells, as detected by anti-cytokeratin 14 antibody, exhibited a cytoplasmic reticular positivity (Figure 2A). Mucous cells, as detected by anti-MUC5AC antibody, showed two different immunolabeling patterns characteristic of mucous vesicles (Figure 2B). The  $\beta$  IV tubulin immunolabeling allowed to clearly identify ciliated structures at the apical side of some cells (Figure 2C).

The spontaneous course of cell differentiation in the wounded area was compared to that of the control area. As shown in Figure 3A, at each time, cytokeratin 14 positivity was significantly higher in the wounded area than in the control area. Cytokeratin 14 was strongly positive in the wounded area at T1 and T2 and significantly decreased at T3. MUC5AC (Figure 3B) and ciliated  $\beta$ IV tubulin immunolabeling (Figure 3C) was always significantly less positive in the wounded area than in the control area. MUC5AC positivity in the wounded area was detected by T1 and remained stable from T1 to T3 (Figure 3B). Ciliated  $\beta$ IV tubulin immunolabeling in the wounded area was weak at T1 and T2 and increased significantly at T3 (Figure 3C).

Wounded epithelial cells were therefore able to differentiate in vitro into mucous cells as early as two days after wound closure, and differentiated into ciliated cells after only 12 days.

### TGF- $\beta$ 1 downregulates MUC5AC and $\beta$ IV tubulin expression in wounded and control areas

TGF- $\beta$ 1 did not significantly modify cytokeratin 14 expression at the various times, in either wounded or control areas (Figure 4A). TGF- $\beta$ 1 supplementation significantly decreased MUC5AC expression, in both wounded and control areas, compared to non-supplemented cultures (Figure 4B). TGF- $\beta$ 1 supplementation significantly decreased  $\beta$ IV tubulin expression (Figure 4C). DAPI staining showed that TGF- $\beta$ 1 supplementation did not decrease the cell number (data not shown).

### TGF- $\beta$ 1 downregulates the expression of DNAI1 and MUC5AC transcripts

The effect of TGF- $\beta$ 1 on DNAI1 and MUC5AC expression is shown in Figure 5. TGF- $\beta$ 1 dramatically downregulated the expression of these two differentiation-specific genes.

## DISCUSSION

Epithelial damage is an important event involved in the pathophysiology of many chronic inflammatory airway diseases (1, 2, 6). Various in vitro models of epithelial wounding have shown that poorly differentiated cells migrate in the airways without proliferating (9, 16, 17). However, none of these studies have followed cell differentiation after wound closure. We show for the first time that, after wound healing, human nasal epithelial cells are able to "redifferentiate" in vitro into mucous and ciliated epithelium.

There are however some limitations to the present study. First, data were obtained in vitro, and cannot therefore be extrapolated to in vivo conditions. Secondly, our in vitro model uses nasal epithelial cells, which may not strictly mimic what might be observed in bronchial epithelial cells. Moreover, using a model of mechanical wounding, the present study does not pretend to reflect the healing events that occur after other airway aggressions. Despite this different bias, this study brings solid information concerning nasal epithelium wound healing and the potential role of TGF- $\beta$ 1.

Immunohistochemical markers were used to characterize and quantify basal (cytokeratin 14), mucous (MUC5AC) and ciliated cell ( $\beta$  IV tubulin) differentiation. Among the various cytokeratins detected in basal cells of airway epithelium, i.e. (5+8), 14, (13+16), 17 and 19 (18, 19), cytokeratin 14 is one of the most intensely expressed in upper airway epithelium (19, 20). As expected, the immunolabeling was observed in the cytoplasm of small cells, resembling basal cells. Among the various mucins expressed in airways, i.e. MUC5AC, 5B and 8 (21, 22), MUC5AC expression appears to be specific for mucous cells in the surface epithelium and is never observed in mucous glands (14). MUC5AC, the first mucin expressed during in vitro mucous differentiation (15), is overexpressed in nasal polyps in response to the epidermal growth factor receptor cascade (22). In our study, the two different patterns of MUC5AC immunolabeling could

represent different amounts of exocytosis: thin vesicles could be secretions accumulated at the apical part in their condensed form, while large spots could correspond to the hydrated expanded form after degranulation (3). We then selected  $\beta$ IV tubulin as a marker of ciliated cells. In mammals,  $\beta$ I and  $\beta$ IV are the only isotypes detected in ciliated airway cells (23, 24). Both are common to cilia but the  $\beta$ IV isotype is the only one containing the axoneme-specific sequence, necessary for cilia and flagella beating (25, 26).

In this study, differentiation was first evaluated under basal conditions in the wounded area just after wound closure. Basal cells were predominant in the wounded area during the first week and after two weeks, and their number decreased as the number of ciliated cells increased. Conversely, mucous cells were detected early in the wounded area and their number remained stable. In theory, ciliated cells could be derived from either basal or mucous cells. On the one hand, our results showing an inverse relation for the number of basal and ciliated cells support the hypothesis that basal cells could differentiate into ciliated cells (2, 20). On the other hand, mucous cells could also differentiate into ciliated cells while renewing themselves, which would explain their stable number (27).

In our model, the cell differentiation process was always less complete in the wounded area than in the control area, in contrast with *in vivo* data showing that epithelium is completely restored after wound healing (5). We can therefore hypothesize that the absence of certain growth factors, produced by epithelial cells, fibroblasts or inflammatory cells that are absent in our culture model, results in incomplete healing (5).

Among the various cytokines overexpressed in inflammatory airway diseases, TGF- $\beta$ 1 could be important for airway remodeling (6, 8, 28, 29). The evaluation of cell differentiation after wound closure, in the presence of TGF- $\beta$ 1, showed a decreasing number of mucous and ciliated cells both in wounded and control areas. Only a few studies have examined the effect of TGF- $\beta$ 1 on epithelial cell differentiation: TGF- $\beta$ 1 inhibits mucous differentiation of gastric epithelial cells from guinea pig (30) and induces squamous cell differentiation in rabbit tracheal epithelial cells (31). None of these studies reported the effect of TGF- $\beta$ 1 on ciliated differentiation. To further confirm the role of TGF- $\beta$ 1 on cell differentiation, we assessed the expression level of MUC5AC and DNAI1, two genes involved in mucous and ciliary differentiation, respectively. As first observed at the protein level, we confirmed that TGF- $\beta$ 1 is able to downregulate expression of the MUC5AC transcript in HNEC. It has been previously shown that the TGF- $\beta$ RII signaling pathway induces downregulation of MUC5AC in human respiratory epithelial cells (32).  $\beta$  tubulin mRNA synthesis was not used to assess ciliary differentiation, as axonemal  $\beta$ IV tubulin is generated by a post-transcriptional modification (24). We therefore chose a gene highly specific for ciliated cells: DNAI1, which encodes an intermediate chain of dynein located in the outer dynein arm of the axoneme (13). We showed for the first time that TGF- $\beta$ 1 induces a dramatic decrease of DNAI1 transcripts.

As activation of the TGF- $\beta$ -Smad signaling pathway has been shown to downregulate nontypeable *Haemophilus influenzae*-induced MUC5AC transcription (32), we therefore looked for Smad binding sites (GTCTgcct and GTCTaggct) (33, 34) in the 2 kb region preceding the first exon of DNAI1 and MUC5AC (Genomatix Suite of sequence analysis tools, MatInspector). Interestingly, we found two putative sites in each promoter region of these genes, suggesting a potential interaction of TGF- $\beta$ 1 with MUC5AC and DNAI1 via Smad signaling pathway in our model.

The high TGF- $\beta$ 1 expression associated with loss of epithelial cell differentiation observed in polyps *in situ* is in accordance with our *in vitro* results. TGF- $\beta$ 1 expression is enhanced in damaged airways in asthma and nasal polyposis (8, 28). TGF- $\beta$ 1 can also disturb epithelial cell function by inhibiting Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) expression (12). Our present data show that, by modifying differentiation, TGF- $\beta$ 1 could impair mucociliary clearance, leading to chronic infections and increased inflammation.

Overall, this study shows that, after wound healing, HNEC are able to differentiate into mucous and ciliated cells *in vitro*, and that this capacity is more efficient in non-wounded than in wounded areas. We also provide evidence that TGF- $\beta$ 1 is able to decrease both mucous and ciliated cell differentiation *in vitro*. These findings are further clues to incriminate TGF- $\beta$ 1 as a decisive protagonist in the epithelial airway remodeling frequently observed in nasal polyposis, but also in asthma or chronic bronchitis where similar studies remain to be conducted.

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## ANNEX 1: List of reagents

Ham's F-12 nutrient medium (F12) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies (Cergy-Pontoise, France). Penicillin, streptomycin, amphotericin B, fetal calf serum (FCS), trypsin, and Ultrosor G were purchased from

GIBCO BRL (Cergy-Pontoise, France). Bovine serum albumin (BSA), Tween-20, dithiothreitol, pronase, and gentamicin were obtained from Sigma (Saint Louis, MO, USA). Human TGF- $\beta$ 1 was obtained from Sigma. Anti-cytokeratin 14, anti-MUC5AC, anti- $\beta$ IV tubulin and anti-TGF- $\beta$ 1 monoclonal antibodies were obtained from Tebu-bio (Le Perray en Yvelines, France), Neomarkers (Fremont, CA, USA), Sigma and Serotec (Cergy Saint-Christophe, France), respectively. Anti-mouse biotinylated secondary antibody and Vectastain Elite ABC kit were purchased from Vector laboratories (Burlingame, USA). Non-immune mouse and goat serum were purchased from Interchim (Montluçon, France). Alexa 488-conjugated goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR, USA).

Inserts used for the culture of the cells (Transwell, Costar, Cambridge, MA) were with 12-mm-diameter polycarbonate micropore membranes coated with type IV collagen (Sigma).

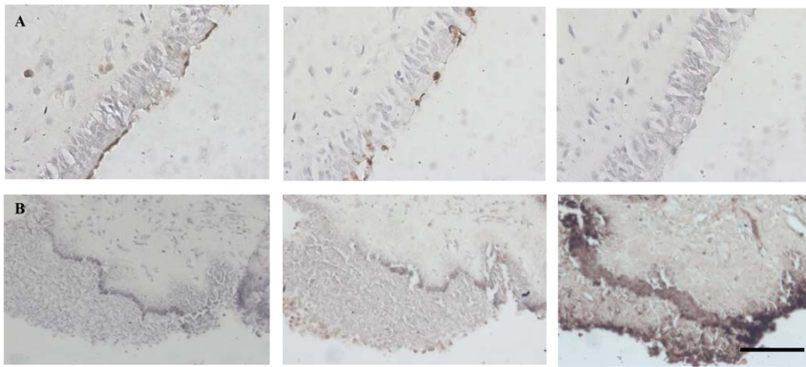
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**Figure 1**

Evaluation of epithelial differentiation and TGF- $\beta$ 1 expression in nasal polyps

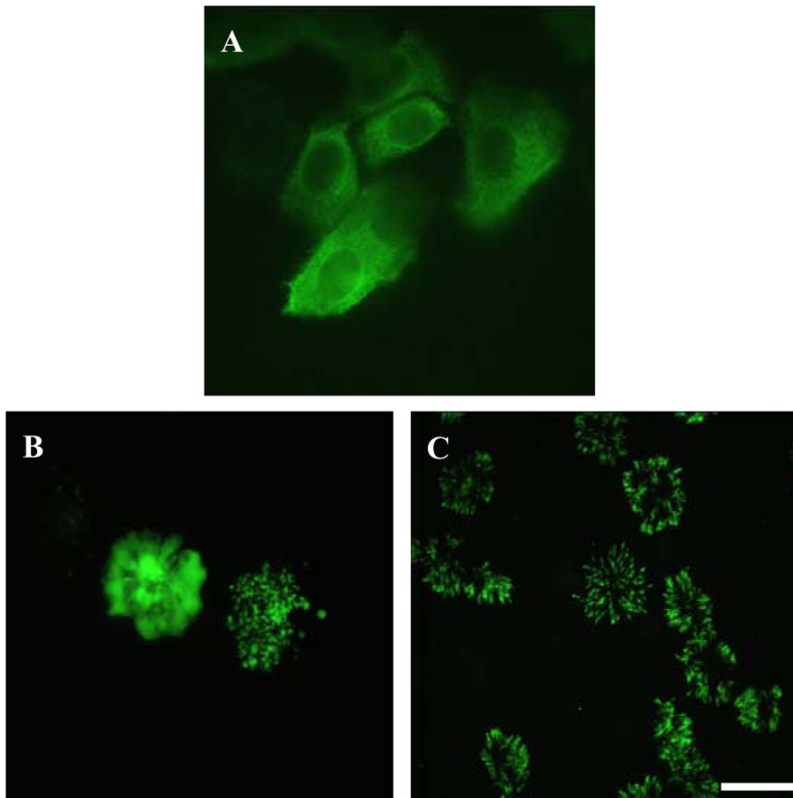
A. Pseudostratified epithelium exhibiting ciliated cells positive for  $\beta$ IV tubulin (left, 1/500 dilution) and mucous cells positive for MUC5AC (middle, 1/500 dilution) with low expression of TGF- $\beta$ 1 (right, 1/500 dilution) B. Loss of epithelial differentiation with rare cells positive for  $\beta$  IV tubulin (left, 1/500 dilution) and MUC5AC (middle, 1/500 dilution) and high TGF- $\beta$ 1 expression (right, 1/500 dilution). Bar = 50  $\mu$ m.



**Figure 2**

Patterns of the different immunolabelings of cytokeratin 14, MUC5AC and  $\beta$  IV tubulin in cultured HNEC, in non-wounded and grown in basal conditions cultures

A. Cytokeratin 14 (1/20 dilution): Immunolabeling was cytoplasmic and reticular corresponding to small, polyhedral cells. B. MUC5AC (1/500 dilution): Two different patterns were detected showing either a large heterogeneous apical spot (left) or multiple thin vesicles at the apical side (right). C.  $\beta$ IV tubulin (1/400 dilution): Ciliated structures were clearly detected at the apical side of the cells. Bar = 25  $\mu$ m.

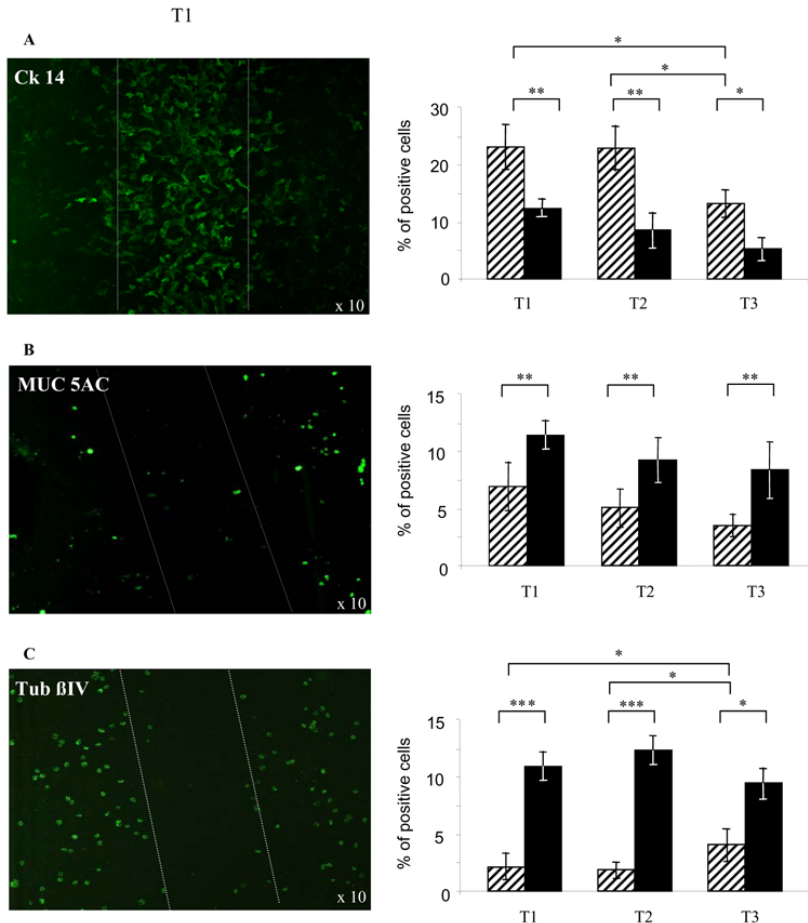




**Figure 3**

Comparative course of epithelial cell differentiation in wounded (hatched) and control (black) areas, under basal conditions, from T1 to T3 (n = 7)

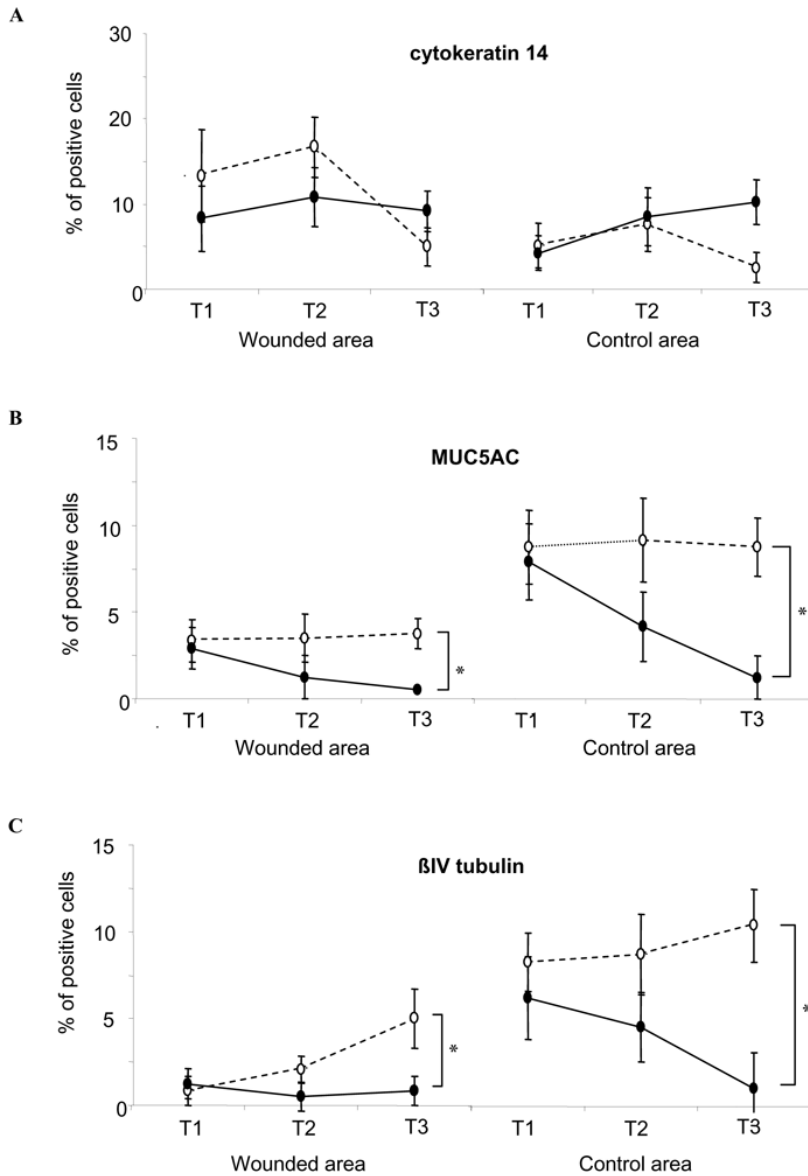
T1 is two days after wound closure, T2 is five days after wound closure and T3 is 12 days after wound closure. Left column: Pictures showing the immunolabeling patterns in the control area and in the wounded area (between the dotted lines) at T1. Images were taken on an inverted microscope (Zeiss, Rueil-Malmaison, France) equipped with epifluorescence illumination and an x10 objective. The wounded area was easily differentiated, as a mark was done on the well. Right column: Percentages of positive cells expressed in relation to the total number of cells determined by DAPI nuclei staining. Data are presented as mean  $\pm$  SEM. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . A. Time-course of cyokeratin 14 immunolabeling: At T1, T2, T3, cyokeratin immunolabeling was significantly higher in the wounded area than in the control area. Cyokeratin immunolabeling was intense at T1 and T2 in the wounded area and significantly decreased at T3. B. Time-course of MUC 5AC immunolabeling: At T1, T2, T3, MUC 5AC immunolabeling was always significantly lower in the wounded area than in the control area. It was detected as early as T1, and remained quantitatively stable until T3. C. Time-course of  $\beta$ IV tubulin immunolabeling (only ciliated patterns were considered): at T1, T2, T3,  $\beta$ IV tubulin immunolabeling was always significantly lower in the wounded area than in the control area. It was weak at T1 and T2 and significantly increased at T3.



**Figure 4**

Comparative time-course of epithelial cell differentiation under basal conditions (open circles) versus TGF- $\beta$ 1 supplementation (black circles), in wounded (left) and control (right) areas, from T1 to T3 (n = 6)

Data are expressed as mean  $\pm$  SEM. \*: 0.01 < p < 0.05. A. Time-course of cytokeratin 14 immunolabeling: no significant difference was observed between non-supplemented and TGF- $\beta$ 1-supplemented cultures. B. Time-course of MUC 5AC immunolabeling: TGF- $\beta$ 1 significantly decreased MUC 5AC immunolabeling in both wounded and control area, from T1 to T3, compared to the course under basal conditions. C. Time-course of  $\beta$ IV tubulin immunolabeling (only ciliated patterns were considered): TGF- $\beta$ 1 significantly decreased  $\beta$ IV tubulin immunolabeling in both wounded and control areas, from T1 to T3, compared to the course under basal conditions.



**Figure 5**

Comparative expression levels of DNAI1 and MUC5AC transcripts under basal conditions (white histograms) and with TGF- $\beta$ 1 supplementation (gray histograms), two days after confluence

Columns show the fold decrease (values <1) in DNAI1 and MUC5AC transcripts relative to 18S values expressed as mean  $\pm$ SEM of the  $2^{-\Delta\Delta}$  Ct values. The mean  $\pm$ SEM of six independent experiments are shown. Mean  $\pm$  2 SEM are considered as significant (\*  $p < 0.05$ ) when less than one.

