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Oral steroids enhance epithelial repair in nasal polyposis via up-regulation of AP-1 gene network

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ABSTRACT

**Background**: Chronic mucosal inflammation, epithelial damage and aberrant tissue remodeling are common features in nasal polyposis (NP). We sought to characterize gene expression profile within NP tissues and to explore the molecular mechanisms underlying the ameliorative effects of glucocorticosteroids (GCs) in NP.

**Methods**: Two sets of NP biopsies, *i.e.*, before the initiation and after GC treatment, were taken from 10 patients with untreated (GC-naïve) bilateral NP. Biopsy of inferior turbinate from 6 patients who underwent surgery for nasal septal deviation was served as nasal mucosal control. DNA microarrays containing 38,500 genes were used to characterize global gene expression profile. Functional network analysis was applied to identify key molecular pathways and genes in response to GC treatment (GC-treated). Selected genes were retested by means of quantitative RT-PCR and immunohistochemistry in the same polyps and control samples.

**Results**: Sixty-four genes were differentially expressed in GC-treated versus GC-naïve NP tissues. Highest scoring network was assembled around activation protein 1 (AP-1), a heterodimer of c-Fos and c-Jun oncoprotein, and 5 AP-1 related genes (COX-2, IL-6, AREG, HBEGF and EGR1) with tissue repair function. Quantitative PCR confirmed that AP-1 and its related genes were markedly repressed in GC-naïve polyps and were up-regulated after GC treatment. Immunohistochemical staining indicated that epithelial restitution in GC-treated polyps were associated with elevated expression of c-Jun protein.

**Conclusions**: This study demonstrates oral steroids promote epithelial repair in NP via up-regulation of AP-1 (especially c-Jun) network and its related genes.
INTRODUCTION

Nasal polyposis (NP), a common chronic inflammatory disorder of the upper airways, is often associated with asthma and other respiratory diseases. The infiltration of various inflammatory cells, epithelial damage and expression of the TH2 cytokines are similar to the pathologic profile of asthma. Hence, from this perspective, NP could be regarded as a paradigm of chronic airway inflammation.

The important features of NP are mucosal inflammation, epithelial damage followed by aberrant tissue repair and structural changes (remodeling). Inflammation in NP is characterized by the increased expression of multiple inflammatory genes regulated by pro-inflammatory transcription factors, such as nuclear factor (NF)-κB and activator protein-1 (AP-1), which binds to and activate coactivator molecules that acetylate core histones and switch on gene transcription. Enhanced immune and inflammatory responses in NP are associated with tissue repair and remodeling in the target tissue and are related to disease severity. Recruited inflammatory cells and activated epithelial cells in NP are the major source of cytokines sustaining the inflammatory reaction and activation, proliferation of fibroblasts and myofibroblast differentiation.

Glucocorticosteroids (GCs) are the most effective anti-inflammatory therapy for NP. The ability of GCs to elicit anti-inflammatory effect is mediated by suppressing the multiple inflammatory genes that are activated in NP, mainly by reversing histone acetylation of activated inflammatory genes through binding of glucocorticoid receptors to coactivators and recruitment of histone deacetylase 2 to the activated transcription complex. Inflammatory insult could lead to a normal reconstruction or a pathological remodeling. An important aspect of GCs is to promote tissue repair and remodeling in NP and asthma through transforming growth factor (TGF)-β and epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) signaling pathway. Previous studies have shown that transcription factors, cytokines, and growth factors also influence wound healing process in airway tissues.

Airway epithelial repair is a well-coordinated process. Epithelial remodeling in NP represents a complex event, which includes migration, proliferation, and differentiation of epithelial cells. This complex process involves groups of interacting molecules such as various transcription factors, cytokines and growth factors in overlapping signaling networks. In this study, we sought to characterize genome-wide transcriptional profile within NP tissues. We also sought to explore the genome-wide interaction network and to identify significant functional modules perturbed in response to GC therapy. In this regard, we compared dysregulation of functional modules in NP before and after oral GCs. Understanding the mechanism of glucocorticosteroids to restore the normal phenotypic appearance within nasal cavity may yield clue about the pathobiology of NP and foster development of novel treatments.

METHOD

Nasal tissues
NP patients (n=10) with refractory nasal obstruction underwent functional endoscopic sinus surgery (FESS). Two sets of polyp biopsies were taken from the same patient, i.e., before the initiation (GC-naïve) and after (GC-treated) the oral prednisone treatment (10 mg thrice per day for 3-5 days). None of the patients had an upper respiratory infection nor undertook any forms of GCs and antibiotics for more than three months before the study. All subjects did not have history of aspirin exposure and asthma. Cases were coded to provide confidentiality. Clinical characteristics, diagnosis and atopic status (determined by serum specific IgE levels) of subjects were noted (Table 1). Biopsy of inferior turbinate was obtained from control subjects (n=6) who underwent surgery for nasal septal deviation. This tissue served as nasal mucosal control. Fresh specimens were dissected into two sections; one section was preserved with RNA later (Ambion Inc, Austin, TX) for gene expression profiling and the other section was fixed in formalin for histological evaluation. Approval to conduct this study was obtained from the Institutional Review Board of the First Affiliated Hospital, Sun Yet-Sen University and the National University of Singapore.

Microarray study and Ingenuity Pathways Analysis (IPA)
Total cellular RNA was isolated from nasal tissues with RiboPure Kit (Ambion). Concentration and integrity of the purified RNA were determined. cRNA was reversely transcribed using 1 µg total RNA. Synthesized cRNA was hybridized onto Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) and processed accordingly (http://www.affymetrix.com/Auth/support/downloads/manuals/expression_ever_manual.zip). Quality of the microarray data was assessed (https://support/downloads/manuals/data_analysis_fundamentals_manual.pdf). This led to the exclusion of 2 NPs and 1 control sample for microarray study. A total of 54,000 probe sets, representing approximately 38,500 genes, were analyzed. Details of analysis are shown in the online data supplement. Functional and network analysis were performed using Ingenuity Pathways Analysis (IPA) tools (Ingenuity® Systems, www.ingenuity.com). Complete information was available in Supplemental Methods.

Quantitative PCR
Selective genes were validated with quantitative PCR by using ABI Prism 7000 Sequence Detection System (ABI Applied Biosystems, Foster City, CA). Relative gene expression was calculated with comparative $2^{-\Delta\Delta C_t}$ method [10] with GAPDH as a reference.

Histological evaluation
Paraffin sections of nasal biopsies were stained with hematoxylin and eosin. Eosinophil infiltration and epithelial integrity of biopsies were evaluated.[11] c-Jun and c-Fos protein was stained with monoclonal antibodies (Dako A/S, Glostrup, Denmark) and evaluated by using a semi-quantitative scoring system.[12] Detailed information was provided in Supplemental Methods.

Statistical analysis
Power sample estimation suggested that a minimum of four biopsies in each study group are required to detect a 2.0-fold difference in gene expression with a 1% significance level and a 90% power (data not published). Hence, we would expect to detect a 2.0-fold
difference in gene expression with \( p < 0.01 \) and with a 90% chance of avoiding a type II error. Median values of fold change of gene expression were analyzed using SPSS version 14 (SPSS Inc., Chicago, IL). Wilcoxon matched pairs sign rank test was used to compare gene expression levels between GC-treated and GC-naïve NP tissue. Mann-Whiney two-tailed test was performed to compare gene expression levels between NP tissues, either GC-treated or GC-naïve, versus nasal mucosal controls. Spearman rank analysis was used to analyze the correlation between the gene expression levels assessed by microarray and those measured by quantitative PCR; and between fold change of c-Jun and other AP-1 related genes. Fisher’s exact test was used to assess protein expression of c-Jun and epithelium integrity in NP tissues. Values were considered significant if \( p < 0.05 \).

**RESULT**

**Genome-wide transcriptional alterations**

Genome-wide transcriptional alterations were measured and analyzed. We detected significant alterations in 2,482 transcripts that were differentially expressed in GC-naïve NP tissues versus nasal mucosal controls while 1,908 transcripts differed in relative expression in GC-treated NP tissues versus nasal mucosal controls (Data not shown). All these transcripts differed in their relative intensities by at least 2.0-fold change. For GC-treated versus GC-naïve NP tissues, 103 differentially regulated transcripts were detected. After filtering the unmapped genes, genes with unknown gene ontology and genes with redundant probe identities, search for informative uncovered that 1,441 and 1,115 genes were differentially expressed in GC-naïve and GC-treated versus nasal mucosal controls, respectively (**Figure 1**). Expressions of 64 genes were different in GC-treated versus GC-naïve NP tissues (**Figure 1, Supplementary Table E1**). Interestingly, among those 64 GC-responsive genes, most of them (n=45, 70.3%) were also differentially expressed in GC-naïve NP tissue as compared to nasal mucosal controls.

**Integrated analysis of molecular pathways**

Sixty-four differentially expressed GC-responsive genes were further analyzed using Ingenuity Pathways Analysis into functional categories and pathways. Our results indicated that differentially expressed molecules regulate critical biological functions like cell death (43 molecules, \( p=3.58\times10^{-15} \)), cellular growth and proliferation (47 molecules, \( p=4.45\times10^{-13} \)), cellular development (36 molecules, \( p=1.51\times10^{-11} \)), and cellular movement (34 molecules, \( p=1.94\times10^{-11} \)) (**Figure 2**).

To further elucidate the global changes in NP tissue and its response to GC treatment, we sought to computationally decipher the principle networks involved. The specificity of connections for each gene was calculated, as defined by the percentage of its direct connections to other genes showing significant transcriptional changes. Our global representation revealed concerted dysregulation of functional modules in NP tissue. Among 4 networks identified by IPA calculation, AP-1 network appeared to be the top-scoring network (score = 39). Notably, 18 of the 64 GC-responsive genes that were differentially expressed have at least one gene in the AP-1 pathway, underscoring its pivotal role in its corresponsive to GC therapy (**Supplementary Table E2**). At the level
of interconnecting functional modules, 2 important AP-1 genes (c-Jun and c-Fos) were the prominent interaction partners (Figure 3 and Supplementary Figure E1). In addition, evaluation of the AP-1 sub-network regions revealed that a group of AP-1 related genes (COX-2, IL-6, AREG, HBEGF and EGR1) were modulated by GCs administration in NP tissues (Supplementary Table E2).

**Quantitative PCR**
To verify microarray results, same starting materials for the microarray study were used for quantitative PCR measurements. Appropriate primers for genes of interested and control were used (Supplementary Table E3). PCR data of AP-1 and its related genes were comparable to the results generated from microarray experiments (Figure 4). The rank order and magnitude of gene expression profile derived from quantitative PCR were in accordance with the microarray data. Two glucocorticoid receptor (GR) isoforms were also measured by quantitative PCR. GRα mRNA was significantly decreased in patients with NP than control tissues. GRβ mRNA was undetectable in all samples (Data not shown).

We investigated the correlation among gene expression in GC-treated NP tissues. c-Jun mRNA is positively correlated with c-Fos and JunB mRNA (Spearman, \( r = 0.924, p < 0.001 \) and \( r = 0.827, p = 0.003 \), respectively; Figure 5A and 5B). Similarly, c-Jun gene expression is positively correlated with AP-1 related genes (COX-2, IL-6 and EGR1) in GC-treated NP tissues (COX-2, \( r = 0.827, p = 0.003 \); IL-6, \( r = 0.662, p = 0.03 \) and EGR1, \( r = 0.893, p = 0.001 \); Figure 5C, 5D and 5E).

**Histological and immunohistochemical studies**
Significant epithelial damage was evident in 8 of 10 GC-naïve NP tissues (Table 1). Subtle epithelial damage (i.e., squamous metaplasia) was also detected in the other 2 patients (NP3 and NP8 in Table 1). The decrease in eosinophil infiltration in GC-treated NP tissue was considerable. In 8 of 10 GC-treated NP tissues, significant improvement in epithelium structure was observed (Table 2). Improvement in epithelium structure (i.e., absence of squamous metaplasia) was also evident in patients NP3 and NP8 after GC therapy. Immunohistochemical staining indicated that c-Jun protein was predominantly expressed in epithelium. c-Jun protein level was markedly lower in GC-naïve NP tissues (Figure 6A and Table 2) as compared to GC-treated NP tissues (Figure 6B and Table 2). Equally important, c-Jun protein level was positively correlated to the epithelial integrity in both GC-naïve and GC-treated NP tissues (Table 2). No difference in c-Fos protein level was observed among GC-naïve, GC-treated NP tissues, and nasal mucosal controls (data not shown).
Table 1. Clinical characteristics of patients undergoing polypectomy surgery for nasal polyposis (NP) or inferior turbinectomy (IT) for septal deviation (SD)

<table>
<thead>
<tr>
<th>Code</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Atopy</th>
<th>c-Jun staining*</th>
<th>Epithelial damage†</th>
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<tbody>
<tr>
<td>NP1</td>
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<td>F</td>
<td>NP</td>
<td>-</td>
<td>3</td>
<td>9</td>
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<tr>
<td>NP2</td>
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<td>M</td>
<td>NP</td>
<td>+</td>
<td>2</td>
<td>9</td>
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<tr>
<td>NP5</td>
<td>32</td>
<td>M</td>
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<td>+</td>
<td>6</td>
<td>9</td>
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<tr>
<td>NP6</td>
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<td>F</td>
<td>NP</td>
<td>+</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>NP7</td>
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<td>+</td>
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</tr>
<tr>
<td>NP8</td>
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<td>9</td>
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<td>-</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
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<td>M</td>
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<tr>
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<tr>
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<td>F</td>
<td>SD</td>
<td>-</td>
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</tr>
<tr>
<td>IT3</td>
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<td>-</td>
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<td>IT4</td>
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<td>+</td>
<td>9</td>
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<td>IT5</td>
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<tr>
<td>IT6</td>
<td>27</td>
<td>M</td>
<td>SD</td>
<td>+</td>
<td>9</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

* c-Jun staining as described in Supplementary Methods: overall score obtained by multiplying the extent score (0 to 3) with intensity score (0 to 3).
† Epithelium evaluation as described in Supplementary Methods: 0, intact epithelium; 1, moderately damaged epithelium; 2, severely damaged epithelium.
N.A., Not applicable.

Table 2. c-Jun protein in GC-naïve and GC-treated nasal polyp epithelium

<table>
<thead>
<tr>
<th>Group</th>
<th>c-Jun protein staining *</th>
<th>Epithelial integrity†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weak</td>
<td>Strong</td>
</tr>
<tr>
<td>GC-naïve NP</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>GC-treated NP</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Damaged Epithelium †</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Intact Epithelium †</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

* Referring to c-Jun protein staining in Table 1: overall score of ≥ 6 defined as “strong” expression, and < 6 as “weak” expression.
† Referring to epithelium damage in Table 1: Grade 0 defined as intact epithelium; Grade 1 and 2 defined as damaged epithelium.
# p-value obtained by Fisher’s exact test.
N.A., Not applicable.
DISCUSSION

Airway epithelium remodeling may represent a repair response to pathological insults such as inflammation, allergens, mechanical damage and environmental pollutants. Oral glucocorticosteroids are currently the most effective anti-inflammatory medications for the treatment of recurrent NP [1]. This is the first in vivo study deciphering the role of AP-1 gene pathway and epithelial remodeling in NP. The key findings are: (1) Epithelial damage is an important feature of NP and a remarkable epithelial restitution is observed after glucocorticosteroid treatment; (2) AP-1 and its related genes appear to be a key molecular network underlying the wound healing in NP; (3) Epithelial c-Jun protein expression is up-regulated by oral steroid therapy and is positively correlated with epithelium restitution in NP tissues.

AP-1 is a heterodimer of Fos and Jun oncoproteins. The most abundant AP-1 heterodimer is c-Fos:c-Jun. AP-1 and its related genes participate in the induction of a wide variety of pro-inflammatory proteins, receptors and mediators.[13] In this aspect, AP-1 induces a variety of growth related genes (e.g. EGF protein family),[14] cytokines (e.g. IL-6),[15] and enzymes (e.g. PTGS2, known as COX-2).[16] Recent evidence suggests a causative relationship between AP-1 and epithelial remodeling in airway diseases.[14,17] The epithelial localization suggests that AP-1 may play an important role in the pathology of NP. We found a significant low level of c-Jun and c-Fos mRNA expression in GC-naïve NP tissues as compared to nasal mucosal controls. c-Jun mRNA and protein level were increased after GC therapy in NP tissue. In this study, c-Fos mRNA was up-regulated in GC-treated NP. Immunohistochemical staining suggests that GC treatment did not improve c-fos protein level in extracted NP tissues. Our results are in disagreement with a previous report in which elevated c-Fos protein was detected in NP tissue and steroid decreased c-Fos protein but did not affect c-Fos mRNA level in NP tissues.[18] Induction of AP-1 in response to GC treatment is highly complex and may be cell-type-specific.[19-20] c-Fos protein was expressed among endothelial cells, lymphocytes, granulocytes, and monocytes. Baraniuk et al. have studied c-Fos mRNA level using a qualitative instead of quantitative measurement. [18] c-Fos mRNA level was normalized by β-actin intensity band, which is not a reliable internal control for GC-treated nasal samples.[21] c-Fos mRNA and protein expression may also be affected by broad alterations in post-transcriptional processes, such as increased turnover of c-Fos protein, negative auto-regulation of c-Fos expression, and mutual degradation between activated GR and c-Fos.[22-23] Other plausible explanations for such a conflicting result are due to variable factors such as choice of different subjects, dosage and duration of GC treatment, different experimental protocols, and tissue types used in their experiments.

The integrated analysis of multi-dimensional transcriptional data has proved informative. Our network analyses have identified a number of AP-1 related genes (COX-2, IL-6, AREG, HBEGF, and EGR1) appeared to modulate airway epithelial remodeling. AP-1 induces transcription of COX-2 which is the key enzyme required for the conversion of arachidonic acid into prostaglandins (PGs). COX-2/PGE2 signaling regulates epithelial remodeling process.[24] Decreased COX-2/PGE2 has been associated with aspirin intolerance in NP patients.[25] We found an up-regulation of COX-2 mRNA in NP
tissues after oral GC treatment. Dworski et al., have shown that treatment with prednisone up-regulates COX-2 mRNA and protein level in atopic subjects.[26] IL-6 is an important AP-1 related gene. AP-1 induces IL-6 expression through CRE binding region,[27] while IL-6-mediated signal pathway enhances c-Jun expression.[28] Inhaled corticosteroid promotes IL-6 production and favors respiratory epithelial cell remodeling in airway diseases.[15,29] EGFR and its ligands (AREG and HBEGF) are direct AP-1 target genes.[30] Activation of EGF/EGFR signaling leads to the induction of AP-1.[14, 17] EGF/EGFR signaling pathway influences epithelial remodeling.[3,6] In this study, mRNA levels of AREG and HBEGF were decreased in NP tissues and were up-regulated by GC treatment. EGR1, another AP-1 related gene, induces c-Jun expression and triggers c-Jun/AP-1 activation.[31] Steroid increases expression of EGR1.[32] EGR1 signals through AP-1/EGF pathways and modulates epithelial remodeling.[33]

There are several limitations to this study that could be rectified in future investigations. We have recruited a relative small number of NP and control subjects in this study. Subsequent follow-up studies in a large pool of patients are needed to verify this important finding. Another potential shortcoming is the analysis of NP versus nasal mucosal control. Since there are various cell types in nasal tissues, some of the findings may reflect the differences in the cellular makeup of the tissues. Additional limitation of the current study is that the functional networks and their essential gene-gene interaction is deduced by an array modeling approach which is based on predetermined database knowledge and can only be considered as a source of hypotheses. Therefore, functional testing will be needed for the rigorous evaluation of individual molecular interactions inferred by our database approach.

CONCLUSIONS
We have explored the molecule mechanisms underlying the pathogenesis of NP and their response to GC treatment. Our data suggest that AP-1 and its related gene network are central molecular effecters of epithelial damage and repair, which can be modulated by GC treatment. Our findings may contribute to a better understanding of key molecular pathways implicated in the pathogenesis of NP. We hypothesize that these immunoregulatory and remodeling effects elicited by steroids might be, at least in part, mediated by AP-1 gene network.
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Figure Legends

Figure 1. Venn diagram shows the overlapping of differentially expressed genes in GC-naïve NP versus nasal mucosal control, GC-treated NP versus nasal mucosal control and GC-treated versus GC-naïve NP.

Figure 2. Twelve significant functional annotations for gene sets differentially expressed in GC-treated versus GC-naïve NP tissues. Statistic \( p \)-value for a given function is calculated by considering 1) the number of functional analysis genes that participate in that function and 2) the total number of genes that are known to be associated with that function in the Ingenuity Pathway Knowledge Base (IPKB). On the y-axis of the diagram, the significance is expressed as the exponent of the \( p \)-value calculated for each function. Dashed line represents the threshold (\( p = 0.05 \)) of the significant level of the functions.

Figure 3. Functional network analysis. Top-scoring network composed of GC-responsive genes was complemented by an unsupervised relevance network learning algorithm without any prior assumptions and post adjustment. The molecules in this network are arranged into their various subcellular compartments: nucleus, cytoplasm, plasma membrane, and extracellular space. An unknown category also exists for molecules which no subcellular localization information is currently available. Nodes represent genes, with their shape representing the functional class of the gene product, and edges indicate the biological relationship between the nodes (see network legend). Nodes are color coded according to their \( d \) score generated by SAM (red, overexpression; green, underexpression) and the color intensity increases with the magnitude of altered regulation. PTGS2, the official symbol of COX-2.

Figure 4. Correlation between quantitative PCR and microarray results. Fold changes were determined by means of PCR and microarray, respectively. Correlation for c-Fos, c-Jun, FosB, JunB, COX-2, IL-6, HBEGF, AREG, and EGR1 was illustrated.

Figure 5. Relationship between mRNA level of c-Jun versus c-Fos (A), JunB (B), COX-2 (C), IL-6 (D), and EGR1 (E) was illustrated. \( \Delta \), fold change of the indicated gene in individual patient prior to versus after GC treatment. Fold changes were determined by PCR.

Figure 6. Expression of c-Jun protein determined by means of immunohistochemistry in the representative NP patient. Plates A and B represent c-Jun protein staining in the patient NP4 prior to versus after GC treatment. Original magnification: 200x.
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Oral steroids enhance epithelial repair in nasal polyposis via up-regulation of AP-1 gene network

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“Online data supplement”
Material and Methods

Microarray data analysis

A total of 54,000 probe sets, representing approximately 38,500 genes on the HG-U133 Plus 2.0™ arrays were analyzed. Three Affymetrix probe-level data sets in CEL files: (1) GC-naïve NP tissue (8 chips) versus nasal mucosal control (5 chips); (2) GC-treated NP tissue (8 chips) versus nasal mucosal control (5 chips); and (3) GC-treated versus GC-naïve NP tissue from the same patient (8 pairs of chips). Each set was normalized by the Robust Multichip Average (RMA) method, available in RMAexpress software version 0.4.1 Release (Free version available in http://rmaexpress.bmbolstad.com/).[1] RMA consists of a background adjustment, quantile normalization, and the median-polish summary method, producing a single normalized expression set for each comparison. RMA transformed data were on the log base 2 scale.

Three RMA normalized data sets were imported to Microsoft Excel spreadsheets, respectively, and then formatted for analysis by the Significant Analysis of Microarray (SAM) software, version 3.00 (Free version available in http://www-stat.stanford.edu/~tibs/SAM/).[2] Differentially expressed genes in each comparison were identified using SAM. SAM method accepts normalized expression data sets and identifies statistically significant changes in gene expression by assigning each gene a score (called “d score”) based on its change in expression relative to the standard deviation of repeated measurements. Genes with scores greater than a threshold are deemed potentially significant. The percentage of such genes identified by chance is the false discovery rate (FDR). SAM uses permutations of empirical measurements to estimate the FDR for the called list in the form of a 90% confidence interval. FDR controls the expected proportion of incorrectly rejected null hypotheses (type I errors). In addition, fold change has also been indicated in each probe ID in SAM generated gene list. The options selected for the SAM analysis: (1). Response type: two-class, unpaired data (comparison of GC-naïve/GC-treated NP versus control); two-class, paired data (comparison of NP without versus with GC treatment); (2). Data logged: logged (base 2); (3). Number of permutations: 5000. Hence, genes identified with FDR of 0.99%/1.14% in the datasets of GC-naïve/GC-treated NP vs. control and with a FDR of 1.72% in dataset of GC-treated vs. GC-naïve NP were deemed significant if they passed a 2.0-fold change filter.

Gene annotations of the analyzed transcripts were verified by means of web-based program NetAffx™ Analysis Center (http://www.affymetrix.com/analysis/index.affx). This resource contains probe sequences and up to date gene annotations and allows researchers to quickly search for gene annotation information, compare and refine results, and export data into Excel-friendly formats. Two filtration steps were performed: Firstly, those transcripts which did not contain identified gene symbols were filtered. Secondly, those genes with redundant probe identities and those transcripts with unknown gene ontology (such as those annotated with “chromosome open reading frame”, “hypothetical protein”, “KIAAs” and “family with sequence similarity”) were filtered.
In HG-U133 Plus 2.0™ arrays, 4 probe sets were designed for the measurement of gene expression level of nuclear receptor subfamily 3, group C, member 1 (NR3C1) that is known as glucocorticoid receptor. Unfortunately, those 4 probe sets were unable to differentiate the sequence variation between GRα or GRβ. Thus, we have specially added these markers in quantitative PCR measurements.

**Ingenuity Pathway Analysis**

**Network analysis**
The list of significant GC-responsive genes identified by SAM, containing Affymetrix probe set ID as clone identifiers as well as d scores, were uploaded into the Ingenuity Pathway Analysis (IPA) tool (version 6.0, Ingenuity® Systems, www.ingenuity.com). Each clone identifier was mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base (IPKB). These so-called focus genes were then used as a starting point for generating biological networks. To start building networks, the application queries the IPKB for interactions between focus genes and all other gene objects stored in the knowledge base, and generates a set of networks. Every resulting gene interaction has supporting literature findings available online. IPA then computes a score for each network according to the fit of the user’s set of significant genes. The score is derived from p-value and indicates the likelihood of the focus genes in a network being found together as a result of random chance. A score of 2 indicates that there is a 1-in-100 chance that the focus genes are together in a network as a result of random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone.

**Function analysis**
Two types of functional analysis can be performed in IPA: Functional Analysis for a dataset and Functional Analysis for a network. The biological functions and/or diseases across GC-responsive genes were identified by referring IPKB. Fisher’s exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that the dataset of GC-responsive genes is due to chance alone. Functional Analysis for a network identifies the significant biological functions and/or diseases which are associated with each of the network composed of GC-responsive genes. Fisher’s exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone. In general, p-values less than 0.05 indicate a statistically significant, non random association.

**Histological evaluation**
Retrieved samples were fixed in formalin, embedded in paraffin and sectioned at 4 µm with Leica microtome (Leica, Wetzlar, Germany). Deparaffinization and rehydration were performed subsequently. To obtain a general impression of the histopathological features of the examined specimens, slides were stained with hematoxylin and eosin and examined with a light microscope (Leica). The results of eosinophil counting were showed in percentage, which were calculated by positive staining cells per 200 cells at 200× magnification in every three fields with high-intensity positive cells. The
percentage of eosinophil was categorized into four grades: Grade 0, none; Grade 1, less than 10%; Grade 2, 10% to 40%; Grade 3, more than 40%.[3]

Slides were examined for assessing epithelial damage as described.[4] Grade 0 indicated intact epithelium which all layers of epithelial cells were present; grade 1 indicated moderately damaged epithelium which 2 or more layers of cells were present; grade 2 indicated severely damaged epithelium which only 1 layer of cells or no epithelial cells (naked basement membrane) remained. In this study, grade 0 was defined as “intact” epithelium, while grade 1 and 2 were defined as “damaged” epithelium.

Protein expression of c-Jun and c-Fos was examined by immunohistochemical staining, using a modified horseradish peroxidase (HRP) technique with the DakoCytomation EnVision+System-HRP (Dako A/S, Glostrup, Denmark). Slides were processed with Target Retrieval Buffer (Dako A/S). Endogenous peroxidase activity was blocked with 3% \( \text{H}_2\text{O}_2 \). Slides were stained with anti-c-Jun monoclonal antibody [isotype, mouse \( \text{IgG}_{2a} \); clone, 3/Jun] (BD Biosciences, San Jose, CA) and anti-c-Fos polyclonal antibody [Rabbit \( \text{IgG} \); clone, K-25] (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:100 and 1:300, respectively. Species- and subtype-matched antibodies were used as negative controls [N-Universal Negative Control for mouse \( \text{IgG} \) and N-Universal Negative Control for rabbit \( \text{IgG} \)] (Dako A/S). The slides were then incubated with DAKO EnVision+System-HRP (Dako A/S) at room temperature for 30 min. Diaminobenzidine was used as substrate for color development. All slides were counterstained with hematoxylin.

Specimen was evaluated by light microscope to estimate the extent of immunoreactivity and staining intensity with a semi-quantitative scoring system.[5] The immunoreactivity of c-Jun or c-Fos within the epithelium region was graded as “0 point” for negative staining, “1 point” for < 15% positive cells, “2 points” for 15-60% positive cells, and “3 points” for > 60%. The intensity of c-Jun or c-Fos staining was graded as “1 point” for weak staining, “2 points” for moderate staining, and “3 points” for strong staining. The overall score was calculated by multiplying the score of immunoreactivity and intensity of staining in each case. Hence, the maximum score for individual case is 9. In this study, the overall score of \( \geq 6 \) was defined as “strong” expression while the overall score < 6 was defined as “weak” expression. To have a standardized histological evaluation of the staining (including both H&E staining and immunostaining), the pathologist independently assessed all cases in a blind fashion.
Table E1, E2, and E3 are listed in the excel file of supplementary tables.

Figure legends

**Figure E1.** Functional network analysis. Networks composed by an unsupervised relevance network learning algorithm without any priori assumptions and post adjustment were generated by IPA. All four networks composed of GC-regulated genes were merged. The highlighted box shows that c-Jun and c-Fos are the core molecules. Edges between c-Jun/c-Fos and their interacting genes are highlighted in light blue. Nodes represent genes, with their shape representing the functional class of the gene product, and edges indicate the biological relationship between the nodes (*see network legend*). Nodes are color coded according to their $d$ score generated by SAM (red, overexpression; green, underexpression) and the color intensity increases with the magnitude of altered regulation.
References
### Table E1 Significant glucocorticoidsteroid (GC)-responsive genes in NP

Up-regulated genes in NP after GC treatment (n=52)

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Score(d)</th>
<th>Fold Change</th>
<th>FDR (%)</th>
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<tbody>
<tr>
<td>209189_at</td>
<td>FOS</td>
<td>v-fos FBJ murine osteosarcoma viral oncogene homolog</td>
<td>6.145</td>
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<td>201041_s_at</td>
<td>DUSP1</td>
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<td>5.315</td>
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<td>233011_at</td>
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<td>Annexin A1</td>
<td>5.260</td>
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<td>201693_s_at</td>
<td>EGR1</td>
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<td>205249_at</td>
<td>EGR2</td>
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<td>243296_at</td>
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<td>206211_at</td>
<td>SELE</td>
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<td>202768_at</td>
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<td>sprouty homolog 4 (Drosophila)</td>
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<td>212099_at</td>
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<td>3.711</td>
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<td>SNC_A</td>
<td>synuclein, alpha (non A4 component of amyloid precursor)</td>
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<td>gene_id</td>
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<td>description</td>
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<td>adjusted p-value</td>
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<td>216834_at</td>
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<td>205266_at</td>
<td>LIF</td>
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<td>222162_s_at</td>
<td>ADA</td>
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<td>3.319</td>
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<td>protein kinase C, iota</td>
<td>2.700</td>
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<td>carbonic anhydrase XII</td>
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<td>amphiregulin (schwannoma-derived growth factor)</td>
<td>3.024</td>
<td>6.474</td>
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<td>212558_at</td>
<td>SPRY1</td>
<td>sprouty homolog 1, antagonist of FGF signaling (Drosophila)</td>
<td>2.859</td>
<td>2.485</td>
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<td>213992_at</td>
<td>COL4A6</td>
<td>collagen, type IV, alpha 6</td>
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<td>PRKCI</td>
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<td>glycoprotein M6A</td>
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## Down-regulated genes in NP after GC treatment (n=12)

<table>
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<tr>
<th>Probe Set ID</th>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Score(d)</th>
<th>Fold Change</th>
<th>FDR (%)</th>
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<td>1566139_at</td>
<td>HOP</td>
<td>HOP homeobox</td>
<td>-5.424</td>
<td>0.420</td>
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<td>201744_s_at</td>
<td>LUM</td>
<td>lumican</td>
<td>-4.714</td>
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<td>202270_at</td>
<td>GBP1</td>
<td>guanylate binding protein 1, interferon-inducible</td>
<td>-4.711</td>
<td>0.475</td>
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<td>227609_at</td>
<td>EPSTI1</td>
<td>epithelial stromal interaction 1</td>
<td>-4.264</td>
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<td>204747_at</td>
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<td>interferon-induced protein with tetratricopeptide repeats 3</td>
<td>-3.241</td>
<td>0.495</td>
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</table>
Figure 1

GC-naïve NP versus control

GC-treated NP versus control

472
924
177

35
10
15

GC-treated versus GC-naïve NP
Figure 3

Network legend

Node types
- Complex
- Cytokine/Growth Factor
- Enzyme
- Group
- Growth factor
- Ligand-dependent Nuclear Receptor
- Peptidase
- Transcription Regulator
- Unknown

Edge Labels
- A Activation / Deactivation
- E Expression
- T Transcription
- LO Localization
- PD Protein-DNA binding
- PP Protein-Protein binding
- PR Protein-RNA interactions
- RB Regulation of Binding

Edge Interactions
- A Binding only
- B Direct interaction
- Direct interaction
- Acts on
- Indirect interaction
- Inhibits
- Notes: "Acts on" and "Inhibits" edges may also include a binding event.
Figure 4

$r = 0.967$
$p < 0.001$

Fold change by means of microarray

Fold change by means of PCR

- COX-2
- Jun
- JunB
- AREG
- HB-EGF
- EGR1
- IL-6
- IL-6
- EGR1
- COX-2
- Jun
- JunB
- AREG
- HB-EGF
- EGR1
- IL-6
- IL-6
Figure 5

(A) Δc-Fos
![Graph showing Δc-Fos against Δc-Jun with r = 0.924 and p < 0.001.]

(B) ΔJunB
![Graph showing ΔJunB against Δc-Jun with r = 0.827 and p = 0.003.]

(C) ΔCox-2
![Graph showing ΔCox-2 against Δc-Jun with r = 0.827 and p = 0.003.]

(D) Δc-IL-6
![Graph showing Δc-IL-6 against Δc-Jun with r = 0.662 and p = 0.03.]

(E) ΔEGR1
![Graph showing ΔEGR1 against Δc-Jun with r = 0.893 and p = 0.001.]

Figure 6

(A) (B)
![Images of tissue sections labeled (A) and (B).]
Oral steroids enhance epithelial repair in nasal polyposis via up-regulation of AP-1 gene network
Chun Wei Li, Wai Cheung, Zhi Bin Lin, Tian Ying Li, Joe Thuan Lim and De Yun Wang

Thorax published online January 21, 2009

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