Methylation of the Human Pendrin Promoter

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Key Words

SLC26A4 • Epigenetics • CpG • Bisulfite modification
• NCI-H292 • HEK-Blue

methylation between the two cell types may determine a cell-specific response to IL-4 in terms of pendrin mRNA expression.

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Abstract

Inspection of the nucleotide sequence of the human pendrin promoter revealed the presence of a CpG island. We investigated the ability of IL-4 to stimulate pendrin message expression in two separate cell lines: the NCI-H292 lung epithelial cell line and the human embryonic kidney (HEK)-Blue cell line. The expression of pendrin mRNA was significantly increased in both cells types after 4, 24, 48 and 72 hours treatment with IL-4, and interestingly, the increase in pendrin mRNA was greater in the NCI-H292 cells. Methylation of CpG sites within the promoter regions of genes can affect activities of gene promoters and have either positive or negative implications on the transcription and mRNA expression of the particular gene. We quantitatively analyzed the methylation status of 35 CpG sites within the human pendrin promoter in both cell lines. The basal methylation pattern was statistically different at multiple CpG sites between the NCI-H292 and HEK-Blue cells. We propose that the difference in basal

Introduction

Epigenetics is the study of changes in phenotype or gene expression resulting from mechanisms other than changes in the underlying DNA sequence [1]. Besides other epigenetic phenomena [2], DNA methylation plays an important role in chromosomal stability, gene expression patterns, proper telomere length and stem cell reprogramming [3]. Recent studies indicated that DNA methylation is central to the aberrant epigenetics of cancer as well as other disease entities. Hypomethylation (manifested as a global loss of methylation) of cytosine-phosphate-guanine (CpG, regions of DNA where a C precedes a G in the linear sequence) sites can lead to the over-expression of thousands of previously silenced or down-regulated genes [4, 5], and is considered to be the epigenetic hallmark of human tumorigenesis [6]. On the

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other hand, hypermethylation is manifested as an increase methylation, and can result in the silencing or down regulation of hundreds of genes, depending on the extent of methylation and the selection process, including tumor-suppressor genes [7] and drug metabolizing enzymes [8].

Mutations in the *SLC26A4* gene encoding pendrin are responsible for the most common form of syndromic sensorineural hearing loss, **Pendred Syndrome (PS)** [9]. Besides the inner ear, pendrin is expressed in a multitude of tissues, including the thyroid gland, kidney, mammary gland, testis, placenta, endometrium and liver [10-14]. In bronchial epithelial cells, pendrin mRNA expression is upregulated following treatment with the T_H2-type cytokines, interleukin (IL)-4 and IL-13 [15-20]. We recently confirmed this finding in the NCI-H292 cell line, which are human lung cells derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma, and showed that a 48 hour treatment with IL-4 increased pendrin mRNA levels in **human embryonic kidney (HEK)-Blue cells** [19], which are stable, human signal transducer and activator of transcription (STAT)6 transformants of HEK293 cells. Both cell lines display epithelial morphology.

In the current manuscript, we show that a 24, 48 and 72 hour stimulation with IL-4 resulted in a greater increase in pendrin mRNA expression in NCI-H292 cells than in HEK-Blue cells. Since the degree of methylation at CpG sites within promoter regions of genes can affect transcription, we also explored whether CpG methylation of the pendrin promoter correlated with differences in IL-4-induced expression of pendrin mRNA between the NCI-H292 and HEK-Blue cells.

Materials and Methods

Materials

Penicillin, streptomycin, glutamine, and base media were from Sigma Aldrich (St. Louis, MO; USA). Fetal bovine serum (FBS) was from Lonza (Cologne, Germany). IL-4 was from Invivogen (San Diego, CA; USA) and prepared in distilled water. Blasticidin, zeocin and the HEK-Blue IL-4/IL-13 (HEK-Blue) cells were also from Invivogen. NCI-H292 cells were from ATCC.

Cell Culture and IL-4 Treatment

All cell lines were cultured at 37°C in a humidified incubator gassed with 5% CO₂ and maintained in plastic petris. HEK-Blue cell media consisted of DMEM high glucose (4.5g/L) base media supplemented with 10% FBS, 10µg/mL

blasticidin, 100µg/mL zeocin, 50U/mL penicillin, 50µg/mL streptomycin and 1.5% NaHCO₃. NCI-H292 cell media consisted of RPMI-1640 base media supplemented with 10% FBS, 4.5g/L D-glucose, 1.5g/L NaHCO₃, 2.38 g/L HEPES, 1mM sodium pyruvate, 50U/mL penicillin and 50µg/mL streptomycin. Media was replaced three times weekly for both cell lines.

HEK-Blue or NCI-H292 cells were seeded in 35mm x 10mm petris for real-time PCR and methylation analyses. 24 hours following seeding, media was replaced with that containing either vehicle (water) or IL-4 (40ng/mL). Total RNA and genomic DNA were simultaneously extracted using the AllPrep DNA/RNA Mini Kit (Qiagen; Hilden, Germany) either 4, 24, 48 or 72 hours later according to the manufacturer's instructions.

PCR and Cloning

cDNA corresponding to 2630 nucleotides of the pendrin gene (positions -2556 through +74, relative to the start codon; GenBank accession number AC078937.1) was generated by PCR from NCI-H292 and HEK-Blue genomic DNA with a forward primer: 5' AAA GGA GAC ACA GTG CTT GCC CTC 3' and a reverse primer: 5' CGG CCG CGA CAC CAT GTA GC 3'. In a second PCR, cDNA corresponding to 1849 nucleotides of the pendrin promoter (positions -1842 through -668, relative to the start codon; GenBank accession number AC078937.1) was generated using the products from the first PCR with the forward primer: 5' CTA CGG TAC CAG ATC TGT TGA AAG CTG TTG AC 3' and the reverse primer: 5' ACT GAA GCT TCT GCC ATG ACC TGC 3'. The cDNA fragments were then cloned individually into the pGL3B vector (Promega) with the KpnI and HindIII restriction sites. The inserted cDNA corresponding to the pendrin promoter for both the NCI-H292 and HEK-Blue cells was then sequenced (Microsynth; Balgach, Switzerland). PCR reactions were carried out with the Novagen® KOD Hot Start Polymerase kit (TOYOBO, EMD Chemicals Inc.)

Real-Time PCR

RNA integrity was analyzed on an agarose gel. 1µg total RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen). RT-PCR was performed using the LightCycler 480 Real-time PCR System and the Universal Probe Library (UPL) System (Roche Applied Science; Indianapolis, IN). Oligonucleotide primers and universal probes specific for human pendrin (forward: 5' TGG CCA CCA CTG CTC TTT 3'; reverse: 5' ATC ATC ACA ATC GCA GCA GA 3'; UPL probe #65), (forward: 5' GTG GAG ACA GAA TAA GAT TGA TGC 3'; reverse: 5' GGC CAG CTA GTA AAC CGA GA 3'; UPL probe #45) and GAPDH (forward: 5' AGC CAC ATC GCT CAG ACA C 3'; reverse: 5' GCC CAA TAC GAC CAA ATC C 3'; UPL probe #60) were designed and selected using the UPL Assay Design Center. Real-time PCR of all samples was performed three times in triplicate in 10µl reactions using the Universal Probe Master Mix according to the manufacturer's instructions (Roche Applied Science). Relative gene expression values were quantified using the comparative Ct method as previously described [21].

Forward Primer Sequence*	Reverse Primer Sequence**	Nucleotide Coordinates of Amplicon	Amplicon Size	CpG Sites Covered	CpG Sites Detected	CpG Units Detected	Strand ***
5'-TTTTTAGTGGTTTTTAATTTATTGGG-3'	5'-AAAAAACTAAAACTATCCTACCTCATCAA-3'	Chr.7 107300202- Chr.7 107300602	401 bp	4	2	2	Forward
5'-TAGAAGGAAGGGTTTGATAAATTAGAATA-3'	5'-AACCCCTTTATTAATAAACCAATAATATACA-3'	Chr.7 107300080- Chr.7 107300514	435 bp	4	3	3	Reverse
5'-TAGTGGGTAAGAAA GGGTTATTTGG-3'	5'-CCAAACCTTATAAACTCCTTAAAAA-3'	Chr.7 107300692- Chr.7 107300995	304 bp	5	3	3	Forward
5'-TTTTGTGGGATTTTTTAAAGAAGAG-3'	5'-TCCCAACAAAAACAAAACTAATA-3'	Chr.7 107300554- Chr.7 107300990	437 bp	5	4	4	Reverse
5'-AGTGGGTAAGAAAGGGTTATTGGA-3'	5'-AAAAAACTAAAACTCTTACTAACCAC-3'	Chr.7 107300693- Chr.7 107301090	398 bp	10	8	7	Forward
5'-GTGGTAGTTAGTAAGAGTTTGTAGTTTTT-3'	5'-TATACTACCCCTACCCACCTAATC-3'	Chr.7 107301062- Chr.7 107301419	358 bp	23	21	14	Forward

Table 1. PCR Primers for Methylation Detection of the Pendrin Promoter. *Forward Primers contain the T7 promoter tag (5' AGG AAG AGA G 3') on the 5' end of the primer sequence, **Reverse primers contain the T7-promoter tag (5' CAG TAA TAC GAC TCA CTA TAG GGA GAA GGC T 3') on the 5' end of the primer sequence, ***Primers are complementary to either the forward (+) or the reverse (-) template strand.

Quantitative Methylation Analysis

1µg of genomic DNA from NCI-H292 and HEK-Blue cells was bisulfite modified (BSM) using the Qiagen EpiTect Kit following the manufacturer's protocol (Qiagen). In the BSM process, single stranded DNA is created from the double stranded DNA template, with the exception that unmethylated cytosine is converted into uracil. Methylated cytosine is protected from the BSM process, and remains unchanged. To confirm complete bisulfite modification, completely unmethylated (0%), untreated DNA from the EpiTect Control DNA set (Qiagen) was bisulfite modified in tandem with cell line DNA using the same protocol. Regions from the pendrin promoter were then PCR amplified using T7-promoter tagged primers specific for single-stranded, bisulfite-modified DNA. A total of 6 primer pairs were designed (EpiDesigner software, Sequenom; San Diego CA) to span the STAT-6 binding region (N₄ GAS motif) through the defined CpG island of the pendrin promoter (Table 1). PCR amplification of each of the six amplicons was performed in a 5 µl reaction volume (1x PCR Buffer, 0.75mM MgCl₂, dNTPs (2.5mM each), 0.18mM each primer, 0.05µl HotStar Taq DNA polymerase (Qiagen) & 2µl bisulfite-modified DNA). All bisulfite-modified samples were PCR amplified in quadruplicate along with two methylation controls (bisulfite-converted, completely methylated (100%) DNA and bisulfite-converted, completely unmethylated (0%) DNA) obtained from the EpiTect Control DNA set (Qiagen) to ensure accurate DNA methylation detection. Unincorporated dNTPs from the PCR reaction were neutralized using shrimp alkaline phosphatase (SAP), and double-stranded cRNA was created from the template by *in vitro* transcription. The cRNA (in which unmethylated cytosines are now adenines and methylated cytosines are now guanines) was then base-specifically cleaved with RNase A, thereby generating fragments (CpG units) containing any number of CpG sites. The cleavage products were then spotted onto silicon chips (SpectroCHIP II, Sequenom) using a nanodispenser. Adenine and guanine differ in mass by ~16 daltons; therefore, each CpG site that is methylated creates a 16 dalton change that is detectable by matrix assisted laser desorption ionization-time

of flight mass spectrometry (MALDI-TOF MS) (MassARRAY® system, Sequenom).

Methylation percentage for CpG sites within a sample of genomic DNA was then calculated with the EpiTYPER software which generates predicted CpG units from the entire process and pre-calculates their mass. The abundance of each fragment (signal/noise level in the spectrum) detected by the MassARRAY® system is indicative of the amount of DNA methylation in the interrogated sequence. It is worthy to note here that the mass of some CpG units might fall outside the detection range of the MassARRAY® system, making methylation analysis of any CpG sites within those units impossible. In addition, CpG sites may lie within the same CpG unit, making them unable to be differentiated. In these cases, multiple CpG sites were considered and analyzed as one site (examples for the pendrin promoter analyzed in this study include sites 79 & 78, 77 & 76, 68 & 67, 66 65 64 & 63, and 61 60 & 59).

Statistics

Significant differences between 2 experimental groups were determined using the two-tailed Student's t-test. Differences were considered statistically significant where the p-value < 0.05. Statistical analyses and the generation of graphs were performed with GraphPad Prism 5.0 software (GraphPad, San Diego CA).

Results

The extent of IL-4-induced increases in pendrin mRNA is cell-specific

We previously showed that a 48 hour treatment with IL-4 resulted in a greater increase in pendrin mRNA in NCI-H292 cells than in HEK-Blue cells [19]. We extended those experiments by analyzing pendrin mRNA with two separate primer sets in each cell line following

stimulation with either vehicle (water) or IL-4 (40ng/mL) for 4, 24, 48 and 72 hours (Fig. 1). In each cell line, IL-4 resulted in a significant increase in pendrin mRNA at each time point analyzed. Closer inspection showed that the NCI-H292 cells were more sensitive to IL-4 than the HEK-Blue cells. Comparing the fold-change of pendrin mRNA at each time-point between the two cell lines revealed that the NCI-H292 cells responded to IL-4 with a significantly greater increase in pendrin message at the 48 and 72 hour time-points. The fold change was also significantly greater in the NCI-H292 cells at the 24 hour time-point with primer set 45 (Fig. 1B), but not with primer set 65 (Fig. 1A).

We also compared the basal (untreated) levels of pendrin mRNA expression in NCI-H292 and HEK-Blue cells, again using the same two primer sets as before. With both primer sets, significantly more pendrin mRNA was detected in the HEK-Blue cells as compared to the NCI-H292 cells ($0.13 \pm 0.014^*$ vs. 0.02 ± 0.005 with primer set #64, and $0.071 \pm 0.008^*$ vs. 0.013 ± 0.002 ; pendrin mRNA was normalized to that of GAPDH, arbitrary units, $*p < 0.0001$ by a two-tailed Student's *t*-test).

Basal methylation levels of the human pendrin promoter are cell-specific

It has previously been reported that the human pendrin promoter contains a CpG island [22], and this region is shown in Fig. 2. CpG sites upstream of the CpG island are heretofore referred to as those contained within the CpG shore. As previously mentioned, methylation at CpG sites within promoter regions of genes can alter transcription activity. cDNA corresponding to the region depicted in Fig. 3 was amplified from NCI-H292 and HEK-Blue cells, sequenced and compared to the human pendrin sequence found in the NCBI database (GenBank accession number AC078937.1). One polymorphism in each cell line was found relative to the NCBI reference sequence. The base change (adenine to cytosine) in the HEK-Blue cells creates a CpG site (90.5), whereas the base change (guanine to adenine) in the NCI-H292 cells destroys a CpG site (59). Basal promoter activity and that induced by IL-4 (48 hrs treatment of 40ng/mL IL-4) were identical for each sequence (data not shown). These data suggest that the difference in the pendrin promoter DNA sequence between the two cell lines does not account for the differences in the extent of IL-4 induced changes in pendrin mRNA observed between the two cell lines.

Fig. 1. HEK-Blue and NCI-H292 cells respond to IL-4 with an increase in pendrin mRNA. HEK-Blue or NCI-H292 cells were treated with vehicle (water) or IL-4 (40ng/mL) for 4, 24, 48 or 72 hours and analyzed for pendrin mRNA expression using two separate primer sets (A) #65 or (B) #45 with real-time PCR. GAPDH was used as a housekeeping gene. The values at each time-point for the vehicle-treated samples were averaged. Each measurement for both vehicle and IL-4 treated cells was then normalized to this average to obtain the fold-increase in mRNA expression (pendrin mRNA/GAPDH mRNA) induced by IL-4. These values from each of the three separate experiments were then averaged and expressed in bar graphs. Therefore, the bars represent mean values $\pm$ SEM of three independent experiments containing three replicates each, where $*p < 0.05$ and $**p < 0.001$ by a two-tailed Student's *t*-test. Asterisks directly above bars are compared to vehicle-treated of that timepoint.

The basal methylation status of CpG sites 91R through 56 was analyzed from NCI-H292 and HEK-Blue genomic DNA. Fig. 4 shows the percent methylation of particular CpG sites from NCI-H292 or HEK-Blue total genomic DNA. Overall, we detected significant differences in methylation between the NCI-H292 and HEK-Blue cells at the following CpG sites: 91R, 90, 87, 86R, 85, 83R, 82, 81, 77-70, 68-62, 58 and 57. The amount

Fig. 2. The human pendrin promoter region contains a CpG island. The 1850 nucleotides immediately 5' to the start codon (ATG) of the human pendrin gene contains 91 CpG sites (indicated by circles). This sequence is from the NCBI database with GenBank Accession number AC078937.1. CpG sites 1 through 84 are within the CpG island, whereas sites 85 through 91 belong to the CpG shore. The masses of those CpG sites that were unable to be detected by the Sequenom are indicated with filled circles. Open circles indicate those CpG sites that were within the detection range. In some cases, no cleavage site was present between CpG sites. Therefore, the methylation status of only the unit (fragment containing multiple CpG sites) was determined. The CpG units include 79 & 78, 77 & 76, 68 & 67, 66 65 64 & 63, and 61 60 & 59. The N₄ interferon- γ activated sequence (GAS) motif that is required for IL-4 stimulated increases in pendrin promoter activity is indicated [19]. The positions of single nucleotide polymorphisms (SNPs) that, if present, would result in the addition or deletion of a CpG site are indicated. The identifiers for each SNP, beginning at the left are rs75343499 (T/C), rs2712228 (A/C), rs73191606 (G/C), rs55980027 (A/G), rs115976606 (C/G), rs60284988 (T/C), rs17154282 (C/G), rs115688927 (G/A), rs74963008 (C/G).

of DNA methylated at CpG sites 91R and 90 in the two cell lines differed profoundly. Specifically, there was more than a 60% difference at CpG site 91R ($69.8 \pm 3.4\%$ of the total genomic DNA from HEK-Blue cells compared to $6.9 \pm 1.3\%$ of the total genomic DNA from NCI-H292 cells) and more than a 70% difference at CpG site 90 ($100.00 \pm 0.0007\%$ for HEK-Blue cells versus $29.64 \pm 2.13\%$ for NCI-H292 cells). The difference in methylation of CpG sites 85, 81, 75, 58 and 57 was modest (24-37%), whereas the methylation of CpG sites 87, 86R, 83R, 82, 77 & 76, 74-70, and 68-62 was minimal (less than 24%) between the two cell lines. Methylation of the remaining CpG sites (89R, 88R, 87R, 84R, 83, 79 & 78, 69 and 56) were not significantly different between the NCI-H292 and HEK-Blue cells.

Discussion

Epigenetics are heritable alterations in gene expression without changes in the DNA sequence [1]. As opposed to the irreversible nature of genetic events, which introduce changes in the primary DNA sequence, epigenetic modifications are reversible and leave the original DNA sequence intact. The complex pattern of silenced/expressed gene regions that exists within the genome of a single cell type is tightly regulated by

Fig. 3. Sequence alignment of the pendrin promoter. cDNA corresponding to positions -1842 through -668, relative to the start codon was sequenced from the NCI-H292 (hPDSpromNCI-H292) and HEK-Blue cells (hPDSpromHEK-Blue), and aligned with a reference human pendrin promoter sequence (GenBank accession number AC078937.1) (hPDSpromNCBI). The CpG sites analyzed in this study are numbered. Roman numerals indicate previously identified single nucleotide polymorphisms (SNPs) that, if present, would either create or destroy a CpG site. The identifier numbers for each SNP are: (i) rs75343499 (T/C), (ii) rs2712228 (A/C), (iii) rs73191606 (G/C), (iv) rs55980027 (A/G), (v) rs115976606 (C/G), (vi) rs60284988 (T/C), (vii) rs17154282 (C/G), (viii) rs115688927 (G/A). The N_4 interferon- γ activated sequence (GAS) motif that is required for IL-4 stimulated increases in pendrin promoter activity is indicated [19].

Fig. 4. The CpG sites in HEK-Blue and NCI-H292 cells are differentially methylated. The percent methylation of total genomic DNA from NCI-H292 (black circles) or HEK-Blue (grey circles) cells at a particular CpG site was analyzed. CpG site names containing an “R” indicate that the methylation status was analyzed from the reverse DNA strand. The methylation status of all other CpG sites were analyzed from the forward DNA strand. CpG sites 90.5, 86, 80, and 61 60 & 59 were not included in the analysis due to poor signal strength. Symbols represent means $\pm$ S.E.M of 8 to 26 measurements from three separate samples of the NCI-H292 cells and two separate samples of the HEK-Blue cells where $^{\circ}p < 0.05$, $^*p < 0.01$, $^{\infty}p < 0.001$ and $^{*}p < 0.0001$ by a two-tailed Student’s *t*-test.

epigenetic modifications including histone deacetylation and DNA methylation. Although the exact sequence of events remains controversial, the link between histone deacetylation and DNA methylation in specific genomic regions represents a powerful mechanism for long-term gene silencing.

Methylation of the cytosine residue is the most common base modification in the eukaryotic genome and is defined as the addition of a methyl group to the 5'-carbon of the pyrimidine ring to generate 5-methylcytosine (5meC) [23]. 5meC is preferentially found in the context of C-phosphate-G (CpG) dinucleotides, with methylation occurring at approximately 80% of all CpG sites located primarily in repetitive sequences and within centromeric repeat regions of chromosomes [24]. The remaining 20% is unmethylated and preferentially found in short sequence stretches which range from 0.5 to 5 kilobases occurring at average intervals of 100 kilobases [24]. These stretches, or CpG islands, are often methylation-free in somatic tissues and, to a large extent, have been maintained through evolution. Current estimates indicate that 50-60% of human genes are associated with a CpG island [25]. The functional relevance of CpG islands derives from the observation that changes in their methylation status results in altered expression of their associated genes. In general, genes associated with methylated CpG islands are either silenced or down-regulated [26]. The function of DNA methylation in normal cells is diverse and necessary for normal development, including silencing of transposable elements, inactivation of viral sequences, maintenance of

chromosomal integrity, X-chromosome inactivation, and transcriptional regulation of a large number of genes [27].

Pendrin is a member of the anion transporter family solute carrier (SLC) 26, which mediates the exchange of anions, including Cl^- , HCO_3^- , OH^- , I^- , SCN^- or HCO_2^- , and is encoded by the *SLC26A4* gene (chromosome 7, chr7:107301206-107302416). Pendrin malfunction leads to PS, an autosomal recessive disorder accounting for 4-10% of inherited hearing losses [9]. While much work has centered on *SLC26A4* genetics and the functional mutations that affect pendrin function, relatively few studies have focused on unraveling the mechanisms controlling its transcription [19, 22, 28-33].

Xing et al. investigated the qualitative methylation status of a small region of the pendrin promoter (corresponding to CpG sites 75, 74, 73 and 72 in our study) in numerous cell line models of thyroid cancer and thyroid tumor DNA samples from humans [22]. They found an inverse relationship between methylation status and pendrin gene expression, at least in normal and thyroid cancer cell line models. We expected the basal methylation pattern in HEK-Blue cells, which express higher basal levels of pendrin transcript, to be lower than that in the NCI-H292 cells. However, we detected exactly the opposite, in that the extent of methylation in the NCI-H292 cells was, at most CpG sites analyzed, less compared to that in the HEK-Blue cells. Therefore in these cells, basal regulation of pendrin transcription may not be heavily impacted by CpG methylation, or may be governed by methylation of certain CpG sites (perhaps ones that are closer to the start codon).

Cytokine regulation of pendrin within the airway epithelium is clinically relevant to respiratory diseases like asthma and chronic obstructive pulmonary disease (COPD). Much work in the past years has focused on revealing the extent to which pendrin may contribute to the pathogenesis of these diseases [17, 18, 20, 34]. We recently confirmed increased pendrin gene expression in the NCI-H292 and HEK-Blue cells by IL-4 and showed that increases in the activity of the pendrin promoter stimulated by this cytokine require binding of STAT6 to an N₄ GAS motif [19]. Interestingly, IL-4 treatment resulted in a greater increase in pendrin mRNA expression in the NCI-H292 cells than in the HEK-Blue cells [19]. This, in conjunction with the limited knowledge of the role of DNA methylation in *SLC26A4* gene expression, prompted us to question the possible involvement of this type of epigenetic regulation with IL-4 stimulated increases in pendrin expression.

The present data showed significant differences in the methylation of multiple CpG sites within the pendrin promoter between the NCI-H292 and HEK-Blue cells. It is of particular interest that significant increases in methylation were detected within the HEK-Blue cells at CpG sites located within the CpG shore (CpG sites 91, 90, 87, 86R, and 85). More and more attention is being given to CpG sites located within CpG shores (in regards to transcriptional regulation), as opposed to those contained within the textbook defined CpG islands [35-38]. Moreover, CpG site 91, which is located in close proximity to the N₄ GAS motif, differs greatly (more than 60%) in methylation between the two cell lines. It is plausible that the difference in methylation at this CpG site may be related to the extent at which IL-4 can increase pendrin mRNA between the two cell lines - perhaps by altering the binding ability of STAT6. CpG methylation has been shown to block the binding of multiple transcription factors like specificity protein (SP)1, cAMP response element-binding (CREB), activator protein (AP)1 and upstream stimulating factor (USF)-1, just to name a few [39-42]. Further experiments are needed to verify this postulation. Differences in methylation of CpG site 90 (more than 70%) and CpG sites 85, 81, 78, 58, and 57 (24-37%) between the two cell types were also detected. There may be other, unidentified transcription factors that bind these areas and are important in the regulation of pendrin promoter

activity; however, this would also require further experimental investigation.

The implication of changes in the nucleotide sequence that either create or destroy a CpG site (thereby creating or destroying a potential site for methylation) or a transcription factor binding site is also unknown at this point. We are confident that changes in the nucleotide sequence that we detected in the HEK-Blue and NCI-H292 cells (refer to Fig. 3) do not create or destroy a transcription factor binding site involved in basal or IL-4 stimulated changes in pendrin promoter activity, since basal and IL-4 stimulated firefly luciferase expression driven by each sequence were identical (data not shown). We would like to note that the present experiments do not exclude other possibilities that may explain the difference in IL-4 sensitivity between the cell types. For example, the activity of transcript degradation machinery may be higher in HEK-Blue cells.

Another question arising from these studies is whether IL-4 itself can alter CpG site methylation of the pendrin promoter. Experiments designed to test this question are currently underway by our group.

While the ultimate triggers and immunopathogenesis of asthma and COPD are largely distinct, it is in clear agreement that genetics plays an important role in susceptibility [43, 44]. What is becoming even more evident is the importance of epigenetic regulation in the future and retrospective lead search and optimization of therapies for these diseases [45-50]. Further functional studies are needed to determine if and how methylation within the pendrin promoter alters *SLC26A4* gene expression, and in what context this type of epigenetic regulation has in pendrin-associated disease. Moreover, quantitative determination of CpG methylation might provide a way of honing transcription factor binding areas important in the regulation of gene promoters.

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References

- 1 Wu C, Morris JR: Genes, genetics, and epigenetics: A correspondence. *Science* 2001;293:1103-1105.
- 2 Bonasio R, Tu S, Reinberg D: Molecular signals of epigenetic states. *Science* 2010;330:612-616.
- 3 Ang YS, Gaspar-Maia A, Lemischka IR, Bernstein E: Stem cells and reprogramming: Breaking the epigenetic barrier? *Trends Pharmacol Sci* 2011;32:394-401.
- 4 Hoelzinger DB, Mariani L, Weis J, Woyke T, Berens TJ, McDonough WS, Sloan A, Coons SW, Berens ME: Gene expression profile of glioblastoma multiforme invasive phenotype points to new therapeutic targets. *Neoplasia* 2005;7:7-16.
- 5 Freije WA, Castro-Vargas FE, Fang Z, Horvath S, Cloughesy T, Liao LM, Mischel PS, Nelson SF: Gene expression profiling of gliomas strongly predicts survival. *Cancer Res* 2004;64:6503-6510.
- 6 Cadieux B, Ching TT, VandenBerg SR, Costello JF: Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. *Cancer Res* 2006;66:8469-8476.
- 7 Issa JP: CpG island methylator phenotype in cancer. *Nat Rev Cancer* 2004;4:988-993.
- 8 Ezzeldin HH, Lee AM, Mattison LK, Diasio RB: Methylation of the DPYD promoter: An alternative mechanism for dihydropyrimidine dehydrogenase deficiency in cancer patients. *Clin Cancer Res* 2005;11:8699-8705.
- 9 Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED: Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat Genet* 1997;17:411-422.
- 10 Alesutan I, Pelzl L, C L, Voelkl J, Nofziger C, Dossena S, Paulmichl M, Lang F: Impact of bicarbonate, ammonium chloride, and acetazolamide on hepatic and renal Slc26a4 transcription. *Cell Physiol Biochem* 2011;28:553-558.
- 11 Bidart JM, Lacroix L, Evain-Brion D, Caillou B, Lazar V, Frydman R, Bellet D, Filetti S, Schlumberger M: Expression of Na⁺/I⁻ symporter and Pendred syndrome genes in trophoblast cells. *J Clin Endocrinol Metab* 2000;85:4367-4372.
- 12 Lacroix L, Mian C, Caillou B, Talbot M, Filetti S, Schlumberger M, Bidart JM: Na⁺/I⁻ symporter and Pendred syndrome gene and protein expressions in human extra-thyroidal tissues. *Eur J Endocrinol* 2001;144:297-302.
- 13 Rillema JA, Hill MA: Pendrin transporter carries out iodide uptake into MCF-7 human mammary cancer cells. *Exp Biol Med* (Maywood) 2003;228:1078-1082.
- 14 Suzuki K, Royaux IE, Everett LA, Mori-Aoki A, Suzuki S, Nakamura K, Sakai T, Katoh R, Toda S, Green ED, Kohn LD: Expression of PDS/pds, the Pendred syndrome gene, in endometrium. *J Clin Endocrinol Metab* 2002;87:938-941.
- 15 Di Valentin E, Crahay C, Garbacki N, Hennuy B, Gueders M, Noel A, Foidart JM, Grooten J, Colige A, Piette J, Cataldo D: New asthma biomarkers: Lessons from murine models of acute and chronic asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L185-197.
- 16 Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ: Dissecting asthma using focused transgenic modeling and functional genomics. *J Allergy Clin Immunol* 2005;116:305-311.
- 17 Nakagami Y, Favoreto S Jr, Zhen G, Park SW, Nguyenvu LT, Kuperman DA, Dolganov GM, Huang X, Boushey HA, Avila PC, Erle DJ: The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model. *J Immunol* 2008;181:2203-2210.
- 18 Nakao I, Kanaji S, Ohta S, Matsushita H, Arima K, Yuyama N, Yamaya M, Nakayama K, Kubo H, Watanabe M, Sagara H, Sugiyama K, Tanaka H, Toda S, Hayashi H, Inoue H, Hoshino T, Shiraki A, Inoue M, Suzuki K, Aizawa H, Okinami S, Nagai H, Hasegawa M, Fukuda T, Green ED, Izuhara K: Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease. *J Immunol* 2008;180:6262-6269.
- 19 Nofziger C, Vezzoli V, Dossena S, Schonherr T, Studnicka J, Nofziger J, Vanoni S, Stephan S, Silva ME, Meyer G, Paulmichl M: STAT6 links IL-4/IL-13 stimulation with pendrin expression in asthma and chronic obstructive pulmonary disease. *Clin Pharmacol Ther* 2011;90:399-405.
- 20 Pedemonte N, Caci E, Sondo E, Caputo A, Rhoden K, Pfeffer U, Di Candia M, Bandettini R, Ravazzolo R, Zegar-Moran O, Galletta LJ: Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: Role of pendrin and anion channels. *J Immunol* 2007;178:5144-5153.
- 21 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402-408.
- 22 Xing M, Tokumaru Y, Wu G, Westra WB, Ladenson PW, Sidransky D: Hyper-methylation of the Pendred syndrome gene SLC26A4 is an early event in thyroid tumorigenesis. *Cancer Res* 2003;63:2312-2315.
- 23 Christman JK: Separation of major and minor deoxyribonucleoside monophosphates by reverse-phase high-performance liquid chromatography: A simple method applicable to quantitation of methylated nucleotides in DNA. *Anal Biochem* 1982;119:38-48.
- 24 Gardiner-Garden M, Frommer M: CpG islands in vertebrate genomes. *J Mol Biol* 1987;196:261-282.
- 25 Larsen F, Gundersen G, Lopez R, Prydz H: CpG islands as gene markers in the human genome. *Genomics* 1992;13:1095-1107.
- 26 Jaenisch R, Bird A: Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nat Genet* 2003;33 S:245-254.
- 27 Egger G, Liang G, Aparicio A, Jones PA: Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004;429:457-463.
- 28 Adler L, Efrati E, Zelikovic I: Molecular mechanisms of epithelial cell-specific expression and regulation of the human anion exchanger (pendrin) gene. *Am J Physiol Cell Physiol* 2008;294:C1261-1276.
- 29 Blomqvist SR, Vidarsson H, Fitzgerald S, Johansson BR, Ollerstam A, Brown R, Persson AE, Bergstrom G, Enerback S: Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxi1. *J Clin Invest* 2004;113:1560-1570.
- 30 Blomqvist SR, Vidarsson H, Soder O, Enerback S: Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility. *EMBO J* 2006;25:4131-4141.
- 31 Dentice M, Luongo C, Elefante A, Ambrosio R, Salzano S, Zannini M, Nitsch R, Di Lauro R, Rossi G, Fenzi G, Salvatore D: Pendrin is a novel in vivo downstream target gene of the TTF-1/Nkx-2.1 homeodomain transcription factor in differentiated thyroid cells. *Mol Cell Biol* 2005;25:10171-10182.
- 32 Jonard L, Niasme-Grare M, Bonnet C, Feldmann D, Rouillon I, Loundon N, Calais C, Catros H, David A, Dollfus H, Drouin-Garraud V, Duriez F, Eliot MM, Fellmann F, Francannet C, Gilbert-Dussardier B, Gohler C, Goizet C, Journel H, Mom T, Thuillier-Obstoy MF, Couderc R, Garabedian EN, Denoyelle F, Marlin S: Screening of SLC26A4, FOXI1 and KCNJ10 genes in unilateral hearing impairment with ipsilateral enlarged vestibular aqueduct. *Int J Pediatr Otorhinolaryngol* 2010;74:1049-1053.

- 33 Yang T, Vidarsson H, Rodrigo-Blomqvist S, Rosengren SS, Enerback S, Smith RJ: Transcriptional control of SLC26A4 is involved in Pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (DFNB4). *Am J Hum Genet* 2007;80:1055-1063.
- 34 Izuhara K, Ohta S, Shiraishi H, Suzuki S, Taniguchi K, Toda S, Tanabe T, Yasuo M, Kubo K, Hoshino T, Aizawa H: The mechanism of mucus production in bronchial asthma. *Curr Med Chem* 2009;16:2867-2875.
- 35 Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R, Herb B, Ladd-Acosta C, Rho J, Loewer S, Miller J, Schlaeger T, Daley GQ, Feinberg AP: Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet* 2009;41:1350-1353.
- 36 Dudzic E, Miah S, Choudhry HM, Owen HC, Blizzard S, Glover M, Hamdy FC, Catto JW: Hypermethylation of CpG islands and shores around specific micornas and mirtrons is associated with the phenotype and presence of bladder cancer. *Clin Cancer Res* 2011;17:1287-1296.
- 37 Portela A, Esteller M: Epigenetic modifications and human disease. *Nat Biotechnol* 2010;28:1057-1068.
- 38 Pollard SM, Stricker SH, Beck S: Preview. A shore sign of reprogramming. *Cell Stem Cell* 2009;5:571-572.
- 39 Liu H, Zhang L, Niu Z, Zhou M, Peng C, Li X, Deng T, Shi L, Tan Y, Li G: Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. *BMC Cancer* 2008;8:253.
- 40 Qiang M, Denny A, Chen J, Ticku MK, Yan B, Henderson G: The site specific demethylation in the 5'-regulatory area of NMDA receptor 2B subunit gene associated with CIE-induced up-regulation of transcription. *PLoS One* 2010;5:e8798.
- 41 Weih F, Nitsch D, Reik A, Schutz G, Becker PB: Analysis of CpG methylation and genomic footprinting at the tyrosine aminotransferase gene: DNA methylation alone is not sufficient to prevent protein binding in vivo. *EMBO J* 1991;10:2559-2567.
- 42 Aoki M, Terada T, Kajiwara M, Ogasawara K, Ikai I, Ogawa O, Katsura T, Inui K: Kidney-specific expression of human organic cation transporter 2 (OCT2/SLC22A2) is regulated by DNA methylation. *Am J Physiol Renal Physiol* 2008;295:F165-170.
- 43 Holgate ST: Pathophysiology of asthma: What has our current understanding taught us about new therapeutic approaches? *J Allergy Clin Immunol* 2011;122:495-505.
- 44 Barnes PJ: New therapies for chronic obstructive pulmonary disease. *Med Princ Pract* 2010;19:330-338.
- 45 Sakao S, Tatsumi K: The importance of epigenetics in the development of chronic obstructive pulmonary disease. *Respirology* 2011;16:1056-1063.
- 46 Koppelman GH, Nawijn MC: Recent advances in the epigenetics and genomics of asthma. *Curr Opin Allergy Clin Immunol* 2011;11:414-419.
- 47 Yang IV, Schwartz DA: Epigenetic control of gene expression in the lung. *Am J Respir Crit Care Med* 2011;183:1295-1301.
- 48 North ML, Ellis AK: The role of epigenetics in the developmental origins of allergic disease. *Ann Allergy Asthma Immunol* 2011;106:355-361; quiz 362.
- 49 Durham AL, Wiegman C, Adcock IM: Epigenetics of asthma. *Biochim Biophys Acta* 2011
- 50 Martino D, Prescott S: Epigenetics and prenatal influences on asthma and allergic airways disease. *Chest* 2011;139:640-647.