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Uropathogenic *E. coli* infection provokes epigenetic downregulation of *CDKN2A* (p16INK4A) in uroepithelial cells

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Host cell and bacterial factors determine severity and duration of infections. To allow for bacteria pathogenicity and persistence, bacteria have developed mechanisms that modify expression of host genes involved in cell cycle progression, apoptosis, differentiation and the immune response. Recently, *Helicobacter pylori* infection of the stomach has been correlated with epigenetic changes in the host genome. To identify epigenetic changes during *Escherichia coli* induced urinary tract infection (UTI), we developed an *in vitro* model of persistent infection of human uroepithelial cells with uropathogenic *E. coli* (UPEC), resulting in intracellular bacteria colonies. Cells inoculated with FimH-negative *E. coli* (N-UPEC) that are not internalized and non-inoculated cells were used as controls. UPEC infection significantly induced *de novo* methyltransferase (DNMT) activity (12.5-fold $P = 0.002$ UPEC *vs* non-inoculated and 250-fold $P = 0.001$ UPEC *vs* N-UPEC inoculated cells) and Dnmt1 RNA expression (6-fold $P = 0.04$ UPEC *vs* non-inoculated cells) compared with controls. DNMT1 protein levels were significantly increased in three uroepithelial cell lines (5637, J82, HT-1197) in response to UPEC infection as demonstrated by confocal analysis. Real-time PCR analysis of candidate genes previously associated with bacteria infection and/or innate immunity, revealed UPEC-induced downregulation of the tumor suppressor gene *CDKN2A* (3.3-fold $P = 0.007$ UPEC *vs* non-inoculated and 3.3-fold $P = 0.001$ UPEC *vs* N-UPEC) and the DNA repair gene *MGMT* (9-fold $P = 0.03$ UPEC *vs* non-inoculated). Expression of *CDH1, MLH1, DAPK1* and *TLR4* was not affected. Pyrosequencing of *CDKN2A* and *MGMT* CpG islands revealed increased methylation in *CDKN2A* exon 1 (3.8-fold $P = 0.04$ UPEC *vs* N-UPEC and UPEC *vs* non-inoculated). Methylation of *MGMT* was not affected. UPEC-induced methylation of *CDKN2A* exon 1 may increase bladder cancer and presage UTI risk, and be useful as a biological marker for UTI susceptibility or recurrence.

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Urinary tract infection (UTI) results in significant morbidity and accounts for about 8 million doctor visits each year in the United States alone.[1,2] Although UTI can be treated with antibiotics, a significant number of patients are prone to recurrent UTI. Recurrent UTI, which is often resistant to antibiotic therapy, causes medical challenges particularly in pediatric or immunocompromised patients. For example, urinary tract abnormalities such as vesicoureteral reflux or a dilated upper urinary tract often increase UTI susceptibility. Even without aggravating anatomic risk factors, it is not known whether the intrinsic biologic propensity to develop a recurrent UTI differs between patients in the general population. The symptoms of UTI are wide ranging and include pain, fever, incontinence, aberrant voiding patterns often inducing chronic bladder dysfunction, renal parenchymal damage, nephron loss and renovascular hypertension. Morbidity, multisystem complications and sepsis requiring hospitalization for febrile UTI commonly burden the provider system. Co-existing immunocompromised states (chemotherapy, transplantation, HIV) can be life threatening, with UTI in some cases precipitating mortality. Furthermore, although still controversial, several studies

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identified a correlation between recurrent UTI and bladder cancer risk.[3,4]

Escherichia coli, belonging to a family of gram-negative bacilli, Enterobacteriaceae, accounts for $>80\%$ of acute UTI in children.[5] Uroepithelial cells lining the entire urinary tract are the first cells of contact for uropathogenic *E. coli* (UPEC). Clinical UTI results from a cascade of events beginning with binding of adherence factors (e.g. FimH) that coat the UPEC surface to uroplakins on the uroepithelial cells surface.[6] This binding results in UPEC invasion into the uroepithelial cells.[7] Internalized UPEC form intracellular bacterial communities, which are self-contained, actively multiplying clusters of UPEC that reside within the cytoplasm of uroepithelial cells. Acute UPEC infection stimulates uroepithelial cell apoptosis resulting in release of intracellular bacteria and infection of adjacent cells. After the initial stages of active infection, a few UPEC escape the host immune response, become dormant and form quiescent intracellular bacteria reservoirs (QIR), which can persist for months inside uroepithelial cells.[8] Reactivation of the QIR may lead to recurrence of UTI.[8] Although the mechanism of QIR maintenance and reactivation has not been clearly delineated, it is likely that UTI responsive cellular mechanisms within the uroepithelial cells are co-opted to have a role.

Susceptibility to UTI, as with other infections, is generally influenced by host factors in response to specific bacterial virulence factors and involves a repertoire of different adherence factors, including hyaluronan, CD44 and uroplakins, which bind the FimH adhesin molecules of the pathogenic bacteria.[9] The host cell response to UTI involves activation of an innate immune response, apoptosis, increased proliferation and differentiation, implicating activation of cell surface receptors such as the Toll-like receptors (TLR) leading to transcription of pro-inflammatory cytokines, such as interleukin-6 (IL6) and interleukin-8 (IL8), ultimately recruiting neutrophils to the site of infection. Recruitment of neutrophils and increased uroepithelial cell apoptosis results in exfoliation of infected uroepithelial cells into the urine thereby decreasing bacteria burden and the risk for re-infection.[10–13]

While UTI is clearly multifactorial in nature,[14] genetic variability in the host may underlie some forms of susceptibility for UTI.[15–17] For example, mutations and polymorphisms in members of the TLR family that recognize pathogen-associated molecular patterns can predispose to UTI in mice,[18,19] and humans.[20,21] Nevertheless, while UTI susceptibility may be influenced by some inherited predisposition, it remains largely sporadic and is most likely a non-Mendelian trait.

Epigenetics is the study of long-term changes in gene function which persist through many cell divisions without changes in the DNA sequence.[22] Epigenetic mechanisms in eukaryotes include DNA methylation, histone modification and expression of small non-coding microRNA sequences, all of which contribute to gene silencing. Since methylation and histone modifications are relatively simple chemical alterations extrinsic to the DNA sequence, they are often viewed as one means by which external or environmental factors bring about differential expression for a given DNA sequence. DNA methylation is the addition of a methyl group to the carbon position 5 on cytosine rings in CpG dinucleotides. It has an important role in development, differentiation, chromatin structure, transcriptional regulation and genomic stability in normal cells. Thus far, three *de novo* methyltransferases (DNMTs), DNMT1, DNMT3a and DNMT3b, have been identified in mammalian cells. DNMT1 is the most critical for controlling the methylation state of DNA in somatic cells.[22] Increased DNA methylation (hypermethylation) is often associated with loss of gene expression, although this paradigm has recently been challenged by the observation that expression of a significant number of genes is downregulated in Dnmt-deficient cells. Nevertheless, methylation-associated downregulation of genes can be seen in host cells in response to viral, parasitic and bacterial infections, including *Schistosoma* infection of the bladder[23] and *Helicobacter pylori* or Epstein–Barr virus infection of the stomach.[24–27] Human papillomavirus E7 and adenovirus E1A proteins have also been shown to directly associate with DNMT1 and stimulate DNMT1 activity.[28] Induction of latent membrane protein 1 of Epstein–Barr virus has been shown to directly activate DNMT1 expression in cultured cancer cells[29] as does Hepatitis B viral infection of a human hepatocellular carcinoma cell line.[30]

In addition to genetic alterations, epigenetic silencing by methylation of the promoter region is well documented for many growth-suppressor genes, such as cyclin-dependent kinase inhibitor 2A (*CDKN2A* (P16INK4A)), mutL homolog 1 (*MLH1*), O6-methylguanine-DNA-methyltransferase (*MGMT*) and death-associated protein kinase 1 (*DAPK1*) resulting in 'suppressing the suppressors'.[31,32] P16INK4A initiates cell cycle arrest by inhibiting cyclin-dependent kinase 4 (*CDK4*) during induced cell stress, for example, by chronic infection. Indeed, methylation-mediated silencing of several of these genes has been implicated in *Schistosoma*-associated bladder carcinoma[23] and in gastric *H. pylori* infection mentioned above.[24–26]

Promoter methylation is also a common mechanism for downregulation of *MLH1* and *MGMT*, two members of the DNA repair system.[33] This system has a major role in maintaining genomic stability and integrity by correcting errors during DNA replication. Defects in the DNA repair system are associated with accumulation of potential mutations or a 'mutator phenotype' especially in the microsatellite regions of DNA. Loss of this system predisposes to unregulated growth. Loss of *MGMT* expression increases the numbers of genetic mutations resulting from incorrect DNA replication[34] thereby contributing to neoplastic progression.

Given the described correlation between DNA methylation and disease progression, we hypothesized that the environmental stress of UTI may lead to modification of the uroepithelial cell epigenome and transcriptome thereby increasing

susceptibility to UTI recurrence and possibly bladder carcinoma. Here, we describe the use of an *in vitro* UTI model to study epigenetic consequences of UTI and show by proof-of-principle a correlation between promoter methylation and loss of gene expression in infected uroepithelial cells.

MATERIALS AND METHODS

Bacteria Strains and Cell Lines

The *E. coli* strains UT189 and SLC2-35-1 were generously provided by Dr Scott Hultgren (Washington University, St Louis, MO, USA). SLC2-35-1 bacteria lack the FimH adhesion and are unable to invade host cells. The HT-1197, J82 and 5637 cell lines were obtained from ATCC. These cell lines were isolated from human uroepithelial carcinoma and are commonly used to induce UPEC infections.[9]

Infection

Cells were cultured in either RPMI-1640 + 10% FCS (5637) or EMEM + 10% FCS (HT-1197, SLC-35-1) at 37°C, 5% CO_2 and humidified atmosphere. In all, 80% confluent cultures were inoculated with two *E. coli* strains: UPEC (UT189, FimH [+]) and N-UPEC (SLC-35-1, Fim H [−]) at the optimized multiplicity of infection (MOI) of 2.5–5 *E. coli* per cell, for 2 h. Post-inoculation, cells were washed with PBS + Gentamycin (100 μg/ml) followed by incubation in Gentamycin (10 μg/ml) containing medium (EMEM, 10% FBS) for up to 6 days. Cells that were not inoculated with bacteria were used as negative control. Cells exposed to N-UPEC (SLC-35-1) were used to control for any non-Fim H-related responses to *E. coli* infection. The term 'inoculation' refers to exposure of cells to UPEC or N-UPEC bacteria. The term 'infection' refers to uptake of UPEC by host cells.

Immunofluorescence and Image Analysis

Cells were cultured and inoculated in chamber slides. Cells were fixed in 4% paraformaldehyde/PBS pH 7.4 for 10 min at room temperature followed by a 5-min wash step with PBS. Incubation with 3% BSA/PBS for 1 h at room temperature was used to block non-specific binding. Anti-DNMT1 and *E. coli* antibodies (Abcam, Cambridge, MA, USA) were used at a 1:100 dilution in 1% BSA/PBS and slides were incubated at 4°C overnight. Incubation with non-immune IgG was used as negative control. Following antibody incubation, slides were washed three times for 5 min with PBS at room temperature. FITC- or TRITC-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used at a 1:200 dilution in 1% BSA/PBS and slides were incubated for 30 min at room temperature. Following secondary antibody incubation, slides were washed three times for 5 min with PBS. Hoechst dye was used as nuclear stain at a 1:2000 dilution and slides were incubated for 10 min at room temperature. Following a 5-min wash step with PBS, slides were mounted with fluorescence mounting medium (Dako, Burlington, Canada). Images were obtained using an inverted confocal microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) and LSM software. Fluorescence was quantified with ImageJ software (http://rsbweb.nih.gov/ij/).

Quantification of DNMT Activity

In all, 5637 cells were inoculated as described above and DNMT1 activity was quantified using EpiQuik DNA Methyltransferase Activity kit (Epigentek, Brooklyn, NY, USA) following the manufacturer's guidelines.

RT–PCR

In all, 5637 cells were inoculated as described above. At 6 days post-inoculation, total RNA was isolated using the RNAeasy kit (Qiagen, Mississauga, ON, USA) following the manufacturer's instructions. cDNA synthesis was performed following the manufacturer's instructions using 0.5 μg of total RNA, SuperScript III Reverse Transcriptase and oligo dT (Invitrogen, Carlsbad, CA, USA). This cDNA was used as template for quantitative real-time PCR utilizing the DyNAmo SYBR green qPCR kit (Finnzymes, Woburn, MA, USA). PCR conditions: 94°C 15 min; 45 cycles of 94°C 30 s; 55°C 30 s; 72°C 30 s; 72°C 5 min. Primer 3 software was used to design forward and reverse primers for each candidate gene (Table 1). Amplification of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was

Table 1 RT–PCR primers

Gene	Sequence	Tm (°C)	Product size (nt)
CDKN2A forward	ATATGCCTTCCCCCACTACC	56	130
CDKN2A reverse	CGTGAGTGCTCACTCCAGAA		
TLR4 forward	TCCATAAAAGCCGAAAGGTG	56	119
TLR4 reverse	GATACCAGCACGACTGCTCA		
MGMT forward	GGCACCGCTGTATTAAAGGA	58	145
MGMT reverse	ATAGAGCAAGGGCAGCGTTA		
MLH1 forward	CAGAGGAAGATGGTCCCAAA	58	116
MLH1 reverse	CAGGTTCCCTTCCTCATCAA		
IL8 forward	TAGCCAGGATCCACAAGTCC	56	117
IL8 reverse	GCTTCCACATGTCCTCACAA		
IL6 forward	GAAAGCAGCAAAGAGGCACT	58	108
IL6 reverse	TTTCACCAGGCAAGTCTCCT		
DAPK1 forward	ATGATCCCACGTCAATCCAT	56	131
DAPK1 reverse	ACCGAAGGCTATGGGTTCTT		
E-cadherin forward	AGCTGCCCAGAAAATGAAAA	56	102
E-cadherin reverse	TTGGCCAGTGATGCTGTAGA		
Dnmt1 forward	ACCAAGCAGGCATCTCTGAC	56	127
Dnmt1 reverse	ACCAGCTTCAGCAGGATGTT		
GAPDH forward	GTCAGTGGTGGACCTGACCT	56	147
GAPDH reverse	TGCTGTAGCCAAATTCGTTG		

used as quantitative standard. Real-time PCR reactions were performed on the DNA Engine Opticon 2 Real-Time detection system (Bio-Rad, Hercules, CA, USA).

Pyrosequencing

Cells were inoculated with UPEC and N-UPEC for 2 h followed by 6 days post-inoculation incubation as described in the cell culture methods. DNA was isolated using the DNeazy tissue extraction kit (Qiagen) following the manufacturer's instructions. DNA bisulfite conversion and pyrosequencing was performed by EpigenDx (Worcester, MA, USA) using Biotage PyroMark RUO instruments (Biotage, Kungsgatan, Sweden) and primers that were recommended by EpigenDx. The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (Biotage).

Statistical Analysis

Student's *t*-test and three-way ANOVA was used for data analysis. $P < 0.05$ was considered to be statistically significant.

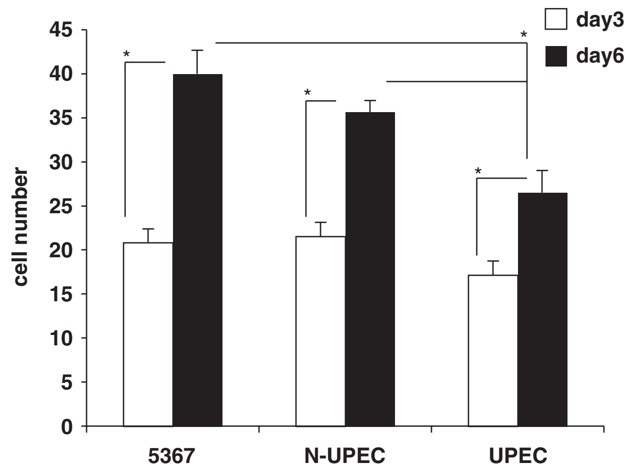

Figure 1 5637 cells inoculated with UPEC or N-UPEC continue to proliferate. In all, 5637 cells were inoculated as described in Materials and methods. Three and 6 days after inoculation, nuclei of DAPI-stained cells were counted. Values represent mean of $N = 3 \pm$ s.e.m. Student's *t*-test was used to calculate *P*-values.

RESULTS

UPEC Persist in Infected Uroepithelial Cells *In Vitro*

Since we were primarily interested in epigenetic changes resulting from prolonged UPEC infection, we established a chronic UTI *in vitro* model. In all 5637, J82 and HT-1197 cells, originally isolated from a bladder carcinoma and commonly used as host cells for infection models,[35] were inoculated with two *E. coli* strains: UPEC (UT189) and N-UPEC (SLC2-35-1). UPEC adhere and internalize into uroepithelial cells whereas uroepithelial cell binding and internalization of N-UPEC does not occur due to lack of functional FimH fimbrae.[36,37] First, we optimized the multiplicity of infection (MOI = number of *E. coli* per number of uroepithelial cells), infection time, as well as duration of post-inoculation incubation. Initially, prolonged (24 h) *E. coli* infection at MOI of 100 *E. coli* per cell was cytolytic (data not shown). Therefore, we reduced the inoculation period to 2 h and the MOI to 2.5 to 5 *E. coli* per cell. To mimic the clinical situation, where patients are treated with antibiotics, as closely as possible, following 2 h of acute infection, cells were treated with Gentamycin. This treatment removed extracellular (non-internalized) bacteria. Since *de novo* DNA methylation requires cell proliferation, it was essential that our infection protocol did not interfere with cell proliferation. To compare cell proliferation of UPEC-infected *vs* non-inoculated or N-UPEC inoculated uroepithelial cells, 2×10^5 cells were inoculated (with UPEC or N-UPEC) at a MOI of 5 for 2 h and cultured post-inoculation in the presence of Gentamycin for up to 6 days. Uroepithelial cell proliferation in response to *E. coli* infection was quantified by cell counting at days 3 and 6 post-inoculation (Figure 1). At an MOI of 5, UPEC-infected cells continue to proliferate between days 3 and 6, although at a reduced rate, demonstrating that the described infection protocol is not cytotoxic. A similarly reduced proliferation rate was seen with N-UPEC inoculated cells.

One of the hallmarks of persistent infection is the presence of intracellular bacterial colonies. To demonstrate that our infection protocol leads to persistence of intracellular bacteria colonies, we stained inoculated cells with anti-*E. coli* antibodies. N-UPEC inoculated and non-inoculated cells were used as controls. UPEC-infected, N-UPEC inoculated and non-inoculated 5637 cells were cultured post-inoculation for 1–6 days, stained with anti-*E. coli* antibodies and analyzed by confocal microscopy. Only infection with UPEC resulted in internalization of bacteria, no bacteria were present inside N-UPEC inoculated or non-inoculated 5637 cells. Image analysis revealed that, although the percentage of infected cells decreased over time (Figure 2a), the number of bacteria per cell significantly increased between days 1 and 2 post-infection and remained constant between days 2 and 5 (Figure 2b), suggesting that UPEC not only persisted but also proliferated inside uroepithelial cells (Figure 2a and b).

UPEC Infection Stimulates DNA Methyltransferase Activity

Since DNMT activity is responsible for increases in DNA methylation, we postulated that changes in DNMT activity would be an indicator for DNA methylation changes. Since cell division is usually associated with methylation of CpG islands by DNMTs,[38] we increased the post-inoculation period to 6 days to allow uroepithelial cell proliferation and DNA methylation to occur. Endogenous total DNMT activity of UPEC and N-UPEC inoculated 5637 cells was analyzed using nuclear extracts. Nuclear extracts of HeLa cells and UPEC were used as positive and negative control, respectively. Infection of 5637 cells with UPEC increased DNMT activity 12.5-fold compared with non-inoculated cells ($P = 0.002$),

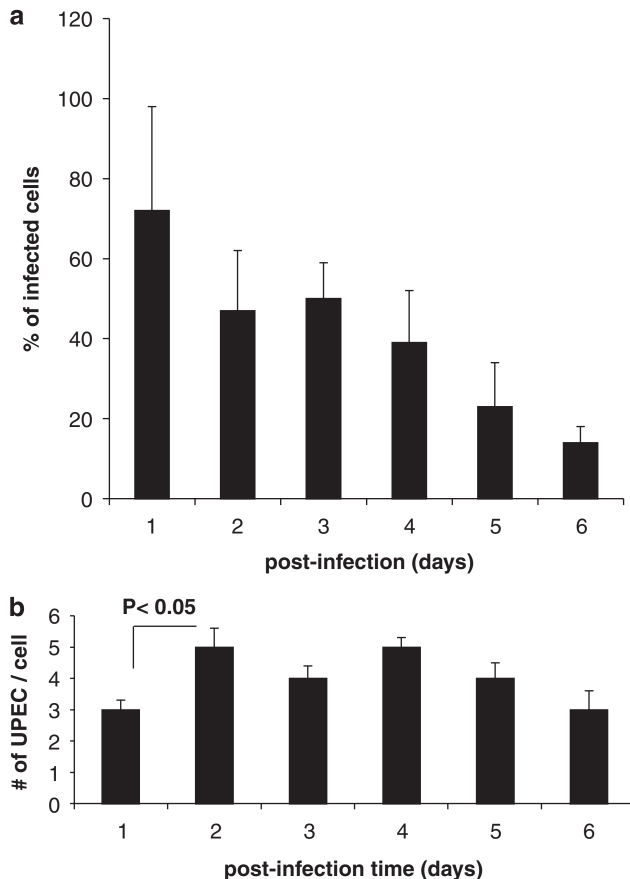

Figure 2 5637 cells internalize UPEC but not N-UPEC. UPEC are internalized by 5637 cells and persist intracellularly for the entire experimental period of up to 6 days. In all, 5637 cells were inoculated with UPEC or N-UPEC as described in Materials and methods. One to 6 days post-inoculation, cells were stained with antibodies against *E. coli*. Internalized bacteria and cells were counted using confocal microscopy images. (**a**) Graph depicts number of infected cells over time. (**b**) Graph depicts number of internalized *E. coli* divided by total number of cells. Values represent mean of $N = 5 \pm$ s.e.m. Student's *t*-test was used to calculate *P*-values.

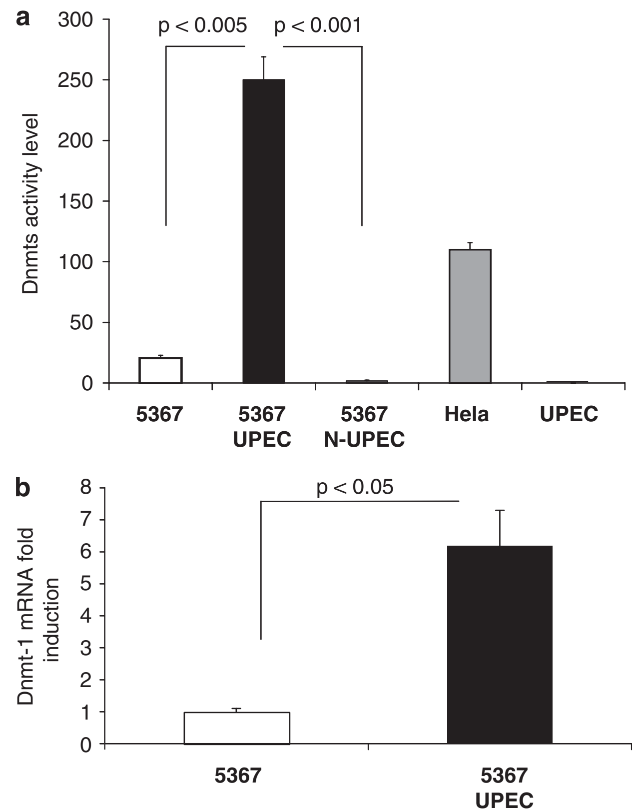

Figure 3 UPEC infection of 5637 cells results in upregulation of endogenous DNA methyltransferase activity and DNMT1 expression. (**a**) In all, 5637 cells were inoculated with either UPEC or N-UPEC as described in Materials and methods. DNMT activity was measured 6 days post-inoculation and was increased 12.5-fold compared with non-inoculated cells, and 250-fold compared with N-UPEC inoculated cells. HeLa cell nuclear extract was used as a positive and UPEC nuclear extracts as a negative control. (**b**) Infection of 5637 cells with UPEC resulted in 6-fold increase in *DNMT1* mRNA expression compared with non-inoculated cells. *DNMT1*and *GAPDH* expression was analyzed by real-time PCR. Quantitative analysis was performed by normalizing the *DNMT1* values to matched *GAPDH* measurements and then calculating the ratio inoculated cells/non-inoculated (control) cells. Values represent mean of $N = 3 \pm$ s.e.m. Student's *t*-test was used to calculate *P*-values.

and 250-fold compared with N-UPEC inoculated cells ($P = 0.001$) (Figure 3a), suggesting that uroepithelial cell DNA might get specifically modified in UPEC-infected cells. Interestingly, uroepithelial cell inoculation with N-UPEC decreased total DNMT activity compared with non-inoculated control cells, suggesting that the presence of N-UPEC has an inhibitory effect on DNA methylation that is independent of *E. coli* internalization.

UPEC Infection Stimulates DNMT1 Expression

Since DNMT1 is recognized as the 'maintenance DNMT' in somatic cells, we investigated whether increased *DNMT1* mRNA expression correlates with the observed high DNMT activity in UPEC-infected cells. In all, 5637 cells were infected with UPEC for 2 h followed by 6 days of post-inoculation incubation. Real-time PCR revealed a 6-fold increase in *DNMT1* mRNA expression compared with non-inoculated uroepithelial cells ($P = 0.04$) (Figure 3b), suggesting that the high DNMT activity seen in UPEC-infected cells is at least partly due to an increase in *DNMT1* mRNA expression.

The increase in host DNMT1 expression could be either directly mediated by the internalized bacteria themselves, or an indirect consequence of factors that are released by infected cells. To gain insight into the mechanism that causes DNMT1 upregulation, we asked whether the *E. coli* infection-induced DNMT1 expression is restricted to infected cells or is also manifested in non-infected cells that are co-cultured with infected cells. DNMT1 expression was analyzed on a single-cell level 6 days post-inoculation using immunofluorescent staining of DNMT1 followed by confocal microscopy and image analysis. Infected cells were identified

by staining with an antibody against *E. coli* proteins. As a ubiquitous protein, DNMT1 localized to the nucleus in UPEC and N-UPEC inoculated cells and in non-inoculated control cells. However, 6 days post-inoculation, DNMT1 staining was 1.6-fold increased in UPEC-infected cells compared with non-inoculated cells ($P = 3 \times 10^{-8}$) and about 1.4-fold increased compared with N-UPEC inoculated cells ($P = 7.3 \times 10^{-5}$) (Figure 4a and b). Furthermore, N-UPEC inoculation increased DNMT1 expression 1.2-fold compared with non-inoculated cells ($P = 0.026$), suggesting that mechanisms independent of bacteria internalization also have a role (Figure 4a and b).

Analysis of DNMT1 levels of single cells within the UPEC-infected cell population revealed that individual *E. coli*-containing cells express higher DNMT1 levels compared with individual cells that do not contain *E. coli* within the UPEC-infected cell population (Figure 4c). Nevertheless, DNMT1 levels in individual cells of the UPEC-infected population remained significantly higher compared with N-UPEC inoculated or non-inoculated cells. These results support paracrine as well as autocrine mechanisms for bacterial infection-induced DNMT1 upregulation. A similar infection-induced increase in DNMT1 levels was observed in two additional uroepithelia cell lines, J82 and HT-1197, suggesting that the effect of UPEC infection on DNMT1 expression is not restricted to one specific cell line but is a more prevalent phenomenon (Figure 4d and e).

UPEC Infection Downregulates *CDKN2A* and *MGMT* Expression

Gene-specific DNA hypermethylation is generally associated with downregulation of gene expression. Since elevated *DNMT1* mRNA transcription and DNMT activity in UPEC *vs* N-UPEC inoculated uroepithelial cells is suggestive of alterations in DNA methylation, we sought downregulation of expression of genes that had previously been implicated in infectious pathogenesis in bladder and gastric cancers. Expression of these genes in UPEC and N-UPEC inoculated 5637 cells was compared using real-time PCR. We initially focused our investigation on genes that are methylated in response to *Schistosoma* infection of the bladder and *H. pylori* infection of the gastric mucosa, such as *CDKN2A*, *MLH1* and *MGMT*, as well as other candidate genes such as *CDH1* (E-cadherin, cell–cell adhesion) and *DAPK1* (death-associated protein kinase 1, apoptosis). Infection of 5637 cells with UPEC resulted in a 3.3-fold decrease in *CDKN2A* (p16INK4A) mRNA expression compared with non-inoculated ($P = 0.007$) or N-UPEC inoculated cells ($P = 0.01$) (Figure 5a). Moreover, infection with UPEC significantly downregulated *MGMT* mRNA expression compared with non-inoculated cells or N-UPEC inoculated cells (Figure 5b). Interestingly, *MGMT* mRNA expression was increased by N-UPEC, concordant with the decrease in DNMT activity observed above, suggesting that it is pathogenic (*vs* non-pathogenic) bacteria that are specifically associated with gene methylation and expression downregulation. In contrast, inoculation with either UPEC or N-UPEC did not decrease mRNA expression for *CDH1*, *MLH1, DAPK1* and *TLR4* (Table 2), suggesting that only specific genes are affected by *E. coli* infection.

Inoculation of 5637 cells with UPEC or *N-UPEC* resulted in 2.3- and 1.87-fold increase in *IL8* mRNA expression, respectively (Table 2), suggesting that some pro-inflammatory factors may be upregulated in response to *in vitro* uroepithelial cell infection, and are not specifically downregulated through UPEC-induced DNA methylation.

UPEC Infection of Uroepithelial Cells Induces CpG Methylation in Exon 1 of *CDKN2A*

Since UPEC infection induces upregulation of DNMT activity and relative silencing of *CDKN2A* (p16INK4A) and *MGMT* mRNA expression, we asked whether DNA hypermethylation may be occurring in candidate CpG islands in these genes. DNA methylation was assessed by pyrosequencing. *CDKN2A* contains three CpG islands in the promoter region and one island in exon 1 (Figure 6a). We quantified DNA methylation of 29 CpGs flanking the translation start site in exon 1 of the *CDKN2A* (p16INK4A) gene (Figure 6a and b).

Methylation of CpG sites located 11 to 98 nt downstream of the translation start site in exon 1 was significantly increased in response to UPEC infection (Figure 7). Methylation of CpG 20 was most significantly affected. Furthermore, we quantified DNA methylation of 8 CpGs located 14 nucleotides downstream of the *MGMT* transcription start site. Although infection with UPEC led to a decrease in *MGMT* transcription, the methylation status of the *MGMT* CpG positions assessed remained unchanged (data not shown).

DISCUSSION

The goal of this study was to demonstrate a proof of principle that infection of host uroepithelial cells, the first cell of contact in UTI, with UPEC can provoke an epigenetic response in host cell DNA. DNA hypermethylation is a relatively stable epigenetic modification that leads to changes in gene expression. DNA methylation is being tested as potential diagnostic marker for a variety of cancers such as prostate, colorectal, cervical, lung, bladder cancer and leukemias.[39–43] Whereas hypermethylation of tumor suppressor genes is a widely accepted mechanism for tumor progression, DNA hypermethylation in response to UTI infection is currently an understudied field and no DNA methylation based biomarker for future UTI infection susceptibility exists. Our goal is to identify candidate regions as potential biomarkers for UTI proclivity, as well as reveal new candidate mechanisms associated with UTI pathogenesis. To study the process *in vitro*, we developed a model of persistent infection of human uroepithelial cell lines with UPEC. Although an *in vitro* infection model requires MOI optimization for each host cell line, variation of DNA methylation is

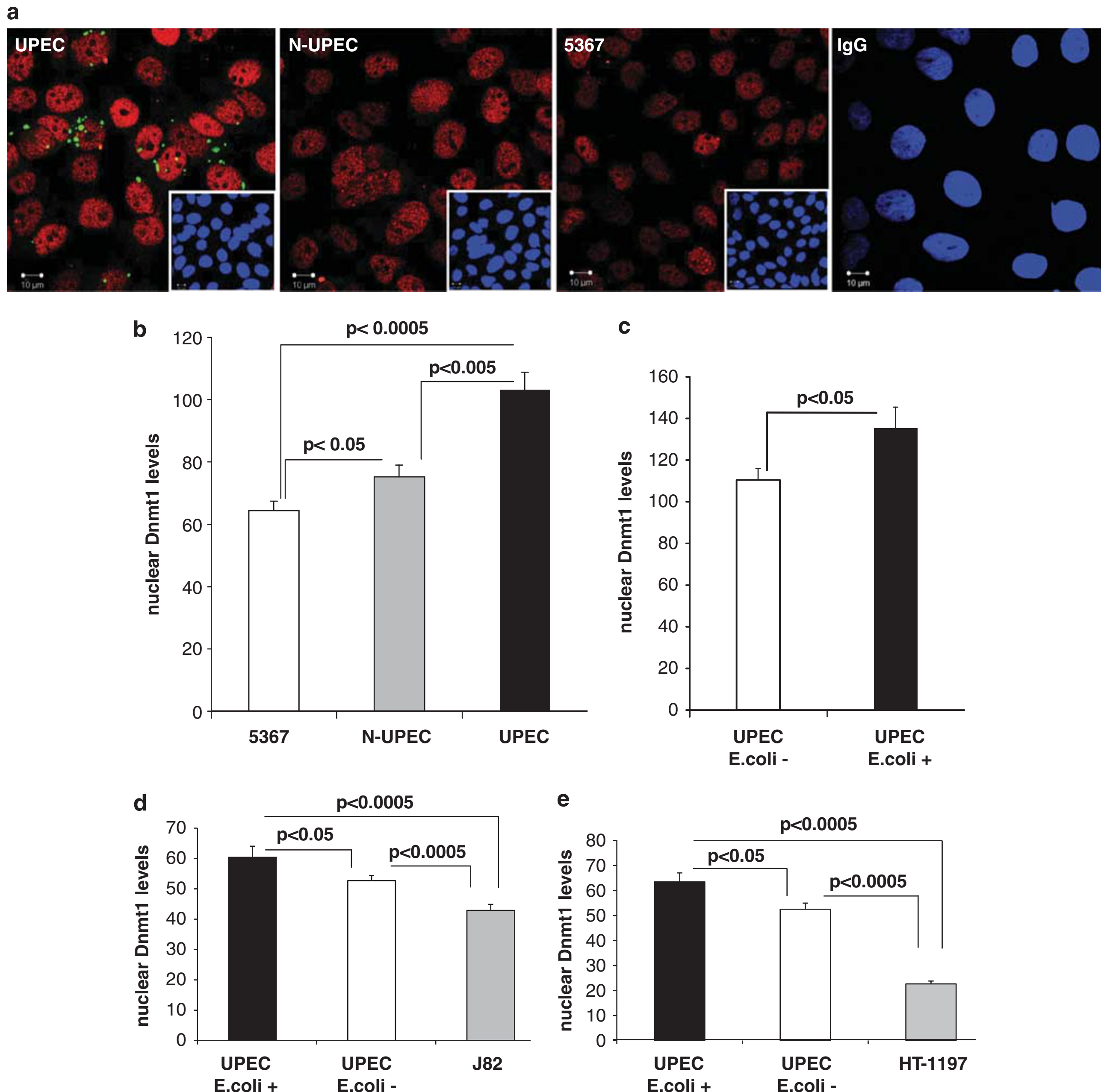

Figure 4 *E. coli* internalization is not required for DNMT1 upregulation. (**a**) UPEC-infected 5637 cells have increased nuclear DNMT1 levels compared with N-UPEC and non-inoculated cells. In all, 5637 cells were inoculated with UPEC or N-UPEC as described in Materials and methods. Six days post-inoculation, cells were double-stained with DNMT1 and *E. coli*-specific Ab. (**b**) Nuclear Dnmt1 levels were quantified by image analysis of confocal microscopy images. The graph compares DNMT1 levels of non-inoculated, N-UPEC and UPEC inoculated 5637 cells. (**c**) Nuclear DNMT1 levels of UPEC-infected cells that are void of internalized *E. coli* are higher compared with non-inoculated cells but slightly lower compared with *E. coli*-positive cells. Infected cells were identified by positive staining with *E. coli*-specific antibody. (**d**) Graph compares nuclear DNMT1 levels of *E. coli*-positive, *E. coli*-negative and non-inoculated J82 cells. (**e**) Graph compares nuclear DNMT1 levels of *E. coli*-positive, *E. coli*-negative and non-inoculated HT-1197 cells. Values represent mean of $N=3\pm$s.e.m. Student's *t*-test was used to calculate *P*-values.

reduced in an immortalized cell line compared with primary cells and patient samples, thereby increasing experimental reproducibility and allowing identification of relatively small methylation changes that may be missed in other experimental settings. Although an *in vitro* infection model has the disadvantage that cells of the immune system are absent, it allows efficient and reproducible analysis of infection-induced epigenetic consequences. Moreover, it allows analysis of infection-induced epigenetic consequences in the first cell of bacterial contact without the presence of

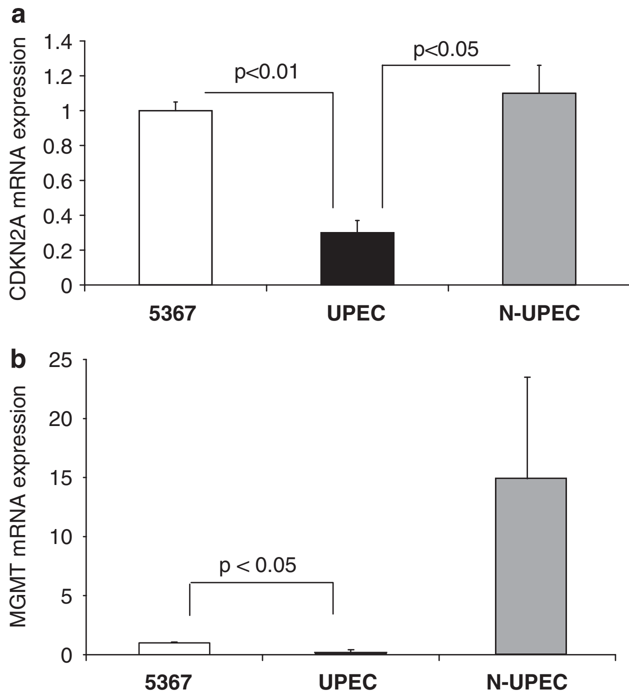

Figure 5 UPEC infection of 5637 cells results in downregulation of *CDKN2A* and *MGMT*. Infection of 5637 cells with UPEC resulted in 3.3-fold decrease of *CDKN2A* mRNA (**a**) and a 9.1-fold decrease of *MGMT* mRNA expression (**b**) compared with non-inoculated cells. *CDKN2A*, *MGMT* and *GAPDH* expression was analyzed by real-time PCR. Quantitative analysis was performed by normalizing the *CDKN2A or MGMT* values to the corresponding *GAPDH* values and then calculating the ratio inoculated cells/non-inoculated (control) cells. Values represent mean of $N=3\pm$ s.e.m. Student's *t*-test was used to calculate *P*-values.

Table 2 Infection of 5637 cells with FimH [+] *E. coli* results in mRNA downregulation of *CDKN2A* and *MGMT* compared to control cells or to FimH [−] *E. coli*-infected cells

Gene name	Control 5637 cells	FimH [+] *E. coli*	FimH [−] *E. coli*	**P*-value	***P*-value
CDKN2A/p16	1	**0.3**	**1.1**	**0.007**	**0.04**
CDH1	1	1.7	1.5	0.16	0.21
MLH1	1	1.2	0.9	0.17	0.55
MGMT	1	**0.11**	15	**0.03**	0.33
DAPK1	1	1.4	1.4	0.19	0.675
IL8	1	2.3	1.87	0.09	0.56
TLR4	1	1	1.4	0.875	0.39

*$P<0.05$ for FimH [+] *E. coli*-infected cells compared with control cells. ***P*-value for FimH [+] *E. coli*-infected cells compared with FimH [−] *E. coli*-infected cells.

Bold numbers indicate significant differences compared with control cells. Results were significant if $P<0.05$. $N=3$.

effects on downstream cell types in the infection cascade on a single-cell level under controlled inoculation and timing conditions.

It is difficult to precisely relate the concepts of acute and persistent infection in a clinical sense to this *in vitro* model. Acute infection implies the early phases of bacterial exposure to host cells. Persistent infection refers to the host cell–bacterial relationship in the days following initial bacterial exposure. Clinically, persistent infection can also imply how bacteria persist following antibiotic treatment—a feature that is best studied in patients. In our model, uroepithelial cells undergo some form of both of these phases: some host cells undoubtedly apoptose *in vitro*. We have been able to establish MOI conditions *in vitro*, however, that demonstrate that host cells also proliferate, as they do during clinical infection.[44] Importantly, our assessments of epigenetic responses begin several days following inoculation to allow for bacterial internalization, which characterizes clinical persistence of infection. In this sense, the model is being studied for responses to persistent infection, as the clinical persistent infection phase is characterized by the presence of intracellular bacteria colonies.[37] These colonies escape the immune system, and clinically can become reactivated and cause recurrent UTI. The resemblance between our *in vitro* model and the actual *in vivo* situation is of course limited because of the absence of an immune system, and 3D organ structure.

We employed a candidate gene approach to compare DNA methylation between cells inoculated with uropathogenic/UPEC and non-pathogenic/N-UPEC, or non-inoculated cells. We reasoned initially that infection pathobiology would most likely be influenced by genes that have a role in host cell/pathogen interactions, cell survival, and innate immunity and concentrated our efforts on genes previously demonstrated to be regulated by DNA methylation in other experimental settings.

Since DNA hypermethylation influences gene expression, we initially quantified expression of these candidate genes in infected and non-infected cells. Using this approach we identified two genes, *CDKN2A*, encoding *p16INK4A*, and *MGMT* that were downregulated in response to infection with UPEC but not in response to inoculation with N-UPEC. In contrast, inoculation with either UPEC or N-UPEC did not decrease mRNA expression for *CDH1*, *MLH1*, *DAPK1* and *TLR4*, although these genes are well known to be inactivated by DNA methylation.[23] TLR4 is a receptor for the bacterial FimH adhesin and LPS and TLR4 activation governs UTI susceptibility via stimulation of the host immunity.[13,45–49] For example, TLR4 activation in epithelial cells leads to chemokine and cytokine production.[45,46,48] Interestingly, UTI clearance requires TLR4 expression by bladder uroepithelial and innate immune cells. Analysis of TLR4 polymorphism revealed that certain TLR4 genotypes correlate with UTI recurrence or persistent asymptomatic infection in adult women and children.[50–52] Also, TLR4 mutant mice develop chronic asymptomatic infections.[49] Although we did not observe changes in TLR4 gene expression in our *in vitro* UTI infection model, TLR4 may still be epigenetically altered by methylation or histone modification

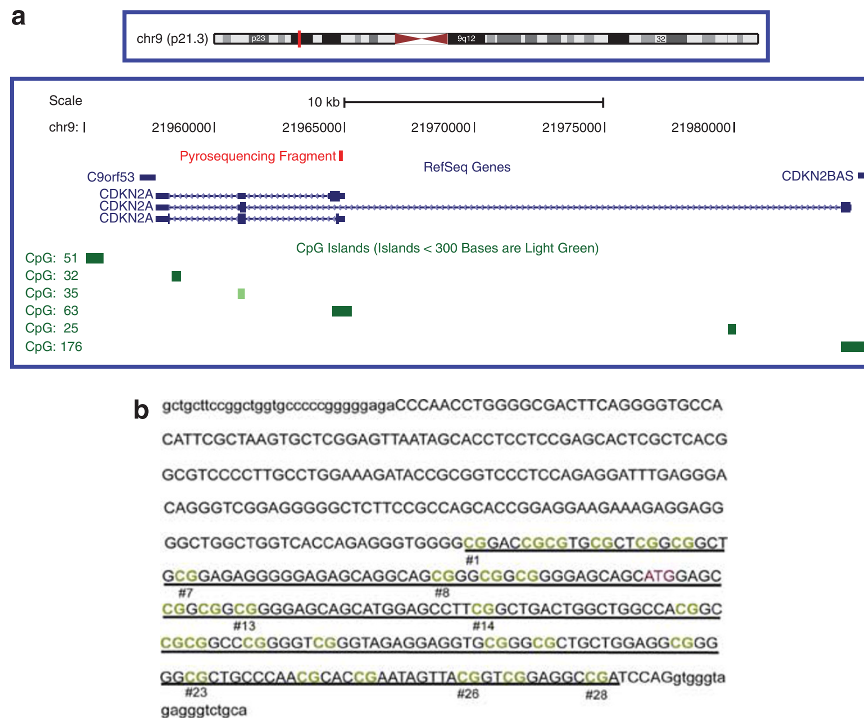

Figure 6 Diagram of *CDKN2A* CpG islands and sequence analyzed by pyrosequencing. (**a**) CpG islands are shown as green boxes, region analyzed by pyrosequencing is shown in red. (**b**) Translation start codon is shown in red, sequence analyzed is underlined and CpG dinucleotides are shown as green letters.

in response to UTI *in vivo*. In addition, future studies that incorporate UTI-induced cell–cell interactions that are present *in vivo*, but absent in our *in vitro* system, may yet reveal epigenetic involvement of TLR4 in UPEC infection.

CDKN2A (p16INK4A) is a well-known tumor suppressor gene and is involved in cell cycle regulation.[53] P16INK4A is a G1-specific cell cycle inhibitor and reduces cell proliferation by inhibiting phosphorylation of the Rb protein by cyclin-dependent kinases CDK4 and CDK6.[53] Although P16INK4A deletion has an undisputed role in transitional bladder carcinoma progression, P16INK4A DNA methylation is highly variable between individual bladder carcinoma patients and the contribution of epigenetic factors to bladder carcinoma risk is still controversial.[54,55] UTI-induced CDKN2A methylation could provide a mechanism for the observed correlation between recurrent UTI and bladder carcinoma risk.[56–58]

After infection, bladder uroepithelial cells undergo apoptosis that results in shedding of infected cells and intracellular pathogens, thereby reducing the overall pathogen load. Cell proliferation of basal cells increases and is followed by cell differentiation to replace lost uroepithelial cells.[59,60] UPEC-induced downregulation of *CDKN2A* may increase uroepithelial cell proliferation, thereby counteracting infection stimulated host uroepithelial cell apoptosis. This may increase pathogen persistence, ultimately increasing UTI recurrence risk.

MGMT is a DNA repair key enzyme that removes alkyl groups from the O^6 position of guanine nucleotides and thereby prevents base transitions that would otherwise result in inactivation of important tumor suppressor genes, such as p53 or activation of oncogenes.[61] It can be speculated that downregulation of *MGMT* in infected uroepithelial cells induces a mutator phenotype; accumulation of mutations could possibly increase pathogen persistence or uroepithelial cell re-infection, thereby increasing UTI recurrence risk. Although the observed effects on gene expression are rather moderate, they are likely additive and have the potential to increase UTI infection risk at multiple levels.

Interestingly, inoculation with N-UPEC upregulated *MGMT* expression, compared with control or UPEC-infected cells. This higher *MGMT* expression in N-UPEC inoculated cells correlated very well with reduced DNMT activity seen in N-UPEC inoculated cells, suggesting that inoculation with N-UPEC might inhibit *MGMT* DNA methylation. Therefore, N-UPEC may have potential for a probiotic effect and counteracting UPEC-induced epigenetic changes in the host genome. A probiotic effect may be one that has beneficial defensive characteristics in the face of pathogenic infection. The expression results may be the epigenetic reflection of such characteristics. Probiotic effects against UTI have already been described for *Lactobacillus rhamnosus* and *L. fermentum*.[62,63]

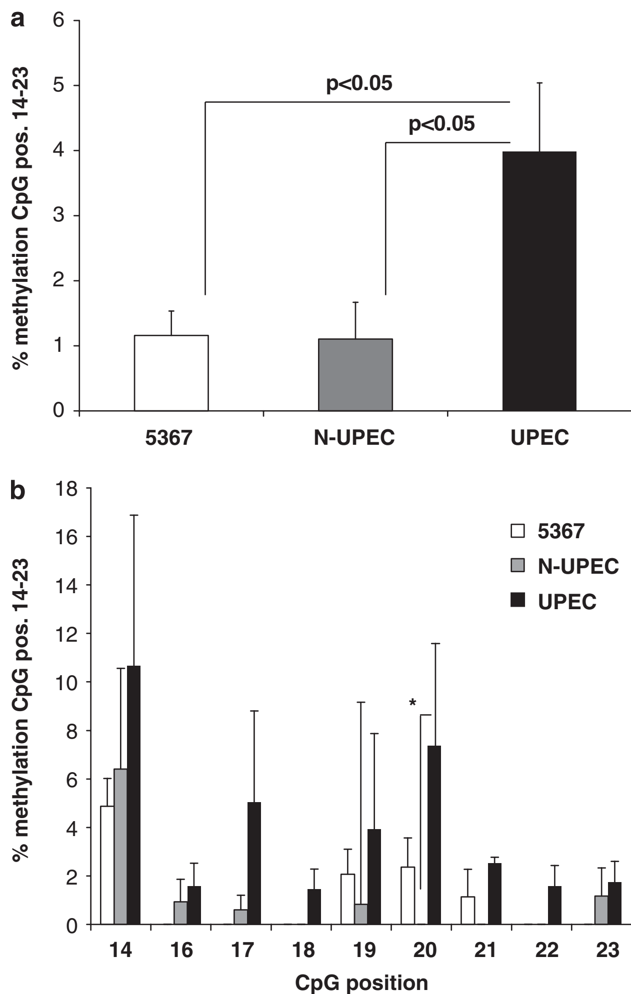

Figure 7 UPEC infection of 5637 cells results in hypermethylation of *CDKN2A* exon 1. In all, 5637 cells were inoculated with UPEC or N-UPEC as described in Materials and methods. Six days post-inoculation, methylation of individual CpG in the *CDKN2A* (p16INK4A) promoter region and exon 1 was quantified by pyrosequencing. (**a**) Graph depicts overall percentage methylation of CpG islands pos. 14–23. (**b**) Graph depicts percentage methylation of individual CpG dinucleotides in the same region as in (**a**). Values represent mean of $N = 3 \pm$ s.e.m. ANOVA was used to calculate *P*-values.

Inoculation with both, UPEC or N-UPEC increased expression of IL8, an important player in the innate immune response. These result are consistent with the clinical situation, providing an exciting validation to our *in vitro* model. Several studies reported suppression of cytokine production in response to UPEC *in vitro* infection.[35,64,65] This discrepancy with our results is likely due to differences in post-infection incubation time and is therefore reflecting differences between acute and persistent phase of infection.

Both, *CDKN2A* (p16INK4A) and *MGMT* are regulated by hypermethylation of CpG islands in other pathological situations. Interestingly, UPEC infection increases methylation of the *CDKN2A* (p16INK4A) exon 1 CpG island 3.8-fold. In contrast, methylation of CpGs in the *CDKN2A* (p16INK4A) or *MGMT* promoter region was not changed. Hypermethylation of the *CDKN2A* (p16INK4A) promoter region correlates with tumor progression and is also a consequence of *H. pylori* infection.[66] Therefore, *CDKN2A* (p16INK4A) DNA methylation patterns (exon 1 *vs* promoter) may depend on the specific factor inducing DNA methylation (*E. coli vs H. pylori* infection).

The observed methylation difference is based on analysis of a heterogeneous cell population consisting of infected and non-infected cells; fold changes in only infected single cells are likely much higher. Furthermore, this study does not represent a comprehensive genome-wide analysis of DNA methylation. Therefore, other genes are likely similarly affected. The final effect of infection-induced hypermethylation will be determined by the combination of all these modifications.

DNMT, the enzyme responsible for *de novo* DNA methylation, is upregulated in response to pathogenic infection in our model and increased DNMT activity correlates with augmented DNMT1 expression. We cannot rule out stimulation of DNMT activity by extra-transcriptional factors, as is the case for Epstein–Barr virus latent membrane protein 1 induced DNMT1 activity[29] and enhanced expression of other members of the DNMT family (DNMT3A, DNMT3B) as mechanism for the observed DNMT activity increase.

Since our experiments did not reveal any changes in *MGMT* promotor CpG methylation, expression of this gene may be more specifically regulated by cell signaling, rather than epigenetic alteration in response to infection. For example, infection has been previously shown to increase interferon release, which potentially induces downregulation of *CDKN2A* (p16INK4A) and *MGMT* expression. Immunocytochemistry in combination with image analysis revealed that infection with UPEC not only affects DNMT1 expression of cells that contain bacteria but also neighboring cells that either escaped infection or cleared the bacteria. Nevertheless, DNMT1 levels were highest in cells that harbor *E. coli*. The observed gene regulation therefore likely results from a combination of downstream effects induced by diffusible factors and direct host cell responses to bacteria adhesion and uptake. Although previous studies found a correlation between DNMT levels and DNA methylation in cancer, more recent studies demonstrated that DNA methylation is not strictly influenced by DNMT levels but also by factors that recruit DNMTs to promoter regions. Consequently, the observed *CDKN2A* (p16INK4A) methylation increase can be a direct result of increased DNMT activity or increased DNMT recruitment. Since the methylation increase is specific for CDKN2A (p16INK4A), without affecting MGMT or overall DNA methylation (data not shown), DNMT recruitment likely has a role in UTI-induced CDKN2A methylation. In summary, this study provides a proof-of-principle that human uroepithelial cells undergo epigenetic modification following pathogenic *E. coli* infection. UPEC infection of uroepithelial cells results in hypermethylation of *CDKN2A*

(p16INK4A) exon 1, which correlates with downregulation of *CDKN2A* (p16INK4A) expression. Although MGMT expression is downregulated in UPEC-infected cells, DNA methylation of this gene was not affected. E-cadherin (*CDH1*), *MLH1* and *DAPK* and *TLR4* were not specifically downregulated by UPEC infection in our model. Furthermore, these results suggest that infection triggers pathogen-specific alterations of the host cell epigenome, and not general microbe-induced gene downregulation.

Although this study is not a comprehensive analysis of DNA methylation and gene expression in UPEC-infected cells, it supports the search for epigenetically regulated biomarker genes for UTI recurrence. In future studies, *CDKN2A* (p16INK4A) methylation will be tested in DNA isolated from shed human urinary uroepithelial cells from patients followed longitudinally for UTI recurrence.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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