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# Differentiation-Induced Uroplakin III Expression Promotes Urothelial Cell Death in Response to Uropathogenic *E. coli*

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## Abstract

Uropathogenic E. coli (UPEC) expressing type 1 pili underlie most urinary tract infections (UTIs). UPEC adherence to the bladder urothelium induces a rapid apoptosis and exfoliation of terminally-differentiated urothelial cells, a critical event in pathogenesis. Of the four major uroplakin proteins that are densely expressed on superficial urothelial cells, UPIa serves as the receptor for type 1-piliated UPEC, but the contributions of uroplakins to cell death are not known. We examined the role of differentiation and uroplakin expression on UPEC-induced cell death. Utilizing *in vitro* models of urothelial differentiation, we demonstrated induction of tissue-specific differentiation markers including uroplakins. UPEC-induced urothelial cell death was shown to increase with enhanced differentiation but required expression of uroplakin III: infection with an adenovirus encoding uroplakin III significantly increased cell death, while siRNA directed against uroplakin III abolished UPEC-induced cell death. In a murine model of UTI where superficial urothelial cells were selectively eroded to expose less differentiated cells, urothelial apoptosis was reduced, indicating a requirement for differentiation in UPEC-induced apoptosis *in vivo*. These data suggest that induction of uroplakin III during urothelial differentiation sensitizes cells to UPEC-induced death. Thus, uroplakin III plays a pivotal role in UTI pathogenesis.

### Keywords

Bladder; Cystitis; Infection

## 1. Introduction

UTIs are the second most common infectious disease in humans, following respiratory tract infections. Approximately 90% of uncomplicated, community-acquired UTIs are caused by uropathogenic *E. coli* (UPEC) infection [1]. Among several virulence factors implicated in UPEC pathogenesis, the best-characterized virulence factor is the type 1 pilus that mediates attachment and colonization of mucosal surfaces [2]. Apoptosis of the bladder urothelium is a key event in the pathogenesis of UPEC in the murine UTI model. The UPEC strain NU14 induced exfoliation of superficial cells within hours [3]. Mulvey and colleagues observed that

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inhibiting apoptosis reduced bladder clearance of NU14, suggesting that urothelial apoptosis is a host defense mechanism that promotes pathogen clearance [3]. Studies demonstrating that UPEC establish intracellular populations within urothelial cells [4] also suggest that UPEC exploit apoptosis of superficial cells to access more compliant underlying cells for the establishment of bacterial reservoirs [5].

The stratified urothelium consists of an undifferentiated basal layer, an intermediate layer of 1-3 cells, and a terminally differentiated superficial or “umbrella cell” layer. Markers of urothelial differentiation include increased expression of several cytokeratins (CK8, CK18, & CK20), decreased expression of cytokeratin CK17, and increased expression of uroplakin proteins [6,7]. The major uroplakin proteins (UPIa, UPIa, UPII, and UPIII) are important for maintaining the bladder transcellular permeability barrier and are primarily expressed in superficial umbrella cells, with reduced uroplakin expression in intermediate cell layers [8]. The tetraspanin uroplakin UPIa also plays a critical role in UTI pathogenesis by serving as a receptor for UPEC type 1 pili [9,10]. UPIa and UPIb form heterodimers with UPII and UPIII, respectively, which assemble into a higher-order complexes and contribute to the permeability barrier [11,12]. UPIII is the only uroplakin protein predicted to have a significant cytosolic domain [13].

Urothelial differentiation has been previously modeled in primary human urothelial cell cultures with a peroxisome proliferator-activated receptor gamma (PPARγ) agonist [14]. PPARγ activation, in combination with epidermal growth factor receptor (EGFR) inhibition, resulted in increased expression of UPIa, UPIb, and UPII but did not induced UPIII. We utilized this model and a complementary serum-dependent model that induces differentiation markers. We demonstrate that UPEC-induced urothelial cell death increases in parallel with the expression of urothelial differentiation markers. We confirmed these results *in vivo* by demonstrating that superficial urothelial cells are more susceptible to UPEC-induced apoptosis than underlying cells. Our results suggest that differentiation sensitizes urothelial cells to UPEC-induced apoptosis through UPIII expression.

## 2. Materials and Methods

### 2.1. Bacterial strains

NU14 is a cystitis isolate of *E. coli*, and NU14-1 is a variant of NU14 that lacks functional type 1 pili [15]. Bacteria were propagated in Luria broth at 37°C under static conditions that promote expression of type 1 pili, and pilus expression was confirmed by mannose-sensitive hemagglutination [16] of guinea pig erythrocytes (Cleveland Scientific). For in vitro infections, bacteria were centrifuged and washed once in cold PBS followed by determination of $O.D._{600}$. Bacteria were resuspended in culture medium to the appropriate multiplicity of infection (MOI) or used for *in vivo* studies as previously described [17].

### 2.2. Mice

Female, specific-pathogen-free C57BL/6 mice were obtained from Jackson Laboratories and housed in barrier facilities at the Center for Comparative Medicine. After a 1-week acclimatization period, 6- to 10-week old mice were anesthetized with isoflurane and inoculated by transurethral catheter with 10 μl of bacterial suspension containing $10^8$ c.f.u. in saline under conditions that minimize reflux to the kidneys [18].

### 2.3. Reagents

CK8 and CK17 monoclonal antibodies were obtained from Sigma, and anti-UPIII monoclonal antibody AU1 was purchased from Research Diagnostics. Additional reagents included biotinylated goat anti-mouse antibody (Invitrogen), goat anti-mouse antibody conjugated to

horseradish peroxidase (HRP; Santa Cruz), anti-rabbit antibody conjugated to HRP (Pierce), Cy2-strepavidin (Amersham), and AlexaFluor 594-strepavidin (Invitrogen). Anti-uroplakin antisera were raised in rabbits against peptides coupled to key limpet hemocyanin (KLH) by Research Genetics/Invitrogen against the following human uroplakin peptides: (KLH)-DTDQGQELTRLWDR (UPIa), QNNSPPNNDDQWKNNGVTKT (UPIb), (KLH)-GTATESSREIPMSTLPRRN (UPII), and (KLH)-QTLWSDPIRTNQL (UPIIIa).

### 2.4. Cell culture and differentiation models

The PD07i cell line was previously established [19], and cultures were maintained in EpiLife medium supplemented with HKGS (Invitrogen). For serum/calcium-induced differentiation, PD07i cells were grown to confluence in EpiLife before culture in E medium (3 parts Dulbecco's modified Eagle's medium to 1 part F12 containing 5% fetal bovine serum and additional supplements) for up to 10 days [20,21]. Cells were cultured in antibiotic-free medium 24 hours prior to bacterial infections. In the alternative approach [14], PD07i cells were grown to approximately 70% confluence and then treated with PD153035 (1 μM, Calbiochem) for 1 hour, followed by addition of troglitazone (1 μM final concentration, Sigma). After 24 hours, the medium was replaced with fresh medium containing only PD153035; cultures were maintained in the presence of PD153035 until analysis at day 6. All cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

### 2.5. Immunoblotting

Cell extracts were prepared in RIPA buffer containing protease inhibitors, fractionated on 4-15% SDS-polyacrylamide gels, and transferred to Immobilon-P membrane (Millipore). After blocking with milk diluent (KPL), membranes were incubated with primary antibody (1:1000) in milk diluent, washed in TBS/Tween, and then incubated with secondary antibody conjugated to HRP (anti-mouse or anti-rabbit for detection of cytokeratins and uroplakins, respectively). Bound antibodies were detected using chemiluminescence (Pierce).

### 2.6. Quantitation of uroplakin gene expression

Total RNA was purified using Trizol (Invitrogen). Primers for human UPIIIa (forward 5′-AGTGTGACTTTCGCCACCAACAAC-3′, reverse 5′-ATTCAGGATCTGTGAGGCCTTGGA-3′) and UPIb (forward 5′–AGCCACCGACAACGATGACATCTA-3′, reverse 5′-ATCTGCTTCAGGAAGAGGTTGGGT-3′) were used to quantify uroplakin gene expression in urothelial cells by reverse transcription with iScript cDNA synthesis kit (Bio-Rad) and real-time PCR using a PTC 200 using iQ SYBR green reagents (Bio-Rad). Results were expressed as relative fold change where Ct values were normalized first to the ribosomal subunit L19 mRNA and an untreated control. Analyses were performed using the ΔΔCt method [22].

### 2.7. Measurement of urothelial cell death

Cell death was assessed by trypan blue dye exclusion. Four hours after NU14 infection (MOI 500), cells were trypsinized and washed with PBS, mixed 1:1 with 0.4% trypan blue, and incubated 3 minutes followed by counting of viable and nonviable cells in a haemocytometer. More than 200 cells in duplicate were assessed blindly in three independent trials. In all experiments, cell death was normalized to untreated cells and expressed as relative cell death (%). To detect apoptosis in tissues, we used TUNEL assay with the *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's protocol. Slides were mounted with medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and TUNEL-positive cells were assessed by epifluorescence.

### 2.8. Measurement of bacterial adherence

PD07i cells were seeded into 24-well plates and infected with NU14 (MOI 10), centrifuged at 500×g for 5 minutes to synchronize bacteria-cell contact, and incubated at 37°C for 2 hours. Cells were washed 4 times with PBS and incubated with 0.05% trypsin/0.1%Triton X-100 for 10 minutes to lyse cells. Cell lysates were harvested with vigorous pipetting, vortexed, and plated onto LB agar at a final dilution of $10^{-3}$. Resulting colonies were counted to determine total number of bacteria associated with the cells.

### 2.9. Adenovirus-mediated uroplakin expression

Human UPIa, UPIb, UPII and UPIII cDNA clones in pMT/BiP/V5-His (Invitrogen) were a kind gift from Dr. Jennifer Southgate. Individual uroplakin cDNAs were cloned into the pAdTrack-CMV shuttle vector for production of recombinant adenoviruses in HEK 293 cells. For over-expression, PD07i cells were incubated in serum-free medium containing adenoviral supernatants (MOI 100, 4 hr), followed by removal of supernatants, addition of fresh media, and overnight culture before subsequent use in experimental assays. UPIII expression was assessed by staining cultures with AU1 monoclonal antibody specific for UPIII and detected using an anti-mouse-HRP secondary antibody and visualized by staining with diaminobenzidine.

### 2.10. Uroplakin III gene silencing

The pSM2 retroviral vector encoding a short hairpin RNA (shRNA) specific for UPIIIa was obtained commercially (clone V2HS_95009, OpenBiosystems) and transfected into BOSC 23 cells. BOSC 23 supernatants were collected and used to infect PA317 cells to generate amphotropic retrovirus. Following selection in puromycin, PA317 supernatants were collected for storage at -70°C. A control virus expressing shRNA targeted against the TNF receptor 1 (TNFR1) was similarly generated [19]. Retroviral supernatants were used to infect PD07i cells followed by selection in puromycin to create stable cell lines. Silencing of UPIII gene expression was confirmed using real-time PCR (see above).

### 2.11. Selective urothelial erosion and murine UTI

Bladder damage was induced in mice using dilute HCl [23]. To remove superficial urothelium but retain intermediate and basal layers, mice (n=3) were anesthetized with isoflurane and instilled via transurethral catheter with 10 mM HCl or PBS for 10 minutes, followed by rinsing with 0.1 M sodium bicarbonate. The bladder was then washed with successive saline instillations. Mice were subsequently infected with NU14 for 4h while maintained under isoflurane anesthesia. Animals were sacrificed, and bladders were collected, fixed in 10% formalin, and embedded in paraffin. Serial sections were examined for apoptosis by TUNEL. Parallel groups of animals were similarly infected with $1\times10^8$ NU14 and then assessed for bacterial colonization at 2h by washing harvested bladders with PBS, homogenizing bladders, and plating tissue homogenates onto selective agar [24]. All protocols were approved by the Northwestern University Institutional Animal Care and Use Committee.

### 2.12. Immunofluorescence

The sections (5 μm) were deparaffinized using standard methods and rehydrated in graded ethanols. Antigen retrieval was performed by treatment with 0.01 M sodium citrate, pH 6.0, at 92°C for 20 min, and sections were treated with blocking solution (1% BSA, 0.1% Triton X-100 in PBS) followed by room temperature incubation with anti-UPIII (clone AU1) in blocking solution for 1 hour. Sections were then incubated with biotinylated goat anti-mouse secondary antibody for 30 minutes followed by incubation with strepatavidin-conjugated Alexa-fluor 594 for an additional 30 minutes. Finally, slides were washed with PBS, mounted with DAPI medium and visualized by epifluorescence.

### 2.13. Statistical analyses

Experiments were performed at least three times. Data were analyzed using Prism (version 4.0, GraphPad) and presented as mean ± SEM. Significance was determined two-tailed *t* test for two groups or one-way ANOVA, followed by Dunnett's post-test comparison. A $p$ value <0.05 was considered significant.

## 3. Results

### 3.1. *In vitro* differentiation of human urothelial cells

We employed complementary *in vitro* urothelial differentiation models. PD07i human bladder urothelial cells [19] were propagated in serum-free medium and transferred into a medium previously shown to promote epithelial differentiation, either “E medium” containing 5% serum and elevated calcium or a serum-free medium containing the PPARγ activator troglitazone and the EGFR inhibitor PD153035 [14]. Following culture, cell extracts were prepared for immunoblotting to detect cytokeratins and uroplakins. We previously found that cytokeratin CK8 was induced by culturing immortalized human urothelial cell lines in 3D organotypic cultures or in semi-solid suspension cultures in E medium [20], and serum induced tight junction markers in stratified cultures of immortalized urothelial cells [25]. Here, we examined CK8 expression in monolayer PD07i cultures and observed a 2-fold increase in CK8 expression upon differentiation with either E medium or troglitazone treatment (Fig. 1A and data not shown). In contrast, CK17 expression decreased upon culture of PD07i cells in either E medium or troglitazone/PD153035 (Fig. 1A), consistent with previous findings that CK17 expression diminishes during urothelial differentiation *in vivo* [6]. Thus, both culture models promote changes in PD07i cytokeratin expression consistent with induction of urothelial differentiation.

We next assessed whether PD07i differentiation induced uroplakin expression (Fig. 1B). Uroplakin expression was undetectable in immunoblots of PD07i cultures maintained in serum-free medium, but UPIa, UPIb, and UPII protein were evident in cells extracts from both E medium- and troglitazone-treated cultures. The band detected by UPIa antiserum exhibited a mass that varied with PD07i culture conditions, suggesting differential UPIa glycosylation in the E medium and troglitazone/PD153035 differentiation model systems. In contrast, UPIII expression was differentially induced by *in vitro* culture approaches. Consistent with previous studies in primary urothelial cultures [14], troglitazone/PD153035 did not induce UPIII expression in PD07i cultures, whereas E medium induced detectable UPIII protein expression (Fig. 1B). The expression of UPIII and UPIb mRNAs in E medium was then examined using quantitative RT PCR (Fig. 1C & D). After normalizing to expression under basal culture conditions in serum-free medium, UPIII mRNA expression peaked at approximately six days of E medium culture and remained stable (Fig. 1C). Similarly, UPIb message peaked at day 6, but the level of induction was much smaller than for UPIII mRNA (compare Figs. 1C and D). The modest accumulation of UPIII protein relative to UPIII mRNA suggests that E medium-induced PD07i differentiation does not completely overcome post-transcriptional hurdles to robust UPIII expression. However, these data indicate that E medium culture of PD07i cells represents a model for uroplakin-dependent physiologic responses to UPEC.

### 3.2. Urothelial differentiation enhances UPEC-induced cell death

Urothelial cells were cultured in E medium for 10 days followed by analysis of cell death in response to UPEC isolate NU14 using trypan blue dye exclusion (Fig. 2). Previous studies established that UPEC-induced apoptosis is dependent upon type 1 pili [3,17,26]. To confirm pilus-dependent cell death, cultures were infected with NU14 or NU14-1, a variant that lacks functional type 1 pili [15]. After 10 days of growth in E medium, treatment of PD07i cultures with NU14 resulted in death of a majority of cells, whereas NU14-1 induced significantly less

cell death (Fig. 2A). We next examined whether E medium culture was associated with altered sensitivity to UPEC-induced death. NU14 caused cell death at all times tested, but PD07i cells maintained in E medium for 6 or more days exhibited significantly elevated death relative to cultures maintained in serum-free medium (Fig. 2B). This effect appeared specific to E medium-induced differentiation because troglitazone/PD153035 did not alter the cell death response to NU14 (Fig. 2C). It is possible that enhanced cell death was an indirect consequence of increased bacterial binding, but we found that E medium did not significantly alter NU14 adherence (Fig. 3C). These results therefore suggest that urothelial cell death is mediated by type 1 pili and modulated by differentiation status. Furthermore, the observation that enhanced sensitivity to UPEC-induced cell death became significant at the same time when differentiation-induced uroplakin mRNA levels plateaued raises the possibility that uroplakins contribute to UPEC-induced urothelial death.

### 3.3. Uroplakin III mediates UPEC-induced cell death

To directly assess the role of uroplakins in UPEC-induced urothelial cell death, we employed adenoviruses encoding individual uroplakins to express uroplakins in undifferentiated PD07i cells. In PD07i cultures maintained in serum-free medium, infection with an adenovirus encoding UPIII significantly enhanced NU14-induced cell death, relative to cells infected with a luciferase control virus (Fig. 3A). Co-infection of PD07i cells with either all four uroplakins or UPIb and UPIII resulted in further enhancement of NU14-induced cell death. These data suggest that UPIII alone or in combination with its heterodimeric partner UPIb is sufficient for mediating NU14-induced cell death. Furthermore, the increased cell death in co-infected cultures (UPIb and UPIII) relative to UPIII alone may indicate that UPIb facilitates enhanced surface expression of UPIII protein. Despite modest expression levels, staining for UPIII expression (Fig. 3D) supports this possibility, for co-expression of UPIb enhanced UPIII staining relative to expressing UPIII alone. Finally, adenovirus-mediated uroplakin expression did not alter TNF-induced cell death [19] in PD07i cells (data not shown), suggesting the UPIII-associated sensitivity to UPEC is not indirectly mediated by increased urothelial cell stress.

We further examined the role of the uroplakins as mediators of NU14-induced cell death using troglitazone/PD153035-treated PD07i cultures. Troglitazone/PD153035 treatment alone did not appear to increase NU14-induced cell death relative to serum-free cultures (compare Figs. 3A and 3B). However, adenovirus-mediated UPIII expression elevated NU14-induced cell death in troglitazone/PD153035-treated PD07i cultures to levels similar to cultures treated with E medium (Fig. 3B). Co-expression of UPIb or UPIa/UPIb/UPII did not result in any further increase in cell death (Fig. 3B), suggesting that UPIII is the limiting uroplakin that mediates NU14-induced cell death in troglitazone/PD153035-treated cultures. Enhanced cell death was not a function of increased receptor expression on urothelial cells because bacterial adherence to PD07i cells was unaltered by troglitazone/PD153035 (Fig. 3C). These data further support the possibility that urothelial differentiation enhances NU14-induced cell death signals through UPIII expression.

As genetic confirmation for UPIII in UPEC-induced cell death, we used RNA interference to disrupt UPIII expression. Stable cell lines were established that knocked-down UPIII or TNFR1, and these cell lines were cultured in E medium for 10 days. Real-time PCR was used to quantify message expression, and UPIII mRNA levels were significantly reduced in PD07siUPIII cells relative to parental cultures (Fig. 4A). The siUPIII was specific for UPIII at the protein level, for immunoblotting revealed decreased UPIII protein in PD07siUPIII cultures relative to PD07siTNFR cultures (Fig. 4B, 54% reduction relative to GAPDH). PD07i cells expressing siUPIII were significantly less sensitive to NU14-induced cell death than either parental PD07i cultures or PD07siTNFR cultures (Fig. 4C). These results further show that UPIII is required for NU14-induced urothelial cell death.

### 3.4. Differentiated urothelial layers are susceptible to NU14-induced apoptosis *in vivo*

To confirm the role of urothelial differentiation on UPEC-induced cell death, we used a previously described mouse model that selectively removes superficial urothelial cells [23]. Treatment with dilute HCl resulted in selective erosion of the superficial layer when compared with saline-treated controls, leaving the underlying, less-differentiated layers largely intact (Fig. 5, compare A&D). Consistent with this histological assessment, normal UPIII immunoreactivity was largely absent in HCl-treated bladders (Fig. 5, compare B&E). These tissues were then stained for apoptotic nuclei using TUNEL assay, and NU14 infection resulted in TUNEL-positive cells in saline-treated controls, signifying apoptosis of the superficial urothelial cells (Fig. 5C). In contrast, TUNEL-positive foci were absent bladder tissues of HCl-treated animals (Fig. 5F). To confirm that the differential sensitivity of urothelial layers was not merely the result of differential bacterial adherence, we examined bladder colonization at two hours following infection. Both saline-treated and HCl-treated groups exhibited similar levels of bacteria (Fig. 5G), indicating that differential sensitivity of urothelial cell layers cannot be attributed to differential bacterial adherence. Thus, these results *in vivo* support our culture models that demonstrate a role for uroplakins in UPEC-induced urothelial cell death.

## 4. Discussion

Apoptosis is a key event in the pathogenesis of urinary tract infections due to UPEC. An intriguing question raised by the results of Mulvey and colleagues [3] is the mechanism underlying the exquisite susceptibility of the superficial epithelium to UPEC-induced apoptosis. In this study, we examined the role of urothelial differentiation as a host factor determining susceptibility to UPEC-induced cell death and demonstrate that urothelial differentiation and UPIII sensitize cells to death.

UPEC-induced apoptosis of urothelial cells is mediated by type 1 pili [3,17,26]. We found that type 1 pili-induced urothelial cell death was enhanced in cells cultured under conditions that promote expression of all four major uroplakins. In a culture model that failed to promote UPIII expression, troglitazone/PD153035, UPEC-induced cell death was only enhanced by heterologous UPIII expression (Figs. 3A and 3B), suggesting that UPIII mediates UPEC-induced cell death. This possibility was confirmed when knockdown of UPIII expression significantly reduced cell death in response to NU14 (Figs. 3 and 4). Together, these data suggest that UPIII or the UPIb/UPIII complex is the limiting factor that mediates cell death and that differentiation-induced UPIII expression sensitizes urothelial cells to UPEC.

These data implicating UPIII in urothelial cell death are the first report of a role for UPIII in UPEC virulence. Interestingly, UPIII is the only uroplakin with an appreciable cytosolic tail that may function in signal transduction [13]. UPIII-deficient mice are severely deficient in normal bladder function due to aberrant formation of the asymmetric unit membrane [27], but the effects on UPEC infection in the murine UTI model have not been described. Recent studies have shown a role for the *Xenopus* homologue of UPIII (xUPIII) in signaling during sperm-egg fusion [28,29]. These signaling events occur upon fertilization or egg activation and lead to rapid *Xenopus* Src activation and phosphorylation of UPIII on a carboxyl-terminal tyrosine residue [28,29]. UPIb also plays an important role in proper regulation of xUPIII-mediated Src activation [30]. These findings support an emerging model that xUPIII complexes transduce extracellular signals, raising the possibility that similar signaling may be initiated by mammalian UPIII homologues. We speculate that UPEC type 1 pilus binding results in UPIII transduction of signals that mediate apoptosis and cell death. Indeed, UPIII-mediated signaling appears important for both UPEC-induced apoptosis and UPEC invasion of urothelial cells (Thumbikat et al, in preparation).

In summary, apoptosis is a critical step in UPEC pathogenesis and our data suggest that urothelial differentiation and expression of UPIII sensitize host cells to UPEC-induced cell death. Disruption of superficial epithelium by type 1 pilus-dependent apoptosis may provide UPEC with access to underlying, more compliant cells, where UPEC reservoirs become established and drive recurrent UTI [5]. Our results suggest therefore that UPEC have evolved to exploit UPIII signaling to further pathogenesis within the urinary tract. Thus, in addition to roles in normal host processes of bladder barrier function and sperm-egg fusion, UPIII plays an important role in UTI pathogenesis.

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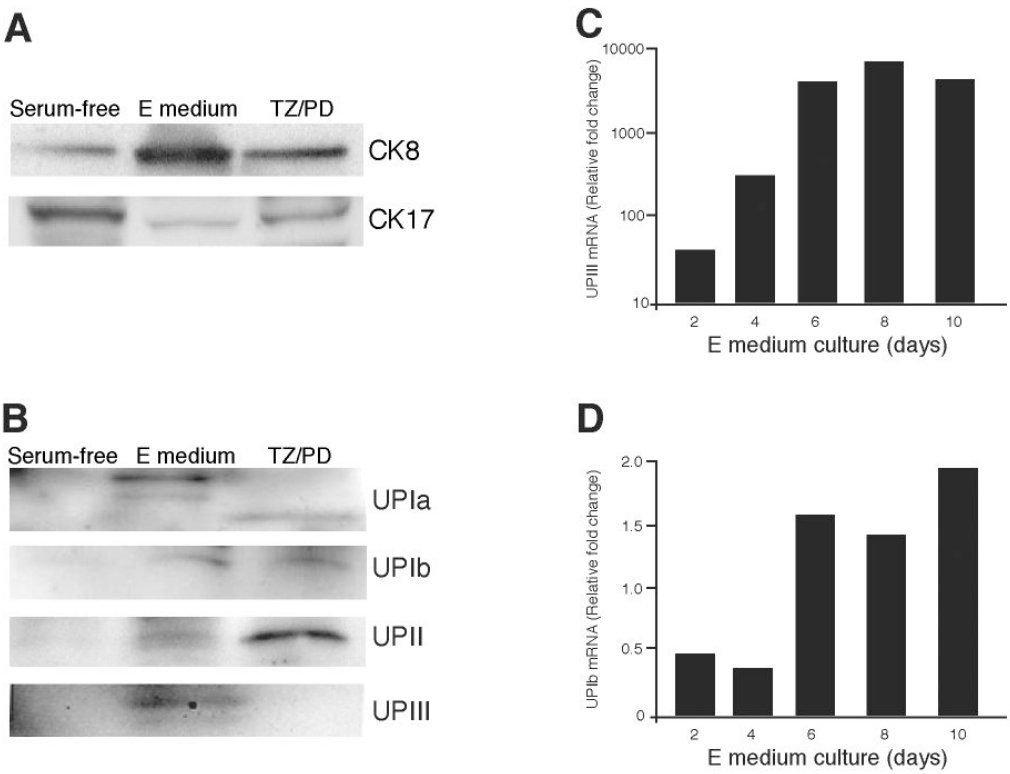


**Figure 1.**

Culture conditions promote induction of tissue-specific differentiation markers in human urothelial cells in vitro. PD07i human urothelial cells were cultured under serum-free conditions, in medium containing serum and elevated calcium (E medium), or in serum-free medium containing the PPARγ agonist troglitazone and the EGFR antagonist PD153035 (TZ/PD; see Methods for details). **A)** Immunoblotting detected increased cytokeratin expression in PD07i cells cultured in CK8 in E medium and troglitazone/PD153035 cultures relative to cells cultures in serum-free medium. Cytokeratin CK17 expression was reduced by culture of PD07i cells in E medium or troglitazone/PD153035. **B)** Immunoblotting revealed uroplakin induction by culture of PD07i cells in either E medium or troglitazone/PD153035. UPIa, UPIb and UPII were induced by culture in troglitazone/PD153035, while E medium induced all four uroplakins. Quantitative RT PCR analysis demonstrated increased expression of UPIII and mRNA **(C)** and UPIb mRNA **(D)** in PD07i cells cultured in E medium. Values reflect fold change relative to PD07i cells maintained in serum-free medium (i.e., Day 0).

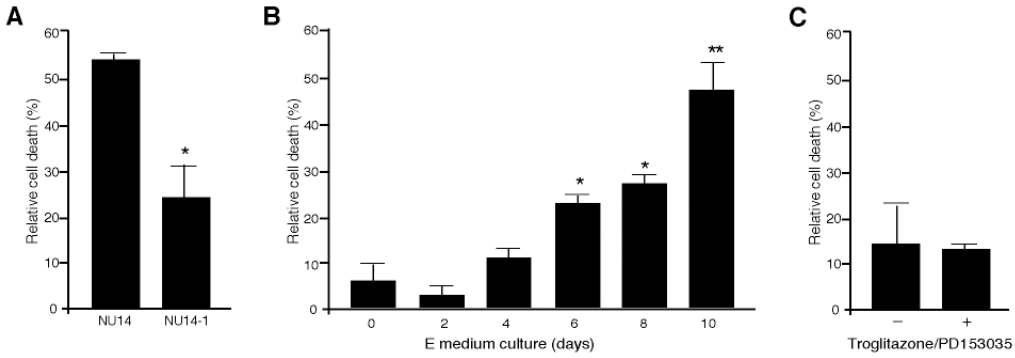


**Figure 2.**

Differentiation sensitizes urothelial cells cell death from UPEC type 1 pili. **A)** PD07i cells were cultured in E medium and infected with NU14 or NU14-1 (MOI 500), and cell death was quantified by dye exclusion assay. Cell death induced by FimH$^-$ NU14-1 was significantly attenuated relative to NU14-induced death (*$p<0.05$). **B)** PD07i cells were cultured for different time periods in E medium and then infected with NU14 (MOI 500). Dye exclusion assay demonstrated significantly increased cell death (*$p<0.05$, ** $p<0.001$) in response to NU14 for cells cultured in E medium for 6 days or longer, relative to cells cultured in serum-free medium (i.e., Day 0). **C)** PD07i cultures were treated with troglitazone/PD153035 (see Methods) and then infected with NU14. Dye exclusion indicated no difference in NU14-induced cell death between troglitazone/PD153035-treated and control cultures. Bars indicate the mean ± SEM after subtraction of baseline cell counts of dye permeability in untreated cultures.

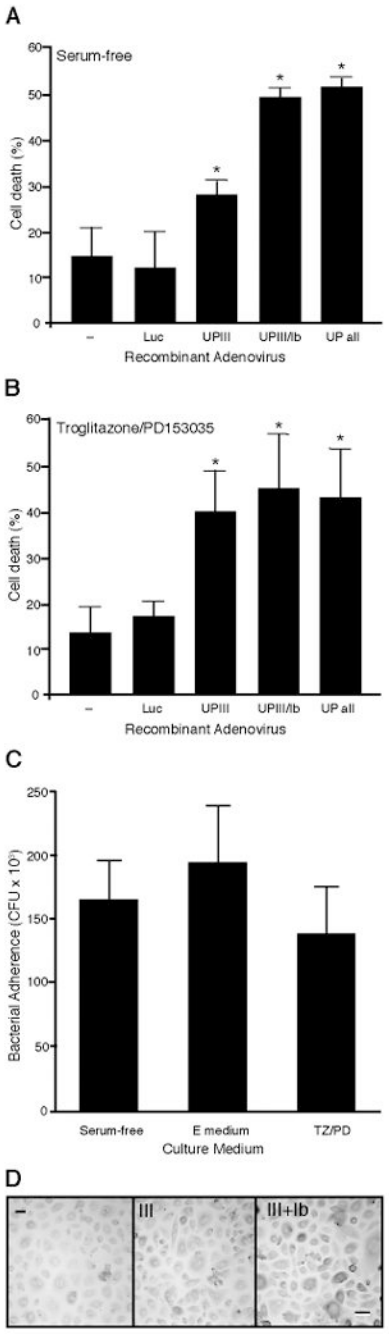


**Figure 3.**

UPEC-induced urothelial cell death is modulated by UPIII expression. PD07i cells were cultured in serum-free conditions or treated with troglitazone/PD153035. Cultures were then infected with recombinant adenoviruses (MOI 100) encoding luciferase (Luc), UPIII, UPIII plus UPIb, or all four major uroplakins overnight, followed by infection with NU14 (MOI 500) and assay for cell death by dye exclusion. **A)** PD07i cells cultured in serum-free medium were significantly more sensitive to NU14-induced death when expressing UPIII alone or in combination with other uroplakins (*$p<0.05$). **B)** Expression of UPIII alone or in combination with other uroplakins significantly enhanced NU14-induced cell death over control adenovirus infection in UPIII-deficient TZ/PD differentiated cells (*$p<0.05$). **C)** PD07i cells were cultured

under different conditions and infected with NU14 (MOI 10). After 2 h, cultures washed and total remaining NU14 (cell surface and invasive bacteria) was determined by plating detergent lysates onto selective agar and counting the resulting bacterial colonies. NU14 adherence was not significantly different between cell cultures treated with serum free media, E medium, or troglitazone/PD153035 (TZ/PD). **D)** PD07i cells were infected with adenoviruses encoding UPIII (III) or UPIII and UPIb (III+Ib) as above and then stained for UPIII expression. Infection with the virus encoding UPIb increased UPIII staining. Bars indicate the mean ± SEM, with each experiment repeated at least three times. Scale bar in D represents 50 μm.

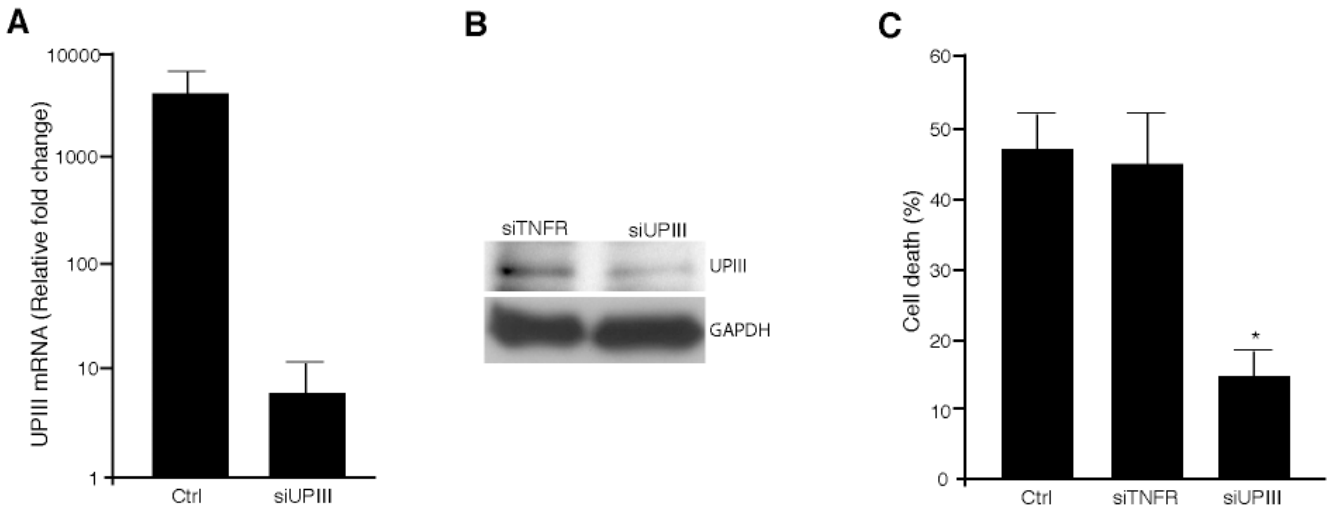


**Figure 4.**

Inhibition of UPIII gene expression abrogates NU14-induced cell death. **A)** PD70i cells expressing siRNA specific for UPIII (PD07siUPIII cells; labeled "siUPIII") were cultured in E medium for 10 days and the assessed for UPIII mRNA expression by quantitative RT PCR. PD07siUPIII exhibited less UPIII mRNA than parental PD07i cultures (data are normalized to respective cultures maintained in serum-free medium). **B)** Immunoblotting revealed less UPIII protein (54% reduction relative to GAPDH by densitometry) in E medium-treated PD07siUPIII cells relative to PD07i cells expressing siRNA specific for TNFR1 (PD07siTNFR). **C)** Parental PD07i cells (Ctrl), PD07siUPIII, or PD07siTNFR were cultured in E medium for 10 days and assessed for cell death following infection with NU14 (MOI 500) by dye exclusion. NU14-induced cell death was significantly lower in PD07siUPIII cells (*$p<0.05$) than in PD07i or PD07siTNFR cells. Bars indicate the mean ± SEM, with each experiment repeated at least three times.

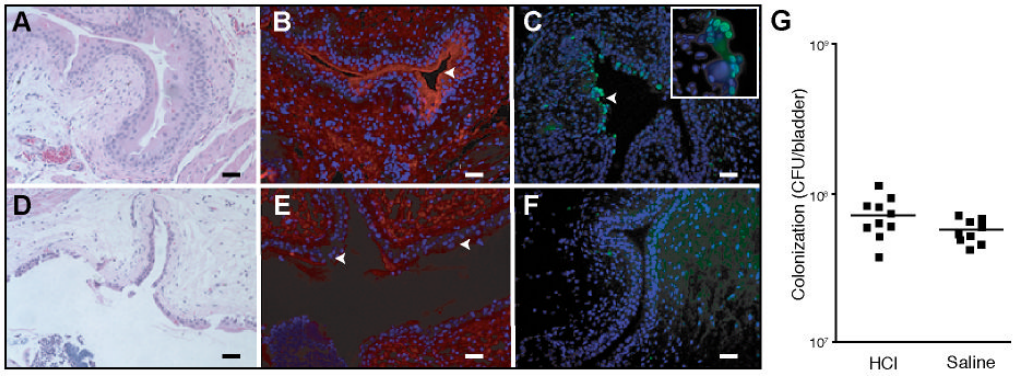


**Figure 5.**

Superficial urothelial cells are sensitive to NU14-induced apoptosis. Bladders of female C57BL/6 mice were denuded of superficial urothelial cells by instillation with 10mM HCl, and mice were subsequently infected with NU14 ($10^8$ CFU in 10μl). Panels **A-C** show representative sections from saline-treated control mice, and panels **D-F** show sections from HCl-treated mice. **A and D)** HCl-treated mice show eroded urothelium (D) relative to saline-treated mice (A) in H&E stained sections. **B and E)** HCl-treated mice lack UPIII immunoreactivity (red) in bladder sections (E), but UPIII staining is evident in the superficial epithelial layer of intact bladders (B). **C and F)** Bladder sections from NU14-infected mice were subjected to TUNEL staining (green) as a marker of apoptosis. Apoptotic cells were evident (green) in the superficial epithelial layer of saline-treated bladders (C, arrowhead and inset) but were absent in sections of HCl-treated bladders (F). **G)** Bacterial colonization was assessed in tissue homogenates of bladders harvested 2 h after infection with NU14. HCl- and saline-treated bladders exhibited similar levels of colonization. Scale bar represents 50 μm, and the nuclear stain DAPI (blue) was used for epifluorescence imaging.