Ribonuclease 7 Shields the Kidney and Bladder from Invasive Uropathogenic Escherichia coli Infection

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ABSTRACT

Background Evidence suggests that antimicrobial peptides, components of the innate immune response, protect the kidneys and bladder from bacterial challenge. We previously identified ribonuclease 7 (RNase 7) as a human antimicrobial peptide that has bactericidal activity against uropathogenic Escherichia coli (UPEC). Functional studies assessing RNase 7’s contributions to urinary tract defense are limited.

Methods To investigate RNase 7’s role in preventing urinary tract infection (UTI), we quantified urinary RNase 7 concentrations in 29 girls and adolescents with a UTI history and 29 healthy female human controls. To assess RNase 7’s antimicrobial activity in vitro in human urothelial cells, we used siRNA to silence urothelial RNase 7 production and retroviral constructs to stably overexpress RNase 7; we then evaluated UPEC’s ability to bind and invade these cells. For RNase 7 in vivo studies, we developed humanized RNase 7 transgenic mice, subjected them to experimental UTI, and enumerated UPEC burden in the urine, bladder, and kidneys.

Results Compared with controls, study participants with a UTI history had 1.5-fold lower urinary RNase 7 concentrations. When RNase 7 was silenced in vitro, the percentage of UPEC binding or invading human urothelial cells increased; when cells overexpressed RNase 7, UPEC attachment and invasion decreased. In the transgenic mice, we detected RNase 7 expression in the kidney’s intercalated cells and bladder urothelium. RNase 7 humanized mice exhibited marked protection from UPEC.

Conclusions These findings provide evidence that RNase 7 has a role in kidney and bladder host defense against UPEC and establish a foundation for investigating RNase 7 as a UTI prognostic marker or non-antibiotic-based therapy.

JASN 30: 1385–1397, 2019. doi: https://doi.org/10.1681/ASN.2018090929

Urinary tract infections (UTI), including cystitis and pyelonephritis, are among the most common infections encountered in clinical practice. In 2007, UTI accounted for >8.6 million office visits, 600,000 hospitalizations, and >$3.9 billion in hospital costs.1,2 In 2011, acute pyelonephritis accounted for over 200,000 emergency department encounters, 64,000 hospital discharges, and $378 million in inpatient hospital costs.3,4 Certain populations have increased UTI susceptibility, including sexually active and pregnant women, people
with indwelling bladder catheters, and the elderly. Acutely, UTI can lead to kidney injury, bacteremia, urosepsis, and death. Long-term UTI complications include hypertension, proteinuria, renal fibrosis, and renal insufficiency.5

Uropathogenic *Escherichia coli* (UPEC) is the causative ur-ropathogen identified in 80% of UTIs.6 To initiate cystitis, UPEC bind, invade, and replicate within the superficial uro-thelial cells of the bladder. The UPEC pathogenic cascade has been characterized extensively in murine models of cystitis.6,7 UPEC strains encode adhesive pili that are tipped with the FinH adhesin. FinH binds uroplakins coating the apical surface of human and murine bladders and facilitates bacterial invasion.8–10 With pyelonephritis, UPEC ascend into the kid-ney’s collecting tubules and attach to the intercalated cells.11,12

Tremendous progress has been made in identifying mech-anism that protect the kidneys and bladder from invading uropathogens. Evidence suggests that the innate immune re-sponse plays a pivotal role in providing front-line defense against potential pathogens.8 Antimicrobial peptides (AMPs), which form a component of the innate immune re-sponse, shield the urothelium from pathogens and have the potential to be developed as new UTI therapies. AMPs are small cationic peptides that are produced and secreted by cell types involved in host defense. Although certain AMPs exhibit a narrow antimicrobial spectrum, others show broad-spectrum antimicrobial activity. In the bladder, AMPs are produced by the urothelium. In the kidney, the collecting tube’s intercalated cells are the main source of AMP pro-daction. Although some AMPs are produced at high basal levels to prevent bacterial attachment, others are rapidly in-duced to facilitate pathogen clearance. A limited number of AMPs have been identified in the urinary tract. When AMP production is disrupted, UTI risk increases.5,13

Our research team identified ribonuclease 7 (RNase 7) as a potent and abundant AMP in the human urinary tract.14,15 RNase 7 is a 14.5-kD peptide that was first identified in the human epidermis and has broad-spectrum antimicrobial ac-tivity.16,17 RNase 7’s catalytic activity is not required for its bacterial killing. Instead, its antimicrobial functions are de-pendent on its capacity to permeate and disrupt bacterial cell membranes.15,18 Our published data suggest that RNase 7 may be an ideal AMP to develop as a UTI therapeutic target because it has potent bactericidal activity against UPEC and multi-drug-resistant (MDR) UPEC strains, minimal toxicity, is pro-duced by the bladder urothelium and kidney’s intercalated cells (cell types targeted by UPEC), and is abundant in human urine.14,15,18 On a per-molar basis, RNase 7 has been identified as one of the most potent human AMPs.20

Currently, there is limited evidence evaluating RNase 7’s antimicrobial activity in model systems. In part, evaluation of RNase 7’s antimicrobial activity is lacking because its ex-pression is absent in the laboratory mouse and is restricted to higher-order vertebrates and humans.21,22 RNase 6, the murine peptide with greatest homology to human RNase 7, is expressed by leukocytes, complicating our ability to evaluate the in vivo role of epithelial-derived RNase 7 in host defense.23

To overcome this knowledge gap, the studies in this manu-script use human urine specimens, newly developed urothelial cultures, and murine models to investigate RNase 7’s contrib-utions to urinary tract antibacterial defenses.

**METHODS**

**Patient Study Approval and Human Specimens**

Human subjects research was performed in accordance with the principles of the World Medical Association’s Declaration of Helsinki. The Nationwide Children’s Hospital (NCH) Institutional Review Board approved this study (IRB07–00383). Informed written consent was obtained from all patients. Written parental/guardian consent and patient assent were obtained for subjects <18 years of age. From November 2017 to April 2018, pediatric and adolescent females (aged 2–19 years) with a history of culture-positive UTI, but not actively infected, were recruited for study participation through NCH Nephrology or Urology Clinics. Culture-positive UTI was defined according to the American Academy of Pediatrics Guidelines.24 Because the incidence of UTI is significantly greater in females compared with males, enrollment was limited to females. In addition, because high-grade vesicoureteral reflux increases UTI risk, en-rollment was limited to females with low-grade vesicoureteral reflux (grade I–II).25 Thus, girls undergoing a voiding cystour-ethrogram were also enrolled from the NCH Radiology Depart-ment. Additional exclusion criteria and clinical variables are outlined in Supplemental Appendix 1 (Supplemental Appendix 2). Control patients had no history of abnormal renal function, structural urinary tract disorders, history of UTI, or recurrent infection. They were evaluated for nonfebrile minor medical complaints.

**RNase 7 Knock-Down by RNA Interference In Vitro**

Pooled small interfering RNA (siRNA) libraries targeting RNASE7 and a nontargeting pool (negative control) were pur-chased (ON-TARGETplus SMARTpool; Dharmacon, Lafayette,
Human 5637 urothelial cells (American Type Culture Collection, Manassas, VA) were cultured in antibiotic-free RPMI 1640 medium with 10% FBS at 75%-80% confluence in 24-well plates and transfected with a mixture of siRNA (2.5 μl/well of 5 μM stock solution), DharmaFECT transfection reagent (formulation no. 1), and serum-free medium per the manufacturer’s recommendations (Dharmacon). Culture medium was replaced 24 hours after transfection. Cells were used for the described experiments 72 hours after transfection.

Generation of RNase 7 Retroviral Constructs and Urothelial Cell Transduction

The PINCO retroviral transfer plasmid was kindly provided by Dr. Jianhua Yu (The Ohio State University, Columbus, OH).26 The PINCO retroviral vector permits RNASE7 expression from the 5’ long terminal repeat as well as the enhanced green fluorescent protein (EGFP) from an internal cytomegalovirus immediate early promoter.27,28 cDNA encoding the RNASE7 open reading frame was cloned into the BamHI and EcoRI sites of PINCO. After confirmation of cloning by bidirectional DNA sequencing, each construct was prepared for virus production by endotoxin-free maxiprep (Qiagen, Carlsbad, CA). RNASE7-overexpressing PINCO retroviral supernatants and PINCO control supernatants were generated by transient transfection of the Phoenix-Ampho packaging cell line with plasmid DNA expressing the vesicular stomatitis virus protein along PINCO-RNASE7 or empty PINCO vector as previously described.27

Human UROtsa cells, which have been previously characterized, were transduced with the resulting RNASE7 overexpression constructs and control PINCO constructs as described.27,29,30 Because of low initial infection efficiency, EGFP+ cells were isolated by FACS using a BD Influx Cell Sorter (BD Biosciences, San Jose, CA) to establish a polyclonal population with >90%-95% EGFP+ expression.

UPEC Attachment and Invasion Assays

Bacterial attachment and invasion assays were performed as described on confluent UROtsa and 5637 cells cultured in 24-well plates.31,32 After replacing fresh medium 1 hour before UPEC challenge (to minimize extracellular UPEC killing), host cells were infected with 2×10^5 CFU/well UPEC for 2 hours at 37°C. UPEC contact with host cells was expedited by centrifugation of plates at 1000 rpm for 5 minutes. Cell integrity was assessed by visual inspection and trypan blue exclusion assays.33 After UPEC incubation, one set of infected cells (three to four wells) was lysed in 0.1% Triton X-100 in PBS and plated onto Luria broth (LB)-agar plates to enumerate the total number of UPEC per well (extracellular and intracellular) to account for bacterial growth over the time period of the experiment. To assess attachment, another set of infected cells (three to four wells) was washed with PBS and then lysed in 0.1% Triton X-100 and plated on LB-agar plates. The percentage of adherent bacteria was determined as the number of CFU recovered after PBS washing divided by the total number of CFU in each well.

UPEC cellular invasion was determined using a gentamicin protection assay.32,34 An additional set of infected cells (three to four wells) was washed with PBS and incubated for an additional 3 hours in gentamicin-containing media (100 μg/ml) to kill any extracellular bacteria, which was confirmed by plating media samples. After PBS washing, cells were lysed and plated onto LB-agar plates. The percentage of intracellular bacteria was determined as the number of CFU recovered after cell lysis after incubation in the presence of gentamicin divided by the total number of CFU.

Generation of RNase 7 Transgenic Mice

All procedures involving mice were approved by NCH’s Institutional Laboratory Animal Care and Use Committee (IACUC, Protocol AR13–00057). Mouse maintenance was performed in strict accordance of the IACUC rules and regulations. RNase 7 transgenic mice were generated at TransViragen (Chapel Hill, NC under the direction of Dr. Dale Cowley). The CRISPR/CAS9 system was used in vitro to allow Cas9-mediated excision of a 23-kb RNASE7 transgene fragment from bacterial artificial chromosome clone CH17–81N4 (BACPAC Resources, Children’s Hospital Oakland Research Institute). The guide RNAs used to excise the fragment were RNASE7-g1 (target sequence: ATCCACCCCTTGTAGGACAGGGG) and RNASE7-g2 (target sequence: GGATGAAGTTAGGTCACTCATGGGGG). The Cas9-digested bacterial artificial chromosome DNA was separated on a 0.5% agarose Tris-acetate-EDTA gel at 25 V overnight and the 23-kb fragment UPEC cellular invasion was determined using a gentamicin protection assay.32,34 An additional set of infected cells (three to four wells) was washed with PBS and incubated for an additional 3 hours in gentamicin-containing media (100 μg/ml) to kill any extracellular bacteria, which was confirmed by plating media samples. After PBS washing, cells were lysed and plated onto LB-agar plates. The percentage of intracellular bacteria was determined as the number of CFU recovered after cell lysis after incubation in the presence of gentamicin divided by the total number of CFU.
RESULTS

Urinary RNase 7 Concentrations are Suppressed in Females with a History of UTI

Given RNase 7’s proposed role in host defense, we postulated that people with increased UTI susceptibility produce less RNase 7. Thus, we quantified urinary RNase 7 concentrations in healthy pediatric and adolescent females and compared them with RNase 7 concentrations in urine specimens from girls and adolescent females with a history of at least one UTI. Compared with controls, females with a UTI history had significantly lower urinary RNase 7 concentrations (Figure 1A, Supplemental Table 1). Although not reaching statistical significance, urinary RNase 7 concentrations negatively correlated with the number of prior UTI (Spearman \( r=-0.823 \), \( P=0.058 \); Figure 1B). These findings suggest that deficient urinary RNase 7 expression may increase UTI susceptibility.

Silencing RNase 7 Expression In Vitro Increases Urothelial Susceptibility to UPEC

To investigate whether suppressed RNase 7 expression affects UPEC susceptibility, RNase 7 was silenced \textit{in vitro} using siRNA. Human 5637 urothelial cells, which express high basal RNase 7 concentrations (unlike T24 cells), were transiently transfected with a RNASE7 or a nontargeting control siRNA pool. Urothelial cells were challenged with UPEC once they were reaching confluency. When RNase 7 was silenced, the percentage of UPEC binding or invading the urothelial cells significantly increased (Figure 1C, D, Supplemental Figure 2, Supplemental Table 2). These results suggest that RNase 7 serves as a front-line shield to protect the urothelium from UPEC.

RNase 7 Overexpression In Vitro Shields the Urothelium from UPEC

To confirm this phenotype, RNase 7 was overexpressed in human UROtsa cells. Unlike 5637 cells, these urothelial cells have minimal endogenous RNase 7 expression. UROtsa cells were stably infected with retroviral constructs expressing full-length RNase 7 or empty vector (Figure 2, A and B). Western blot confirmed cellular RNase 7 overexpression and ELISA quantitated RNase 7 secretion into the culture media (Figure 2, C and D). Next, a series of experiments were performed to evaluate if RNase 7 overexpression affects extracellular UPEC survival, urothelial attachment, and invasion.

1. Extracellular killing: To evaluate if secreted RNase 7 increases UPEC killing \textit{in vitro}, we collected 3-day-old culture media from confluent RNase 7–overexpressing cells and control cells. Isolated media was inoculated with UPEC. After 2 hours incubation, UPEC survival significantly decreased in media isolated from RNase 7–overexpressing cells (Figure 2E).

2. UPEC attachment: To assess if cellular RNase 7 shields the urothelium from bacterial attachment, confluent UROtsa cells were challenged with different clinical UPEC isolates, including strain UTI89, MDR-UPEC, and the avirulent mutant UTI89Δ\( {\text{fimH}} \). Compared with controls, the percentage of UPEC or MDR-UPEC adhering to RNase 7–overexpressing cells was significantly reduced (Figure 2F, Supplemental Figures 2 and 3). The attenuated UTI89Δ\( {\text{fimH}} \) showed reduced urothelial binding but attachment ratios were comparable to UTI89, suggesting that RNase 7 does not interfere with type 1 pilus-mediated bacterial adhesion (Supplemental Figure 3).

3. UPEC invasion: In parallel, gentamicin protection assays were performed. The addition of gentamicin to the culture media was sufficient to render 99.9% of UPEC non-culturable (data not shown). Gentamicin protection assays demonstrate that RNase 7 overexpression prevents UPEC invasion (Figure 2F, Supplemental Figure 2). Together, these results demonstrate that secreted and cellular RNase 7 protect the urothelium from UPEC and suggest that bacterial killing is its major upstream mechanism.

To further validate that RNase 7 protects the urothelium from pathogens, we challenged UROtsa cells with UPEC and evaluated cellular toxicity \textit{via} lactate dehydrogenase (LDH) release. After bacterial challenge, LDH release was significantly diminished in RNase 7–overexpressing cells, confirming that RNase 7 contributes to cellular defense by shielding the urothelium from UPEC (Figure 2G). LDH release did not differ between control and RNase 7–overexpressing cells, supporting prior data showing that RNase 7 lacks cellular toxicity.

Generation and Characterization of Humanized RNase 7 Transgenic Mice

To complement these \textit{in vitro} findings and investigate RNase 7’s functions \textit{in vivo}, we developed humanized RNase 7 transgenic mice by integrating a 23-kb RNASE7 transgene fragment into the mouse genome. This transgene fragment includes the human RNASE7 gene with 7397-bp 5’ and 13,601-bp 3’ flanking regions, excluding the presence of neighboring genes whose coexpression might affect the phenotypes of the transgenic mice (Figure 3A). Three founder lines were generated. Founders were crossed with C57BL/6 mice to generate hemizygous RNase 7+ mice. In their F2 offspring, we identified one founder line with significantly greater urinary tract RNASE7 expression. This founder line was selected for further characterization and, for consistency, data from this founder line (4000) are presented here. Data were normally distributed and are presented as means±SEM. Treated and control cells were compared by one-way ANOVA with the Tukey procedure. Additional methods are provided as Supplemental Appendix 1.
from the other founders are shown as supplementary information (Supplemental Figure 4).

Compared with littermate controls, RNase 7 transgenic mice had normal development, fertility, serum chemistries, and urinary tract histology (Supplemental Figures 5 and 6, Table 1). Quantitative RT-PCR and Western blot confirmed RNase 7 mRNA and peptide expression in different murine tissues with highest expression in the skin, esophagus, bladder, and kidney (Figure 3, B and C). This transgene expression pattern mirrors RNase 7 expression in humans.16,17 RNASE7 mRNA expression did not differ between male and female mice (data not shown). Western blot confirmed urinary RNase 7 expression, with urinary levels comparable to humans (Figure 3D). As observed in humans, immunostaining localized RNase 7 expression to the urothelium of the bladder and kidney’s intercalated cells (Figure 3E, Supplemental Figures 5 and 6).14

To determine if the RNase 7 transgene affects murine immune profiles, we performed complete blood counts with differential (Table 1), profiled the expression of 84 inflammatory cytokines and their receptors in noninfected kidney tissue (Figure 3F), and quantified 23 cytokines in serum and bladder homogenates (Supplemental Tables 3 and 4). Compared with controls, significant differences were not identified in erythrocyte, leukocyte, or cytokine profiles in RNase 7 transgenic mice. These results confirm that RNase 7 overexpression does not suppress endogenous murine innate immune responses.

Because of the widespread RNase 7 expression observed in these mice, we assessed whether RNase 7 expression induced gastrointestinal (GI) tract dysbiosis. A healthy GI microbiota maintains homeostasis with the host immune system to minimize GI inflammation while preventing unwanted pathogen colonization, including UPEC.37 Significant differences were not identified in the α- or β-diversity of the GI microbial communities between RNase 7 transgenic mice and littermate controls (Figure 3G). Also, diversity differences were not identified between male (n=5 mice/genotype) and female (n=4 mice/genotype) littermate controls.
mice/genotype) mice (data not shown). These results suggest that RNase 7 expression does not disrupt the GI microbiota.

Previously, we demonstrated that RNase 7 expression is greater in infected human urine samples and pyelonephritis kidney biopsy specimens. In contrast, we did not detect significant RNase 7 induction when primary human urothelial cells were challenged in vitro with UPEC. Thus, we subjected female RNase 7 transgenic mice to experimental UTI. After transurethral UPEC challenge, kidney, bladder, and urinary RNase 7 expression significantly increased (Figure 4), validating the need for an in vivo model to study this potent human AMP.

**RNase 7 Shields the Urothelium from Invasive Bacterial Infection In Vivo**

To assess whether RNase 7 expression affects UTI susceptibility, 6- to 8-week-old female hemizygous RNase 7 transgenic mice

Figure 2. RNase 7 overexpression prevents UPEC attachment and invasion in vitro. Human UROtsa cells were stably infected with retroviral constructs expressing full-length RNase 7 or empty vector. (A) Representative FACS plots show the sequential gating strategy used to enrich PINCO-infected EGFP+ cells (expressing RNase 7 or empty vector). Representative percentages of positive events are shown below each graph. P1 was used to enrich for whole cells, P2 for singlet events, and EGFP+ purified the PINCO-infected cells. After FACS, an aliquot of EGFP+ cells was analyzed to check for purity (right-most panel, 91.29% EGFP+). SSC, side scatter; FSC, forward scatter. (B) Representative micrographs of noninfected and infected EGFP+ cells. Scale bars represent 200 μm. (C) Representative Western blot, probed for RNase 7 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), confirms cellular RNase 7 overexpression. (D) ELISA-quantified RNase 7 expression in culture media. Graphs show the mean and SEM. Results are from four independent experiments performed in triplicate (n=4). (E) Extracellular UPEC kill assays were performed using isolated culture media from empty vector and RNase 7 retrovirus-infected cells as described in the methods. Graphs show the mean UPEC (strain UTI89) survival and SEM. Results are from four independent experiments performed in quadruplicate (n=4). (F) UPEC attachment and invasion assays were performed as outlined in the methods. Shown are the percentage of UPEC (strain UTI89) adhering to the cellular surface or invading the UROtsa cells. Graphs show the mean and SEM. As positive controls, gentamicin (gent) or ceftriaxone (ctx) were added to the media of empty vector–infected cells. Results are from four independent experiments performed in quadruplicate (n=4). (G) Cytotoxicity assays measured LDH release into culture media in untreated and UPEC-challenged cells. Empty vector–infected cells are depicted by the gray bars and RNase 7 infected cells by the open bars. Results are from four independent experiments performed in quadruplicate (n=4). Asterisks denote significant P values for the pairwise comparisons; (D) t test, (E–G) one-way ANOVA with Tukey modification. Graphs show the mean and SEM. *P<0.05, **P<0.01, ***P<0.001.
and littermate controls were transurethrally infected with UPEC. At 6 hours postinfection (HPI), significantly fewer intracellular bacterial communities (IBCs) were enumerated in bladders from RNase 7 transgenic mice (Figure 5A). At 24 and 48 HPI, RNase 7 transgenic mice had nearly a 99% reduction (2-log) in urinary and bladder UPEC burden compared with controls (Figure 5B). When the other RNase 7 transgenic mouse founder lines were challenged with UPEC, bacterial clearance correlated to relative RNASE7 expression (Supplemental Figure 4).

Additionally, we directly infected the bladders of male mice using a surgical inoculation method to analyze the bacterial activity of RNase 7 in the kidney. With this technique, mice develop higher kidney bacterial titers.36 After UPEC inoculation, RNase 7 transgenic mice had greater urinary UPEC clearance and significantly less bladder and kidney bacterial burden (Figure 5C). Nonmetric multidimensional scaling (NMDS), based on Bray–Curtis dissimilarity (right), identified no significant difference in β-diversity between genotypes in either the ileum or stool.
Table 1. Biometric data in RNase 7 transgenic mice and littermate controls

<table>
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<tr>
<th>Characteristics</th>
<th>RNase 7 (−) (n=6) Mean±SEM</th>
<th>RNase 7 (+) (n=6) Mean±SEM</th>
<th>P Value</th>
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<tr>
<td>Weight (g)</td>
<td>25.3±0.31</td>
<td>25.8±0.77</td>
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<td>Serum chemistry</td>
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<td>BUN (mg/dl)</td>
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<td>Creatinine (mg/dl)</td>
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<td>Glucose (mg/dl)</td>
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<td>167±17</td>
<td>0.84</td>
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<td>Complete blood count and differential</td>
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<td>WBC (K/μl)</td>
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<td>PMN (%)</td>
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<td>Lymphocyte (%)</td>
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<td>Eosinophil (%)</td>
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<td>0.79±0.26</td>
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Body weight, serum chemistry, and complete blood count values with differential leukocyte profiles in control (−) and RNase 7 transgenic mice (+). P values assess differences between genotypes (unpaired t test). WBC, white blood cell.

burden (Figure 5C). Together, these results suggest that RNase 7 has in vivo bactericidal activity and protects the upper and lower urinary tract from UPEC challenge.

To validate RNase 7’s antimicrobial activity, we isolated and cultured bladder urothelium from RNase 7 transgenic mice and littermate controls ex vivo. Once the cultured urothelial cells reached confluency, culture media was replaced. Three days later it was collected and inoculated with UPEC. Compared with controls, UPEC survival significantly decreased in media collected from the urothelium of RNase 7 transgenic mice (Figure 5D). The addition of a RNase 7–neutralizing antibody increased UPEC survival, confirming that increased UPEC killing is specific to RNase 7’s antimicrobial activity in these mice.14,33

**DISCUSSION**

Here, we provide compelling evidence that RNase 7 is an innocuous and necessary AMP that prevents UPEC infection (Figure 6). Novel findings in this manuscript demonstrate: (1) Pediatric and adolescent females with a UTI history have lower urinary RNase 7 concentrations compared with healthy controls. (2) Silencing urothelial RNase 7 expression in vitro increases UPEC susceptibility. In contrast, RNase 7 overexpression shields the urothelium from UPEC via different mechanisms: cellular and extracellular killing, inhibiting UPEC and MDR-UPEC attachment, and preventing bacterial invasion. These results suggest that RNase 7’s bactericidal activity is its major upstream mechanism. (3) RNase 7 transgenic mice show comparable RNase 7 expression profiles to humans and display limited signs of toxicity. RNase 7’s in vivo antibacterial activity prevents IBC formation and facilitates UPEC clearance. The following paragraphs highlight how these new findings, when coupled with previously published RNase 7 data, establish a foundation to develop RNase 7 as a UTI prognostic or nonantibiotic-based UTI therapy. Also, we review the role(s) of AMPs in UTI prevention and highlight the benefits of a RNase 7 in vivo model.

**RNase 7 as a UTI Prognostic**

In a cohort of girls and adolescent females with a UTI history, we show that urinary RNase 7 concentrations are suppressed and negatively correlate with the number of prior UTI. These are the first data to suggest that reduced RNase 7 expression may affect UTI susceptibility. In support of this hypothesis, we demonstrate that silencing urothelial RNase 7 production in vitro increases susceptibility to UPEC challenge. Furthermore, we previously published that inhibiting RNase 7’s antibacterial activity in human urine facilitates UPEC replication.14 Together, these findings potentially give cause to why some people have increased UTI risk and suggest that decreased urinary or cellular RNase 7 production is a risk factor for UTI. To fully address this hypothesis, a large prospective analysis is needed to determine if people with suppressed RNase 7 expression have an increased propensity to develop recurrent UTI, more severe UTI (i.e., pyelonephritis), or complicated infections. Also, because our data suggest that RNase 7 increases with acute infection, prospective studies are needed to determine if people with heightened UTI susceptibility have a delayed or lack of RNase 7 induction during an acute infection. These prospective studies may identify at-risk populations, with suppressed RNase 7 profiles, who may benefit from close UTI surveillance, antibiotic prophylaxis, or novel strategies to boost endogenous the murine innate immune capacity to confer marked resistance against UPEC. With the female transurethral UTI model, this finding was evinced by reductions in IBC formation as well as decreased UPEC titers in urine and bladder. Similarly, after direct bladder inoculation, male mice were protected from pyelonephritis. We used the direct bladder inoculation model because previously published work from our research group and others demonstrates that it is difficult to establish pyelonephritis by
transurethrally infecting nonrefluxing mice. Here, the direct bladder inoculation model is advantageous in that it allowed us to inoculate male mice, maximized the usage of generated mice (i.e., using male mice for UTI which may otherwise be euthanized), and provided an avenue to investigate the role of RNase 7 during upper urinary tract infection. Although our current experimental design does not evaluate sex-specific UTI response, our results using both models show that RNase 7 contributes to upper and lower urinary tract defense against UPEC.

Interestingly, when comparing UPEC burden in the different transgenic mouse lines after experimental UTI, bacterial clearance correlated to relative RNase 7 expression. This finding, when coupled with the data from our clinical urine specimens, further validates our hypothesis that reduced RNase 7 expression increases UTI risk. Additionally, it suggests that enhancing RNase 7 production to treat or prevent UTI may represent a paradigm shift away from the use of systemic antibiotic treatment. Potentially, this may be achieved by targeting signaling pathways or transcription factors that regulate RNase 7 expression. For example, we have shown that insulin enhances RNase 4 and RNase 7 expression via PI3K/AKT signaling. Thus, future studies may have clinical impact investigating how the insulin-PI3K/AKT-RNase 7 axis can be used to prevent or treat infections. Similarly, another potential strategy is to combine antibiotic therapy with novel immune modulators that enhance RNase 7 expression. However, this mode of therapy may not be universally applicable and may need personalization to account for patient demographics, genetic profiles, immune competence, and pathogen virulence.

**The Utility of In Vivo Models to Evaluate AMPs and UTI Defense**

Our findings provide an endorsement for the role of AMPs in UTI defense. Recent in vivo studies suggest that host defense
peptides and proteins can prevent UTI. Mice deficient in ur-nomodulin, the bactericidal AMP cathelicidin, or the iron-regulatory peptides hepcidin and lipocalin 2 are slower to eradicate UPEC infection. However, the contributions of other AMPs to urothelial defense remain unclear. Regenerating islet-derived 3 knockout mice have comparable UPEC and Gram-positive UTI susceptibility as wild-type controls.35 Mice deficient in β-defensin-1 exhibit increased rates of spontaneous Staphylococcus bacteriuria, yet transurethral UPEC inoculation of β-defensin-1 knockout mice did not demonstrate differences in renal or bladder bacterial burden compared with controls.35,46

Although many of these studies suggest that disrupted expression of selected AMPs increases UTI susceptibility, they do not directly implicate the AMP’s antibacterial activity to diminished in vivo bacterial killing. A limitation to the loss-of-function (i.e., knockout) approach for establishing in vivo bactericidal function is that the targeted genes and their corresponding peptide or protein, may have other physiologic roles that indirectly contribute to infection susceptibility.47

Figure 5. RNase 7 shields the urothelium from in vivo UPEC challenge. (A) Female RNase 7 (R7) transgenic mice and littermate controls were transurethrally infected with UPEC. IBCs were enumerated 6 HPI. (B and C) In separate experiments, (B) female and (C) male mice were infected with UPEC (strain UTI89). At the indicated time points postinfection, urine was collected, organs were harvested, and UPEC colonies were enumerated. Each point depicts UPEC burden in a different mouse. The horizontal line indicates the geometric mean of each group. Asterisks denote significant P values for the indicated pairwise comparisons (Mann–Whitney). (D) Isolated bladder urothelium from RNase 7 transgenic mice and littermate controls was cultured ex vivo to confluency. Representative Western blots probed for RNase 7, uroplakin 2 (Upk 2, an apical urothelial cell marker), uroplakin 3a (Upk 3a, an apical and intermediate urothelial cell marker), cytokeratin 14 (Krt 14, a basal urothelial cell marker), and glyceraldehyde 3-phosphate dehydrogenase (GADPH). Isolated culture media from control (−) and RNase 7 transgenic (+) urothelial cells was incubated with and without anti-RNase 7 antibody (anti-R7 Ab) or an irrelevant antibody (IgG) before UPEC inoculation. After 90 minutes incubation, the number of CFU was enumerated. Columns represent the mean and SEM. Results are from three independent experiments performed in quadruplicate (n=3). Asterisks identify significant P values for the indicated pairwise comparison, as determined by one-way ANOVA with Tukey modification. *P<0.05, **P<0.01.
Here, we use a gain-of-function model which provides a more direct approach to evaluate RNase 7’s in vivo antibacterial function. Our findings, as suggested by others, highlight the need for additional humanized models to identify human-specific factors and responses involved in UTI susceptibility or prevention. To our knowledge, this is the second published gain-of-function study showing that a human AMP shields the host from in vivo bacterial challenge. Transgenic mice expressing human defensin 5 in Paneth cells are markedly resistant to Salmonella typhimurium oral challenge.

To conclude, when our observations are combined with prior reports, they suggest that AMPs, including RNase 7, play key roles in host defense. Moreover, they underscore the potential of AMPs as UTI biomarkers and augmenting AMP production for the prevention and treatment of common infections like UTI. As the mechanisms regulating RNase 7 expression are clarified, strategies to induce RNase 7 production to prevent or treat UTI may be developed to decrease antibiotic overuse.

ACKNOWLEDGMENTS

We thank Dr. Dale Cowley (Transviragen, Inc.) for generating thease 7 transgenic mice. We thank Dr. Sheryl Justice for providing the UTI89Δ fimH strain. We acknowledge Ms. Lisa Feurer for assistance with the artwork in the abstract and Figure 6.

Dr. Spencer supervised the project and contributed to the design and interpretation of all experiments. Mr. Eichler contributed to the design and interpretation of all experiments, performed the PINCO experiments, murine urothelial isolations, and Western blot analysis. Ms. Bender, Dr. Murtha, and Ms. Metheny characterized thease 7 mouse. Ms. Bender, Dr. Murtha, and Mr. Li performed experimental UTI. Ms. Metheny managed the mouse colony, breeding, and genotyping. Dr. Bailey and Dr. Schwartz performed the microbiome studies and 16S ribosomal RNA sequencing analysis. Dr. La Perle reviewed the murine histopathology. Dr. Mosquera collected the human urine specimens and managed the Institutional Review Board protocol. Dr. Gupta and Dr. Ching performed the IBC assays. Dr. Becknell and Dr. Schwartz
contributed to the design of in vivo and/or in vitro studies and the design of the these 7 transgenic mouse. Dr. Spencer, Mr. Eichler, and Ms. Bender wrote the manuscript with input from all coauthors.

DISCLOSURES

None.

FUNDING

This work is supported by the National Institutes of Health (NIH) National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) R03 DK109242, R01 DK114035 and R01 DK115737 (awarded to Dr. Spencer). Becknell is supported by the NIH NIDDK K08 DK102594 and R03 DK118306. The Ohio State University Comparative Pathology and Mouse Phenotyping Shared Resource is supported by the NIH National Cancer Institute P30 CA016058.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2018090929/-/DCSupplemental.

Supplemental Appendix 1: Methods
Supplemental Table 1. Patient clinical demographics and laboratory studies.
Supplemental Table 2. UPEC susceptibility to antibiotics.
Supplemental Table 3. Serum cytokine profiles in non-infected and UPEC infected mice.
Supplemental Table 4. Bladder cytokine profiles in non-infected and UPEC infected mice.
Supplemental Figure 1. RNASE7 Transgene Purification.
Supplemental Figure 2. In vitro UPEC (UTI89) attachment and invasion assays (non-normalized data).
Supplemental Figure 3. In vitro UPEC attachment assays with MRD-UPEC and UTI89A fimH.
Supplemental Figure 4. RNASE7 mRNA expression in founder transgenic mouse lines.
Supplemental Figure 5. Murine bladder histology and RNase 7 urothelial localization.
Supplemental Figure 6. Murine kidney histology and RNase 7 localization.
Supplemental Appendix 2: References

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