Loss of Dicer Exacerbates Cyclophosphamide-Induced Bladder Overactivity by Enhancing Purinergic Signaling

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microRNAs (miRNAs) have regulated the expression and function of genes implicated in many pathological settings, but their impact on the pathoetiological characteristics of overactive bladder (OAB) largely remains unknown. We have generated a mouse model in which adult mice can be induced for detrusor deletion of Dicer, an enzyme essential for miRNA processing. Targeted deletion of Dicer did not lead to a significant change for detrusor functionality under physiological conditions; however, loss of Dicer exacerbated cyclophosphamide-induced OAB, manifested by the higher severity of altered detrusor contractile force and sensitivity, abnormal urodynamics, and enhanced macrophage infiltration. Mechanistic studies revealed that loss of Dicer may impair the expression of miRNAs that are capable of targeting P2x mRNAs. As a result, mice deficient in Dicer manifest enhanced P2X expression in the detrusor on cyclophosphamide treatment, predisposing to the increased risk for OAB development. More important, studies using bladder biopsy samples of patients with OAB also demonstrated similar results as those found in animals. Taken together, our results suggest that miRNAs modulate OAB susceptibility by regulating purinergic signaling, in which the pathogenic insult induces the expression of miRNAs capable of targeting P2X mRNAs to suppress OAB symptoms. (Am J Pathol 2012, 181:937–946; http://dx.doi.org/10.1016/j.ajpath.2012.05.035)

Dysfunctional voiding refers to the abnormalities in either the storage or the emptying phase of micturition and is associated with voiding disorders, such as urgency, frequency, enuresis, dysuria, or incontinence.1,2 Overactive bladder (OAB), usually associated with detrusor overactivity (DO), is a common voiding disorder, affecting approximately 12% to 17% of males and females in the general population.3,4 In line with its high prevalence, treatment of patients with OAB has been a huge economic burden in the current society. For example, in 2007, approximately $65.9 billion was expended on the health care of patients with OAB in the United States alone.5,6 Furthermore, patient quality of life is substantially affected because social, psychological, occupational, physical, and sexual functioning are all affected.7,8 Despite decades of extensive studies, the pathoetiological characteristics underlying OAB remain poorly understood.

microRNAs (miRNAs) are species of short noncoding RNAs that regulate gene expression post-transcriptionally. They are matured from longer primary RNA precursors transcribed from the genome by the RNase III enzymes, Drosha and Dicer.9 Extensive studies for the past few years highlighted a role for miRNAs in the regulation of growth, differentiation, and functionality of cardiovascular smooth muscle cells.10 As a result, altered miRNA

Supported by grants from the National Natural Science Foundation of China (Key Project Award 81130014 and Career Development Award 81100538), the European Foundation for the Study of Diabetes/Chinese Diabetes Society/Lilly Program for Collaborative Diabetes Research between China and Europe, and Bridge Funding from Tongji Hospital (hgy201002).

Accepted for publication May 30, 2012.

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expressions have been implicated in the pathogenesis of hypertension, vascular calcification, and artherosclerosis. Given that urinary detrusor is also composed of smooth muscle cells, these findings support that miRNAs could also modulate the susceptibility for OAB development. We, therefore, hypothesize that miRNAs are implicated in maintaining the homeostasis of detrusor sensitivity/contractility and dysregulation of miRNAs in the urinary detrusor, thereby predisposing to OAB susceptibility.

Because Dicer plays an essential role for miRNA processing, we, thus, generated an inducible smooth muscle–specific Dicer knockout model in a B6 background by crossing Dicerflox/flox mice with SM-CreERT2 transgenic mice to test the previously described hypothesis. Dicer deficiency exacerbates cyclophosphamide (CYP)–induced OAB, characterized by altered detrusor functionality, along with abnormal urodynamics and enhanced macrophage infiltration. Mechanistic studies revealed that targeted Dicer deletion enhances the expression of purinergic receptors, which could be because of impaired expression of miRNAs capable of targeting purinergic receptor mRNAs. More important, similar results are obtained from studies using bladder biopsy samples of patients with OAB. Together, these data suggest that miRNAs modulate the risk for OAB development by regulating purinergic signaling.

Materials and Methods

Mice and Human Bladder Samples

Dicerflox/flox mice were obtained from The Jackson Laboratory (Bar Harbour, ME). SM-CreERT2 transgenic mice with selective expression of recombinant Cre in the smooth muscle after tamoxifen induction were kindly provided by Dr. Robert Feil from the Technische Universität München (München, Germany). SM-CreERT2 mice were backcrossed with Dicerflox/flox mice to generate SM-CreERT2-Dicerflox/flox mice. Induction of Dicer deficiency in the detrusor was performed by i.p. injection of tamoxifen (25 mg/kg of body weight, dissolved in corn oil) for 5 consecutive days (defined as smDicer<sup>−/−</sup>). Littermates injected with corn oil served as controls (defined as smDicer<sup>+/+</sup>). To induce DO, 8-week-old smDicer<sup>−/−</sup> or smDicer<sup>+/+</sup> mice were i.p. injected with CYP (40 mg/kg of body weight) for 9 consecutive days. The mice were tested for OAB 3 days after last injection. All studies were conducted in compliance with the Animal Care and Use Committee guidelines in Tongji Hospital (Wuhan, China) and Georgia Health Sciences University (Augusta, GA). Bladder biopsy samples were collected from patients diagnosed as having OAB in Tongji Hospital, and normal bladder samples were obtained from three cadaveric donors that did not have dysfunctional voiding. A consent form was obtained from all subjects, and the studies were approved by the Tongji Hospital Human Assurance Committee.

Reagents and Antibodies

Antibodies for Dicer (H-212), monoclonal acetylcholine (ACh) receptor M3 (L-18), P2X1 (H-100), P2X2 (Y-25), P2X3 (H-60), P2X1, P2X3, F4/80 (Cl: A3-1), mast cell tryptase (FL-275), and β-actin (ACTBD11B7) were obtained from Santa Cruz (Santa Cruz, CA). Fluorescein isothiocyanate–labeled anti-CD2 was obtained from BD Biosciences (San Jose, CA). Mouse Ly6B.2 antibody was obtained from AbD Serotec (Raleigh, NC). CYP, DAPI, ACh, atropine, and tamoxifen were obtained from Sigma-Aldrich (St. Louis, MO).

Detrusor Contractility Assays

The mice were euthanized by halothane, and urinary bladders were removed and placed in ice-cold Dulbecco’s modified Eagle’s medium. After removing the surrounding adipose and connective tissues, the bladder body was separated from the base at the level of the urethral orifices. Four longitudinal 1.5 × 2.5-mm urinary bladder strips were then cut from the bladder wall. The strips were next mounted to a four-chamber MyoMED myograph system (Catamount, St Albans, VT) to measure detrusor contractility. They were first equilibrated at a resting load from initial 1 mN to final 3 mN in Krebs solution for 60 minutes, and then induced for contraction with 1 × 10<sup>−7</sup> to 1 × 10<sup>−3</sup> mol/L ACh. To measure electrical field stimulation (EFS)-induced contraction, the strips were subjected to EFS for 2 seconds, which was delivered with increasing frequency at 1, 2, 4, 8, 16, 32, and 64 Hz, respectively. Stimulations were delivered with a 20-V amplitude, alternating polarity between pulses, a 0.2-millisecond stimulation width, and at a 3-minute period.

Urodynamic Analysis

The mice were anesthetized with isoflurane (1% to 3% in O<sub>2</sub>, inhaled). A lower midline abdominal incision was made to expose the urinary bladder, and a polyethylene catheter (PE-10) was inserted into the dome of the urinary bladder and secured in place using a purse string suture. The bladder catheter was sealed and routed s.c. to the back of the neck, where it was coiled and stored in a skin pouch. After a 3- to 7-day recovery, the bladder catheter was exteriorized and opened. The animal was placed in a Small Animal Cystometry Lab Station (MED Associates, Inc., St Albans, VT) for urodynamic measurements. The catheter was connected to one port of a pressure transducer using a 22-gauge needle stub and a short piece of PE-50 adapter tubing. The other port of the pressure transducer was connected to a syringe pump. Sterile isotonic saline (0.9% NaCl, room temperature) was continuously infused into the bladder at a rate of 40 μL/minute. The amount of urine voided during continuous cystometry was measured by an analytical balance beneath the wire-bottom animal cage. A single cystometrogram (CMG) was defined as the simultaneous recording of infused volume, intravesical pressure, and voided volume during a single filling-voiding cycle. The capacity for bladder was measured by the amount of saline infused into the bladder until a micturition commenced. Residual volume for each CMG was defined as the difference between the infused volume and the voided volume. Minimum pressure was recorded as the lowest pressure observed during the
filling phase of a CMG. Threshold pressure was noted as the intravesical pressure right before micturition. Micturition pressure was defined as the peak intravesical pressure during voiding. Nonvoiding contractions were measured as increase in intravesical pressure that exceeds 5 mmHg, but without voiding of urine.

**Histological Analysis**

Bladders were first fixed in 4% paraformaldehyde overnight, followed by treatment with 15% sucrose for 4 hours and 30% sucrose for 4 hours, respectively. Afterwards, the specimens were placed into a mold containing OCT and then cooled at 4°C for 20 minutes. After maintaining at −80°C for at least 2 hours, tissue blocks were divided into sections (16 μm thick) with a Leica HM-325 rotary microtome (Nussloch, Germany). All sections were subjected to H&E staining, as previously described, and images were captured at ×100 magnification under a light microscope.

**Immunostaining of Bladder Sections**

Tissue sections prepared from OCT-embedded bladders were subjected to immunostaining of infiltrated immune cells. The slides were first blocked with 3% bovine serum albumin for 10 minutes, followed by staining with indicated primary antibodies against T cells (CD2), mast cells (mast cell tryptase), neutrophils (Ly6B.2), and macrophages (F4/80) for 2 hours at room temperature; this was followed by a fluorescence or biotin-labeled secondary antibody. Nuclei were stained blue by DAPI (0.2 μg/mL). After washes, the slides were treated with 50% glycerol and mounted using Faramount aqueous mounting medium (Dako, Carpenteria, CA). Images were captured at ×100 magnification under a fluorescent/light microscope.

**Western Blot Analysis**

Total proteins were prepared from bladder detrusor using radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitors (Sigma-Aldrich, St. Louis). Western blot analysis was performed as reported by probing the blots with an indicated primary antibody, followed by a horseradish peroxidase–conjugated secondary antibody. The reactive bands were visualized using an ECL PlusTM Western blot kit (PIERCE, Rockford, IL), as instructed.

**Statistical Analysis**

All data are presented as mean ± SEM. Individual comparisons were conducted with a two-tailed, unpaired,
Student's t-test. Comparisons between groups for detrusor contractility and urodynamic data were accomplished by one-way analysis of variance using SPSS 17.0 for Windows. In all cases, $P < 0.05$ was statistically significant.

Results

Dicer Is Dispensable for Detrusor Functionality under Physiological Conditions

Because loss of Dicer led to embryonic lethality, we generated SM-CreER<sup>T2</sup>-Dicer<sup>flox/flox</sup> (smDicer) mice by cross-breeding SM-CreER<sup>T2</sup> and Dicer<sup>flox/flox</sup>, in which detrusor-specific Dicer deficiency (smDicer<sup>−/−</sup>) could be induced in adult mice by tamoxifen injection. To confirm Dicer deficiency in the detrusor, 8-week-old male smDicer mice were i.p. injected with tamoxifen (25 mg/kg body weight) for 5 consecutive days, and detrusor lysates were then prepared 1 week after last induction for Western blot analysis. smDicer mice injected with vehicle (corn oil) were used as a control. As expected, Dicer was detected in the detrusor of corn oil–injected mice, but it was absent in tamoxifen-induced mice (Figure 1A).

We first sought to define the role of Dicer in detrusor functionality under physiological conditions. Interestingly, adult mice deficient in Dicer in the detrusor did not show a discernable abnormality for urinary voiding, as manifested by the similar voiding frequency and volume as their control counterparts (data not shown). Consistently, smDicer<sup>−/−</sup> mice displayed similar bladder body mass and weight as their control littermates 2 weeks after tamoxifen induction (Figure 1B). To further determine the impact of Dicer deficiency on detrusor contractile force and sensitivity, we measured detrusor contractility evoked by Ach and EFS. For this purpose, detrusor strips were prepared from both smDicer<sup>−/−</sup> and control mice and then mounted to a four-chamber MyoMed myograph system (Catamount), as described. To our surprise, detrusor strips originated from smDicer<sup>−/−</sup> mice showed comparable levels of contractile force as those of wild-type mice after Ach stimulation (Figure 1, C and D). Similarly, we failed to detect a significant difference for detrusor contractility between two groups of mice induced by EFS (Figure 1, E and F). Together, our data suggested that Dicer was dispensable for detrusor functionality under physiological conditions.

Loss of Dicer Enhances Detrusor Sensitivity and Contractile Force after CYP Induction

We next aimed to dissect the impact of Dicer on detrusor function under disease conditions, in which the mice were

Figure 2. Targeted Dicer deletion enhances CYP-induced DO. Both smDicer<sup>−/−</sup> and control mice are treated with CYP for 9 consecutive days, as described, and detrusor strips are prepared 3 days after last injection. A: Mice deficient in Dicer show significantly higher bladder body mass and weight after CYP induction. B and C: Analysis of Ach-induced detrusor contractile force and sensitivity after CYP treatment. B: A typical result for Ach-induced detrusor contractile force and sensitivity. C: Data collected from all mice studied. No perceptible differences are observed between CYP-induced control and smDicer<sup>−/−</sup> mice. D and E: Loss of Dicer promotes CYP-induced DO evoked by EFS. D: A representative result for EFS-evoked detrusor contractile force and sensitivity. E: Data derived from all mice analyzed in each group. F: EFS-evoked contractile force and sensitivity in smDicer<sup>−/−</sup> strips are even enhanced after blockade of muscarinic receptors by 1 μmol/L of atropine. The data are presented as mean ± SEM, and five mice are analyzed for each group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. 

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CYP-induced OAB. A polyethylene catheter was inserted into the dome of the urinary bladders of smDicer−/− and control mice 1 day after last CYP injection, as previously described. After 1 week of recovery, the catheter was connected to a Small Animal Cystometry Lab Station (MED Associates, Inc.) for urodynamic analysis. Consistent with the previously described results, smDicer−/− mice showed significantly higher bladder baseline pressure (18.1 ± 0.85 versus 15.3 ± 0.59 mmHg; P < 0.05), threshold pressure (26.0 ± 0.8 versus 21.4 ± 1.0 mmHg; P < 0.05), and micturition pressure (39.6 ± 1.0 versus 35.2 ± 1.2 mmHg; P < 0.05) compared with their control counterparts after CYP induction (Figure 3D). In sharp contrast, we observed a significantly lower voiding interval (2.7 ± 0.2 versus 5.6 ± 1.1 minutes; P < 0.01; Figure 3E) and micturition volume (55.5 ± 3.5 versus 112.8 ± 22.8 mg; P < 0.01; Figure 3F) in smDicer−/− mice than those of control mice. Furthermore, nonvoiding contraction (the increase of intravesical pressure exceeded 5 mmHg, without voiding of urine) was found in both control and smDicer−/− mice; it was much more frequent in the smDicer−/− mice (Figure 3, A–C). Altogether, our data demonstrated that mice deficient in Dicer in the detrusor were more susceptible to CYP-induced OAB.

**Dicer Deficiency Promotes CYP-Mediated Pathological Changes in the Bladder**

In the physiological conditions, smDicer−/− mice displayed a similar transitional epithelial layer, lamina propria, and detrusor layer as those of wild-type mice, and the epithelial layer was fully covered by the membrane plaque (Figure 4, A and B). CYP induction significantly changed bladder layers, both in smDicer−/− (Figure 4C)

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**Figure 3.** Dicer deficiency exacerbates CYP-induced OAB. Urodynamic analyses were conducted 8 days after CYP induction, as described. A typical result for a single CMG, defined by simultaneous recording of infused volume (A), intravesical pressure (B), and voided volume (C) during a filling-voiding cycle. D–F: Bar graph showing the data from three mice in each study group, with 10 micturition cycles. D: Bar graphs showing the differences for the baseline pressure (BP), threshold pressure (TP), and micturition pressure (MP). E: Bar graphs presenting the differences of time for voiding interval (VI). F: A graphic figure displaying the differences for micturition volume (MV). All data are presented as mean ± SEM. *P < 0.05, **P < 0.01.

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**Figure 4.** Histological data for bladder sections. The bladder sections are prepared and subjected to H&E staining, as described. All sections are evaluated under a light microscope. Original magnification, ×100. A: A representative bladder section from control mice. B: A representative bladder section from smDicer−/− mice. C: A bladder section for control mice after CYP induction. D: A bladder section derived from CYP-induced smDicer−/− mice. Insets: A higher magnified image is shown for each indicated location. Loss of Dicer does not result in a significant histological change for the bladder under physiological conditions. However, more significant changes are observed after CYP induction in smDicer−/− mice. The data are representative of three animals analyzed for each study group.

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**Dicer Deficiency Exacerbates CYP-Induced OAB**

Urodynamic assays were next conducted to further determine the effect of targeted Dicer deletion in the detrusor on treated with CYP to induce OAB. To this end, smDicer−/− and control male mice 2 weeks after tamoxifen induction (aged 10 weeks) were subjected to CYP treatment (40 mg/kg body weight) for 9 consecutive days. The mice were sacrificed 3 days after last treatment for analysis of detrusor functionality. Unlike the mice under physiological conditions, a significantly higher bladder body mass and weight were noted in smDicer−/− mice after CYP induction compared with that of control mice (Figure 2A). Next, detrusor strips were prepared and subjected to contractility assays, as previously described. Surprisingly, we failed to detect a significant difference for detrusor sensitivity and contractile force evoked by Ach between two groups of mice, in which they showed similar responses as those under physiological conditions (Figure 2, B and C). Nevertheless, detrusor strips derived from smDicer−/− mice displayed significantly higher sensitivity and contractile force induced by EFS at different frequencies (Figure 2, D and E). More interestingly, the differences were even enhanced after blockade of muscarinic receptors by 1 μmol/L of atropine (Figure 2F). Collectively, our data suggested that loss of Dicer enhanced CYP-induced detrusor sensitivity and contractile force, which was likely independent of Ach-mediated cholinergic signaling.
Loss of Dicer Enhances CYP-Induced Purinergic Receptor Expression in the Detrusor

Next, we sought to dissect the underlying mechanisms by which Dicer deficiency promoted CYP-induced OAB. Previous studies revealed that detrusor contractility was primarily under the control of the parasympathetic nervous system through ACh acting on its muscarinic receptors on the detrusor surface, and the muscarinic receptor subtype M3 played a predominant role in mediating detrusor contraction. We, therefore, first examined the expression of the M3 receptor in the detrusor. To our surprise, we failed to detect a significant difference between wild-type and smDicer−/− mice, both under physiological and disease conditions (Figure 6A). Given the fact that nonadrenergic, noncholinergic bladder contractions induced by ATP are also implicated in OAB pathoetiology, we compared the expression levels for P2X receptors (P2X1, P2X2, and P2X3) in the detrusor between wild-type and smDicer−/− control mice. Interestingly, P2X1, P2X2, and P2X3 were almost undetectable in both wild-type and smDicer−/− control mice under physiological conditions (Figure 6B). However, there was a significant increase on CYP induction, but their expression levels were significantly higher in smDicer−/− mice compared with their control counterparts (Figure 6B).

To further confirm the previously described results, we next examined their human counterparts, P2RX1 and P2RX3, in bladder biopsy samples derived from patients with OAB. As shown in Figure 6C, moderate levels of P2RX1 and P2RX3 were detected in the normal bladder samples, but biopsy samples that originated from patients with OAB showed significantly higher levels of P2RX1 and P2RX3 expression. Collectively, these results suggested that OAB was associated with altered P2X expression, and loss of Dicer enhanced CYP-induced P2X expression in the detrusor.

Loss of Dicer Impairs the Expression of miRNAs Capable of Targeting P2X mRNAs

To further explore how Dicer deficiency promoted CYP-induced P2X expression, we comparatively analyzed bladder miRNA expression profiles between normal and CYP-induced mice. Two miRNAs, mmu-miR-34a and mmu-miR-25, were significantly up-regulated in CYP-induced mice. Indeed, real-time PCR confirmed a 4.2-fold (Figure 7A) and a 2.5-fold (Figure 7B) increase for mmu-miR-34a and mmu-miR-25 in the detrusor in CYP-treated mice, respectively.

To obtain additional evidence, we then selectively analyzed hsa-miR-34a, a highly conserved miRNA between human and mouse, by using bladder biopsy samples
derived from patients with OAB. Similar to the studies in animals, a 2.4-fold increase for hsa-miR-34a was noted compared with that of healthy controls (Figure 7C). These results prompted us to analyze the potential targets for mmu-miR-34a and mmu-miR-25. Interestingly, a region within the 3’-untranslated region (UTR) of the mouse P2X1 gene was highly conserved between the human P2RX1 gene, which can be potentially targeted by mmu-miR-34a and hsa-miR-34a (Figure 7D). Similarly, the 3’-UTR sequence in the P2X3 gene can be potentially targeted by mmu-miR-25 (Figure 7E). Taken together, these data suggested that loss of Dicer may impair the expression of miRNAs (eg, mmu-miR-34a and mmu-miR-25) capable of targeting P2X mRNAs; as a result, mice deficient in Dicer showed enhanced P2X expression on CYP induction, which then exacerbated OAB development.

Discussion

OAB constitutes a common form of dysfunctional voiding in society and affects both men and women equally. Despite the significant impact this condition has on patients’ quality of life, OAB remains underrecognized and undertreated as a result of patient embarrassment and reluctance to seek medical help.22 Therefore, it is important to dissect the underlying mechanisms leading to OAB and to further develop novel and effective approaches for treatment of this devastating disorder. Previous large population-based studies and twin studies have consistently suggested genetic factors in OAB susceptibility, as manifested by the presence of a familial predisposition in the general population,23,24 and higher rates of probandwise concordance in monozygotic twins compared with dizygotic twins.23,25 However, genetic factors alone cannot explain such high prevalence of this disorder in the general population, suggesting additional factors, such as epigenetic factors, in disease etiology. Indeed, the impact of epigenetic factors was noted early in the past century,26 and it has recently received increased attention from the urologic society.27 For example, common factors associated with epigenetic changes, such as social stress, anxiety, depression, and living environment, affect voiding in both animal models28,29 and human patients.30–33 Consistent with earlier discoveries, the defining symptom for OAB, urgency, was independently associated with a significant increase in depression scores and sleep problems.22 Although these findings are important, experimental evidence demonstrating epigenetic modulation of OAB susceptibility is needed.

Interestingly, epigenetic mechanisms, such as DNA methylation and histone modification, regulate the expression of protein-encoding genes and miRNAs, and miRNAs, in turn, are capable of targeting genes that control epigenetic pathways.34,35 These findings support the notion that miRNAs act as a bridge to convey the effect of epigenetic pressure on the risk for OAB development. We, therefore, used an inducible detrusor-specific Dicer knockout model to dissect the role of miRNA in modulation of OAB susceptibility after CYP induction. Unexpectedly, adult mice deficient in Dicer neither exhib-
of the P2x3 samples are prepared from three cadaveric donors absent of dysfunctional voiding. miRNAs from patients with DO/OAB are prepared using three pooled bladder biopsy samples, and three pools are analyzed. miRNAs from healthy bladder

...Figure 7. Real-time PCR and bioinformatics results for miRNA analyses. A: CYP treatment induces a 4.2-fold increase for mmu-miR-34a expression in smDicer\(^{-/-}\) detrusor. B: Real-time PCR results for mmu-miR-25. CYP induces a 2.5-fold increase for mmu-miR-25 expression in smDicer\(^{-/-}\) mice compared with control vehicle-treated mice. Five mice are analyzed for each group. C: Patients with OAB show a 2.4-fold increase for hsa-miR-34a expression than that of controls. miRNAs from patients with DO/OAB are prepared using three pooled bladder biopsy samples, and three pools are analyzed. miRNAs from healthy bladder samples are prepared from three cadaveric donors absent of dysfunctional voiding. D: miRNAs are capable of targeting the

...or without CYP treatment. The M3 receptor is involved in smooth muscle contraction. CYP treatment induces a 2.5-fold increase for mmu-miR-25 expression in smDicer\(^{-/-}\) mice compared with control vehicle-treated mice. Five mice are analyzed for each group. C: Patients with OAB show a 2.4-fold increase for hsa-miR-34a expression than that of controls. miRNAs from patients with DO/OAB are prepared using three pooled bladder biopsy samples, and three pools are analyzed. miRNAs from healthy bladder samples are prepared from three cadaveric donors absent of dysfunctional voiding. D: miRNAs are capable of targeting the 3'-UTR sequences of the P2x3 gene. Dicer software is used for analysis of miRNA targets, as instructed (http://www.microRNA.org, last accessed January 28, 2012). **P < 0.01, ***P < 0.001.

...carcinic receptor M3 is predominantly responsible for bladder contraction essential for micturition under physiological conditions, as manifested by the fact that detrusor lacking the M3 receptor cannot contract as efficiently as that in wild-type mice.\(^{39}\) We, therefore, first sought to examine whether Dicer deficiency affected M3 receptor expression. Unfortunately, Dicer deficiency did not result in a significant change in the expression levels of M3 receptor, both under physiological and CYP-induced disease conditions (Figure 6A). Consistent with this observation, detrusor strips derived from smDicer\(^{-/-}\) mice showed similar contractile force and sensitivity as that of control counterparts under physiological (Figure 1C) and disease (Figure 2B) conditions after Ach stimulation. These results support the hypothesis that Dicer deficiency alters CYP-induced OAB susceptibility, probably independent of cholinergic signaling.

...neurons is the main transmitter for mediating bladder contraction and emptying in humans and mice, and the muscarinic receptor M3 is predominantly responsible for bladder contraction essential for micturition under physiological conditions, as manifested by the fact that detrusor lacking the M3 receptor cannot contract as efficiently as that in wild-type mice.\(^{39}\) We, therefore, first sought to examine whether Dicer deficiency affected M3 receptor expression. Unfortunately, Dicer deficiency did not result in a significant change in the expression levels of M3 receptor, both under physiological and CYP-induced disease conditions (Figure 6A). Consistent with this observation, detrusor strips derived from smDicer\(^{-/-}\) mice showed similar contractile force and sensitivity as that of control counterparts under physiological (Figure 1C) and disease (Figure 2B) conditions after Ach stimulation. These results support the hypothesis that Dicer deficiency alters CYP-induced OAB susceptibility, probably independent of cholinergic signaling.

...other than the muscarinic receptors, purinergic receptors (eg, P2X1, P2X2, and P2X3) are also present on urinary bladders.\(^{37,38}\) In addition, there is evidence that P2X receptors are implicated in bladder disorders of patients with carcinoma or interstitial cystitis.\(^{38–40}\) and studies in animals revealed that loss of P2x1 impaired detrusor contractile force.\(^{41,42}\) We, therefore, explored whether loss of Dicer affected P2X expression on the bladder detrusor. Interestingly, P2X1, P2X2, and P2X3 were almost undetectable for both smDicer\(^{-/-}\) and control mice, whereas they were induced on CYP treatment, but their expression levels were much higher in smDicer\(^{-/-}\) mice than those of control mice (Figure 6B); this enhanced expression was concordant with disease severity (Figure 3). These re-
sults suggest that loss of Dicer enhances CYP-induced P2X expression in the detrusor, which could then predispose animals with increased risk for CYP-induced OAB.

To further dissect the mechanisms underlying increased P2X expression in mice deficient in Dicer on CYP induction, we performed a comparative analysis of miRNA expression profiles in the detrusor between CYP-treated and control mice. CYP induced a 4.2- and a 2.5-fold increase for mmu-miR-34a (Figure 7A) and mmu-miR-25 (Figure 7B), respectively. More important, bioinformatic analysis revealed that mmu-miR-34a is capable of targeting the 3’-UTR sequence within the p2x1 gene (Figure 7D). Similarly, the 3’-UTR sequence within the P2x3 gene is a potential target for mmu-miR-25 (Figure 7E). Given the essential role Dicer played in processing miRNA maturation, these results support the notion that loss of Dicer impairs the expression of mmu-miR-34a and mmu-miR-25, which are capable of targeting P2x1 and P2x3 mRNAs, respectively. As a result, mice deficient in Dicer manifest enhanced P2X expression in the detrusor on CYP treatment, which then predisposes animals with increased susceptibility to OAB development.

Although multiple CYP injections are most commonly used to establish the animal models for OAB, a discrepancy may exist between these animal models and the clinical observations in human patients. To exclude this possibility, we validated some of our data from animal studies in the clinical setting of patients diagnosed as having OAB. We first examined P2RX1 and P2RX3 expression using bladder biopsy samples derived from patients with OAB, the two human homologous counterparts for the mouse p2x1 and P2x3 genes, respectively. Consistent with our observations in animal studies, patients with OAB displayed significantly higher levels of P2RX1 and P2RX3 expression compared with healthy controls (Figure 6C). We next analyzed hsa-miR-34a, the human homolog of mouse mmu-miR-34a, and demonstrated a 2.4-fold higher hsa-miR-34a expression in patients with OAB than that of healthy controls (Figure 7C). Similarly, hsa-miR-34a is capable of targeting the 3’-UTR sequence within the P2RX1 gene (Figure 7D). These data support the hypothesis that the capacity between individuals for induction of some miRNAs, such as hsa-miR-34a, may contribute to the differences for OAB susceptibility in the general population, in whom induced miRNAs suppress OAB symptoms by targeting P2X mRNAs in the detrusor.

In summary, the present report provides convincing evidence supporting the implication of miRNAs in OAB pathoetiological characteristics. Loss of Dicer exacerbates CYP-induced OAB in animals, which is associated with increased P2X expression in the detrusor. Dicer deficiency may impair the expression of miRNAs that are capable of targeting P2X mRNAs, which then predisposes animals with increased risk for CYP-induced OAB. Given that studies in bladder biopsy samples of patients diagnosed as having OAB revealed a similar mechanism, our data further strengthen the argument that the capacity between individuals for induction of some miRNAs that are capable of targeting detrusor P2X mRNAs may determine the differences for OAB susceptibility in the general population.

Acknowledgements

We thank Dr. Robert Feil for providing us the SM-CreER<sup>72</sup> transgenic mice, Drs. Bo Chen and Hongping Huang for their assistance with miRNA real-time PCR assays, and Dr. Yan Chen for the technical support on immunohistochemistry.

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