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Intravesical Delivery of Rapamycin Suppresses Tumorigenesis in a Mouse Model of Progressive Bladder Cancer

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Abstract Early-stage bladder cancer occurs as two distinct forms: namely, low-grade superficial disease and high-grade carcinoma *in situ* (CIS), which is the major precursor of muscle-invasive bladder cancer. Although the low-grade form is readily treatable, few, if any, effective treatments are currently available for preventing progression of nonmuscle-invasive CIS to invasive bladder cancer. Based on our previous findings that the mammalian target of Rapamycin (mTOR) signaling pathway is activated in muscle-invasive bladder cancer, but not superficial disease, we reasoned that suppression of this pathway might block cancer progression. To test this idea, we performed *in vivo* preclinical studies using a genetically engineered mouse model that we now show recapitulates progression from nonmuscle-invasive CIS to muscle-invasive bladder tumors. We find that delivery of Rapamycin, an mTOR inhibitor, subsequent to the occurrence of CIS effectively prevents progression to invasive bladder cancer. Furthermore, we show that intravesical delivery of Rapamycin directly into the bladder lumen is highly effective for suppressing bladder tumorigenesis. Thus, our findings show the potential therapeutic benefit of inhibiting mTOR signaling for treatment of patients at high risk of developing invasive bladder cancer. More broadly, our findings support a more widespread use of intravesical delivery of therapeutic agents for treatment of high-risk bladder cancer patients, and provide a mouse model for effective preclinical testing of potential novel agents.

Bladder cancer is a common malignant disease in the United States, with an estimated incidence of 71,000 cases in 2009, accounting for over 14,000 deaths (1). Although the vast majority of these cases are urothelial in origin, bladder cancer remains a heterogeneous disease with 70% of patients initially presenting with superficial disease [i.e., stages T_a, T_1, or carcinoma *in situ* (CIS)] and 30% presenting with muscle-invasive disease (i.e., stages T_2-T_4). Although usually not life threatening, nonmuscle-invasive bladder cancer recurs in as many as 50% to 70% of patients, and approximately 10% to 20% of these will eventually progress to muscle-invasive disease, which has a 5-year survival rate of <50% (2–4).

The high rate of recurrence and potential for progression is a feature of nonmuscle-invasive bladder cancer that requires close follow-up and effective management. The use of intravesical therapy (i.e., delivery of pharmacotherapeutics directly to the bladder lumen) has been used to reduce morbidity and mortality associated with bladder cancer. In particular, patients with high-risk nonmuscle-invasive bladder cancer (i.e., high grade T_a, T_1, or CIS) are frequently managed by intravesical delivery of the immunomodulator, Bacillus Calmette Guerin (BCG). Although BCG has been shown to be effective with respect to reducing disease recurrence, its efficacy for preventing disease progression remains uncertain (5). Furthermore, patients who become refractory to BCG treatment (called BCG refractory disease) are prone to rapid tumor recurrence and are at high risk of developing muscle-invasive bladder cancer (6). Concerns about progression of patients with BCG refractory disease has prompted some clinicians to offer early radical cystectomy to patients as an intervention approach before the occurrence of muscle-invasive disease; however, this management strategy subjects many patients to radical surgery who may never have progressed to invasive disease. Clearly, there is a need for more effective management of patients at risk for developing muscle-invasive bladder

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cancer, such as those with BCG refractory disease, which would obviate the need for radical surgery without sacrificing cancer control and survival.

We have been investigating new therapeutic approaches for preventing the progression of nonmuscle-invasive bladder cancer to invasive disease by pursuing preclinical studies in a genetically engineered mouse model that recapitulates key molecular events in human bladder tumorigenesis. We have previously shown that targeted deletion of two key tumor suppressor genes, namely *p53* and *Pten*, in the bladder epithelium of genetically engineered mutant mice results in the development of invasive bladder tumors that share histologic and molecular features of the human disease and display metastases as are prevalent in human bladder patients (7). Most notably, combinatorial disruption of *p53* and *PTEN* in human bladder tumors is associated with poor survival outcomes and is correlated with activation of the mammalian target of Rapamycin (mTOR) signaling pathway (7). In the current study, we now show that this genetically engineered mouse model recapitulates progression from nonmuscle-invasive CIS to muscle-invasive bladder cancer. Using this mouse model for *in vivo* preclinical analyses, we further show that Rapamycin effectively suppresses disease progression, particularly when delivered intravesically directly into the bladder lumen. Our findings suggest that mTOR inhibition may be effective for suppressing progression of high-risk bladder cancer patients, and establish a new mouse model for testing novel intravesical therapies for this high-risk patient group.

Materials and Methods

All studies using animals have been approved by the institutional review board at Columbia University Medical Center. The genetically engineered mouse model of bladder cancer used for this study has been described previously (7). Briefly, this model is based on floxed alleles for *p53* (8) and *Pten* (9), which were obtained from the National Cancer Institute Mouse Models of Human Cancer Consortium[3] and mated to compound homozygosity ($p53^{flox/flox}$; $Pten^{flox/flox}$). To achieve targeted gene deletion in the bladder epithelium, an adenovirus expressing Cre recombinase (Adeno-Cre) was obtained from the University of Iowa's Vector Core Facility (Ad5CMVCre).[4] The virus was delivered by surgical injection into the bladder lumen of $p53^{flox/flox}$; $Pten^{flox/flox}$ mice as described by Puzio-Kuter et al. (7). We have previously shown that this results in stochastic deletion of *p53* and *Pten* in bladder epithelium resulting in bladder tumors, and that deletion of both alleles of both genes is essential for the generation of such tumors.

For the current studies, Adeno-Cre was injected at 2 mo of age and mice were then monitored for up to additional 6 mo to monitor tumor growth. Alternatively, for analyses of the preinvasive phenotype, mice were sacrificed 6 wk subsequent to Adeno-Cre delivery. Unlike our previous study in which mostly male mice were analyzed, for this study, we used primarily female mice because they can be catheterized for intravesical therapy (10); however, we have shown previously that both female and male mice develop bladder tumors following deletion of *p53* and *Pten* (7). Furthermore, in the current study, the consequences of systemic treatment of Rapamycin were evaluated using both male and female mice.

For preclinical studies, Rapamycin (LC Labs) was dissolved in 100% ethanol to make a working stock of 25 mg/mL, which was then diluted to 1.25 mg/mL in 5.2% Tween 80, 5.2% PEG400 as described previously (11). Rapamycin was delivered by i.p. injection (i.e., systemically) at a dose of 10 mg/kg or through intravesical catheter (24 G Angiocatheter Jelco 4073) at a dose of 15 mg/mL in DMSO. I.p. injections were delivered five times weekly; intravesical delivery was done twice weekly with a dwell time of 1 h.

At the conclusion of the study, experimental and vehicle control mice were sacrificed; although some of the vehicle group with large tumors had to be sacrificed earlier for humane purposes. Bladders were documented by photographic inspection, their sizes and weights were determined, and processed for histologic and immunohistochemical analyses as described previously (7, 11). Antibodies used for immunohistochemical analyses were as follows: p-S6 Ribosomal Protein (Cell Signaling Ser235/236 rabbit polyclonal, 2211; 1:200), p-Akt (Cell Signaling Ser473 rabbit monoclonal, 3787; 1:50), and Ki-67 (NovoCastra rabbit polyclonal, NCL-Ki67-p; 1:2,000). Quantification of proliferating cells was done as described previously (11). Results are expressed as the percentage of KI67-labeled epithelial cells relative to the total epithelial cells. The human bladder cancer tissue microarray was obtained from Dominion/Pharmakine and includes 70 cases of T_2 muscle-invasive bladder tumors. The tissue microarray was stained with p70 S6 Kinase (Rabbit polyclonal, 9202; 1:75) from Cell Signaling and scored for positivity as in (7).

Results

Modeling early stages of bladder cancer in mutant mice

We have previously shown that combinatorial deletion of *p53* and *Pten* in the bladder epithelium by injection of Adeno-Cre into the bladder lumen of $p53^{flox/flox}$; $Pten^{flox/flox}$ mice results in bladder tumors with >95% penetrance by 6 months of age, which requires deletion of both alleles of both genes and is accompanied by metastases in the most advanced cases (7). These bladder tumors share histologic features in common with human bladder cancer including the occurrence of CIS (7). Thus, we reasoned that, if analyzed before the occurrence of overt tumors, these mutant mice may display features of nonmuscle-invasive bladder cancer.

To evaluate this possibility, we examined the bladder epithelial phenotype of $p53^{flox/flox}$; $Pten^{flox/flox}$ mutant mice from 2 weeks to 3 months subsequent to injection of Adeno-Cre, which according to our previous analyses should be subsequent to cancer initiation but before the occurrence of overt bladder tumors (7). We found that by 6 weeks of age, the majority (9 of 10) of the Adeno-Cre–injected mice but none of the control mice (0 of 10) displayed histologic features of CIS, which include a marked expansion of the bladder epithelium, many and prominent mitotic figures as well as dysplastic cells, and a pronounced underlying stromal reaction (Fig. 1A-C). The CIS phenotype was evident by histologic analyses, as well as by the presence of a 2-fold increase in proliferating cells in both the epithelium and stroma (4% Ki67-positive cells), compared with the bladder epithelium of the control mice (2% Ki67-positive cells; Fig. 1D-F). Since these Adeno-Cre–injected $p53^{flox/flox}$; $Pten^{flox/flox}$ develop a nonmuscle-invasive CIS phenotype by 6 weeks of age, which ultimately progresses to muscle-invasive bladder cancer, we conclude that these mice effectively recapitulate the development and progression of human bladder cancer.

Strategy for intravesical treatment for suppressing bladder cancer progression

We have previously shown that activation of mTOR signaling is an important feature of muscle-invasive bladder cancer

[3] http://mouse.ncifcrf.gov/
[4] http://www.uiowa.edu/~gene

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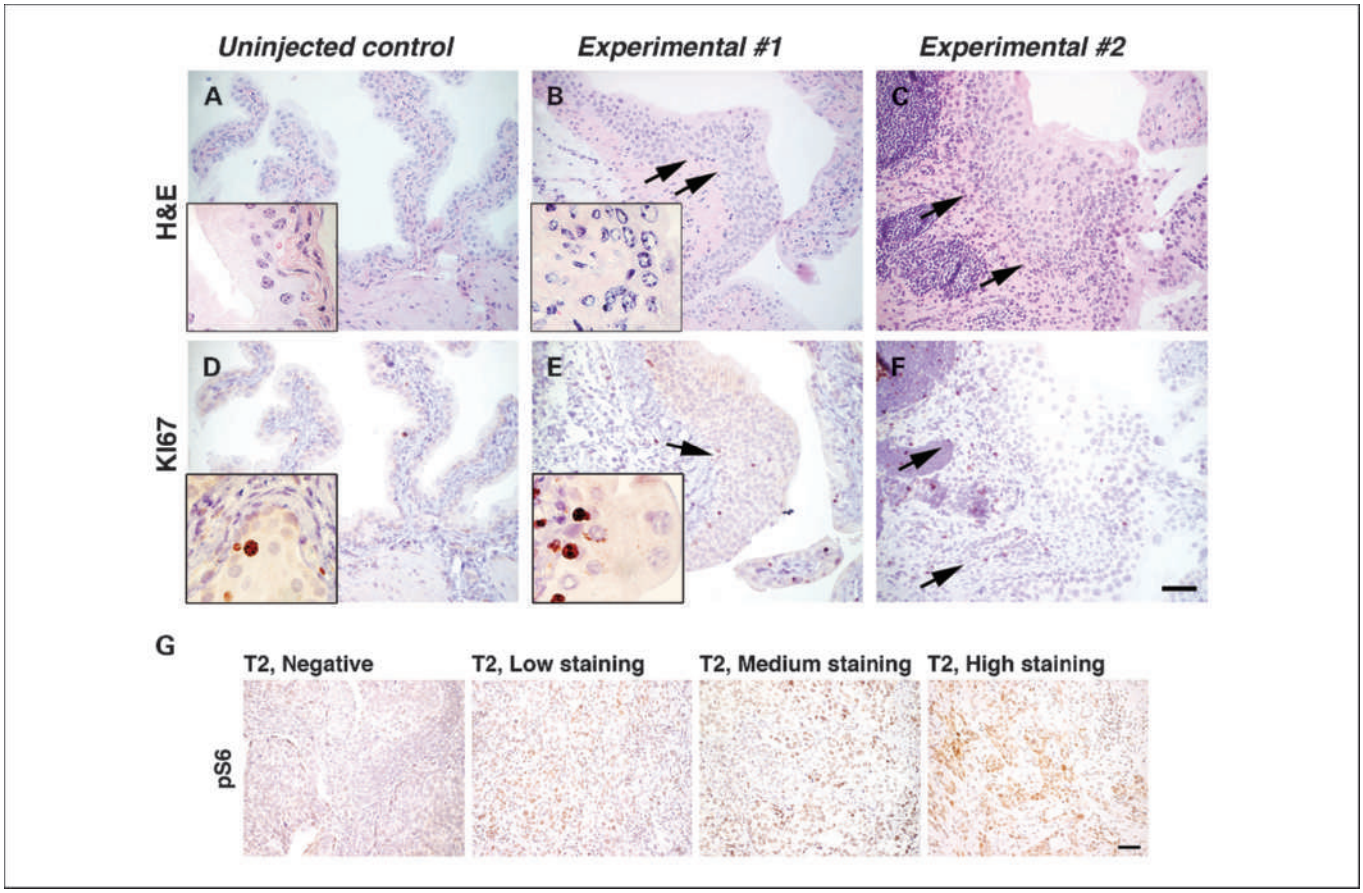

Fig. 1. Modeling early-stage bladder cancer in mutant mice. *A* to *C*, representative H&E-stained sections from bladders of a control mouse (uninjected $p53^{flox/flox}$; $Pten^{flox/flox}$) or two representative experimental mice (Adeno-Cre–injected $p53^{flox/flox}$; $Pten^{flox/flox}$) 6 wk subsequent to delivery of Adeno-Cre (or mock injection for the control). Shown are representative histologic sections from a total of 10 mice in each of the experimental and control groups. Note that the experimental, but not control, mice have a dysplastic and expanded epithelium (*arrows*) as well as abnormal stroma. *Inset*, high power view. *D* to *F*, KI67-immunostained adjacent sections show elevated proliferation in the bladder epithelium of the experimental mice relative to the control mice. *Inset*, high power view. *G*, representative images from a human bladder cancer tissue microarray showing examples of T_2 muscle-invasive tumors that are negative or have varying levels of pS6K immunostaining. *Scale bars*, 100 μm.

in both human patients as well as in the *p53; Pten* bladder cancer mouse model of (7, 3). We now further show that mTOR signaling, as evident by activation of pS6-kinase, is activated in 54 of 70 cases of T_2 muscle-invasive bladder tumors (Fig. 1G). Therefore, we next sought (*a*) to evaluate whether inhibition of mTOR signaling in mice with nonmuscle-invasive CIS could prevent its progression to muscle-invasive disease and (*b*) to investigate the consequences of delivering an mTOR inhibitor intravesically into the bladder lumen. Indeed, the bladder is one of the few organs in which agents can be delivered directly to the vicinity of the precancerous/cancerous lesions, which in rodents is achieved by insertion of a catheter into the urethra for delivery of agents directly into the bladder lumen (10). In principle, intravesical delivery offers the potential benefit of providing agents directly to the intended target cells thereby bypassing harmful effects of systemic delivery (12); however, until now, this approach has not been widely explored for delivery of new agents that target molecular pathways of bladder tumors.

Therefore, we designed a preclinical study to test the ability of an mTOR inhibitor to prevent progression of CIS to muscle-invasive bladder cancer. For this purpose, Rapamycin, an mTOR inhibitor, was delivered to $p53^{flox/flox}$; $Pten^{flox/flox}$ mice beginning at 6 to 8 weeks subsequent to tumor initiation (i.e., gene deletion through Adeno-Cre injection) and continuing up to 6 months later (Fig. 2A). As discussed above, by 6 weeks of age, the experimental (but not the control) mice develop CIS, which over time progresses to muscle-invasive bladder cancer. Therefore, the preclinical trial strategy was intended to evaluate the potential for Rapamycin to block progression from CIS to overt bladder tumors.

Furthermore, the second major goal of the study was to compare the efficacy of Rapamycin when delivered systemically (through i.p. injection) versus locally (through intravesical delivery). Thus, the Adeno-Cre–injected $p53^{flox/flox}$; $Pten^{flox/flox}$ mice were randomized into four groups (Table 1; Fig. 2A): (*a*) the systemic group consisting of vehicle and Rapamycin-treated mice (which included male and female mice), in which agent was delivered through i.p. injection five times weekly for the duration of the preclinical trial (i.e., up to 6 months); and (*b*) the intravesical group consisting of vehicle and Rapamycin-treated mice (which included only female mice), in which agent was delivered by insertion of a catheter into the urethra twice weekly for the duration of the preclinical trial (up to 6 months). All mice were monitored throughout the experiment for signs of distress and loss of body weight, which was a minimal side effect of Rapamycin delivery (<10%; data

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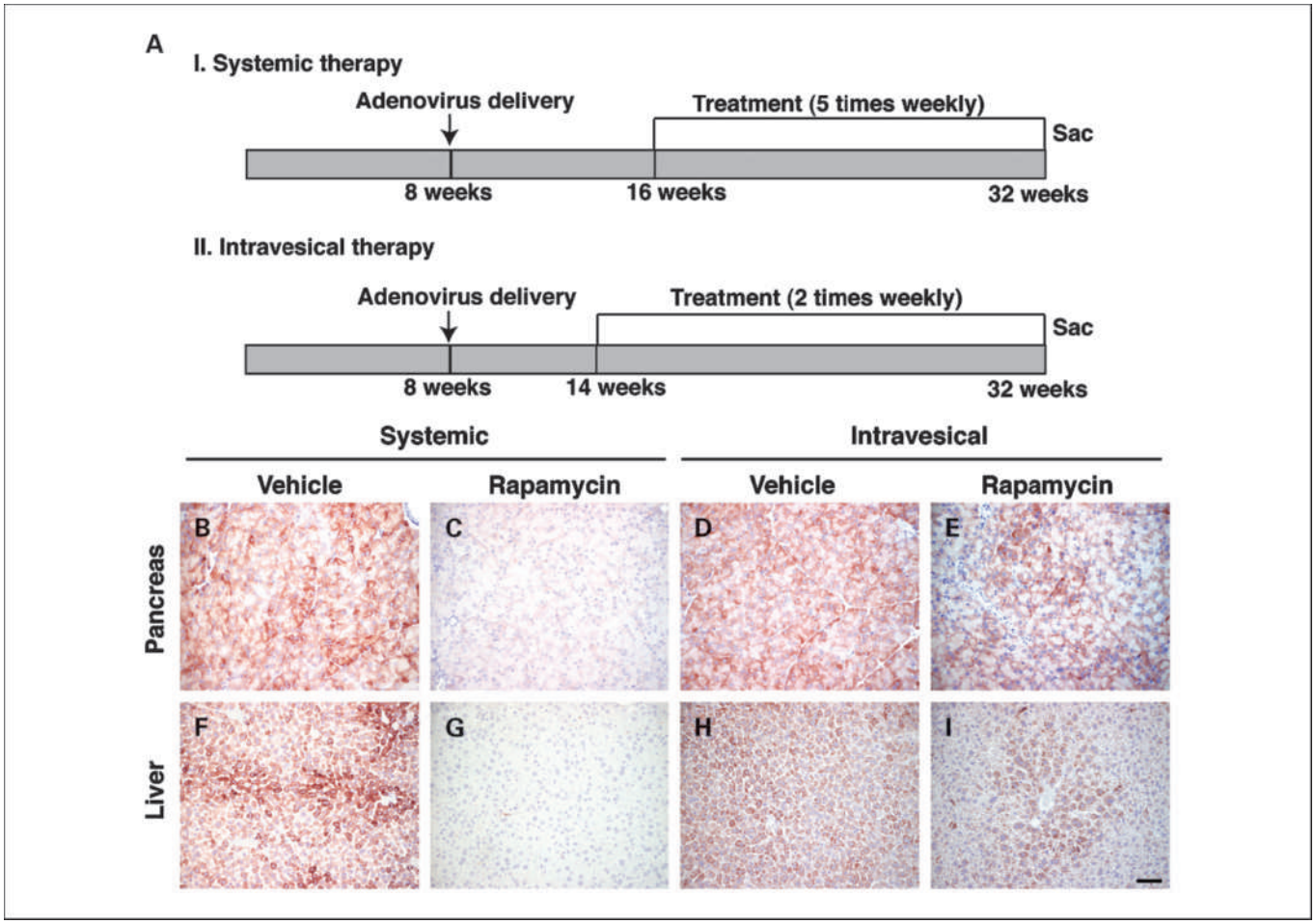

Fig. 2. Experimental strategy for inhibition of mTOR signaling in bladder cancer progression. *A,* diagram of the experimental strategy. Compound mutant mice (*p53*$^{flox/flox}$*; Pten*$^{flox/flox}$) were injected with Adeno-Cre at 8 wk of age and randomized into the systemic or intravesical groups. Beginning at 6 wk (for intravesical) or 8 wk (for systemic) later and continuing until the conclusion of the study, mice were delivered Rapamycin or vehicle through i.p. injection (systemic) or through a catheter into the bladder lumen (intravesical). At the conclusion of the study, mice were sacrificed for analyses of the bladder phenotype. *B* to *I,* intravesical delivery of Rapamycin does not inhibit mTOR activation in distant tissues. To address the potential concern that the consequences of intravesical delivery of Rapamycin are due to its absorption into the blood stream rather than its effect on the bladder epithelium, we evaluated the expression of p-S6 in the liver and pancreas. Shown are p-S6 immunostaining of the indicated tissues. Note that although i.p. delivery of Rapamycin profoundly inhibited p-S6 staining in the liver and pancreas, only a modest reduction in its expression is observed in the intravesical group. *Scale bars,* 100 μm.

not shown); therefore, the treatment was well tolerated when administered either by i.p. or intravesical delivery.

As the bladder lumen is highly vascularized, and given that the tight junctions between the umbrella cells may be disrupted by the chemical and physical trauma of the intravesical delivery, we considered whether Rapamycin delivered intravesically would indeed remain localized to the bladder epithelium or whether it might also be taken up into the blood stream and thereby have an effect on distant organs as well as the bladder. The relative efficacy of Rapamycin in the target tissue (i.e., bladder) versus other tissues is an important consideration for interpreting its effects on

Table 1. Data summary

Experimental Group	No. of mice	Tumor weight		Proliferation	
		In grams	*P*	% of total epithelial cells	*P*
Paradigm 1: systemic therapy					
Vehicle	7	2.60	0.03	50%	<0.0001
Rapamycin	10	0.45		<0.1%	
Paradigm 2: intravesical therapy					
Vehicle	16	1.80	0.001	46%	<0.0001
Rapamycin	16	0.35		2%	

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cancer progression, and also has implications for intravesical delivery of other agents, which may have more toxic effects than Rapamycin if absorbed systemically. Therefore, to evaluate whether intravesical delivery of Rapamycin remains localized to the bladder, we examined mTOR activation by immunohistochemistry in two distant tissues, namely the liver and the pancreas, which typically display robust levels of mTOR activation (Fig. 2B and F).

We found that, as expected, i.p. (systemic) delivery of Rapamycin resulted in complete inhibition of pS6 expression, a marker of mTOR activation, in the liver and the pancreas (n = 5/group; Fig. 2B, C, F, and G). In contrast, intravesical delivery of Rapamycin resulted in only a modest inhibition of p-S6 expression in the pancreas and no detectable inhibition in the liver (n = 5/group; Fig. 2B), although it results in a complete inhibition of mTOR activity in the bladder (Fig. 3K and L). These results support the idea that the effects of intravesical delivery of Rapamycin do not reflect its significant systemic absorbtion. However, as we observe some inhibition of mTOR activity in the pancreas, a small amount of Rapamycin may be absorbed; therefore, the potential for systemic absorption would need to be carefully evaluated on a case by case basis for considering intravesical delivery of other agents. Nonetheless, it is likely that potential adverse side effects of even more toxic agents can be minimized or reduced by their intravesical rather than systemic delivery, which can be evaluated in preclinical studies using the mutant mouse model described herein.

Intravesical delivery of rapamycin effectively inhibits bladder cancer progression

Having established the experimental parameters for the preclinical study, we next evaluated the consequences of

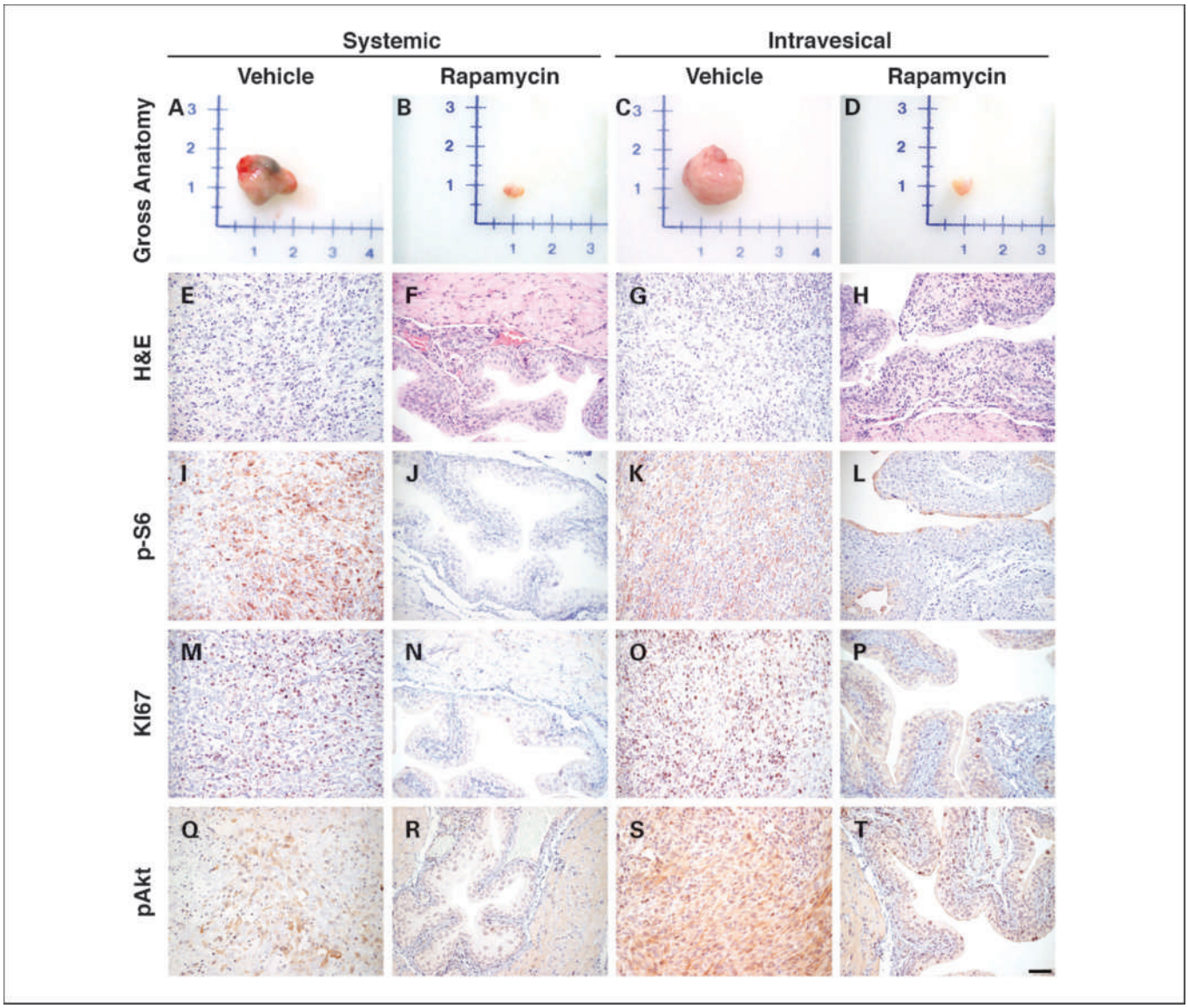

Fig. 3. Rapamycin inhibits tumor bladder tumor progression. Representative examples from the vehicle or Rapamycin-treated mutant mice showing the following: *A* to *D,* gross anatomy of bladder tissues/tumors; *E* to *H,* H&E-stained sections; or *I* to *T,* immunostained sections using the indicated antibodies. In all groups, gross analyses of bladder tissues and H&E analyses were done on all control and experimental mice in each group; immunohistochemistry was done on a minimum of four animals from each group; representative data are shown. *Scale bars,* 100 μm.

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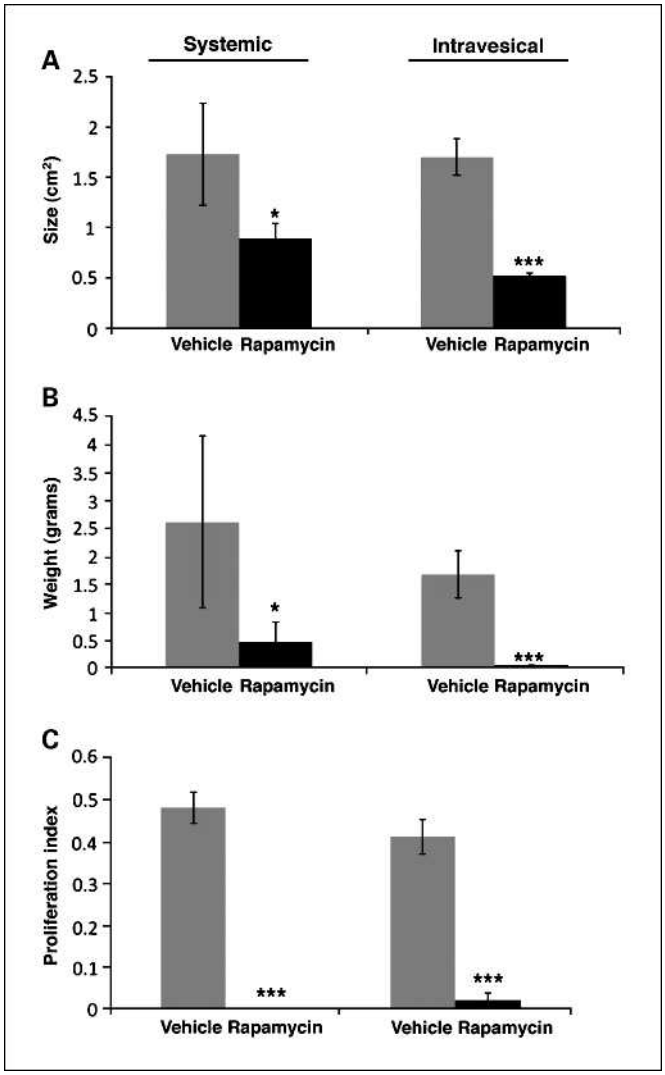

Fig. 4. Comparison of systemic and intravesical therapy with Rapamycin. For the systemic and intravesical groups treated with vehicle or Rapamycin, as indicated, shown are the following: *A*, bladder size; *B*, bladder weights; *C*, percentage of proliferating cells as determined by Ki67 staining. In all cases, the SEM is shown with *P* value (*). Data are summarized in Table 1.

Rapamycin treatment for inhibiting bladder cancer progression when delivered systemically (i.e., i.p.) versus intravesically (Table 1; Figs. 3 and 4). We found that in both delivery modes, Rapamycin was highly effective for prevention of bladder tumors as evident by visual inspection of the bladders, by quantification of their weights and sizes, by histologic analyses, and by evaluation of the proliferative index (Table 1; Figs. 3 and 4).

In particular, in contrast to the vehicle-treated mutant mice, which characteristically develop large, highly proliferative, invasive bladder tumors by 6 months (7), the bladders from the Rapamycin-treated mice were significantly smaller in size and weight and displayed histologic features more typical of normal bladder epithelium than of bladder cancer (Fig. 3A-H). Notably, the bladder weights and sizes were significantly reduced in all cases and the proliferative index of the Rapamycin-treated mice was virtually negligible relative to the profound proliferation observed in bladder tumors from the vehicle-treated control group (Figs. 3M-P and 4). The efficacy of Rapamycin for inhibiting mTOR signaling was evident from the marked inhibition of pS6 immunohistochemistry in the Rapamycin-treated mice relative to vehicle-treated ones (Fig. 3I-L). Notably, mTOR delivery was not accompanied by a complete reduction of pAkt (Fig. 3Q-T); however, because the treated mice are virtually devoid of tumor cells, we cannot be completely exclude feedback activation of pAkt as these results may reflect a virtual absence of relevant (i.e., tumor) cells. Together, these findings show that Rapamycin is highly effective for preventing the progression from nonmuscle-invasive CIS to muscle-invasive bladder cancer and suggest that activation of Akt signaling may not be an adverse side effect of this treatment.

Furthermore, although both treatment paradigms inhibited bladder tumor progression, comparison of the efficacy of Rapamycin delivered systemically versus intravesically revealed that the intravesical delivery was more effective in all end points evaluated (Table 1; Figs. 3 and 4). Importantly, the enhanced efficacy of intravesical delivery of Rapamycin was evident despite the fact that it was given twice weekly whereas the i.p. delivery was done five times weekly (Fig. 2A). In summary, these studies show that Rapamycin is highly effective for inhibition of progression from nonmuscle-invasive CIS to muscle-invasive bladder cancer, particularly when delivered directly into the bladder lumen.

Discussion

In this report, we have pursued *in vivo* preclinical studies in a mouse model of bladder tumorigenesis to investigate the efficacy of mTOR inhibition for preventing progression from nonmuscle-invasive CIS to muscle-invasive bladder cancer. We have shown that Rapamycin, a widely used inhibitor of mTOR signaling, effectively blocks the occurrence of bladder tumors when delivered to mutant mice that have developed CIS. Furthermore, we show that Rapamycin is particularly effective when delivered directly into the bladder lumen intravesically. Thus, our findings support the evaluation of Rapamycin as a therapeutic agent for high-risk patients with nonmuscle-invasive bladder cancer to prevent or delay its progression to muscle-invasive disease. Furthermore, these findings support the idea that intravesical delivery of Rapamycin should be evaluated in this high-risk patient group. More broadly, our findings raise the possibility that intravesical delivery of targeted agents may be an effective way to prevent progression of bladder cancer, and describe a genetically engineered mouse model to investigate potential therapeutic approaches delivered by this method.

Our findings support a growing body of data that have shown the efficacy of targeting mTOR signaling for treatment or prevention of multiple types of cancer (13, 14). Notably, this study extends our previous observations regarding the role of mTOR signaling in muscle-invasive bladder cancer (7), which provided the rationale for the present work. The findings presented herein, namely, the striking efficacy of Rapamycin for prevention of invasive bladder cancer, suggests that mTOR signaling may play a critical role in early stages of disease progression. Along these lines, it is noteworthy that although mTOR signaling is elevated in many tumors, the efficacy of Rapamycin as a

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single agent is limited in most tumor types (13), whereas in our preclinical analyses of bladder cancer, Rapamycin is highly effective as a single agent. This may reflect a tissue-specific role of mTOR signaling in bladder tumorigenesis, although the present data do not exclude the possibility that the findings may be a property of the genetically engineered mouse model used herein. Clearly, these studies warrant a detailed analysis of the activation of mTOR signaling in early-stage high-grade human bladder cancer, as well as the establishment of clinical trials to evaluate efficacy of Rapamycin for prevention of disease progression. Furthermore, although this study has focused on Rapamycin as an agent for mTOR inhibition, there are now several new Rapalogs (Rapamycin analogues) that display increased specificity and/or affinity for the two distinct mTOR complexes, mTORC1 and mTORC2 (14); the efficacy of these agents and their potential for intravesical delivery can be evaluated in the mutant mouse model described herein to further refine the design of clinical trials.

The risk of recurrence of patients with nonmuscle-invasive bladder cancer treated with surgical resection alone ranges from 45% to 80% (15, 16). Intravesical BCG therapy has been shown to reduce the risk of recurrence by ~40% (17), whereas recurrence rates following BCG are 46% and 69% with 5- and 10-year follow-up, respectively (18). When the currently available intravesical agents fail, patients are faced with the prospect of radical cystectomy and urinary diversion. This is a life-altering procedure that can be fraught with significant metabolic and surgical complications, and reluctance to accept this intervention is often hinged on an expected decrease in quality of life or comorbid medical conditions that preclude major surgery. Because there are currently no beneficial intravesical salvage chemotherapeutic alternatives for the estimated 50 to 60,000 patients that relapse after BCG therapy each year in the United States, identification of other efficacious treatments is paramount. In addition, because of the long natural history of the disease and the need for routine monitoring and recurrent treatment of relapse, the per patient cost of bladder cancer from diagnosis to death is the highest of all cancers in the United States, ranging from $96,000 to $187,000 per patient. The high incidence, significant progression rate, mortality risk, and health care economic burden make nonmuscle-invasive bladder cancer an ideal target for testing of effective mechanism-based targeted therapies. The current study provides the rationale for a novel treatment option for this high-risk patient group, as well as the support for the concept of targeting molecular signaling pathways for prevention of bladder cancer recurrence and progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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