Hyperactivation of Ha-ras oncogene, but not Ink4a/Arf deficiency, triggers bladder tumorigenesis

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Although ras is a potent mitogenic oncogene, its tumorigenicity depends on cellular context and cooperative events. Here we show that low-level expression of a constitutively active Ha-ras in mouse urothelium induces simple urothelial hyperplasia that is resistant to progression to full-fledged bladder tumors even in the absence of Ink4a/Arf. In stark contrast, doubling of the gene dosage of the activated Ha-ras triggered early-onset, rapidly growing, and 100% penetrant tumors throughout the urinary tract. Tumor initiation required superseding a rate-limiting step between simple and nodular hyperplasia, the latter of which is marked by the emergence of mesenchymal components and the coactivation of AKT and STAT pathways as well as PTEN inactivation. These results indicate that overactivation of Ha-ras is both necessary and sufficient to induce bladder tumors along a low-grade, noninvasive papillary pathway, and they shed light on the recent findings that ras activation, via point mutation, overexpression, or intensified signaling from FGF receptor 3, occurs in 70%–90% of these tumors in humans. Our results highlight the critical importance of the dosage/strength of Ha-ras activation in dictating its tumorigenicity—a mechanism of oncogene activation not fully appreciated to date. Finally, our results have clinical implications, as inhibiting ras and/or its downstream effectors, such as AKT and STAT3/5, could provide alternative means to treat low-grade, superficial papillary bladder tumors, the most common tumor in the urinary system.

Introduction

Cell growth is normally under the tight control of positive regulators (protooncogenes and growth factors) and negative regulators (senescence- and apoptosis-inducing molecules and tumor suppressors) of cell-cycle progression. It is the intricate balance between these 2 opposing forces that ensures the necessary tissue renewal while maintaining homeostasis (1). Such a balance is not static, but rather is quite dynamic, particularly when cells are called upon to respond to various pathogenic insults. For instance, when an oncogene is mutationally activated, affected cells can turn up senescence and tumor suppressor genes to counter the oncogenic effects, thereby regaining cell-cycle control and putting a brake on cell proliferation (2). Failure to do so could lead to uncontrolled proliferation and tumorigenesis. Hence, it is not surprising that oncogene activation is frequently found to be cooperative with tumor suppressor inactivation in inducing tumors in humans and animal models. However, exactly what type(s) of collaborative partners are involved and how they interact with one another to trigger tumorigenesis in a specific tissue may depend on the cellular context (3). Moreover, evidence remains scant as to whether the strength of oncogene activation per se can tip the balance between pro- and antiproliferative forces, thus altering the course of tumor development. Historically, far more attention has been paid to qualitative alterations (i.e., mutations and deletions) of genes involved in tumorigenesis than to quantitative ones (i.e., overexpression and amplification), even though the latter are equally prevalent in a variety of human tumors (4, 5).

Ras, discovered in 2 urothelial cancer cell lines, was the first named human oncogene (T24 and EJ) in the early 1980s (6–8). Since then, it has become one of the most frequently identified oncogenes in human tumors, but ironically, its precise role in urothelial tumorigenesis has continued to be controversial. Although Ha-ras (as opposed to Ki-ras and N-ras) remains the main target of mutational activation in bladder tumors, the mutation frequency in different patient cohorts ranges from 0% to 84%, with no satisfactory explanation for such a wide variation (9–12). Some studies have shown an association of the Ha-ras mutations with low-grade, noninvasive superficial papillary urothelial tumors (10), while others have suggested that the mutations play a role in bladder tumor invasion (13, 14); and still others found no correlation between Ha-ras mutations and tumor progression (15). Another unsettled issue is whether ras activation in the absence of activating mutations, e.g., via either overexpression of the ras gene and/or increased signaling by upstream receptor tyrosine kinases (RTKs), can lead to bladder tumorigenesis. Indeed, more than half of the human bladder tumors overexpress ras mRNA and protein (16, 17). In addition, several RTKs are believed to be constitutively active in human bladder tumors. Of particular interest is a recent flurry of independent studies revealing that point mutations in the FGF receptor 3 (FGFR3) gene that cause various forms of skeletal development disorders (18) occur in up to 80% of the low-grade, noninvasive alterations (i.e., mutations and deletions) of genes involved in tumorigenesis than to quantitative ones (i.e., overexpression and amplification), even though the latter are equally prevalent in a variety of human tumors (4, 5).

Nonstandard abbreviations used: PTEN, phosphatase and tensin homolog; RTK, receptor tyrosine kinase; UPII, urolplakin II.

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avasive papillary bladder tumors. Together, these data suggest that ras 
activation in urothelial hyperproliferation as well as in low-grade, 
noninvasive superficial papillary tumors from the heterozygous mice. The level of 
p16 and p19 mRNA is expressed as a ratio in reference to a simultaneously amplified internal 
control, GAPDH. Total urothelial proteins used for Western blotting (C) were extracted from 
wild-type mice exhibiting normal urothelia (lane 1); heterozygous UPII/Ha-ras–transgenic 
mice exhibiting urothelial hyperplasia (6 months old; lanes 2 and 3); and heterozygous mice 
exhibiting low-grade, superficial papillary tumors (15 months old; lanes 4 and 5). Urothelial 
proteins from UPII/SV40T-transgenic mice were used as positive controls for p16 and p19 anti 
odies (lanes 6 and 7). MAPK was used as a loading control. Note the marked induction of 
p16, but not p19, in ras-induced urothelial hyperplasia as well as in low-grade, superficial papil 
ary tumors. (D) Histochemical detection of senescence-associated β-galactosidase activity 
(SA-β-gal; stained at pH 6.0) and lysosomal, non–senescence-associated β-galactosidase 
activity (adjacent sections stained at pH 4.0). Urothelial tissues were from wild-type mice exhibiting 
normal urothelium, heterozygous transgenic mice exhibiting simple urothelial hyperplasia, 
and transgenic mice exhibiting papillary urothelial tumors. Note the absence of senescence 
associated β-galactosidase activity in all of the urothelial lesions. Original magnification, ×200.

Figure 1
Expression of senescence-associated markers during urothelial hyperproliferation and tumori 
genesis in UPII/Ha-ras–transgenic mice. (A–C) Expression of Ink4a gene products, p16Ink4a 
(p16) and p19Arf (p19), as assayed by real-time quantitative PCR (A and B) and Western 
blotting (C). n, number of animals assayed in each group; Normal, normal urothelia from wild 
type mice; +/- S/HP, simple urothelial hyperplasia from heterozygous transgenic mice; +/- T, 
low-grade, noninvasive superficial papillary tumors from the heterozygous mice. After a long (>10-month) incubation, about 60% of transgenic mice develop low-grade, 
noninvasive papillary bladder tumors. Such a long tumor latency and incomplete pen 
trance suggest that a rate-limiting step is present during persistent simple urothelial 
hyperplasia and that a cooperating event, such as the loss of a tumor suppressor gene, must take place in order to efficiently transform the urothelium. A notable can 
didate is the Ink4a gene, which encodes 2 protein products, p16Ink4a and p19Arf, acting through Rb 
and p53 tumor suppressor pathways, respectively (2, 25). Enforced expression of activated ras in various types of 
cultured cells often leads to an induction of p16 and/or p19, followed by premature senescence — effects that have recently been reproduced in vivo as well (26–29). Inactiv 
vating these senescence-inducing mol 
ecules has been shown to be a prerequisite for transfected cells to escape senescence and 
become transformed. Indeed, such synergistic effects have been extended into compound mice that express an activated ras while lacking a functional Ink4a gene. For instance, it has been demonstrated that 
the loss of 1 or 2 alleles of the Ink4a gene can potentiate with activated ras to induce melanoma formation in transgenic mice 
(30). Similar synergism has been observed in lung, pancreas, and brain tumorigenesis 
models (31–33). In human bladder tumors, deletion of the Ink4a locus (9p21) is one of the most common and earliest findings, occurring even in precancerous lesions 
(such as hyperplasia) and normal-appearing urothelia adjacent to the tumors (34, 
35). Although point mutation of the Ink4a gene is uncommon in human bladder cancer 
(36), loss of heterozygosity and promot 
er methylation account for more than 50% of the cases examined (22, 34, 37). Little is 
known, however, about whether ras activation 
can induce senescence-associated mol 
ecules in urothelium in vivo; if so, whether 
such a compensatory induction is sufficient to halt ras-induced proliferative effects; and, more importantly, whether Ink4a defi 
ciency can promote ras-induced bladder tumor formation.

Emerging evidence suggests that tumorigenicity of activated ras 
in a specific tissue also depends on the spectrum and the extent 
of the signaling pathways that ras exploits. The signal of activat 
ed ras is propagated mainly through 3 structurally distinct, but 
functionally overlapping, signaling cascades: (a) the Raf/Mek/ 
Erk(MAPK) cascade, which promotes cell proliferation; (b) the 
PI3K/AKT cascade, which mediates cell survival; and (c) the Ral
Because p16Ink4a induction by an activated Ha-ras in lung epithelial cells (2, 28). Thus, Ink4b (also named CDKN2B) and Dec1 (also named BHLHB2) were strongly induced in the low-grade, noninvasive papillary bladder tumors (Table 1).

Table 1

Expression of senescence-associated markers in activated Ha-ras-induced urothelial lesions by microarray analyses

<table>
<thead>
<tr>
<th>Marker</th>
<th>HP</th>
<th>T1</th>
<th>T2</th>
</tr>
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<tbody>
<tr>
<td>Ink4a (CDKN2A)</td>
<td>N/C</td>
<td>1↑</td>
<td>N/C</td>
</tr>
<tr>
<td>Arf</td>
<td>N/C</td>
<td>N/C</td>
<td>N/C</td>
</tr>
<tr>
<td>Ink4b (CDKN2B)</td>
<td>N/C</td>
<td>2.1↑</td>
<td>1.9↑</td>
</tr>
<tr>
<td>Dec1 (BHLHB2)</td>
<td>N/C</td>
<td>3.6↑</td>
<td>4.0↑</td>
</tr>
<tr>
<td>DcR2 (TNFRSF10D)</td>
<td>N/C</td>
<td>N/C</td>
<td>N/C</td>
</tr>
</tbody>
</table>

Values shown are signal ratios (log base 2) of urothelial lesions versus normal urothelium. HP, urothelial hyperplasia pooled from the bladders of 5 independent UPI1/Ha-ras-M-transgenic mice. ↑T1, tumor 1. ↑N/C, no change. ↑ denotes overexpression.

guanine nucleotide exchange factor (RalGEF)/Ral family GTPase, which was recently shown to be important for transformation and tumorigenesis (38, 39). Thus far, there is virtually no information about whether ras-mediated urothelial tumor initiation requires the preferential activation of one, a combination, or all of the effectors pathways. It is also not known whether ras downstream effectors can crosstalk with other signals such as the STAT pathway, whose activation is indispensable for tumorigenesis (40). Last, it is unclear whether the level and spectrum of downstream signal activation are dependent on the strength or level of ras activation.

In this study, we examine the biological responses of in vivo urothelial cells to ras activation; the role of Ink4a/Arf gene in suppressing urothelial proliferation; the effect of increasing the dosage of activated Ha-ras on urothelial tumorigenesis; and the signaling effectors that are required to initiate urothelial tumors. Our data indicate that it is the hyperactivation of ras-signalizing pathways, but not the lack of Ink4a/Arf, that triggers urothelial tumor formation along a low-grade, noninvasive papillary pathway. These data highlight the critical importance of the effects of quantitative changes on oncogene activation in defining the oncogene potency. They also shed light on the molecular effectors involved in the earliest stages of urothelial tumorigenesis and suggest that selectively inhibiting ras downstream effectors may be of therapeutic value in treating and preventing the recurrence of low-grade, noninvasive papillary bladder tumors, the most common form of tumors in the urinary tract.

Results

Ha-ras activation in urothelium selectively induced senescence-associated molecules without histological evidence of senescence. Enforced expression of activated Ha-ras in primary cultured fibroblasts often triggers an upregulation of prosenescence molecules, the most prominent of which are tumor suppressors p16Ink4a and p19Arf (27, 41). These molecules play critical roles in provoking premature cellular senescence in vitro, although little is known about whether such an effect occurs in urothelial cells in vivo and, if so, whether it leads to cellular senescence. By analyzing a transgenic mouse model that specifically expresses a constitutively active Ha-ras oncogene in the urothelium, we found that simple urothelial hyperplasia was associated with a slight increase in expression of p16Ink4a and that late-onset low-grade, noninvasive papillary bladder tumors were associated with a marked increase in expression of p16Ink4a, on RNA as well as protein levels (Figure 1, A and C). In contrast, p19Arf was completely unaffected in these lesions (Figure 1, B and C).

DNA microarray analyses not only provided consistent results, but identified several additional senescence-associated markers that were recently described in transgenic mice expressing an activated Ki-ras in lung epithelial cells (2, 28). Thus, Ink4b and Dec1 were strongly induced in the low-grade, noninvasive papillary bladder tumors (Table 1). No increase in expression was observed, however, with another marker, DcR2 (or TNFRSF10D), in simple urothelial hyperplasia or tumors (Table 1) or with ras-induced senescence 1 molecule (RIS1; ref. 42; data not shown). Despite the significant upregulation of several senescence-associated molecules, histological evidence of cellular senescence was not observed in Ha-ras–expressing urothelial cells, as indicated by the lack of enzyme activity of senescence-associated β-galactosidase in any of the urothelial lesions examined (Figure 1D; ref. 43). Therefore, it appears that both the spectrum of affected senescence-associated molecules and the biological consequences of ras activation are cell type dependent. Nevertheless, the increase in the expression of certain prosenescence molecules may represent an induction of compensatory mechanisms employed by the urothelial cells in response to Ha-ras activation in order to curtail cell growth and proliferation.

Ink4a/Arf deficiency failed to cooperate with Ha-ras activation in initiating urothelial tumors. Because p16Ink4a induction by an activated Ha-ras may represent a tumor-inhibiting, host-defense mechanism, we hypothesized that Ink4a/Arf deficiency, which occurs frequently in human urothelial hyperplasias (44), might accelerate ras-induced urothelial tumorigenesis. To test this hypothesis, we crossed transgenic mice expressing a constitutively active Ha-ras mutant under the control of the UPI1 promoter (UPI1/Ha-ras-M mice) with the Ink4a/Arf-knockout mice (Figure 2A). Consistent with our previous observations, UPI1/Ha-ras-M–transgenic mice developed simple urothelial hyperplasia (Figure 2B; ref. 24). Ink4a/Arf-knockout mice exhibited normal urothelia up to 8–11 months of age (Figure 2B and Table 2), at which point the mice developed fatal melanomas and soft-tissue sarcomas (data not shown) that prevented further observation (45). Surprisingly, mice transgenic for the UPI1/Ha-ras-M that were also heterozygous or nullizygous for the Ink4a/Arf gene (Figure 2B and Table 2) exhibited only simple urothelial hyperplasia, without developing any full-fledged urothelial tumors. Thus, Ink4a/Arf deficiency did not affect tumor latency in urothelia expressing an activated Ha-ras and did not cooperate with Ha-ras activation to initiate urothelial tumor formation. Due to the lethality of the Ink4a/Arf-knockout, it remains unclear whether Ink4a/Arf deficiency might promote muscle invasion of ras-induced tumors at older ages (>11 months).

High-level expression of activated Ha-ras per se was sufficient to induce rapid urothelial tumorigenesis. While Ink4a/Arf deficiency failed to accelerate urothelial tumorigenesis in transgenic mice expressing an activated Ha-ras, simply doubling the transgene dosage of the activated Ha-ras by converting the heterozygous UPI1/Ha-ras-M mice into homozygous ones (Figure 3A), which roughly doubled the mRNA and protein levels of the mutant Ha-ras (Figure 3, B–D), resulted in early-onset and rapidly growing bladder tumors. The tumors arose as early as 2 months, and by 6 months, all homozygous mice had developed full-blown bladder tumors (Figure 3F). This exceedingly short latency was in striking contrast to the long latency seen in the heterozygous mice, in which the great majority of tumors occurred after 18 months of age. In spite of the difference in tumor latency, tumors in both heterozygous and homozygous mice were consistently of low histological grade and were super-
Urothelial abnormalities in compound mice

<table>
<thead>
<tr>
<th>Urothelial phenotype</th>
<th>Ha-ras&lt;sup&gt;WT&lt;/sup&gt; Ink4a&lt;sup&gt;+/+&lt;/sup&gt; (n = 12)</th>
<th>Ha-ras&lt;sup&gt;WT&lt;/sup&gt; Ink4a&lt;sup&gt;+/–&lt;/sup&gt; (n = 10)</th>
<th>Ha-ras&lt;sup&gt;WT&lt;/sup&gt; Ink4a&lt;sup&gt;−/−&lt;/sup&gt; (n = 9)</th>
<th>Ha-ras&lt;sup&gt;WT&lt;/sup&gt; Ink4a&lt;sup&gt;−/−&lt;/sup&gt; (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>11</td>
<td>0</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Tumor</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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*Urinary bladders of compound mice (8–11 months) were examined histopathologically. n, total number of animals analyzed. *WT denotes mice heterozygous for the Ha-ras mutant.

Coactivation of AKT and STAT pathways during urothelial nodular hyperplasia and tumor formation. To determine the molecular effectors whereby Ha-ras leads to urothelial tumorigenesis, we compared the level and activation status of several major signaling molecules that act downstream of ras between age-matched hetero- and homozygous mice. Normal urothelium of wild-type mice expressed MAPK, but mostly as the inactive (underphosphorylated) form (Figure 5, A and B, upper panels). This is consistent with results of previous studies using tritium thymidine labeling indicating that most urothelial cells are in a quiescent state under normal conditions (46). Ha-ras activation in urothelia did not upregulate MAPK but markedly increased its phosphorylation, hence its activity in both hetero- and homozygous mice (Figure 5A). Some of the phosphorylated MAPK appeared to have translocated into the nuclei (Figure 5B, middle panel). Thus, MAPK activation is important for urothelial proliferation but is insufficient
to induce urothelial tumor formation. Profound differences were observed, however, in AKT expression and phosphorylation status between hetero- and homozygous animals. Increased expression of AKT, accompanied by increased phosphorylation of both Thr308 and Ser473, was seen primarily in the homozygous mice with nodular hyperplasia and papillary tumors (Figure 5A), with the phosphorylated form of Ser473 prominently detected in bladder tumors of the homozygous mice (Figure 5B, bottom row). In accordance with AKT activation, major downstream components of the AKT pathway, including glycogen synthase kinase 3β (GSK3B) and forkhead transcription factor forkhead in human rhabdomyosarcoma (FKHR), were preferentially activated in nodular hyperplasia and tumors from the homozygous mice (Figure 5A). These results strongly suggest a critical role for PI3K/AKT pathway activation in urothelial tumor initiation.

Because of the potential crosstalk between ras effectors and other signaling cascades, and because of the critical importance of the STAT pathway, particularly STAT3 and STAT5, in tumor cell/matrix crosstalk prior to and during tumorigenesis (40), we examined the activation status of these molecules during urothelial tumorigenesis. An intense upregulation of STAT3 and markedly increased phosphorylation — the latter of which has been shown to facilitate STAT3 tetramerization, nuclear translocation, and increased target transcription — were observed only in the homozygous mice (Figure 6, A and B, top row). While the level of STAT5 remained relatively constant in the transgenic mice, regardless of the zygosity, phosphorylation of the molecule was also seen primarily in the homozygous mice (Figure 6A). The activation of STAT3/5 in the homozygous mice paralleled a marked induction of VEGF and the appearance of a fibrovascular core inside the proliferative urothelial cells (Figure 6B, bottom row), suggesting a role for the stromal cells in activating the STAT pathway. Together, these data indicate that both AKT and STAT signaling occurs only when Ha-ras is hyperactivated and is strongly associated with nodular urothelial hyperplasia and tumor formation.

Phosphorylation of the C terminus of phosphatase and tensin homolog coincided with AKT and STAT activation. Since phosphatase and tensin homolog (PTEN) is a key negative regulator of AKT and STAT (47–49), we examined whether overactivation of these molecules could be attributed, at least in part, to the inactivation of PTEN.
First, the activated partner can be cell type specific (3). Precisely why activated Ink4a/Arf is not a cooperative partner of Ha-ras in urothelial tumor initiation (53). However, our result does support the important concept that not only the tumorigenicity of an oncogene but also its cooperative tumor suppressor partner can be cell type specific (3). Precisely why activated Ha-ras and Ink4a/Arf deficiency are not synergistic in the urothelium is presently unclear, but the following scenarios could be involved. First, the activated Ha-ras may require a cooperative partner other than Ink4a/Arf. For example, it cannot be ruled out that the deletion of the 9p21 locus, where Ink4a/Arf resides, actually reflects more of a loss of the adjacent Ink4b gene rather than of the Ink4a/Arf gene per se. Ink4b encodes a CDK inhibitor that is critical in negatively regulating G1/S-phase transition. Although it has not been as well studied as the Ink4a gene, there was reduced expression of the p15Ink4b product in one cohort, occurring in as many as 66% of early-stage bladder tumors (54). In our study, we observed a robust increase in expression of the Ink4b gene in response to Ha-ras even in the hyperplastic phase of urothelial proliferation (Table 1), suggesting that the loss of Ink4b might cooperate with Ha-ras activation in accelerating urothelial tumorigenesis. It would be interesting to test this hypothesis by crossing the Ink4b knockouts with the ras transgenics. Other collaborative partners of Ha-ras may also play a urothelium-specific role. For instance, we recently demonstrated that the loss of p53 significantly shortened the latency of ras-induced urothelial tumors, converting urothelial hyperplasia to full-fledged bladder tumors (55). The fact that p19Arf acts in the p53 pathway but failed to exert the same effect during Ink4a/Arf deficiency (Figure 2 and Table 2) as does p53 raises the possibility that the biological effects resulting from the deficiency of these 2 tumor suppressor genes are not equivalent.

Second, as far as the “partnership” is concerned, it is also possible that, in urothelium, Ink4a/Arf deficiency requires the cooperation of an oncogene other than Ha-ras for tumor initiation. However, even if this were the case, it would only account for a minority of the early-stage bladder tumors, because an overwhelming majority of the early-stage tumors in humans have ras pathway activation in one form or another (see below). Third, while Ink4a/Arf deficiency does not appear to be involved in bladder tumor initiation, it remains possible that it could cooperate with activated Ha-ras or another genetic event at later stages to promote bladder tumor progression. We attempted to test this hypothesis via the hyperphosphorylation of its C terminus — an event known to functionally inactivate this protein (50, 51). Unlike p16Ink4a, which was induced by Ha-ras activation, PTEN levels remained relatively constant before and after ras activation (Figure 6A). However, this protein was heavily phosphorylated at the C-terminal Ser380, most profoundly in homozygous mice (Figure 6, A and B, middle row). The result raised the interesting possibility that one of the mechanisms that allows ras hyperactivation of AKT and STAT to proceed is the functional disabling of the upstream inhibitor PTEN by hyperphosphorylation of its C terminus.

Discussion

Ink4a/Arf is not a cooperative partner of Ha-ras in urothelial tumor initiation. A surprising finding of the present study is the apparent lack of synergism between activated Ha-ras and Ink4a/Arf deficiency in urothelial tumorigenesis. The germline loss of one, or even both, alleles of the Ink4a/Arf gene failed to accelerate urothelial tumor formation in mice expressing a constitutively active Ha-ras in the urothelium (Figure 2). This result stands in stark contrast to the demonstrated synergism between these 2 genetic events in other cell types, including melanocytes, astrocytes, and lung and pancreatic epithelial cells (31–33, 52). Our result does not support the idea that since deletion of the Ink4a/Arf locus occurs frequently in the precancerous lesions of the bladder, it must be invariably involved in urothelial tumor initiation (53). However, our result does support the important concept that not only the tumorigenicity of an oncogene but also its cooperative tumor suppressor partner can be cell type specific (3). Precisely why activated Ha-ras and Ink4a/Arf deficiency are not synergistic in the urothelium is presently unclear, but the following scenarios could be involved. First, the activated Ha-ras may require a cooperative partner other than Ink4a/Arf. For example, it cannot be ruled out that the deletion of the 9p21 locus, where Ink4a/Arf resides, actually reflects more of a loss of the adjacent Ink4b gene rather than of the Ink4a/Arf gene per se. Ink4b encodes a CDK inhibitor that is critical in negatively regulating G1/S-phase transition. Although it has not been as well studied as the Ink4a gene, there was reduced expression of the p15Ink4b product in one cohort, occurring in as many as 66% of early-stage bladder tumors (54). In our study, we observed a robust increase in expression of the Ink4b gene in response to Ha-ras even in the hyperplastic phase of urothelial proliferation (Table 1), suggesting that the loss of Ink4b might cooperate with Ha-ras activation in accelerating urothelial tumorigenesis. It would be interesting to test this hypothesis by crossing the Ink4b knockouts with the ras transgenics. Other collaborative partners of Ha-ras may also play a urothelium-specific role. For instance, we recently demonstrated that the loss of p53 significantly shortened the latency of ras-induced urothelial tumors, converting urothelial hyperplasia to full-fledged bladder tumors (55). The fact that p19Arf acts in the p53 pathway but failed to exert the same effect during Ink4a/Arf deficiency (Figure 2 and Table 2) as does p53 raises the possibility that the biological effects resulting from the deficiency of these 2 tumor suppressor genes are not equivalent.

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Evidence is accumulating that overactivation of Ha-ras occurs concurrently in about 10% of the bladder tumors by means of alternative splicing of the last intron of the mutated Ha-ras gene (11, 59). Another mechanism may involve transcriptional upregulation of the mutated Ha-ras gene, as suggested by several independent studies that show that more than half of all human bladder tumors overexpress the ras gene (16, 17). Based on a conservative estimate that 30%–40% of the bladder tumors harbor Ha-ras mutations (59), there is reason to believe that ras mutation and overexpression overlap in a significant number of cases. Furthermore, activation of the ras pathway, via upstream-acting RTKs, such as FGFR3, EGFR, and Erb family proteins, are extremely prevalent in human bladder tumors (19–22). It is highly likely that these RTKs can functionally overactivate the ras pathway in the presence or absence of ras mutations.

Whether the enhanced tumorigenicity from overexpression of an activated Ha-ras applies to other oncogenes is currently unknown, but it has been documented that amplification and/or overexpression of oncogenes are common features of many human tumors (4). Hence, it seems likely that what we have learned here about the gene-dosage dependence of oncogene activation is not limited to Ha-ras but can be extended to other oncogenes. Further experimental studies are needed to investigate this idea.

The mechanism whereby overactivated Ha-ras transforms the urothelium may lie in the imbalance of positive and negative pressures exerted on cell-cycle progression. Under this paradigm, either the increase in positive forces or the decrease in negative forces could tilt the balance toward cell-cycle progression. This concept has been proven valid with tumor suppressors, in which reduced gene dosage per se, via the loss of 1 tumor suppressor gene allele by generating compound mice homozygous for activated Ha-ras and nullizygous for Ink4a/Arf; this approach failed, however, due to the infertility of the ras-homozygous mice (data not shown). Finally, Ink4a/Arf deficiency could be involved in a tumorigenic pathway that is distinct from the one involving the activated Ha-ras. It has been well established that human urothelial tumors are a mixture of biologically, phenotypically, and genetically different entities (23, 56–58). It is possible then that Ink4a/Arf and Ha-ras can act separately in divergent pathways of urothelial tumorigenesis. Definitive answers to these unresolved issues will come from further experimental analyses using both animal models and human tumor specimens.

**Tumorigenicity of Ha-ras: critical role of quantitative differences.** We were also surprised by the finding that doubling the gene dosage of the activated Ha-ras in the ras-homozygous background, even in the presence of the wild-type Ink4a gene, triggered early-onset, rapidly growing, and fully penetrant urothelial tumors throughout the urinary tract (Figures 3 and 4). This finding strongly suggests that while the low-level expression of a constitutively active Ha-ras is insufficient, even in collaboration with Ink4a/Arf deficiency, to initiate urothelial tumors, overexpression of the constitutively active Ha-ras is both necessary and sufficient to initiate urothelial tumor formation. These results highlight an important mechanism of Ha-ras activation that has not been fully appreciated to date and underscore the importance of evaluating not only structural but also quantitative aspects of Ha-ras activation when assessing the tumorigenic potential of activated Ha-ras oncogene.

Evidence is accumulating that overactivation of Ha-ras occurs in human urothelial tumors. Thus, findings reported here are relevant to human urothelial tumorigenesis as well. Czerniak et al. showed that mutation and overexpression of Ha-ras occurred concurrently in about 10% of the bladder tumors by means of alternative splicing of the last intron of the mutated Ha-ras gene (11, 59). Another mechanism may involve transcriptional upregulation of the mutated Ha-ras gene, as suggested by several independent studies that show that more than half of all human bladder tumors overexpress the ras gene (16, 17). Based on a conservative estimate that 30%-40% of the bladder tumors harbor Ha-ras mutations (59), there is reason to believe that ras mutation and overexpression overlap in a significant number of cases. Furthermore, activation of the ras pathway, via upstream-acting RTKs, such as FGFR3, EGFR, and Erb family proteins, are extremely prevalent in human bladder tumors (19–22). It is highly likely that these RTKs can functionally overactivate the ras pathway in the presence or absence of ras mutations.

Whether the enhanced tumorigenicity from overexpression of an activated Ha-ras applies to other oncogenes is currently unknown, but it has been documented that amplification and/or overexpression of oncogenes are common features of many human tumors (4). Hence, it seems likely that what we have learned here about the gene-dosage dependence of oncogene activation is not limited to Ha-ras but can be extended to other oncogenes. Further experimental studies are needed to investigate this idea.

The mechanism whereby overactivated Ha-ras transforms the urothelium may lie in the imbalance of positive and negative pressures exerted on cell-cycle progression. Under this paradigm, either the increase in positive forces or the decrease in negative forces could tilt the balance toward cell-cycle progression. This concept has been proven valid with tumor suppressors, in which reduced gene dosage per se, via the loss of 1 tumor suppressor gene allele...
Unlike most epithelial tumors that follow a single path of evolution from benign to malignant stages, we observed that during the clinical course, with more than 50% of tumors eventually metastasizing to distant organs, despite the complete surgical removal of the bladder. There is compelling evidence from clinicopathological and longitudinal studies that most muscle-invasive tumors are not derived from the low-grade, noninvasive papillary tumors. Rather, they seem to arise de novo or are derived from high-grade, flat, carcinoma-in-situ lesions. Genetic studies of muscle-invasive bladder tumors from humans suggest that more than half of these lesions harbor structural and functional defects in the components of p53 and/or Rb pathways (62, 63). Therefore, the current consensus is that these p53/Rb defects may underlie urothelial tumor development along the invasive pathway. By contrast, the genetic defect(s) that drive urothelial tumor formation along the low-grade, noninvasive pathway have been long sought after, with little success, due partly to the controversy surrounding the role of Ha-ras activation. In this study, we demonstrated that hyperactivation of Ha-ras induced urothelial tumors that were consistently of low pathological grade, papillary, and noninvasive. These data provide direct and strong experimental evidence indicating that hyperactivation of the ras signaling pathway is responsible for the low-grade, noninvasive papillary bladder tumors (Figure 7).

In direct support of our transgenic data is the recent finding that humans with Costello syndrome, which is caused by germline mutations in the Ha-ras gene, are highly predisposed to developing early-onset bladder tumors (64). Interestingly, all 3 cases of bladder tumors reported so far are of low pathological grade, papillary, and recurrent (65–67). These data strongly and independently support the role of ras activation in this distinct phenotypic pathway of bladder tumorigenesis.

Coactivation of AKT and STAT pathways is required for urothelial tumorigenesis. We observed that during Ha-ras hyperactivation, the preferential activation of certain signaling pathways is essential for urothelial tumor initiation. While activation of the MAPK pathway was sufficient to induce simple urothelial hyperplasia, as observed in heterozygous mice, this pathway alone did not lead to urothelial tumor initiation (Figures 3–6 and Figure 7). In contrast, the AKT pathway underwent profound changes only in the homozygous mice that exhibited severe nodular hyperplasia and urothelial tumors (Figure 5). These changes in AKT included its phosphorylation at both Thr308 and Ser473, nuclear translocation, and activation of its downstream targets AKT and STAT pathways, leading to the formation of nodular hyperplasia and low-grade, noninvasive urothelial tumors. The activation of the STAT pathway that is unique to nodular hyperplasia and urothelial tumors, both of which contain mesenchymal components, may result from signaling from growth factors (GFs) and cytokines (CTKs) produced by the tumor mesenchyme. The activation of AKT and STAT pathways may also be facilitated by the functional inactivation of their upstream inhibitor, PTEN, via its C-terminal hyperphosphorylation. FGFR3 mutations, which occur in up to 80% of low-grade, noninvasive human bladder tumors, are likely to transmit the signals along pathways similar to those described above. C, carboxyl terminus.

Figure 7
Schematic diagram of signaling effectors underlying the genesis of low-grade, noninvasive papillary urothelial tumors. Low-level expression of a constitutively active ras-GTPase (ras-GTP) activates the MAPK pathway and converts quiescent normal urothelial cells into simple urothelial hyperplasia, which is persistent for an extended period (8–10 months) without progressing to urothelial tumors. In contrast, overactivation of the ras-GTPase profoundly activates survival and angiogenesis signals along PI3K/AKT and STAT pathways, leading to the formation of nodular hyperplasia and low-grade, noninvasive urothelial tumors. The activation of the STAT pathway that is unique to nodular hyperplasia and urothelial tumors, both of which contain mesenchymal components, may result from signaling from growth factors (GFs) and cytokines (CTKs) produced by the tumor mesenchyme. The activation of AKT and STAT pathways may also be facilitated by the functional inactivation of their upstream inhibitor, PTEN, via its C-terminal hyperphosphorylation. FGFR3 mutations, which occur in up to 80% of low-grade, noninvasive human bladder tumors, are likely to transmit the signals along pathways similar to those described above. C, carboxyl terminus.
context of Ha-ras hyperactivation. Although ras itself is capable of activating the AKT pathway by directly interacting with the regulatory subunit of PI3K (69), inactivating a potent upstream inhibitor, PTEN, within the signaling pathway could be regarded as an additional safeguard employed by tumor cells to free themselves from the “check and balance” and to allow for signal amplification to go forward. Similarly, the cooperativity between ras activation and loss of PTEN allele(s) has recently been shown from transgenic models of skin and ovarian tumorigenesis (70, 71), suggesting that this mechanism is not confined to the urothelium. In human bladder tumors, while somatic mutations of PTEN are rare, loss of heterozygosity of the PTEN locus is fairly common, occurring in about 25% of the cases (72–74). Our study suggests yet another mechanism whereby PTEN can be inactivated during urothelial tumor formation. This underscores the importance of studying the functional status of PTEN when assessing the involvement of this protein in the tumorigenesis of bladder and other tissues (Figure 7).

In addition to AKT overactivation, we also observed a profound activation of STAT3 and STAT5, as evidenced by their elevated expression and phosphorylation, almost exclusively in the homozygous Ha-ras mice (Figure 6). As members of the latent STAT gene family, STAT3 and STAT5 are considered oncogenes that are persistently activated in a variety of tumors in response to growth factors and cytokines produced by the extracellular matrix (40). Phosphorylation of STAT3 and STAT5 promotes homodimerization and nuclear translocation of the proteins and increases transcription of targets critical for cell growth, survival, and angiogenesis (40). The fact that these 2 proteins were not activated at all during simple hyperplasia of the heterozygous ras mice but were markedly activated in nodular hyperplasia and tumors where a substantial amount of extracellular matrix comes in close contact with the tumor cells reflects the importance of signaling crosstalk between transformed urothelial cells and their microenvironment in establishing and maintaining tumor growth (Figure 7). Another possible mechanism of STAT activation may be related to the functional inactivation of PTEN, which normally inhibits not only the PI3K/AKT pathway, but also the STAT pathway (48, 75). Given the remarkable degree of AKT and STAT activation in low-grade, noninvasive urothelial tumors, selectively inhibiting these molecules and/or restoring the function of their upstream antagonist such as PTEN will likely have an important therapeutic effect in treating and preventing the recurrence of this type of bladder tumor.

**Methods**

Transgenic and knockout mice. UPII/Ha-ras-M transgenic mice were produced to express a constitutively active rabbit Ha-ras protooncogene (codon Q61L) under the control of a 3.6-kb mouse UPII promoter (24). This ras mutant was previously shown to share all the functional characteristics of codon 12 and 13 mutants and to be fully capable of transforming cultured NIH 3T3 cells (76). Urothelium-specific expression of the transgene mutant was established by RT-PCR, Western blotting, and immunohistochemical staining. One of the transgenic lines that harbored 1 copy of the UPII/Ha-ras-M transgene (previously referred to as a “low-copy” line; ref 24) was used as a starting material for intra- and intercrossing in this study. Southern blotting of Neo-digested tail DNA using a probe located at the 3’ end of the UPII promoter was used to identify a transgene fragment (1.7 kb) and an endogenous UPII gene fragment (1.4 kb). The X-films containing the 2 gene fragments were scanned to quantify the transgene dosage in reference to the endogenous gene. In heterozygous mice, the ratio of the transgene to the endogenous UPII gene was approximately 1:2. Intercrossing heterozygous mice produced homozygous mice, which harbored twice as much transgene as the heterozygous counterparts, as evidenced by the 1:1 ratio of the transgene to the endogenous UPII gene in the homozygotes.

Inbk4a/Arf knockout mice were obtained from Ronald DePinho (Harvard Medical School, Boston, Massachusetts, USA) (45). Since exons 2 and 3 of the Inbk4a gene in these mice were replaced by a neo gene, the mice did not express p16Ink4a or p19Arf, 2 spliced variants of the Inbk4a gene. Genotyping was performed using Southern blotting of Psf-digested tail DNA with “probe A,” which detected a 9.2-kb wild-type allele and a 6.0-kb knockout allele. For analysis of the potential synergism between ras activation and Inbk4a/Arf deficiency, UPII/Ha-ras-M heterozygous mice were crossed to homozygous Inbk4a/Arf knockout mice. Offspring heterozygous for both genotypes were backcrossed to the Inbk4a/Arf knockout mice to yield mice heterozygous for the UPII/Ha-ras-M transgene and nullizygous for Inbk4a/Arf. All experiments on animals received prior approval from the institutional animal care and use committee of New York University School of Medicine and conformed to the standards set by appropriate regulatory agencies.

**Real-time quantitative PCR.** The expression of p16Ink4a, p19Arf, and rabbit Ha-ras transgene mutant in mouse urothelia was quantified on an mRNA level by real-time quantitative PCR using a LightCycler RNA amplification kit (Roche Diagnostics). Total RNAs were extracted using an RNA extraction kit (Promega) from mouse urothelia exhibiting normal morphology, simple urothelial hyperplasia, or low-grade superficial papillary tumors; reverse-transcribed; and used as templates. Standards for quantification were generated for each gene in the form of plasmids. Thus, p16Ink4a and p19Arf cDNAs were obtained using RT-PCR from high-grade bladder tumor cells of double transgenic mice expressing both an activated Ha-ras and a SV40 large T antigen. The cDNAs were sequence verified and cloned into a pCRII TA cloning vector (Invitrogen). For rabbit Ha-ras quantification, a plasmid harboring the UPII/Ha-ras-M transgene (24) was used. These 3 plasmids were serially diluted (at 2 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, and 0.1 pg/μl) and used as standards to generate amplification curves during real-time PCR. Oligonucleotide primers used were: p16-forward, 5′-AGTCCCGCTGACAGACGGT-3′; p19-forward, 5′-CTTG- GGTCACTGTTAGGATTCC-3′; p16- and p19-reverse (common primer), 5′-GGGGAAGAGTTAGTGGGTGTC-3′; rabbit Ha-ras–forward, CGGC- GGTGATGCAAGACGGC-3′; and rabbit Ha-ras–reverse, 5′-TCCTTG- GCCGAGGTCTCTGAGTA-3′. PCR-amplified products for p16Ink4a were detected by 2 specific internal probes labeled with FITC (5′-GCGTGGTG-GCATGAGTGT-FITC-3′) or with LCRed640 (5′-LCRed640-GGCACCAGCCAATCACCCACCTGCTCCAC-3′) and LCRed640-GAGGTGCTGCTTGCGCT-3′. PCR products for Ha-ras were detected by direct incorporation of an intercalating fluorescent dye (SYBR Green I; Roche) into newly amplified DNA during PCR. Housekeeping gene product GAPDH was amplified in parallel as a normalization control by direct SYBR Green I incorporation using 2 specific primers (forward: 5′-TCCCGGAGACCCAGGGAACAC-TCC-3′ and 5′-LCRed640-GAGGTGCTGCTTGCGCT-3′). Product of rabbit Ha-ras was detected by direct incorporation of an intercalating fluorescent dye (SYBR Green I; Roche) into newly amplified DNA during PCR. Housekeeping gene product GAPDH was amplified in parallel as a normalization control by direct SYBR Green I incorporation using 2 specific primers (forward: 5′-TCCCGGAGACCCAGGGAACAC-TCC-3′ and 5′-LCRed640-GAGGTGCTGCTTGCGCT-3′). Product of rabbit Ha-ras was detected by direct incorporation of an intercalating fluorescent dye (SYBR Green I; Roche) into newly amplified DNA during PCR. Housekeeping gene product GAPDH was amplified in parallel as a normalization control by direct SYBR Green I incorporation using 2 specific primers (forward: 5′-TCCCGGAGACCCAGGGAACAC-TCC-3′ and 5′-LCRed640-GAGGTGCTGCTTGCGCT-3′). Product of rabbit Ha-ras was detected by direct incorporation of an intercalating fluorescent dye (SYBR Green I; Roche) into newly amplified DNA during PCR.
cRNA was then hybridized to the GeneChip Mouse Genome 430 2.0 arrays (Affymetrix). A single 430 2.0 array contains 45,000 probe sets representing more than 39,000 transcripts and variants from more than 34,000 well-characterized mouse genes as well as uncharacterized expressed sequence tags (ESTs). Each gene is represented by at least 11 probe sets, thus allowing multiple independent measurements for high accuracy and reproducibility. Data analyses for over- and underexpressed genes were done by Genome Explorations using GeneChip Operating Software (Affymetrix).

Histochemical detection of senescence-associated β-galactosidase. Freshly dissected mouse bladders from wild-type and transgenic mice were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.0), embedded in OCT medium, and frozen in liquid nitrogen. Ten-micrometer-thick cryosections were prepared from the frozen blocks and were incubated at 37°C overnight in a staining solution containing 1 mg/ml X-gal (Sigma-Aldrich), 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)15, 150 mM NaCl, and 2 mM MgCl2 in PBS (pH 6.0). Control experiments were carried out simultaneously using adjacent sections to detect non-senescence-associated β-galactosidase activity with the same solution made in acidic pH (4.0; ref. 43). The sections were counterstained with 1% neutral red and examined by light microscopy.

Western blot analysis. Urinary bladders from wild-type and transgenic mice were turned inside out, and the urothelial cells were scraped off using a chemical spatula into phosphate-buffered saline. The cells were collected by centrifugation, and a protein extraction buffer was added to a final concentration of 20 mM HEPES buffer (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM leupeptin, 1.5 mM MgCl2, 10% glycerol, 10 mM sodium pyrophosphatase, 50 mM NaF, and 2 mM sodium orthovanadate. For protein extraction from bladder neoplasms, tumors were removed from bladder surface and homogenized using a Polytron in the same extraction buffer mentioned above. Protein concentration was determined using BCA reagent (Pierce Biotechnology) with BSA as a standard. One hundred micrograms of the total urothelial proteins were reconstituted with SDS sample buffer and resolved by 10% SDS-PAGE, transferred onto a PVDF-Immobilon membrane, and blotted with various antibodies (see below). After incubation with secondary antibodies conjugated with HRP, the blots were developed in an ECL detection solution (ECL; Amersham Biosciences) and exposed to an X-ray film. Primary antibodies against p16Ink4a, p19Arf, and p21ras were purchased from EMD BioSciences; those against MAPK, p-MAPK (Thr202/Tyr204), AKT, p-AKT (Thr308), p-AKT (Ser473), p-GSK3B (S9), p-FKHR (S256), STAT3, p-STAT3 (Tyr705), STAT5, p-STAT5 (Tyr694), PTEN, p-PTEN (Ser380) were purchased from Cell Signaling Technology; and antibody against β-actin was purchased from Sigma-Aldrich.

Histology and immunohistochemistry. Bladder tissues were fixed in 10% buffered formalin and processed routinely for paraffin embedding, sectioning, and staining by H&E. For immunohistochemical staining, deparaffinized sections were microwaved in citrate buffer (pH 6.0) at maximum power output for 10 minutes and stained with primary antibodies (see above; rabbit anti-VEGF from Santa Cruz Biotechnology Inc.) and secondary antibodies conjugated with HRP. The staining was visualized by developing the tissue sections in a solution containing 3,3’-diaminobenzidine tetrahydrochloride, followed by light counterstaining in a hematoxylin solution.

Statistics. Statistical analyses were performed using the Web-based SPSS software. Two-tailed Student’s t test was employed to assess the statistical difference in activated rabbit Ha-ras expression between homo- and heterozygous mice. Tumor-free rate curves for hetero- and homozygous mice were constructed according to the Kaplan-Meier method. P values less than or equal to 0.05 were considered statistically significant.

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