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# Overexpression of Epidermal Growth Factor Receptor in Urothelium Elicits Urothelial Hyperplasia and Promotes Bladder Tumor Growth[1]

**Jin Cheng, Hongying Huang, Zhong-Ting Zhang, Ellen Shapiro, Angel Pellicer, Tung-Tien Sun, and Xue-Ru Wu[2]**

*Departments of Urology [J. C., H. H., Z-T. Z., E. S., T-T. S., X-R. W.], Microbiology [X-R. W.], Pathology [A. P.], Dermatology [T-T. S.], and Pharmacology [T-T. S.], Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, New York 10016, and Veteran Affairs Medical Center New York, New York 10010 [X-R. W.]*

## ABSTRACT

**Although urothelium is constantly bathed in high concentrations of epidermal growth factor (EGF) and most urothelial carcinomas overexpress EGF receptor (EGFr), relatively little is known about the role of EGFr signaling pathway in urothelial growth and transformation. In the present study, we used the uroplakin II gene promoter to drive the urothelial overexpression of EGFr in transgenic mice. Three transgenic lines were established, all expressing a higher level of the EGFr mRNA and protein in the urothelium than the nontransgenic controls. The overexpressed EGFr was functionally active because it was autophosphorylated, and its downstream mitogen-activated protein kinases were highly activated. Phenotypically, the urinary bladders of all transgenic lines developed simple urothelial hyperplasia that was strongly positive for proliferative cell nuclear antigen and weakly positive for bromodeoxyuridine incorporation. When coexpressed with the activated Ha-*ras* oncogene in double transgenic mice, EGFr had no apparent tumor-enhancing effects over the urothelial hyperplastic phenotype induced by Ha-*ras* oncogene. However, when coexpressed with the SV40 large T antigen, EGFr accelerated tumor growth and converted the carcinoma *in situ* of the SV40T mice into high-grade bladder carcinomas, without triggering tumor invasion. Our studies indicate that urothelial overexpression of EGFr can induce urothelial proliferation but not frank carcinoma formation. Our results also suggest that, whereas EGFr and Ha-*ras*, both of which act in the same signal transduction cascade, stimulated urothelial hyperplasia, they were not synergistic in urothelial tumorigenesis, and EGFr overexpression can cooperate with p53 and pRB dysfunction (as occurring in SV40T transgenic mice) to promote bladder tumor growth.**

## INTRODUCTION

The EGFr[3] is an important member of the receptor tyrosine kinase family and plays critical roles in cell growth, differentiation, motility, and survival (1, 2). The receptor encodes a $M_r$ 170,000 cell surface transmembrane protein that is capable of interacting with several ligands including EGF, transforming growth factor-$\alpha$, and amphiregulin. Ligand/receptor interaction leads to the autophosphorylation of the tyrosine residues of the cytoplasmic domain of the receptor, which serves as the docking sites for a variety of intracellular signal transducers. Of the multitude of EGFr-induced pathways, the most important and best understood is the Ras-MAP kinase pathway, typified by the Erk1 and Erk2 cascade. This signaling pathway has been shown to be critical for EGFr-induced cell proliferation (1, 2).

Mammalian urothelium is one of the slowest growing epithelia under normal conditions with a [$^3$H]thymidine labeling index of 0.02–0.05% (3). However, when provided with proper growth stimuli, this epithelium can undergo tremendous growth, as occurring during wound healing, *in vitro* culture, and carcinogenesis (4–7). One of the key *in vivo* growth signals for urothelium has been suspected to be EGF because of its natural presence at nanomolar quantities in the urine (8, 9). Nevertheless, because EGFr is primarily expressed by the basal cells of the urothelium (10, 11), the physical barrier imposed by the superficial umbrella cell layer should normally prevent the EGF ligand from interacting with its urothelial receptor. Urothelial tumorigenesis can drastically change this situation, however, because of the breakdown of the urothelial permeability barrier coupled with the overexpression of the EGFr, thus allowing ligand/receptor binding to occur. Indeed, patients with bladder cancer contained a significantly lower urinary EGF concentration than the noncancer controls, a finding consistent with ligand-uptake by the tumor receptors (12, 13).

A role for EGFr in urothelial tumor growth is also supported by the observation that 40–60% of human bladder tumors overexpress EGFr mRNA and protein (9, 14, 15). In addition, there is a strong correlation between EGFr overexpression and the late-stage, invasive urothelial carcinomas, suggesting that EGFr signaling may play a role in tumor progression. Finally, in animal chemical carcinogenesis experiments, EGF significantly increased the frequency of bladder tumor formation in (heterotopically transplanted) rat urinary bladders as induced by *N*-methyl-*N*-nitrosourea (16). These data support a role for EGFr signaling in urothelial tumor development and progression.

Despite these studies, there is no direct evidence that EGFr overexpression can induce urothelial tumorigenesis. Our recent identification of a urothelium-specific promoter has provided an excellent opportunity to drive the urothelial overexpression of EGFr in transgenic mice and to determine its effects on urothelial growth and tumorigenesis (17). Using this approach, we have recently established two transgenic mouse models of bladder tumorigenesis, one expressing the SV40T and another expressing an activated Ha-*ras* (18, 19). Interestingly, these models developed distinctive bladder tumors closely resembling the two major phenotypic variants of human bladder cancer. The SV40T mice, particularly those harboring multiple copies of the transgene, produced bladder CIS, which then progressed to form invasive and metastatic bladder carcinomas (18). In contrast, the Ha-*ras* mice developed urothelial hyperplasia leading to the formation of low-grade, superficial, papillary bladder tumors (19). These results strongly support the idea that bladder tumors can develop via two distinctive genetic and phenotypic pathways (19–25). In addition, these transgenic models provide excellent tools for examining the potential synergistic effects among different oncogenic events.

Herein, we report the generation of transgenic mice overexpressing the EGFr in the urothelium. Our results demonstrate that the activation of EGFr signaling pathway can elicit urothelial proliferation leading to urothelial hyperplasia. Although both EGFr and Ha-*ras* mutant, when singly expressed, induced urothelial hyperplasia, their coexpression did not enhance urothelial tumorigenesis. In contrast, the coexpression of EGFr and SV40T significantly enhanced tumor growth, causing CIS to become high-grade bladder carcinomas, thus establishing the synergistic roles of the two genetic events. These results define the *in vivo* role of EGFr overexpression on urothelium and

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[2] To whom requests for reprints should be addressed, at Department of Urology, New York University School of Medicine, 550 First Avenue, New York, NY 10016. Phone: (212) 951-5429; Fax: (212) 951-5424; E-mail: xue-ru.wu@med.nyu.edu.

[3] The abbreviations used are: EGFr, epidermal growth factor receptor; MAP, mitogen-activated protein; Erk, extracellular signal-regulated kinase; SV40T, SV40 large T antigen; CIS, carcinoma *in situ*; RT-PCR, reverse transcription-PCR; UPII, uroplakin II; PCNA, proliferating cell nuclear antigen; BrdUrd, 5-bromo-2′-deoxyuridine.

provide new opportunities for studying the mechanisms of bladder tumorigenesis.

## MATERIALS AND METHODS

**Construction of Chimeric Gene and Production of Transgenic Mice.** The UPII/EGFr chimeric gene was constructed by linking the mouse *UPII* promoter with the EGFr cDNA (Fig. 1*A*). Briefly, a 4.1-kb PCR product containing the full-length, human EGFr cDNA was amplified from the EGFR-PRK5 plasmid (courtesy of Dr. Joseph Schlessinger, New York University Pharmacology Department, New York, NY; Ref. 26). DNA sequencing of the cloned PCR product showed 100% match with the original sequence. The PCR product, supplemented with *Sal*I and *Spe*I at the 5′ and 3′ ends, respectively, was subcloned downstream of the 3.6-kb mouse *UPII* promoter, which was inserted previously onto the *Apa*I site of the pBluescript (18). After verification of the orientation by restriction digestion and sequencing, the 7.7-kb UPII-EGFr chimeric gene was retrieved from the plasmid by *Kpn*I and *Spe*I digestion, purified by agarose gel electrophoresis and chromatography before being injected into the fertilized eggs of FVB/N inbred mice for transgenic mouse production (27).

**Southern Blot Analysis.** Transgenic mice were identified by Southern blot analysis of the genomic DNA isolated from mouse tail biopsies. DNA was digested with *Nco*I, resolved by gel electrophoresis, and hybridized with a 550-bp *Bam*HI-*Stu*I fragment located at the 3′ end of the mouse *UPII* promoter, which allowed the detection of both endogenous *UPII* gene and transgene.

**RT-PCR.** The expression of the transgene in mouse urothelium was examined by RT-PCR. Total RNA was isolated from urothelial cells scraped from the mouse bladder mucosa using RNAgents Total RNA Isolation System (Promega Corp., Madison, WI). After reverse transcription of 2 $\mu$g of the total RNA, PCR was performed using four pairs of oligonucleotide primers, two specific for human EGFr and another two specific for mouse EGFr. The primers were: human sense primer 1 (HS1), 5′-GGCCGGGGCAGCGCTC-CTGGCGC-3′; human antisense primer 1 (HAS1), 5′-TGCTCTCCACGTT-GCACAGGGCA-3′; HS2, 5′-AAACAGCTGCAAGGCCACAGG-3′; HAS2, 5′-CGGGATCTTAGGCCCATTCGT-3′; mouse S1 (MS1), 5′-CGC-GAGAACCACACTGCTGGTGT-3′; MAS1, 5′-TAGTATCCATATTGCA-GAGGATG-3′; MS2, 5′-GAAAGACTGCAAGGCCGTGAA-3′; and MAS2, 5′-TGGTATCTTTGGCCCAGATGG-3′. One tenth of the transcribed double-stranded cDNA was mixed with 0.1 $\mu$M of each pair of the primers, 200 $\mu$M deoxynucleotide triphosphate, 1:10 volume of PCR buffer, and 1.5 units of Taq polymerase. The reaction conditions were: first cycle at 94°C for 5 min, 62°C for 1 min, and 72°C for 2 min; 35 cycles at 94°C for 2 min, 60°C for 1 min, and 72°C for 2 min; and last cycle at 94°C for 2 min, 62°C for 1 min, and 72°C for 8 min. The PCR products were analyzed by agarose gel electrophoresis.

**Northern Blot Analysis.** Total RNA (10 $\mu$g/lane) was resolved by agarose-formaldehyde gel, transferred onto nylon membrane, and reacted with a human EGFr cDNA probe. The probe was stripped, and the membrane was rehybridized with a $\beta$-actin probe for loading normalization.

**Preparation of Urothelial Proteins and Western Blot Analysis.** For preparation of membrane and cytoplasmic proteins, urothelial cells were scraped from the mouse bladder mucosa and were dissolved in a lysis buffer containing 1% Triton X-100, 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM $MgCl_2$, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, and 10% glycerol. For assays of phosphorylated EGFr and MAP kinases, the buffer was supplemented with phosphatase inhibitors including 50 mM sodium fluoride, 2 mM sodium orthovanadate, and 10 mM sodium $PP_i$. After centrifugation at 12,000 $\times$ *g* for 15 min, the soluble proteins were quantified using the Bradford method (Bio-Rad, Hercules, CA; Ref. 28). Sixty $\mu$g of the total proteins were resolved in 8% SDS-PAGE, electrotransferred onto Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MA) and reacted with primary and secondary antibodies. The reaction was visualized by an enhanced chemiluminescence (ECL) detection system (DuPont NEN, Boston, MA) according to the manufacturer's instructions. The primary antibodies included anti-EGFr (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine (1:1000; Sigma Chemical Co., St. Louis, MO), antiphosphorylated MAP kinases (antiactivated Erk1/2; 1:1000; CST, Bevering, MA), anti-Erk1/2 (1:500; CST), anti-PCNA (1:20; Dako, Carpinteria, CA), and anti-uroplakin Ia (1:5000; the authors' laboratory, Wu *et al.* 29). For preparation of nuclear proteins, urothelial cells were dissolved in a lysis buffer containing 10% SDS, 20 mM Tris/HCl (pH 7.4), 50 mM NaCl, 5 mM $\beta$-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml aprotinin, and 1 $\mu$g/ml leupeptin.

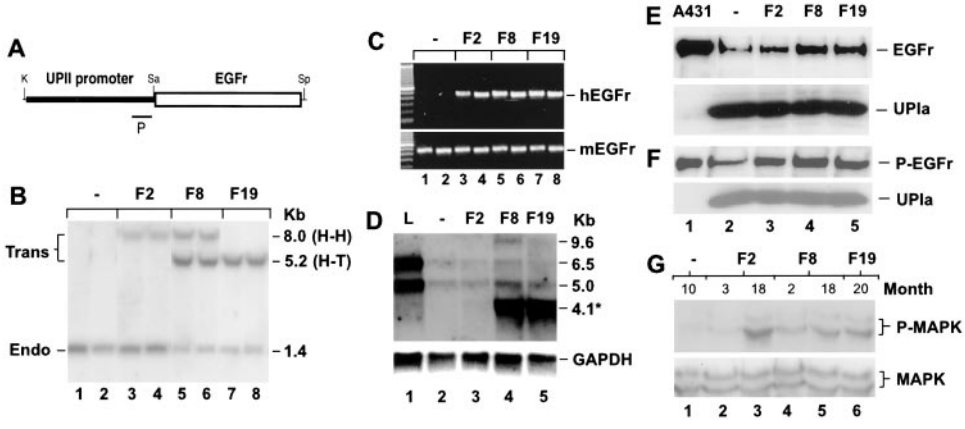


Fig. 1. Generation and characterization of UPII/EGFr transgenic mice. *A*, a chimeric gene consisting of a 3.6-kb mouse *UPII* gene promoter and a 4.1-kb human EGFr cDNA. Restriction sites shown are *Kpn*I (*K*), *Sal*I (*Sa*), and *Spe*I (*Sp*). *P*, the probe for Southern blotting. *B*, Southern blot analysis. Genomic DNA from tail biopsies of the $F_1$ animals were restriction digested and hybridized with a DNA probe located at the 3′ end of the *UPII* promoter (*P* in *panel A*). The probe detected an endogenous *UPII* gene fragment (1.4 kb) in all mice and two transgene fragments at 8.0 and 5.2 kb, which most likely represented the head-to-head (*H-H*) and head-to-tail (*H-T*) transgene orientation, respectively. Note that the transgene in line 2 assumed the H-H orientation, in line 19 the H-T orientation, and in line 8 both orientations; and that the nontransgenic controls contained no transgenes. *C*, RT-PCR analysis of transgene expression in mouse urothelium. Two pairs of adjacent primers specific for human EGFr produced a 467-bp (*Lanes 3*, *5*, and *7*) and a 450-bp (*Lanes 4*, *6*, and *8*) PCR product in all three transgenic lines but not in transgene-negative mouse controls (*Lanes 1* and *2*), whereas another two pairs of primers specific for mouse EGFr (located at the same region as the human primers) produced a 467-bp and a 450-bp product in all mice. *D*, Northern blot analysis. Total RNA of mouse urothelia was hybridized with a human EGFr cDNA probe, which detected a 4.1-kb mRNA species strongly in lines 8 and 19, weakly in line 2, and none in the urothelium (−) or liver (*L*) of the control mice. The probe also detected several other mRNA species at 9.6, 6.5, and 5.0 kb in all tissues analyzed, most likely representing the cross-reacting, alternatively spliced endogenous EGFr mRNAs. The same blot was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe illustrating similar sample loading (*lower panel*). *E–G*, Western blot analysis of the expression and functional status of the transgenic human EGFr. Total urothelial proteins (60 $\mu$g/lane) were resolved by SDS-PAGE and immunoblotted with antibodies against EGFr (*E*), phoshotyrosine (*F*), and phospho-MAP kinase (*G*). Note in *E* the elevated urothelial expression of EGFr protein in three transgenic lines ($F_2$, $F_8$, and $F_{19}$) over the nontransgenic control mouse (−), which expressed only the endogenous EGFr. A total protein extract from A431 cells (6 $\mu$g/lane) was used as a positive control for EGFr. Immunoblotted uroplakin Ia served as a loading control (*lower panel*). Note in *F* the increased levels of the phosphorylated EGFr. A total protein extract from A431 (3 $\mu$g/lane) was used as a positive control. Note in *G* a significantly increased level of phosphorylated MAP kinases (Erk1/2) detected in transgenic mice, particularly in old mice (*Lanes 3* and *5 versus Lanes 2* and *4*).

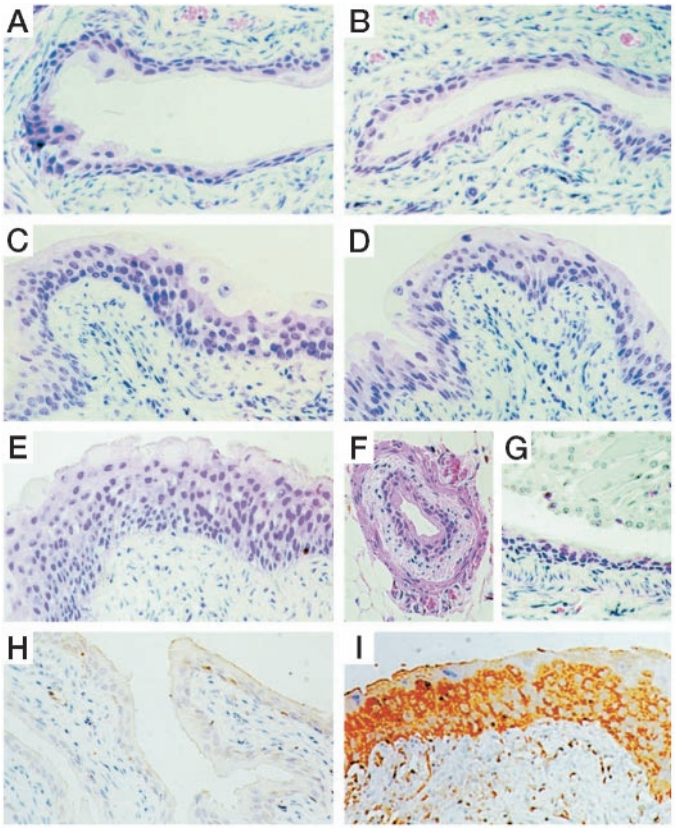


Fig. 2. Urothelial hyperplasia and overexpression of EGFr in the lower urothelial cell layers of the UPII/EGFr transgenic mice. *A*, cross-section of the bladder of a 10-month-old nontransgenic mouse showing three to four urothelial layers. *B*, the urothelium of a 3-month-old EGFr transgenic mouse (line 19) showing normal histology. *C–E*, the urothelia of transgenic mice (*C*, line 19, 13 months; *D*, line 2, 13 months; and *E*, line 8, 19 months) showing urothelial hyperplasia with significantly increased number of urothelial cell layers (five to eight layers). *F* and *G*, ureter and renal pelvis of the line 8 mouse (compare *panel E*) showing the lack of urothelial hyperplasia. *H*, immunohistochemical staining of the urothelium of an 11-month-old normal (control) mouse with anti-EGFr antibody showing a low level of EGFr. *I*, EGFr staining of the urothelium of an age-matched EGFr transgenic mouse (line 8) showing a high level of EGFr in the basal and intermediate layers; note the lack of superficial cell staining. All panels are of the same magnification (×200).

**Histopathology and Immunohistochemistry.** Bladder tissues were freshly dissected from normal and transgenic animals and fixed in 10% buffered formalin. The fixed tissues were embedded in paraffin, sectioned at 4 μm, and routinely stained with H&E. Alternatively, the sections were stained with anti-EGFr and anti-PCNA and counterstained with hematoxylin.

**BrdUrd Incorporation Assay.** Nuclear incorporation of BrdUrd (Sigma) was carried out by i.p. injection of a 100 mg/kg solution in PBS. Because preliminary experiments with single BrdUrd injection failed to produce any positive results in the urothelia of the transgenic and nontransgenic mice, a modified protocol was used that included five injections of BrdUrd (100 mg/kg) at 2-h intervals. Two h after the last injection, the urinary bladders of the transgenic and nontransgenic controls were removed, fixed in 10% formalin, and processed for immunohistochemical staining with a peroxidase-conjugated anti-BrdUrd antibody (Chemicon, Temecula, CA).

**Generation of Double Transgenic Mice.** Two cross-breeding experiments were carried out, one between UPII/EGFr mice and UPII/Ha-*ras* mice, and another between UPII-EGFr mice and UPII/SV40T mice. Offspring from the cross-breedings were analyzed using Southern blotting for their inheritance of the various transgenes (see "Results" for details).

## RESULTS

**Production and Characterization of the UPII-EGFr Transgenic Mice.** To assess the *in vivo* effects of EGFr overexpression on urothelial growth and tumorigenesis, we generated transgenic mice in which EGFr expression was under the control of a urothelium-specific, *UPII* promoter (Fig. 1*A*; Ref. 17). Three independent transgenic mouse lines were obtained (Fig. 1*B*), each expressing the exogenous human EGFr mRNA in their urothelia as assayed by RT-PCR (Fig. 1*C*). Lines 8 and 19 expressed 10–20-fold more of the transgene-encoded, 4.1-kb EGFr mRNA species than line 2 (Fig. 1*D*). This mRNA species was undetectable in normal mouse urothelium. An anti-EGFr antibody, which reacts with both endogenous and transgenic EGFr proteins, detected a 3–6-fold overexpression of the $M_r$ 170,000 EGFr in transgenic mice over the nontransgenic controls (Fig. 1*E*). There was no direct correlation between the level of mRNA overexpression (Fig. 1*D*) and the EGFr protein level (Fig. 1*E*), possibly because of the relative instability of the EGFr mRNA and/or the increased protein degradation in some urothelial cells (see later).

We next examined whether the overexpressed EGFr protein was functionally active by assessing its phosphorylation status and the activation of one of its downstream effectors, MAP kinases. As expected, an antiphosphotyrosine antibody reacted with the $M_r$ 170,000 EGFr protein more strongly in transgenic mice than in the negative controls (Fig. 1*F*). In addition, the UPII-EGFr transgenic mice contained appreciably higher levels of phosphorylated MAP kinases than the nontransgenic controls (Fig. 1*G*). Within the same transgenic line, older mice displayed a higher level of the activated MAP kinases than the younger mice. These results show that the overexpressed human EGFr was functionally activated in the mouse urothelium.

**Induction of Urothelial Hyperplasia by EGFr Overexpression.** Histological survey of the transgenic mice revealed urothelial hyperplasia in all three transgenic lines. In contrast to normal urothelium, which had only three to four cell layers (basal, intermediate, and superficial; Fig. 2*A*), the urothelia of the UPII-EGFr transgenic mice were significantly thickened, with five to eight layers (Fig. 2, *C–E*). Nevertheless, the cells exhibited little atypia with normal nuclear: cytoplasmic ratio and were arranged in normal polarity with prominent superficial umbrella cells. These morphological features resembled simple urothelial hyperplasia that occurs in human bladders under certain pathological conditions (30–32). Although urothelium also lines the ureters and renal pelvis, thus expressing equally strongly the uroplakin and the EGFr (data not shown), urothelia in these areas showed no hyperplasia (Fig. 2, *F* and *G*; also see "Discussion"). The hyperplastic state of the bladder urothelia in transgenic mice correlated well with the extent of EGFr overexpression (Fig. 2*I*). Both membrane-associated and cytoplasmic EGFr were seen. The superficial umbrella cells contained relatively little EGFr; this was surprising because the *UPII* promoter should be most active in umbrella cells, which should have expressed the highest level of transgene-encoded EGFr. This could be because of aberrant EGFr synthesis/transport in the highly specialized umbrella cells or because of an increased degradation of endocytosed urinary EGF/EGFr complex (33, 34). With the exception of line 8, the frequency of urothelial hyperplasia increased with age (Table 1). In line 2, 2 of 6 (33%) mice younger than 10 months had hyperplasia, whereas 7 of 10 mice (70%) older than 10 months had hyperplasia. The same age trend held true for line 19. It is unclear why there was a lack of age-related rate increase of urothelial hyperplasia in line 8; but this could be because of the

Table 1 *Urothelial hyperplasia in UPII/EGFr transgenic mice*

| Lines | <10 mo | >10 mo |
|---|---|---|
| 2 | 2/6 | 7/10 |
| 8 | 5/13 | 5/14 |
| 19 | 1/7 | 5/16 |
| WT | 0/8 | 0/5 |

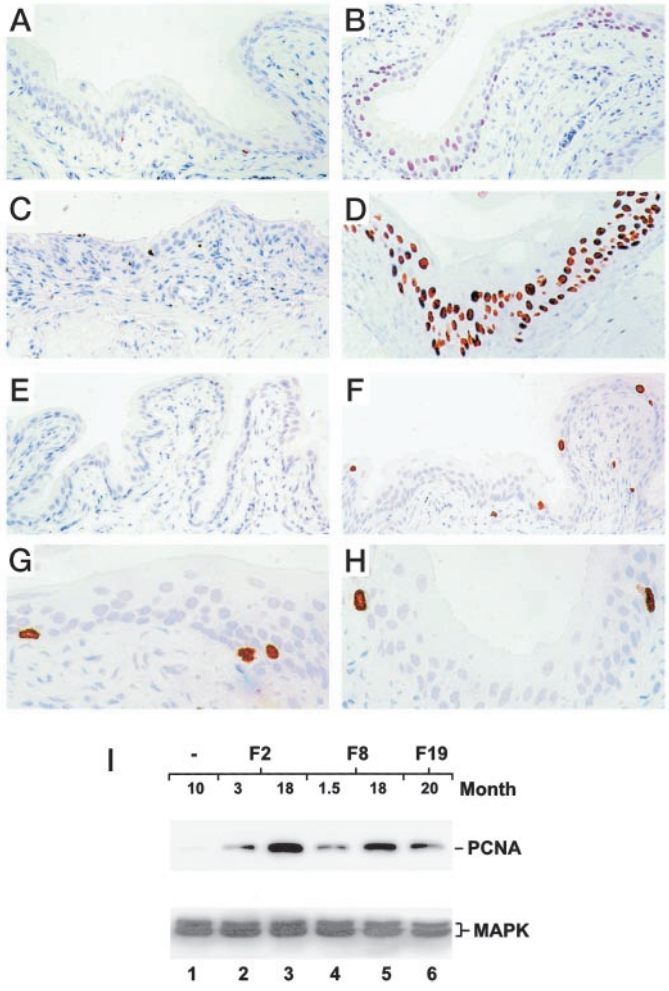


Fig. 3. Overexpression of the PCNA and increased incorporation of BrdUrd in the EGFr-overexpressed urothelia. Urothelia of normal (*A*, 2 month, and *C*, 11 month) and transgenic mice (*B*, line 19–2 month and *D*, line 8–15 month) were stained with anti-PCNA antibody and counterstained with hematoxylin. Note that although normal urothelium expressed little PCNA, almost all cells of the lower urothelial layers of the EGFr transgenic mice expressed this cell proliferation marker strongly. Also note that old mice (*D*), which exhibited more urothelial proliferation, expressed more antigen. *E–H*, anti-BrdUrd staining of normal (*E*) and transgenic mice (*F* and *G*, line 2–20 month; and *H*, line 8–18 month) that had been injected with BrdUrd (see "Materials and Methods"). Note the absence of BrdUrd incorporation in the normal urothelium and the increased BrdUrd-incorporating cells in the EGFr-expressing transgenic mouse urothelium. Magnifications are ×200 for *A–F* and ×400 for *G* and *H*. *I*, Western blot demonstration of the overexpression of PCNA in the urothelia of the EGFr transgenic mice. Total urothelial proteins (100 μg/lane) of normal (−) and three lines of EGFr transgenic mice were resolved by 8% SDS-PAGE, electrotransferred onto Immobilon-polyvinylidene difluoride membrane and reacted with an anti-PCNA antibody, followed by enhanced chemiluminescent visualization method. The same membrane was stripped and reblotted with an anti-MAPK antibody as a loading control. Note the overexpression of PCNA in all transgenic lines (*Lanes 2–6*) as compared with the control (*Lane 1*). Also note that within the same transgenic line, older mice expressed more PCNA than the younger animals (*Lanes 3* and *5 versus Lanes 2* and *4*).

relatively small sample size or certain intrinsic differences, such as transgene insertion sites, among different transgenic lines. Although urothelial hyperplasia was a consistent finding in all transgenic lines, no urothelial tumors were observed throughout the 19-month observation period.

Although normal urothelial cells rarely express the PCNA (Fig. 3, *A* and *C*), the nuclei of almost all of the urothelial cells of the UPII-EGFr transgenic mice expressed this proliferative cell marker (Fig. 3, *B* and *D*). The intensity of the PCNA staining correlated well with the degree of urothelial hyperplasia. The basal and intermediate cells were much more strongly stained than the superficial cells, consistent with the fact that the former contained more EGFr proteins. The increased PCNA synthesis in transgenic mice was confirmed with Western blotting (Fig. 3*I*). The proliferative state of the urothelium was also assessed by a modified BrdUrd incorporation assay in which the reagent was injected five times instead of the regular single injection. Under this condition, urothelium of the transgenic mice contained 7–20 BrdUrd-positive cells/cross-section (representing a positive rate of ~0.3–1% of total urothelial cells; Fig. 3, *F–H*), whereas normal urothelium remained completely negative for BrdUrd incorporation (<0.02%; Fig. 3*E*). Most of the positive cells were basally located, although intermediate cells were occasionally labeled. The relatively low rate of BrdUrd incorporation and high rate of PCNA staining of the urothelial cells may reflect the fact that the majority of the cells had proliferation potential but were not undergoing active DNA synthesis (see "Discussion").

**The Cooperative Relationship between EGFr and Oncogenes.** We have recently generated two transgenic mouse models, one expressing the SV40T and another expressing the activated Ha-*ras* oncogene in the urothelium. The former developed bladder carcinoma *in situ*, some of which, particularly those harboring high transgene copies, progressed to invasive bladder cancer (18). The latter developed urothelial hyperplasia, which over time evolved into low-grade, superficial papillary tumors (19). To study whether EGFr overexpression can cooperate with these oncogenes in promoting urothelial tumor progression, we generated double transgenic mice by breeding line 8 of the EGFr mice that expressed high levels of EGFr mRNA and protein with line 5 of the UPII/Ha-*ras* transgenic mice that harbored low transgene copies and consistently developed urothelial hyperplasia (19).

Four different genotypes were identified, including those without any transgene, those with each of the original transgene (EGFr or Ha-*ras*) and those with both transgenes (Fig. 4*A*). The 3-month-old, EGFr-only mice exhibited normal urothelial morphology (Fig. 5*A*) and Ha-*ras*-only mice exhibited severe urothelial hyperplasia (Fig. 5*B*; Ref. 19). No superficial papillary tumors were observed in the EGFr/Ha-*ras* double transgenic mice during the 10-month observation period (Fig. 5*C*), indicating that a combination of Ha-*ras* and EGFr did not increase cell proliferation.

The second set of the double transgenic mice was generated by breeding line 8 of the EGFr mice with line 19 of the UPII-SV40T that harbored low transgene copies (Fig. 4*B*; Ref. 18). As mentioned

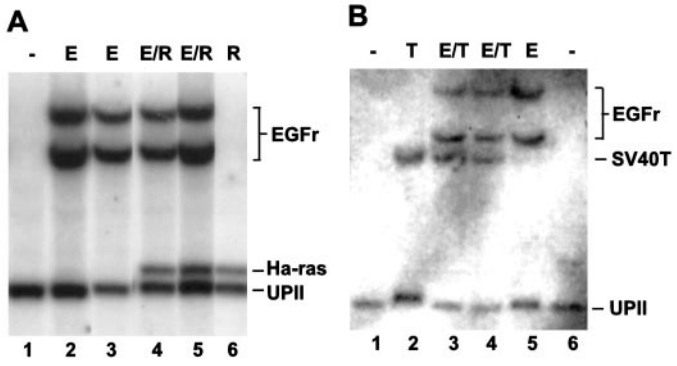


Fig. 4. Generation of the double transgenic mice. *A*, offspring derived from cross-breeding between the UPII/EGFr and UPII/Ha-*ras* transgenic mice were subjected to Southern blotting using a UPII probe. The unique restriction fragments allowed the identification of several genotypes including the negative mice (−, *Lane 1*), the EGFr single transgenic mice (*E*, *Lanes 2* and *3*), the Ha-*ras* single transgenic mouse (*R*, *Lane 6*), and the EGFr and Ha-*ras* double transgenic mice (*E/R*, *Lanes 4* and *5*). *B*, offspring derived from cross-breedings between UPII/EGFr and UPII/SV40T transgenic mice. The genotypes included the negative mice (−, *Lanes 1* and *6*), the SV40T single transgenic mouse (*T*, *Lane 2*), the EGFr single transgenic mouse (*E*, *Lane 5*), and the SV40T/EGFr double transgenic mice (*E/T*, *Lanes 3* and *4*).

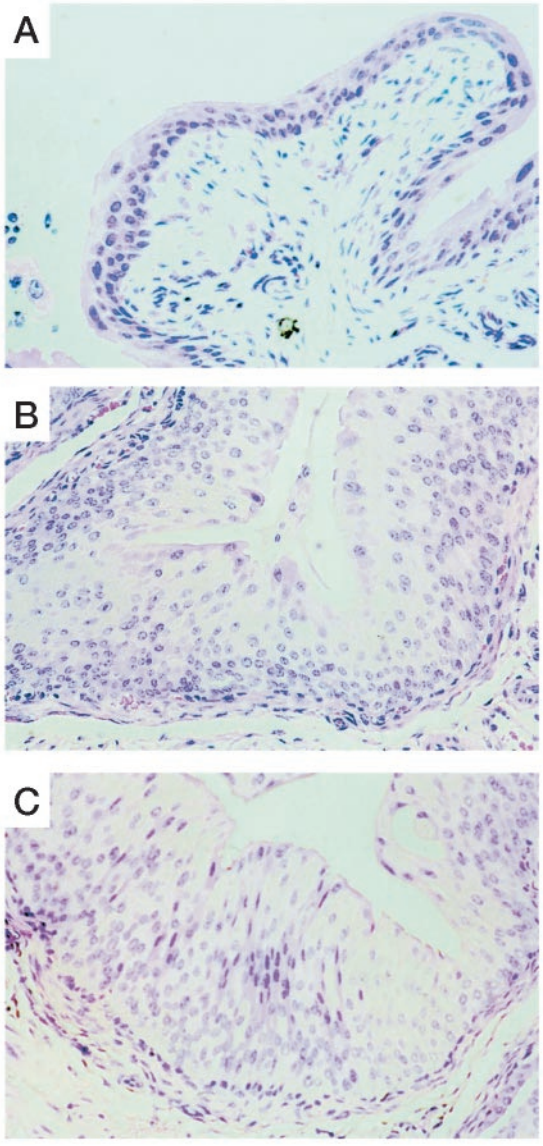


Fig. 5. Histopathology of transgenic mice (all 3 months of age) from EGFr and Ha-*ras* cross-breeding. *A*, An EGFr mouse showing normal urothelial histology. *B*, a Ha-*ras* mouse showing urothelial hyperplasia. *C*, an EGFr/Ha-*ras* double transgenic mouse showing urothelial hyperplasia similar to *B*. All panels are of the same magnification (×200).

earlier, the urothelium of the EGFr mice remained morphologically normal (Fig. 6*A*), and the SV40T-expressing urothelium developed CIS (Fig. 6*B*; Ref. 18). However, the age-matched EGFr/SV40T double transgenic mice showed strikingly enhanced tumor growth leading to the formation of high-grade bladder carcinomas (Fig. 6, *C–F*). Nevertheless, serial sections of the urinary bladders of the double transgenic mice showed that none of these mice developed invasive tumors during the 10-month observation period. These results indicated that, although EGFr promoted tumor growth, it did not have a significant role in tumor invasion.

## DISCUSSION

**The Role of EGFr Overexpression in Urothelial Growth and Tumorigenesis.** Although it is well known that urine contains a high concentration of EGF and that EGFr is overexpressed in human bladder cancer (9), very little experimental evidence was available on the role of EGFr signaling in urothelial growth and tumorigenesis. In this study, we show that the overexpression of EGFr in urothelium can accelerate cell growth of otherwise exceedingly slow-growing urothelium, leading to urothelial hyperplasia but not to frank carcinoma (Figs. 2 and 3). This finding strongly suggests that, although EGFr overexpression promotes urothelial growth, it is insufficient for urothelial tumor formation and therefore is unlikely to be an initiating event in bladder tumorigenesis. However, EGFr significantly increased tumor growth in the EGFr/SV40T double transgenic mice converting CIS into high-grade bladder carcinomas (Fig. 6). This result demonstrates that EGFr can cooperate with appropriate oncogenes or the dysfunctional tumor suppressor genes to accelerate urothelial tumor growth. It is interesting, however, that the accelerated tumor growth did not lead to invasion (Fig. 6), suggesting that the latter event requires additional genetic or epigenetic alterations. These may include the inactivation of additional tumor suppressor genes, activation of other oncogenes, down-regulation of cell adhesion molecules, and the overexpression of vascular growth factors, cyclooxygenases, and matrix-degrading enzymes (35–43). Our results are therefore consistent with the multifactorial concept of bladder tumor progression.

The fact that young mice did not develop urothelial hyperplasia seems to suggest extremely tight growth control and/or suppression in young animals. Alternatively, the development of urothelial hyperplasia *per se* might also require the accumulation of cooperative genetic and epigenetic events.

EGFr overexpression has been thought to play an important role in mammary tumor formation. Approximately half of the human breast cancers overexpress EGFr, a condition often associated with poor prognosis (44). When EGFr was overexpressed in the mammary gland of the transgenic mice under the control of Moloney murine leukemia virus-long terminal repeat or the β-lactoglobulin promoter, virgin mice developed mammary epithelial hyperplasia that upon lactation progressed to dysplasia and tubular adenocarcinoma (45). These data

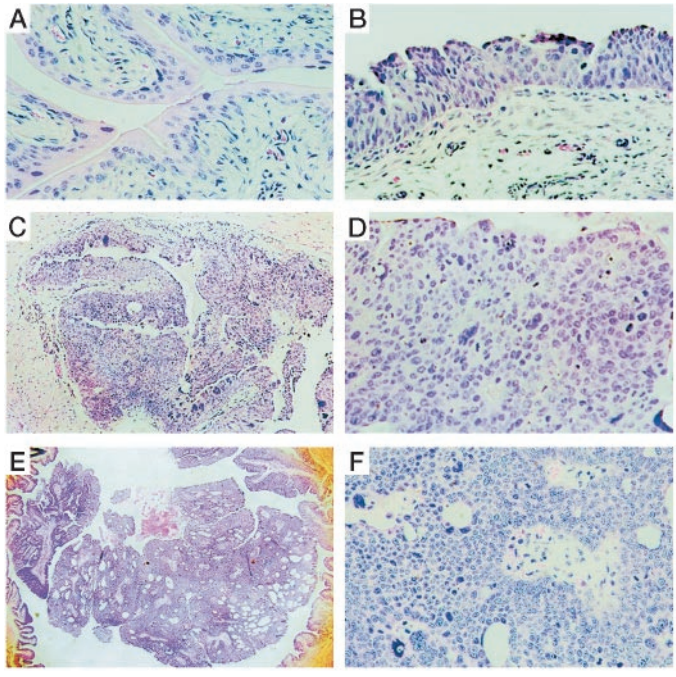


Fig. 6. Histopathology of single and double transgenic mice (all 3 months of age) from EGFr and SV40T cross-breeding. *A*, an EGFr mouse showing normal histology. *B*, an SV40T mouse showing severe urothelial dysplasia/CIS. *C* and *E*, two EGFr/SV40T double transgenic mice showing high-grade superficial papillary bladder tumors. *D* and *F*, high-powered views (of *C* and *E*) showing relatively poorly differentiated urothelial carcinomas. Magnifications are ×200 for *A*, *B*, *D*, and *F* and ×40 for *C* and *E*.

suggest a role of EGFr overexpression in epithelial proliferation and the requirement of other collaborating growth stimuli (the lactating hormones in the case of the mammary gland) to fully transform the epithelial cells (46). The fact that the urothelial overexpression of EGFr alone did not produce any tumors may reflect the lack of lactation-equivalent, cooperating events in the urothelium. It may also reflect the different (intrinsic) susceptibility of different epithelia to EGFr overexpression. Given its extremely low normal self-renewal rate, the urothelium may have a very high threshold for the amount of growth signals that are required for complete cellular transformation.

Although bladder urothelium of the transgenic mice developed hyperplasia, the urothelia of ureters and renal pelvis exhibited normal morphology (Fig. 2), although they also expressed uroplakins, hence, the transgene-encoded EGFr (data not shown). This may be because of the more prolonged exposure of bladder urothelium to urinary EGF or other growth-stimulating signals (47). Similar phenomena have been observed in UPII-SV40T mice and UPII-Ha-*ras* mice, where bladder urothelium often exhibits more advanced tumor lesions than ureteral urothelium.[4] More studies are needed to elucidate the mechanisms underlying the regional differences in response to genetic events.

One puzzling aspect regarding the proliferative state of the urothelium in the UPII/EGFr mice relates to the fact that this urothelium had a high level of PCNA expression but a low level of BrdUrd incorporation (Fig. 3). Although both PCNA and BrdUrd are commonly used as markers for cell proliferation, we showed clearly that most PCNA-expressing urothelial cells were not incorporating BrdUrd and thus were not undergoing active DNA synthesis. It is well known that DNA replication requires the coordinated expression of a whole host of genes including *cyclin D1, E2F, DNA topoisomerase I, DNA polymerase* $\alpha$*, PCNA,* and *thymidine kinase* (48, 49). Earlier studies in cultured 3T3 cells showed that EGF alone can induce PCNA gene expression without inducing the *thymidine kinase* gene (50), indicating that PCNA expression does not always lead to immediate cell proliferation. Indeed, PCNA has been found in noncycling matrix cells surrounding transplanted tumor cells, presumably because of the paracrine EGF stimulation of the tumor (51). Taken together, these results indicate that PCNA is a better marker for proliferative potential and that BrdUrd incorporation is a much more reliable indicator for active cell proliferation.

**EGFr Overexpression and the Two Phenotypic Pathways of Bladder Tumor Formation.** Bladder cancer arises and progresses via two distinctive phenotypic pathways that are believed to be caused by unique genetic defects (20–22). The majority (70–80%) of the bladder cancer presents at diagnosis as superficial, papillary lesions that are of low pathological grade (52). These tumors have a high frequency of recurrence, but few progress to invasive carcinomas. Genetic analyses revealed a close correlation between these tumors and the dysfunction of *p16* tumor suppressor gene, by either deletion or methylation (22, 53). The remaining 20–30% of the bladder cancer presents as invasive carcinomas at diagnosis and are believed to be derived from CIS or arise *de novo* (54). These bladder carcinomas are of high pathological grade with a significant risk of metastatic disease and are frequently found to have dysfunctional *p53* and *pRb* tumor suppressor genes (23–25). Our data from transgenic mouse models clearly established that the urothelial expression of SV40T, which functionally inactivates *p53* and *pRb,* induced CIS and invasive bladder carcinomas (18), whereas the expression of an activated Ha-*ras* oncogene induced urothelial hyperplasia and superficial papillary tumors (19). These results strongly support the concept that different genetic defects are responsible for the two phenotypic pathways of bladder cancer.

The present study showed clearly that EGFr overexpression is insufficient for the bladder carcinoma to become invasive (Fig. 6). However, because EGFr converts CIS into high-grade bladder carcinomas, it can act synergistically in the invasive pathway by accelerating tumor growth. Our results also suggest that at least some of the high-grade, papillary tumors can be derived from CIS lesions. Interestingly, the high-grade, papillary tumors also occur in humans, the majority of which are thought to be originated from CIS (32, 55, 56). This is yet another example that our transgenic mouse models bear striking similarities with human bladder cancers.

EGFr overexpression may also play a role in the superficial, papillary pathway because it can induce urothelial hyperplasia (Fig. 2), which is an important precursor of papillary tumors (57). The fact that EGFr, acting upstream of H-*ras*, can elicit urothelial hyperplasia lends further support to the importance of the *ras* signaling pathway in the low-grade, superficial papillary pathway of bladder cancer. Indeed, studies in human bladder cancer showed that EGFr overexpression is closely correlated with the recurrence of the superficial papillary tumors (58, 59). Although EGFr alone induces urothelial hyperplasia, it did not exhibit a collaborative role with activated Ha-*ras* in shortening the latency of superficial papillary tumor formation in UPII/Ha-*ras* transgenic mice (Fig. 5). This is perhaps not surprising because the constitutive activation of Ha-ras, which acts downstream of the EGFr, is so potent that it overwhelms the effects of additional upstream EGFr activation (1, 2). Collectively, these findings suggest that EGFr could play a role in enhancing tumor growth in both pathways of bladder cancer formation.

In summary, overexpression of EGFr in mouse urothelium leads to urothelial hyperplasia. EGFr alone is insufficient to transform the urothelium, but it can potentiate with certain genetic defects, such as *p53* and *pRb* mutations, to accelerate tumor growth without promoting tumor invasion. These results define the *in vivo* role of EGFr overexpression on urothelium, and our transgenic mouse models provide new opportunities for studying the roles of specific genetic alterations in bladder tumor formation and progression.

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