An EGFR-ERK-SOX9 Signaling Cascade Links Urothelial Development and Regeneration to Cancer

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Abstract

Like many carcinomas, urothelial carcinoma (UroCa) is associated with chronic injury. A better understanding of this association could inform improved strategies for preventing and treating this disease. We investigated the expression, regulation, and function of the transcriptional regulator SRY-related high-mobility group box 9 (Sox9) in urothelial development, injury repair, and cancer. In mouse bladders, Sox9 levels were high during periods of prenatal urothelial development and diminished with maturation after birth. In adult urothelial cells, Sox9 was quiescent but was rapidly induced by a variety of injuries, including exposure to the carcinogen cyclophosphamide, culture with hydrogen peroxide, and osmotic stress. Activation of extracellular signal-regulated kinases 1/2 (ERK1/2) was required for Sox9 induction in urothelial injury and resulted from activation of the epidermal growth factor receptor (Egfr) by several Egfr ligands that were dramatically induced by injury. In UroCa cell lines, SOX9 expression was constitutively upregulated and could be suppressed by EGFR or ERK1/2 blockade. Gene knockdown showed a role for SOX9 in cell migration and invasion. Accordingly, SOX9 protein levels were preferentially induced in invasive human UroCa tissue samples (n = 84) compared with noninvasive cancers (n = 56) or benign adjacent urothelium (n = 49). These results identify a novel, potentially oncogenic signaling axis linking urothelial injury to UroCa. Inhibiting this axis is feasible through a variety of pharmacologic approaches and may have clinical utility. Cancer Res; 71(11); 3812–21. ©2011 AACR.

Introduction

Cancer growth and spread requires coordinated cell migration, proliferation, and stromal remodeling. Similar programs operate in both organogenesis and injury repair (1). Repeated injury repair vastly increases the risk of epithelial cancers (carcinomas), particularly bladder cancer (2, 3). The process of injury repair recapitulates aspects of normal organogenesis (4, 5), with transient reactivation of certain genes that are active in embryonic organogenesis and quiescent in mature tissues. Chronic injury, however, may lead to sustained activation of these genes, and such perseverative signals may lead, in turn, to carcinogenesis (1).

In investigating the molecular links between injury and cancer, transcription factors are appealing targets because they have distinctive and dynamic expression profiles and can themselves coordinate complex genetic programs. These properties are illustrated by SRY-related high-mobility group (HMG) box (Sox) 9 (Sox9). Sox9 belongs to group E (SOX8, SOX9, and SOX10) of the SOX transcription factor family (6) defined by a common HMG box domain originally identified in SRY, the sex-determining gene on the Y chromosome. Sox9 has roles in epithelial invasion, migration, and proliferation as shown in developing prostate (7–9), and similar roles in prostate cancer (8). In chondrocyte development, Sox9 is a master chondrogenic factor whose expression is induced by receptor tyrosine kinase (RTK) signaling (10). Sox9 induction by RTKs requires activation of mitogen-activated protein kinase [p44/42 mitogen-activated protein kinase (MAPK) or Erk1/2; ref. 10]. In this study, we investigate RTK induction of Sox9 in urothelial development, regeneration, and cancer.

Here we identify Sox9 as a molecular link between urothelial injury and urothelial cancer. Sox9 expression coincides with urothelial proliferation during bladder organogenesis, is quiescent in adult urothelium, and is reactivated during acute bladder injury. Sox9 induction occurs through ligand-stimulated activation of epidermal growth factor receptor (EGFR) and subsequent MAPK pathway activation. In contrast to benign bladder, urothelial carcinomas (UroCa) show constitutive SOX9 induction through autonomous EGFR activation, and SOX9 was significantly upregulated in invasive carcinomas. SOX9 knockdown significantly impaired UroCa cell migration and invasion, suggesting its role in UroCa pathogenesis.
data identify a novel link between urothelial development, regeneration, and cancer.

Materials and Methods

Cell lines and cell cultures

BTC905 (German Collection of Microorganisms and Cell Cultures) was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen) with 10% FBS (Sigma) and 1% penicillin/streptomycin (Invitrogen). Human SCaBER bladder cancer cells [American Type Culture Collection (ATCC)] were cultured in DMEM with 10% FBS. UROtsa (11) cells provided by S.H. Garrett (University of North Dakota, Grand Forks, ND), were cultured in DMEM with 2 g/L glucose and 5% FBS. The mouse UroCa line MB49 (12) was provided by Dr. Yi Luo (University of Iowa, Iowa City, IA) and cultured in RPMI1640 with 10% FBS. Cell line identity was assured by using within 6 months of receipt from ATCC or short tandem repeat confirmation by using reference material provided by the contributor (UROtsa cells) or by an online database maintained by the Deutsche Sammlung von Mikroorganismen und Zellkulturen repository.

Compounds and reagents

Erlotinib was purchased from Johns Hopkins Hospital Pharmacy. All other chemicals were purchased from Sigma, unless otherwise indicated. EGF and Matrigel were purchased from BD Pharmingen and Collagen I from Invitrogen.

Antibodies and immunoblotting

Antibodies against EGFR, phospho-EGFR (Tyr1068), Akt, phospho-Akt (Ser473), p44/42 MAPK (Erk1/2; Thr202/Tyr204), and anti-phospho-STAT3 were purchased from Cell Signaling Technology, Inc. Immunoblotting was carried out as previously described (13). Antibodies against human EGFR, β-actin (#A5316), and GAPDH (#sc-32233) were purchased from Dako, Sigma, and Santa Cruz Biotechnology, respectively. Anti-SOX9 antibody (#AB5335) was purchased from Chemicon. Cells were cultured in serum-free medium overnight (16 hours), pretreated with inhibitors for 3 hours, and then with EGF or heparin-binding (HB)-EGF for 24 hours.

Animals and mouse bladder injury model

C56BL6 mice (age, 6–8 weeks) were obtained from The Jackson Laboratory. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University. Mice were randomly selected for a single 0.2 mL intraperitoneal (i.p.) injection of 250 mg/kg body weight of cyclophosphamide (CPA) or PBS (control). This dose is similar to that used in humans receiving high dose CPA (14).

Explant culture

Bladder strips were laid flat on tissue culture inserts (Millipore) floated in DMEM/F-12 (1:1) serum-free media with 1% ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL sodium selenite; Sigma; refs. 15, 16). Inhibitors of EGFR (erlotinib) or MEK/ERK kinase [(MEK1/2 U0126)] were added and tissues were cultured for 1 day before processing for histology.

RNA extraction, reverse transcription, and real-time PCR

RNA was extracted by using Trizol (Invitrogen) followed by RNAeasy mini kit cleanup (Qiagen). RNA was reverse transcribed with Superscript III (Invitrogen). Primer sequences are shown in Supplementary Table S1. Taq SYBR green Supermix with Rox dye (Bio-Rad) was used for real-time PCR and reactions were carried out in triplicate. Quantification of target transcripts was calculated relative to hypoxanthine phosphoribosyltransferase (Hprt1) by using the ΔΔCt method with values from injured (CPA exposed) bladders normalized to values from uninjured (PBS) controls. Data were expressed as mean ± SEM and the Student t test was used to compare the difference in means between control and CPA-treated samples.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out as previously described (13). Briefly, tissue sections were serially incubated in PBS/3% H2O2 (10 minutes at 22°C), PBS (rinse), 10% goat serum (block; 30 minutes at 22°C), and anti-phospho-STAT3 (1 hour at 22°C) rabbit anti-SOX9 antibody (1 hour at 22°C), PBS (rinse), goat anti-rabbit biotinylated secondary antibody (DAKO; 30 minutes at 22°C), PBS (rinse), streptavidin-HRP (DAKO; 30 minutes at 22°C), and PBS (rinse). Staining was visualized with 3,3’-diaminobenzidine tetrahydrochloride (Zymed).

Growth factor and inhibitor treatment

Cells cultured in fresh complete or serum-free media overnight (16 hours) were treated with inhibitors of one of the following targets: phosphoinositide 3-kinase (LY294002), MEK1/2 (U0126), p38 MAPK (PD 169316), EGFR (erlotinib), or vehicle control (DMSO) in fresh serum-free medium for 2 hours, then EGF (10 ng/mL), or HB-EGF (10 or 50 ng/mL) for an additional 24 hours.

SOX9 knockdown

Lentiviral vector-based SOX9 and control microRNA-adapted short hairpin small interfering RNA (shRNA mir) constructs were obtained from Open Biosystems. shRNAs were transfected into human BTC905 UroCa cells by using Arrest-In reagent (Open Biosystems), followed by puromycin (10 µg/mL) selection for 4 weeks to generate stable clones. Clonal colonies isolated were validated by Western blot for SOX9.

Proliferation assay, scratch wound healing, and invasion assay

Proliferation assays were carried out in 96-well plates according to manufacturer’s instruction (ATCC) by using MTT colorimetric assay kit. Plates were read by using a SpectraMax plate reader (Molecular Devices Corp.) at 570 nm with a reference wavelength of 650 nm.

Wounds in 6-well plates were produced with a modified P1000 pipette tip and monitored daily. Wound area was...
measured by using the measurement function in the Analysis Tab of Adobe Photoshop CS4 Extended and then exported as an Excel file for statistical analysis.

In vitro invasion assays were done with the use of 24-well transwell Boyden chambers (17). Polycarbonate membrane inserts (Costar) were precoated with a mixture of growth factor–reduced Matrigel (Invitrogen) and DMEM (1:1 ratio) or of Collagen I (Invitrogen) and DMEM (1:1 ratio). Bottom chambers were filled with DMEM containing 10% FBS as a chemoattractant. A total of 5×10⁴ cells were seeded on the top chamber and incubated for 24 hours. Invasion was quantified as described (13). Aliquots of the same culture were also plated in 24-well plate for MTT assay on the same day.

Tissue microarrays, IHC staining, and scoring

Construction and composition of the 2 tissue microarrays (TMA) used in this study have been previously described (18, 19). Cases were included on the basis of available tissue and follow-up data. Control samples and cancer samples were deemed informative when they contained morphologically recognizable benign urothelium or cancer cells, respectively. The noninvasive cohort (18) included biopsies of benign urothelium, paired with corresponding low- (n=30) and high- (n=30) grade noninvasive papillary carcinomas evaluated at our institution between 1971 and 1995. Of these, 28 low-grade and 28 high-grade carcinomas were deemed informative. In addition, 14 cases had paired benign controls available for analysis. The invasive cohort (19) comprised 132 cystectomies carried out in our institution between 1994 and 2002. Of these, 84 invasive and 15 adjacent CIS lesions were deemed informative. Four-micron TMA sections were stained for SOX9 by IHC as above. Pathologic stages for informative invasive UroCa cases were pT1 (n=4), pT2 (n=26), pT3 (n=40), and pT4 (n=12). Intensity of SOX9 nuclear staining was evaluated and assigned an incremental 0, 1+, 2+, 3+ score. Distribution of staining was categorized as absent, focal (1%–25% of cells), multifocal (25%–75%), or diffuse (>75%). To integrate intensity and distribution of staining, an H-score for SOX9 score was calculated by multiplying the intensity score and the distribution score as previously described (19). H-scores were compared by using the 1-way ANOVA test with the Bonferroni’s post hoc pairwise comparison test. A 2-tailed P<0.05 was required for statistical significance. Data were analyzed by using PASW Statistics version 18.0 (IBM Inc.). Upregulation (Fig. 6A) was calculated separately for each TMA as the ratio of H-score in cancer to H-score in benign.

Results

Induction of Sox9 during urothelial organogenesis

The rudiment of the mouse urinary bladder forms at embryonic day 14 (e14). Urothelium proliferates and differentiates until the perinatal period (e17-birth; ref. 20), giving rise to the mature relatively quiescent trilaminar state consisting of basal stem cells, intermediate transit amplifying cells, and fully differentiated superficial/umbrella cells (21, 22). In rapidly growing epithelium (e14), Sox9 protein induction was detected by IHC staining in the basal and intermediate layers, but not in the superficial layer, with occasional staining seen in stromal cells (Fig. 1A). Sox 9 staining diminished near term (e18, Fig. 1B), was barely detectable by postnatal day 2 (P2; Fig. 1C), and was undetectable by P7 (Fig. 1D). SOX9 was also quiescent in human urothelium from organ donors ranging from age 4 to 40 (data not shown).
the onset of such injury in mice, we found that Sox9 levels rose and fell in parallel with the urothelial repair program. Urothelial necrosis, sloughing, and regeneration began shortly after CPA administration, with peak urothelial proliferation at 36 hours and return to baseline by 7 days, when injury had healed (refs. 23, 24; Supplementary Fig. S1). Sox9 was undetectable by IHC in adult controls. Within 20 hours of CPA injection, Sox9 protein was readily detected in all 3 urothelial cell layers. By 40 hours, we observed Sox9 expression only in the basal cell layer, and by 7 days, the protein was undetectable (Fig. 2A). Thus, induction of Sox9 is a tightly regulated event with temporal kinetics that track closely with urothelial injury repair.

**EGFR pathway induction in urothelial injury repair**

Given its known roles in injury repair (25, 26) and proposed roles in the urothelium (26, 27), EGFR and its family members are likely candidates to induce Sox9. To separately examine epithelial and stromal responses to injury, we rapidly separated the 2 compartments for analysis at the time of harvest after mice had been injected with PBS or CPA for 20 or 40 hours. By reverse transcriptase (RT)-PCR analysis, Egfr, Her2, and Her3 were readily detected at equivalent levels in urothelium (mucosa) and in the muscular bladder wall (stroma), whereas Her4 was nearly undetectable in mucosa (Fig. 3A). Injury caused little change in receptor transcripts (Fig. 3A). In contrast, levels of Egfr ligands were markedly induced (up to 42-fold, \( P < 0.001 \)) in urothelial mucosa. Amphiregulin (Areg), HB EGF-like growth factor, epiregulin, and epigen were the most highly induced ligands, with peak induction within 20 hours of injury (Fig. 3B; \( P < 0.001 \)). Because urothelial basal cells express EGFRs (27, 28), the induction of EGFR ligands in urothelial cells by injury suggests an autocrine/paracrine mechanism through which injury could lead to Sox9 induction.

**EGFR induces Sox9 through ERK1/2 signaling**

We confirmed that Egfr induces Sox9 expression through Erk1/2 signaling by using cultured strips of mouse bladder ( explant culture), with the cut tissue edges simulating urothelial injury (29). Unlike intact bladder, urothelial Sox9 expression was readily detected (Fig. 3C), but this induction was completely blocked by culture with erlotinib (Fig. 3C), confirming Egfr-dependent activation of the pathway. Sox9 induction was also blocked by a MEK1/2 inhibitor, U0126 (Fig. 3C), indicating that canonical Egfr signaling through the Erk1/2 pathway is required for induction of Sox9 by injury.

We confirmed and further investigated this pattern of SOX9 regulation in benign immortalized human UROtsa cells. This line phenocopies the basal cells from which it was derived, as evidenced by the expressions of EGFR and p63 (Supplementary Fig. S2; ref. 30). Under normal culture conditions, SOX9 protein was undetectable (Fig. 4A; Supplementary Fig. S2B and C). Proteolysis through the ubiquitin-proteasome pathway likely contributes to such low SOX9 levels, because treatment with the proteosome blocker MG132 increased the levels of SOX9 protein (Supplementary Fig. S4A). SOX9 induction also resulted from culture with EGFR ligands, including EGF, which is physiologically enriched in urine (27), and HB-EGF, which is physiologically synthesized by urothelial cells (31) and upregulated in urothelial cells following injury (Fig. 3A).
When mediated by EGF or HB-EGF, SOX9 induction was detectable within 6 hours of ligand exposure (Supplementary Fig. S4B), consistent with the kinetics seen with CPA exposure in vivo (data not shown). Induction in this case likely resulted from enhanced de novo synthesis of the SOX9 protein rather than enhanced protein stability because the protein translation inhibitor cycloheximide blocked SOX9 induction (Supplementary Fig. S4B). Consistent with results from organ culture (Fig. 3C), SOX9 induction in cell lines was completely blocked by ERK1/2 or EGFR inhibition (Fig. 4A). Thus, the urothelial EGFR-ERK1/2-SOX9 activation in cell lines was completely blocked by ERK1/2 or EGFR inhibition (Fig. 4A). Therefore, the urothelial EGFR-ERK1/2-SOX9 pathway seems to operate similarly across mouse and human epithelial interactions.

Injurious chemicals present in urine, such as NaCl and urea (32), or released from injured cells, such as H₂O₂ (33) and ATP (34), are known to activate EGFR. In UROtsa cells, treatment with H₂O₂, NaCl, or ATP-γ-S (a nonhydrolyzable form of ATP) induced coordinate EGFR phosphorylation and elevated levels of SOX9 (Fig. 4B; Supplementary Fig. S5A). Interestingly, treatment with urea affected neither EGFR phosphorylation (Supplementary Fig. S5B), nor SOX9 levels (data not shown), suggesting that distinct pathways may mediate responses to different injuries. Thus, a variety of physiologically relevant injuries induce SOX9 in urothelial cells, and such induction may involve activation of EGFR.

**Autocrine EGFR signaling maintains constitutive SOX9 elevation in carcinomas**

In contrast to undetectable levels of SOX9 found in benign urothelial cells, SOX9 protein levels were high in 8 of 9 UroCa cell lines tested (Supplementary Fig. S2B). As shown in human BFTC905 (Fig. 4A; Supplementary Fig. S6B), J82 (Supplementary Fig. S6A) and murine MB49 UroCa cells (Supplementary Fig. S6B), and confirmed in human SCaBER squamous bladder carcinoma cells (Supplementary Fig. S6A), treatment with the EGFR inhibitor or with the MEK1/2 inhibitor effectively suppressed SOX9 to undetectable levels. In contrast, effective pharmacologic inhibition (Supplementary Fig. S4) of Akt, p38 MAPK, STAT3, c-MET, insulin-like growth factor-IR, or platelet-derived growth factor did not significantly alter SOX9 expression (Fig. 4; Supplementary Fig. S6A and B). SOX9 expression in urothelial cancer cells was further increased by treatment of EGF or HB-EGF, but not when cells were pretreated with erlotinib or U0126 (Fig. 4A; Supplementary Fig. S6A). In addition to UroCa, we found evidence for an active EGFR/ERK1/2/SOX9 signaling axis in a variety of other human carcinomas, including those arising in lung, prostate, oropharyngeal mucosa, and skin (Supplementary Fig. S7A). These data indicate that constitutive activation of SOX9 expression through EGFR and ERK1/2 is a common feature of carcinomas. This constitutive activation is also present in vivo as shown in the corresponding xenografts of UroCa and some other carcinomas (Supplementary Figs. S2D and 7B).

![Figure 3. Upregulation of Sox9 in urothelial injury by Egfr family receptors and ligands. A, real-time RT-PCR shows the levels of mRNA transcripts encoding Egfr, Her2, Her3, and Her4 receptors in the bladder wall (stroma, right) and urothelial mucosa (left), compared with controls. The inductions were not significant, except for Her4, which showed decreased expression in CPA-injured mouse urothelial mucosa. *, P < 0.05. B, transcripts encoding Egfr family ligands Areg, Hb-egf, Epigen, (Epigrn), and Epiiregulin (Ereg), were markedly induced in urothelial mucosa (left) by CPA injury, but not in bladder wall (stroma, right). The quantitative data shown in A and B were averaged from 5 bladders in each condition (PBS control and CPA injury). Values are expressed as mean ± SEM. *, P < 0.001. C, photomicrographs (bar = 50 μm) show intense nuclear IHC staining for Sox9 in surgically injured bladder explants cultured in control media (vehicle). Twenty-four-hour culture with erlotinib or U0126 resulted in undetectable Sox9 levels. Representative images from 6 independent experiments (n = 6).](image-url)
Constitutive SOX9 expression may result from autocrine/paracrine signaling by HB-EGF, a phenomenon previously observed to promote growth in urothelial cell cultures (31). Heparin binds EGFR ligands, HB-EGF, and AREG with high affinity. Heparin reduced SOX9 expression (Supplementary Fig. S6C), suggesting that UroCa cells produce EGFR ligands, HB-EGF, and AREG, to sustain constitutive SOX9 expression.

**Requirement for SOX9 in cell migration and invasion, but not proliferation**

Urothelial injury repair likely requires coordinated proliferation and differentiation of basal cells as they migrate to cover the wound and reconstitute the urothelial barrier. To address potential roles of SOX9 in this process, we used stable expression of 2 different shRNAs to generate human BFTC905 UroCa clones deficient in SOX9 (Supplementary Fig. 8A & B). Comparing clones with low (clone 16), intermediate (clone 5), and high (control) levels of SOX9 (Fig. 5A; Supplementary Fig. S8B), effective reduction of SOX9 elicited no significant change in growth rate (Fig. 5B). However, in proportion to the effectiveness of SOX9 reduction, SOX9-deficient BFTC905 cells showed markedly impaired abilities to migrate and cover a wound scraped across a culture dish (Fig. 5C). Consistent with a migration defect, SOX9-deficient BFTC905 cells displayed decreased abilities to invade Matrigel- and collagen-coated membranes in transwell (Boyden) chamber assays (Fig. 5D; Supplementary Fig. S9). This phenotype was confirmed by using separate shRNAs targeting 2 distinct regions of the SOX9 transcript, indicating that the migration effect is an "on-target" effect. By using transient SOX9 knockdown, this effect was further confirmed in another human UroCa cell line, UM-UC-3 (Supplementary Fig. S10). T24 UroCa cells, in contrast, were unaffected, indicating that they use substitute or redundant pathways for migration. Although not universal, these results confirm a role for SOX9 in UroCa invasion and migration.

**Sox9 is reexpressed in UroCa**

Expression analysis in primary human samples suggests a general role for SOX9 in UroCa, particularly in invasive cancers. UroCa arises through 2 divergent pathways (reviewed in ref. 35). The majority originates in an indolent form with papillary formations that extend into the bladder lumen. A minority originates as flat or invasive forms, metastasizes early, and is often lethal. In analysis of SOX9 IHC by H-score (intensity × proportion of stained cells), cancers scored higher than benign urothelium (P = 0.0001). Compared with noninvasive papillary cancers, SOX9 induction was more dramatic in more aggressive flat/invasive cancers (P < 0.03). SOX9 induction was 7-fold for CIS and 14-fold for invasive cancers (Fig. 6A). Induction was observed frequently. Compared with benign urothelium, SOX9 staining was elevated in 75% of CIS cases and 89% of invasive cases (data not shown). However, SOX9 staining was as high in early stage (pathologic stage pT1) invasive carcinomas as in advanced (pT3 or greater) cases (data not shown). Thus, SOX9 induction seems to be a general property of UroCa, particularly of the more aggressive flat/invasive pathway, consistent with the notion that the protein is induced early in the course of urothelial carcinogenesis.

**Discussion**

In embryonic urothelium and urothelium undergoing injury repair, Sox9 is expressed in basal and intermediate cells, but not in terminally differentiated superficial cells. This expression pattern suggests that the transcription factor may antagonize urothelial differentiation, a role consonant with that seen in preadipocytes (36), chondrocytes (37), pyloric sphincter epithelial cells (38), and early differentiation of prostate bud epithelia (7).

Sox9 expression is limited to the invasive front of epithelial buds in developing prostate (7–9) and lung (39) as the buds...
and injury repair. whereas the migratory role is common to both organogenesis and embryonic growth versus injury repair, it may be that the proliferative role of SOX9 operates mainly in primitive embryonic cells whereas the migratory role is common to both organogenesis and injury repair.

In normal undamaged bladder, an overlying urine-blood barrier formed by superficial cells (20, 40) is hypothesized to protect the EGFR-enriched basal cell layer (27, 28, 41) from contact with urinary EGFR ligands (especially EGF). In addition to the possibility that basal urothelial cells might respond to urinary ligands, urothelium and adjacent smooth muscle can produce EGFR ligands in response to injury, including TGFα (42), HB-EGF, and Epiregulin (43). It has not been previously shown that injury to urothelial tissue activates EGFR signaling, and the sequelae of such activation have not been previously determined. Here we provide new evidence that HB-EGF and AREG are significantly induced in UroCa compared with noninvasive tumors or benign urothelium (Fig. 6A). The role of SOX9 in cell migration is also consistent with the notion that urothelial cells are very mobile during injury repair and need to migrate to the superficial layer and to differentiate to heal. In UroCa cells, aberrant expression of EGFR receptors and ligands that lead to constitutive induction of SOX9 also support the invasive migratory phenotype of these cells. This notion is further supported by our recent finding that EGFR ligands, HB-EGF, and NRG2, were highly expressed in a highly tumorigenic basal cell compartment in UroCa (44) that is also enriched for EGFR (Supplementary Fig. S2A) and SOX9 expression (Supplementary Fig. S2D).

Our findings have implications for bladder injury repair and carcinogenesis. Cancer is long been thought as a chronic wound that does not heal (45). The ultimate source of cells for repairing injured tissue is stem/progenitors cells. For tissues with a slow turnover like urothelium, stem/progenitor cells remain quiescent. However, when injury occurs, cells can rapidly migrate (a process known as epithelial restitution), proliferate, differentiate, and remodel to heal the wound. A common trait of both cancer and repair is the activation of signaling pathways best known for their roles in embryonic growth and patterning. We (1, 46) hypothesize that the proliferative role of SOX9 operates mainly in primitive embryonic cells whereas the migratory role is common to both organogenesis and injury repair.

We have discovered a role for SOX9 in cancer cell migration and invasion, indicating that SOX9 might mediate EGFR-induced cancer spread. We further provide evidence for this hypothesis by showing that SOX9 has significantly higher expression in the flat/invasive pathway of UroCa compared with noninvasive tumors or benign urothelium (Fig. 6A). The role of SOX9 in cell migration is also consistent with the notion that urothelial cells are very mobile during injury repair and need to migrate to the superficial layer and to differentiate to heal. In UroCa cells, aberrant expression of EGFR receptors and ligands that lead to constitutive induction of SOX9 also support the invasive migratory phenotype of these cells. This notion is further supported by our recent finding that EGFR ligands, HB-EGF, and NRG2, were highly expressed in a highly tumorigenic basal cell compartment in UroCa (44) that is also enriched for EGFR (Supplementary Fig. S2A) and SOX9 expression (Supplementary Fig. S2D).

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that chronic injury acts on tissue stem cells/progenitors to permanently activate survival, proliferation, and migration, all of which are prominent features of cancer. As part of this process, we propose that urothelial cells become cell autonomous for EGFR activation during urothelial injury, and that such activation induces SOX9 expression to support epithelial migration and wound repair. In chronic injury, unknown genetic or epigenetic mechanisms could lock this signaling circuit in the active state, contributing to malignant transformation of urothelial cells. Future studies will be needed to expand this pathway upstream to identify mediators of sustained EGFR ligand expression and downstream to discover the molecular links between SOX9 and the migration machinery.

Effective treatment of any cancer will likely require combinations of targeted therapies that overcome resistance mechanisms and redundant signaling circuits. A better understanding of inducers and effectors of this newly recognized EGFR-ERK1/2-SOX9 pathway has the potential to aid in this effort.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Figure 6. SOX9 is overexpressed in human bladder cancer tissues.**

A, IHC SOX9 staining showed undetectable or low levels in benign urothelium (n = 49; representative section in A) staining (both distribution and intensity) was slightly elevated in noninvasive carcinomas (n = 56; staining not shown), more widespread and intense in the majority of high-grade flat carcinoma in situ (Flat) lesions (n = 15; staining not shown) and invasive human UroCas (n = 84; A, middle panel). Overall, mean SOX9 scores for cancer exceeded those for benign urothelium (*, P = 0.0001), and mean scores for invasive cancers exceeded those for noninvasive cancers († P < 0.03). Compared with benign urothelium, SOX9 scores were slightly elevated in low-grade papillary noninvasive cancers (not significant) but became increasingly elevated in high-grade and invasive tumors (*, P < 0.05). B, model of EGF ligand-receptor-ERK1/2-SOX9 induction by injury. Note that the system turns off in response to completion of injury repair. With chronic injury, the system may remain activated and facilitate cancer formation.


An EGFR-ERK-SOX9 Signaling Cascade Links Urothelial Development and Regeneration to Cancer

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