Tyrosine Kinase Etk/BMX Is Up-regulated in Human Prostate Cancer and Its Overexpression Induces Prostate Intraepithelial Neoplasia in Mouse


Abstract
The nonreceptor tyrosine kinase Etk/BMX was originally identified from the human prostate xenograft CWR22. Here, we report that Etk is up-regulated in human prostate tumor specimens surveyed. Knocking down Etk expression by a specific small interfering RNA (siRNA) in prostate cancer cells attenuates cell proliferation, suggesting an essential role of Etk for prostate cancer cell survival and growth. Targeted expression of Etk in mouse prostate epithelium results in pathologic changes resembling human prostatic intraepithelial neoplasia, indicating that up-regulation of Etk may contribute to prostate cancer development. A marked increase of luminal epithelial cell proliferation was observed in the Etk transgenic prostate, which may be attributed in part to the elevated activity of Akt and signal transducers and activators of transcription 3 (STAT3). More interestingly, the expression level of acetyltransferase cyclic AMP–responsive element binding protein–binding protein (CBP) is also increased in the Etk transgenic prostate as well as in a prostate cancer cell line overexpressing Etk, concomitant with elevated histone 3 acetylation at lysine 18 (H3K18Ac). Down-modulation of Etk expression by a specific siRNA leads to a decrease of H3 acetylation in prostate cancer cell lines. Our data suggest that Etk may also modulate chromatin remodeling by regulating the activity of acetyltransferases, such as CBP. Given that Etk may exert its effects in prostate through modulation of multiple signaling pathways altered in human prostate cancer, the Etk transgenic mouse model may be a useful tool for studying the functions of Etk and identification of new molecular markers and drug targets relevant to human diseases.

Introduction
Prostate cancer is the most commonly diagnosed cancer among men in the Western world. The lifetime risk of having microscopic evidence of prostate cancer for a 50-year-old man is ~40%. Prostate cancer is a complex disease and displays extensive heterogeneity both genetically and morphologically (1, 2). Despite intense investigations in the past decades, the molecular basis underlying prostate cancer development and progression is not yet completely understood. Numerous genetic and epigenetic alterations, including loss of tumor suppressors (e.g., Pten, Nkx3.1, p53, and Rb) and amplification of oncogenes (e.g., Myc, FGFR8b), have been reported to associate with prostate cancer (3, 4). A number of experimental approaches have been used to assess the contribution of a given genetic/epigenetic alteration to prostate cancer development and/or progression. Although a series of genetically modified animal models for prostate cancer have been established (5, 6), other animal models that recapitulate human disease process are still in demand to fully understand the molecular basis of prostate cancer.

Etk/BMX is a member of the Tec family of nonreceptor tyrosine kinases. We originally identified Etk from a human prostate tumor xenograft CWR22 (7). The gene encoding Etk/BMX kinase is located on human chromosome Xp22 (8). Etk and its family members have emerged as major modulators of signaling pathways initiated by various extracellular stimuli, including growth factors, cytokines, extracellular matrix, and hormones (9). The versatile roles of Etk and its family members are reflected by their multiple conserved signaling modules. Etk contains an NH2-terminal pleckstrin homology domain, a Src homology 3 domain, a Src homology 2 domain, and a COOH-terminal tyrosine kinase domain. Etk has been implicated in various biological processes, including proliferation, differentiation, apoptosis, and cell migration. Etk is also found to be highly expressed in aggressive metastatic prostate and breast tumor cell lines (10). Activation of Etk kinase activity can be achieved by interaction of its pleckstrin homology domain either with the phosphotyidylinositol 3-kinase product phosphatidylinositol 3,4,5-triphosphate or the FERM domain of FAK (9, 10), which leads to plasma membrane translocation of Etk. The interaction between Etk and FAK is involved in integrin signaling and may play a role in tumor metastasis of prostate cancer cells (10, 11). Etk is activated by interleukin-6 (IL-6) in prostate cancer cells through the phosphotyidylinositol 3-kinase pathway and has been implicated in neuroendocrine differentiation (7). Etk can also be stimulated by neuropeptides, such as bombesin and neurotensin, and its kinase activity is required for the androgen-independent growth of prostate cancer cells induced by these factors (12). More recently, we have shown that Etk can form a complex with another nonreceptor Ser/Thr kinase Pim-1 (13), which is overexpressed in >50% of prostate cancer samples surveyed (14). The synergism...
between Etk and Pim-1 is essential for IL-6–induced androgen-independent activation of androgen receptor (AR) in prostate cancer cells (13). Etk has been implicated in activation of signal transducers and activators of transcription 3 (STAT3) and Akt in several cell types (15–18). We also showed that a direct physical interaction occurs between Etk and tumor suppressor p53 and such interaction results in a bidirectional inhibition of the functions of both proteins (19). Overexpression of Etk in prostate cells confers resistance to chemotherapy drugs. Therefore, Etk may play a prominent role in antiapoptosis signaling in prostate cancer cells.

In this report, we show that Etk is up-regulated in human prostate tumor specimens. Knocking down Etk expression by a specific small interfering RNA (siRNA) in prostate cancer cells attenuates cell proliferation. To assess the contribution of Etk in prostate cancer development, we generated transgenic mice overexpressing Etk in the prostate epithelium. The epithelial Etk transgene induces a pathologic change resembling human prostatic intraepithelial neoplasia (PIN), suggesting a causal role of Etk in prostate cancer development. Our study reveals that Etk can regulate multiple signaling pathways in the transgenic prostate, which may contribute to the development of prostate cancer.

Materials and Methods

**Tissue microarray, immunohistochemical, and statistical analysis.** Four tissue microarrays were purchased from Zymed (South San Francisco, CA; 75–4063) and Biomax (Rockville, MD; BC19111, PR8901, and PR802). These arrays included a total of 59 benign prostate tissue samples, and a total of 171 localized prostate cancer samples (Gleason grades ranging from 6 to 10). The Vectastain Elite ABC kit, purchased from Vector Laboratories (Burlingame, CA), was used for immunohistochemical analysis. Briefly, slides were deparaffinized with xylene and rehydrated through graded alcohol washes followed by antigen retrieval by microwave irradiation for 30 minutes in sodium citrate buffer (10 mmol/L, pH 6.0). Slides were then incubated in 0.3% hydrogen peroxide to quench endogenous horseradish peroxidase (HRP) for 30 minutes. The slides were then blocked by incubation in normal goat serum (dilution 1:10) in PBS (pH 7.4) and subsequently incubated for 60 minutes with anti-Etk antibody. Slides were then treated with biotin-labeled anti-rabbit IgG and incubated with preformed avidin-biotin peroxidase complex. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Immunohistochemical staining was assessed independently by two pathologists (X.K. and J.M.), and a consensus of grading was reached. Immunostaining was evaluated manually and graded using a two-score system based on intensity score and proportion score as described previously (20). Intensity score was scored on the following scale: 0, negative; 1, weak; 2, moderate; 3, strong. Distribution of immunopositive tumor cells was scored on a scale of 0 (0%), 1 (0.1–1%), 2 (2–10%), 3 (11–33%), 4 (34–66%), and 5 (67–100%). The immunoreactivity score was determined by the sum of intensity score and proportion score. The nonparametric Wilcoxon rank sum test was done using standard procedures. The immunohistochemistry analysis was carried out as described above with the following antibodies diluted in PBS: anti-Ki67 (DAKO), anti-Nkx3.1 (24), and anti-p63 (Neomarker, Fremont, CA). Negative controls were included in each assay. To quantify the immunohistochemical staining, a total of 500 cells were counted from five independent fields for each slide and the percentage was presented as the number of cells positively stained with the indicated antibodies per 100 total nucleated cells.

**Western blot analysis.** Prostate tissues were extracted by using the T-PER protein extraction reagent (Pierce, Rockford, IL). The equal amounts of protein extracts were resolved on a SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Immunoblotting was done as described previously (19). Anti-Etk was used at 1:2,000, whereas anti-pAktS473 and anti-pSTAT3Y705 (Cell Signaling Technology, Danvers, MA) were used at 1:1,000. Anti-AR, anti-Akt, and anti-signal transducers and activators of transcription 3 (STAT3; Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:2,000.

**Cell culture and lentiviral infection.** LNCaP was purchased from the American Type Culture Collection (Manassas, VA) and CWR-R1 was kindly provided by Drs. Christopher Gregory and Elizabeth Wilson (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC; ref. 25). LNCaP was maintained in RPMI 1640 with 10% fetal bovine serum. LNCaP cells overexpressing T7-Etk was described previously (19). The lentivirus infections were carried out as previously (19). The cells were transfected with FuGENE 6 (Roche) or LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following the instructions of the manufacturers.

Results

**Etk/BMX is overexpressed in human and mouse prostate carcinomas.** To examine the expression of Etk in human prostate tumor tissues, immunohistochemical analysis was done on four tissue arrays containing a total of 230 prostate specimens, including 59 benign and 171 prostate tumor samples. Etk immunoreactivity is dramatically increased in the cytoplasm of prostate tumor samples (6.5 ± 0.1) compared with that in benign samples (3.6 ± 0.3; P < 0.0001). Figure L.4 shows the representative views...
of our tissue arrays. Etk staining was predominantly detected in the cytoplasm of prostate tumor cells and, to a much lesser extent, in the luminal epithelial cells in benign samples. Such alteration in Etk staining pattern was often observed in the tissue section from the same patient (Supplementary Fig. S1). There is a positive correlation of Etk immunoreactivity with the Gleason grades of the tumor samples (Pearson correlation coefficient $p = 0.15$, $P < 0.05$). The elevated Etk protein level in prostate tumors was further confirmed by Western blot analysis on three pairs of tumor tissues and surrounding benign samples. Prostate tumor and surrounding benign tissues were derived from radical prostatectomy of three prostate cancer patients (Gleason grade ranged from 7 to 9). The total tissue lysates were subjected to immunoblotting (IB) with anti-Etk antibody. Actin served as a loading control. Etk expression in the mouse prostate tissues. Total protein was extracted from the prostate dissected from the 10-week-old WT and the homozygous Pten-null (Pten–/–) mice, respectively. Etk expression was determined by Western blot using anti-Etk antibody. Actin served as a loading control.

**Generation of transgenic mice expressing Etk in prostate epithelium.** To better understand the role of Etk in prostate cancer, we generated Etk transgenic mouse by using the modified probasin promoter, ARRP2PB, which has been shown to direct target gene expression in prostate epithelial cells (Fig. 2A). The presence of the Etk transgene in the genomic DNA from two transgenic founders was confirmed by PCR and Southern blot (Fig. 2B). As predicted, the elevated expression of Etk protein was primarily detected in the luminal epithelial cells of the transgenic prostate by immunohistochemical staining (Fig. 2C) and Western blot (Fig. 2D). To further confirm prostate-specific Etk expression in prostate epithelial cells, the prostate from wild-type (WT) mice and Etk transgenic mice at the age of 12 weeks were carefully dissected, and targeted Etk expression was examined by immunohistochemistry and Western blot analysis. Immunohistochemistry staining and Western blot with Etk antibody showed prostate epithelial cell–specific expression of the transgene (Fig. 2C and D). Therefore, we have established a transgenic mouse model with targeted expression of Etk in prostate epithelium.

**Targeted expression of Etk in mouse prostate epithelium leads to pathologic changes resembling human PIN.** Our histologic examination revealed no obvious abnormalities in the prostate glands of Etk transgenic mice at 2 to 4 months of age. However, at the age of 5 to 6 months, 31 of 59 (52.5%) Etk transgenic mice displayed epithelial hyperplasia in the dorsolateral lobes of prostate and to a lesser extent in the ventral lobes and the...
anterior lobes. At 1 year of age, 21 of 59 (35.6%) Etk transgenic mice developed the pathologic changes resembling human PIN (Fig. 3A). The hallmark for PIN are the atypical proliferating luminal epithelial cells within preexisting prostatic ductules/acini and the expanded stroma surrounding the lumen. The marked increase in the cellularity and thickness of the stroma was evident in Fig. 3B. We further characterized these PIN-like lesions by examining the expression of several well-characterized biomarkers of human PIN. Ki67 is an index of proliferation, which is elevated in human PIN. Ki67-positive cells are rarely detected in the luminal compartment. However, in the Etk transgenic prostate, the number of Ki67-positive cells is dramatically increased (Fig. 3B). The proliferation indexes of Etk transgenic prostate are 4-fold higher than the age- and genetic background–matched WT controls (Fig. 3B). This suggested that Etk can promote proliferation of prostatic luminal epithelial cells. NKX3.1 is a prostate-specific tumor suppressor gene located at chromosome 8p21, which frequently displays loss of heterozygosity at early stages of human prostate cancer, and genetic deletion of NKX3.1 in adult mouse prostate resulted in PIN lesions (27–30). Immunohistochemical study showed that Nkx3.1 protein expression was present but significantly decreased in Etk transgenic prostate (Fig. 3B). These data, taken together, suggest that Etk transgenic prostates develop lesions that histopathologically resemble human PIN.

Mechanisms of action of Etk in mouse prostate. To investigate the molecular mechanisms by which Etk induces the PIN-like lesion in the mouse prostate, we examined the status of several known Etk downstream effectors, such as STAT3 and Akt. As shown in Fig. 4A and B, the level of phospho-STAT3 in the dorsolateral lobes of prostate epithelial cells was significantly increased in the Etk transgenic mice compared with their nontransgenic littermates. Previous studies suggested that Etk can activate Akt in cultured cell lines (15, 18). The level of phospho-Akt is significantly up-regulated in Etk transgenic prostates (Fig. 4A and B). These results further confirmed that Etk transgenic prostates develop lesions that histopathologically resemble human PIN.
and B). These data together indicated Etk overexpression leads to activation of both STAT3 and Akt pathways in mouse prostates, which may contribute to the elevated cell proliferation in the transgenic prostate. In our PCR-select differential screening to uncover differentially expressed genes in Etk-overexpressing LNCaP cells, a histone acetyltransferase cyclic AMP–responsive element binding protein–binding protein (CBP) was revealed as one of potential targets that are up-regulated by Etk (Supplementary Fig. S2). We therefore examined the level of CBP in Etk transgenic prostate. Figure 5A and B shows that the level of CBP protein is increased in the Etk transgenic prostate compared with its normal littermate assessed by both immunohistochemistry and Western blot. Recently, it has been reported that the level of acetylation of histone H3 at lysine 18 (H3K18Ac) is positively correlated with tumor grades in human prostate cancer, and CBP is known to induce histone H3 acetylation at K18 (34, 35). We therefore examined the level of H3K18Ac in these mice. As shown in Fig. 5B, the level of H3K18Ac is dramatically increased in the Etk transgenic prostate concomitant with the increase of CBP. The increase of CBP protein level in the Etk-overexpressing LNCaP cells was also confirmed by Western blot (Fig. 5C), which is accompanied with hyperacetylation of K18 of histone 3. Furthermore, knocking down Etk expression in LNCaP cells resulted in a decreased acetylation of H3K18 (Fig. 5C) and attenuated cell proliferation (Fig. 5D). These data taken together showed that Etk can modulate multiple signaling pathways in prostate cells.

Discussion

We initially identified Etk/BMX in a human prostate tumor xenograft. Since then, much work has been done in tissue culture systems to understand its role in signal transduction in various cancer cells. However, its role in prostate cancer still remains elusive. In this report, we, for the first time, showed that Etk is up-regulated in human prostate tumors. The possible involvement of Etk in prostate cancer development was further supported by its increased expression in the prostate of Pten-conditional knockout mice. To better understand the role of Etk in prostate cancer, we have established a transgenic mouse model in which Etk is targeted and expressed in mouse prostate epithelial cells by using a well-defined prostate-specific promoter (ARR 2PB). We found that targeted Etk expression in mouse prostate epithelial cells results in pathologic changes resembling human PIN. The lesions in the Etk transgenic prostates displayed a marked increase of epithelial cell proliferation. This is consistent with the result obtained in the skin BMX/Etk transgenic model (36). In addition, knocking down Etk expression in a prostate cancer cell line by a specific siRNA attenuated cell proliferation in vitro. Our results...
further support a role of Etk for promoting cell proliferation and survival in prostate cells.

Although the detailed molecular mechanisms by which Etk transgene induces the PIN-like lesion in mouse prostate remain to be further investigated, some of the observations made in vivo with this transgenic model give us some clues. Consistent with previous studies carried out in cell lines, Etk can induce Akt and STAT3 activity in mouse prostate, which may account for, at least in part, the increase of epithelial cell proliferation observed in this transgenic model. More interestingly, the expression level of acetyltransferase CBP is moderately increased in the Etk transgenic prostate. This was further confirmed by the in vitro study that overexpression of Etk leads to the increase of CBP expression level in LNCaP cells. It has been shown that p300 and CBP play an important role in prostate cancer cell proliferation as well as prostate cancer progression (37, 38). This raises the possibility for Etk to participate in regulation of chromatin remodeling by modulating these histone-modifying enzymes. Indeed, the increase of acetylated histone H3 (H3K18Ac) was detected in Etk transgenic prostates. Recently, it has been reported that H3K18Ac is positively correlated with increasing grade of human prostate cancer. Our results suggest that the PIN-like lesion in this model may be partly due to in vivo activation of the Etk→CBP→H3K18Ac pathway. We also showed that Etk up-regulates AR protein levels in both the transgenic prostate and LNCaP cells in culture. The regulation of AR by Etk may not occur at the transcriptional level because we did not detect an increase in AR mRNA level in Etk-overexpressing LNCaP cells by real-time reverse transcription-PCR. It is most likely through a posttranslational mechanism such as phosphorylation and/or protein-protein interaction as we were able to detect physical interaction between Etk and AR in prostate cancer cells and increased tyrosine phosphorylation of AR in the Etk transgenic prostate.8 We also showed that the level of Nkx3.1 protein is decreased in the Etk transgenic prostate consistent with the increased proliferation. Loss of Nkx3.1 in mouse prostate leads to development of high-grade PIN (27, 28). Therefore, down-regulation of Nkx3.1 may be attributed, at least in part, to the PIN-like lesion in the Etk transgenic prostate. It has yet to be determined how Nkx3.1 is suppressed by Etk. Further experiments are necessary to investigate the signaling network regulated by Etk using this transgenic model and possibly in combination with other currently available prostate models.

It is noteworthy that although Etk expression is mainly targeted in the epithelial cells in our model, the stroma surrounding the epithelium also display profound changes, possibly under the influence of abnormal changes that occurred in the epithelium. One possible scenario could be that Etk can up-regulate soluble factors, such as vascular endothelial growth factor (VEGF), which in turn modulate the stroma, as previous studies showed that Etk can promote epithelial and endothelial cell proliferation in culture by up-regulating VEGF expression (15). A recent study showed that STAT3 and CBP are components of a transcriptional complex for regulating VEGF expression in several carcinoma cell lines (39). It is likely that Etk may also promote VEGF production via STAT3 and CBP in prostate tissue. Alternatively, Etk may also modulate cell surface molecules, such as integrins, to influence the changes in stroma cells as modulation of integrins by Tec family kinases was reported previously (40).

Further experiments are required for elucidating how Etk modulate the epithelia-stroma interaction in prostate and how such interaction contribute to prostate cancer development. Although it was reported that no obvious abnormal phenotype was found in the BMX/Etk knockout mice (41), it is possible that other members of the Tec family kinases may provide redundant functions during embryogenesis. However, targeted expression of BMX/Etk in epidermal keratinocytes induces skin hyperplasia (36). Our Etk transgenic model showed that overexpression of Etk may play an important role in prostate cancer development by modulating multiple signaling pathways. These transgenic models further support that deregulation of Etk in its target tissues may lead to abnormal proliferation of the target cells and result in premalignant lesions. However, overexpression of Etk alone in the target tissues seems not sufficient to drive cancer formation at least in skin and prostate, which is similar to the phenotype observed in the Akt transgenic model (42). Therefore, additional genetic alteration(s) are necessary for the disease progression to cancer. Perhaps, the potential of Etk transgenic model could be greatly enhanced by seeking genetic synergy between Etk transgene with other mutated genes or transgenes.

In summary, we have shown that tyrosine kinase Etk is up-regulated in human prostate tumors. To recapitulate such alteration in the mouse prostate, we have also established a transgenic model. Our initial characterization of this mouse model has revealed that Etk exerts its effects in prostate by modulating multiple signaling pathways that are altered in human prostate cancer. Therefore, the Etk transgenic mouse model established in this study may be a useful tool for further elucidation of the functions of Etk in prostate and identification of new molecular markers and drug targets relevant to human prostate cancer.

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