Cooperation between FGF8b Overexpression and PTEN Deficiency in Prostate Tumorigenesis

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Abstract

Two commonly occurring genetic aberrations of human prostate cancer [i.e., overexpression of a mitogenic polypeptide (fibroblast growth factor 8, isoform b or FGF8b) and loss of function of PTEN tumor suppressor] were recapitulated into a new combinatorial mouse model. This model harboring the Fgf8b transgene and haploinsufficiency in Pten, both in a prostate epithelium–specific manner, yielded prostatic adenocarcinoma with readily detectable lymph node metastases, whereas single models with each of the defects were shown earlier to progress generally only up to prostatic intraepithelial neoplasia (PIN). In addition to late age-related development of typical adenocarcinoma, the model also displayed a low incidence of mucinous adenocarcinoma, a rare variant type of human prostatic adenocarcinoma. The cooperation between FGF8b activation and PTEN deficiency must be linked to acquisition of additional genetic alterations for the progression of the lesions to primary adenocarcinoma. Here, we identified loss of heterozygosity at the Pten gene leading to biallelic loss, as a necessary secondary event, indicating that a complete loss of PTEN function is required in the development of invasive cancer in the model. Analyses of expression of downstream mediators phospho-AKT (p-AKT) and p27KIP1, in various types of lesions, however, revealed a complex picture. Although PIN lesions displayed relatively strong expression of p-AKT and p27KIP1, there was a notable heterogeneity with variable decrease in their immunostaining in adenocarcinomas. Together, the results further underscore the notion that besides activation of AKT by loss of PTEN function, other PTEN-regulated pathways must be operative for progression of lesions from PIN to adenocarcinoma.

Introduction

There is currently a strong interest on the genetic alterations that are frequently encountered in human prostate cancer in the design of the mouse models (1, 2). The goal is to recapture the pathophysiological characteristics of the human disease in a “natural” manner in immunocompetent mice to facilitate exploration of the molecular mechanism underlying prostate cancer as well as for development or testing of new-targeted therapies. There is now ample evidence that mouse prostate can lead to stages of prostate tumorigenesis that are dependent on the selection of the gene target. For example, transgenic mouse models that represent activation of androgen receptor (AR; ref. 3), fibroblast growth factor 8, isoform b (FGF8b; ref. 4), FGFR1 (FGFR1; refs. 5, 6), or SKP2 (6) in the prostatic luminal epithelium, and those that address genome-wide knockout of a target gene, such as Nkx3.1 (7, 8) or conditional prostate-specific gene inactivation of Nkx3.1 (9) or Ruxz (10), generally display a pattern of increasing degree of phenotypic abnormalities, beginning with epithelial hyperplasia followed by presentations of preneoplastic lesions. Other types of modeling, like disruption of the master tumor suppressor gene Pten (11–13), overexpression of a strong proto-oncogene c-Myc (14), or that of a mutant AR (15), in the prostate epithelium, lead to development of invasive adenocarcinoma of the prostate. Evidence has been collected to show that in a Pten homozygous conditional deletion model, the primary cancer further progresses with metastases into lymph nodes and lung (11, 16).

Considering that carcinogenesis is related to accumulation of multiple genetic aberrations, it is likely to be better understood when a set of relevant genetic changes are present in a single model. In this regard, because low or absent expression of cyclin-dependent kinase inhibitor p27KIP1 correlates with poor prognosis of prostate cancer (17), and PTEN controls p27KIP1 through regulation of AKT kinase activity, crosses between Pten+/− and p27kip1+/− mice were made. Although Pten+/− mice display prostatic intraepithelial neoplasia (PIN) lesions, and p27kip1+/− display only mild hyperplasia, the double mutant mice exhibit multiple tumors in several different organs, including the prostate (18). Loss of function of PTEN also seems to cooperate with Nkx3.1 deficiency in mice. Increased incidence of high-grade PINs is detected in the Nkx3.1+/−;Pten+/− and Nkx3.1−/−;Pten−/− prostases, relative to Nkx3.1−/−;Pten+/− prostases (19, 20). Furthermore, the majority of Nkx3.1−/−;Pten−/− mice, >1 year of age, have invasive prostatic adenocarcinoma with lymph node metastases (21). Another example of synergy is noted when conditional Pten and p53 deletions were combined. Although no prostate tumors were found from conditional inactivation of the p53 gene, its combination with Pten deletions led to early onset of invasive cancer turning to lethality by 7 months of age (22). It is suggested that the growth arrest induced by p53-dependent cellular senescence pathway under PTEN deficiency may be rescued by simultaneous loss of p53 function. We hypothesized a cooperativity of FGF8b overexpression and PTEN deficiency based on the signaling pathways in which they are involved. Activation of FGFRs leads to activation of signal transducers and activators of transcription 1 (STAT1) and STAT3, Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase Cγ pathways (refs. 23–26; reviewed in ref. 27). PTEN, a lipid phosphatase, mainly exerts a negative regulation via antagonism of the PI3K/AKT pathway. With reduction or loss of its function, activated AKT persists to phosphorylate a number of key points.
intermediate signaling molecules, leading to increased cell metabolism, growth, and survival. However, because AKT activation alone induces only PIN in the prostate (28), PTEN must also function through some AKT-independent mechanisms (29–31). In this report, we describe evidence from combinatorial modeling in mice that defects in FGFB8 and PTEN expression, both of which are “natural” aberrations commonly found in human prostate cancer, can indeed strongly cooperate in the induction of prostatic adenocarcinoma and its metastatic progression.

Materials and Methods

Generation of compound mice with monoallelic Pten inactivation and overexpression of FGFB8. To generate mice with one conditionally deleted Pten allele, one wild-type (WT) Pten allele, and carrying the Fgf8b transgene (cPten<sup>+/−</sup> F mice), male mice of ARR2Pb promoter-driven (32), prostate epithelium–specific Cre line PB-Cre4 (33) on C57BL/6xDBA2 background were first crossed to homozygous floxed Pten (Pten<sup>1/2</sup>) mice (34) on the 129/BALB/c background. The male offspring were then crossed to female ARR2Pb-driven, Fgf8b transgenic line (4) on a C57BL/6xDBA2 background, and only F2 generation of male offspring was used in the study.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were sectioned (5 mm), placed on Superfrost slides, and stained with H&E. For immunohistochemistry, the sections were incubated at 65°C for 20 minutes and rehydrated, and antigens were retrieved via incubation of slides in 3% hydrogen peroxide in PBS for 30 minutes followed by incubation with primary antibodies at 4°C overnight. After three washings in PBS, avidin and biotin blocking was done. For polyclonal antibodies, SuperBlock blocking buffer (Pierce, Rockford, IL) was used. Slides were then allowed to cool at room temperature for 2 hours. After washing in deionized water and PBS, the slides were incubated in 3% hydrogen peroxide in PBS for 10 minutes to remove endogenous peroxidase activity.

For monoclonal antibodies, sections were blocked with M.O.M. mouse immunoglobulin blocking reagent (Vector Laboratories, Burlingame, CA) followed by incubation with primary antibodies at 4°C overnight or at room temperature for 30 minutes. For polyclonal antibodies, SuperBlock blocking buffer (Pierce, Rockford, IL) was used. Slides were then incubated with primary antibody at 4°C overnight. After three washings in PBS, avidin and biotin blocking was done. For phospho-AKT (p-AKT; Cell Signaling Technology, Beverly, MA), p63 and synaptophysin (Santa Cruz Biotechnology, Santa Cruz, CA), cytokeratin 18 (Chemicon, Temecula, CA), and K67 (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). Mouse pancreatic tissue (islets) was used as a control for synaptophysin immunostaining. Positive and negative controls were stained appropriately.

Reverse transcription-PCR. The RNA from the prostate tissues was extracted using Total RNA Purification kit (Genta, Minneapolis, MI) according to the protocols of the manufacturer followed by DNase treatment (Genta). The expression of Fgf8b transgene was determined by following the same reverse transcription-PCR (RT-PCR) protocols and primers as described earlier (4).

Microdissection and real-time PCR. Cancer cells were dissected from 10-μm H&E sections using the PALM micro-laser system, and DNA was prepared from catapulted samples according to the manufacturer’s protocols (P.A.L.M. Microlaser Technologies, Bernried, Germany). For PCR amplification, 1 μl of the solution was used for tubulin and 11 μl for the WT Pten allele. Primer sequences were as follows: tubulin: forward primer AGCCGACGCTGATCTAGAG and reverse primer GATG-TAGACGTGGTTGCTT; WT Pten allele, forward primer (WT-RR-F) GTTCACTACCAAGTGAAGTCT and reverse primer (WT-RR-R) GTTCACTACCAAGTGAAGTCT. The PCR conditions were 95°C for 10 minutes followed by 96 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 20 seconds. Real-time PCR was done on the Stratagene MX3000P system using SYBR Green Brilliant Mastermix (Stratagene, La Jolla, CA).

Statistical analysis. The significance of differences in tumor incidences was analyzed by Fisher’s exact test. A probability level of P < 0.05 was considered statistically significant.

Results

Combination of monoallelic inactivation of Pten and overexpression of FGFB8 leads to metastatic prostate cancer. We collected the prostates of a cohort of 31 cPten<sup>+/−</sup> F mice at age groups ranging from 6.5 to 19.1 months, as well as that of 11 cPten<sup>+/−</sup> littermates at age groups between 8.0 to 20.0 months, and evaluated histologically. In combined mutants, we identified hyperplasia and focal low-grade PIN (LGPIN) lesions by the time we started our examination (at 6.5 months of age). The hyperplastic epithelial lesions were multifocal, and PIN was detected in many ducts. Epithelial hyperplasia appeared as increased numbers of epithelial cells with or without atypia and was almost always associated with increased gland size. Atypical cells in LGPINs were generally larger with enlarged nuclei and hyperchromatic; there was only mild pleomorphism, and the nuclear/cytoplasm ratio was not appreciably increased. At ages >7.5 months, high-grade PINs (HGPIN) were detected. The characteristics of HGPIN included an increased nuclear/cytoplasm ratio, marked nuclear atypia, hyperchromasia, and prominent nuclei similar to what we described before for the Fgf8b transgenic line (4). In human pathology, HGPIN is considered a putative precursor of prostatic adenocarcinoma. From 9 months onward, we began to detect adenocarcinoma in some animals with the frequency increasing with further aging. Adenocarcinoma was found in all lobes, although more often in the dorsolateral and ventral lobes than the anterior lobe. An example of a poorly differentiated adenocarcinoma is shown in Fig. 1A. In addition to adenocarcinoma, in 2 of the 31 animals examined by histopathology, a rare form of adenocarcinoma (i.e., mucinous adenocarcinoma) was detected for the first time in a mouse model (Fig. 1B). Mucinous adenocarcinoma accounts for about 0.4% of human prostate adenocarcinoma. It is diagnosed when at least 25% of the examined tumor composed of lakes of extracellular mucin (35).

Consistent with previous reports, the mice with only monoallelic conditional inactivation of Pten (cPten<sup>−/−</sup>) displayed PIN lesions, although they generally did not progress to PIN before 16 months. Of the four mice that we kept for a longer period (18-20 months), three displayed HGPINs, and, interestingly, in the other, a single focal adenocarcinoma in the anterior lobe was detected in the midst of mostly HGPINs. The mice with overexpression of FGFB8 only displayed hyperplasia and PIN, similar to the phenotype described in our previous report and did not progress to development of adenocarcinoma (4). In contrast to these single mutants, the combined mutants developed adenocarcinoma (Fig. 1C). Although none of the four combined mutants of age between 6.5 and 7.7 months had cancer, 6 of 17 of 9.0 to 16.2 months and 9 of 10 of 18.2 to 19.1 months harbored adenocarcinomas. Clearly, the combination of FGFB8 overexpression and monoallelic Pten inactivation resulted in the onset of tumorigenesis in a temporal fashion with a high level of penetrance. In the course of the study, to derive monoallelic inactivation of Pten in the presence of Fgf8b transgene, we also accumulated animals with Fgf8b transgene as well as biallelic conditional deletion of Pten (cPten<sup>−/−</sup> F). Relative to conditional homozygous Pten deleted (cPten<sup>−/−</sup>) mice, the cPten<sup>−/−</sup> F mice depicted a general trend of increased size of all the prostate lobes. A
typical picture of such an enlarged prostate found in a 10-month-old cPten+/F along with an age-matched control animal is shown in Fig. 1D. Although the left anterior lobe was of similar size to that usually found in cPten−/−, the right anterior lobe, however, was extremely enlarged in this case. There was abundant inflammation in this tumor histologically (data not shown).

To determine whether expression of the Fgf8b transgene was maintained in the combined mutants, ventral lobes from animals of three different age groups (7.6, 9.0, and 18.2 months) were examined for transgene expression by RT-PCR (4). Although the prostate from the 9.0- and 18.2-month-old animals contained adenocarcinoma, tumors were not detected in the 7.6-month-old by histopathology. As illustrated in Fig. 2A, the expression of the transgene could be readily detected in all the prostate tissues, irrespective of the detection of tumors. It should be noted that because the primers for the detection of transgene expression were designed to flank an intron in SV40 polyadenylic acid signal sequence, besides the 550-bp product, an additional band of 485-bp long was expected to be amplified if the transcribed transgene was spliced (4). The difference in the incidence of detection of adenocarcinoma in 15 of the 31 total number of cPten+/−/F mice examined, compared with the detection of a single adenocarcinoma lesion in just 1 of the 11 animals of the cPten+/− control group, was determined to be significant (P = 0.030). Similarly, within the cPten−/− group, the temporal increase in incidence of adenocarcinoma between ages 9.0 to 16.2 and 18.2 to 19.1 months (P = 0.014) was statistically significant.

For assessing metastatic potential in the cPten+/−/F mice, we limited our analyses to the lumbar and pelvic lymph nodes of the animals found to have adenocarcinoma. The metastatic cells within the lymph nodes seemed to form glands and, as anticipated, they were positive for the expression of AR as determined by immunostaining with anti-AR antibodies (Fig. 2B). Of the six adenocarcinoma-bearing mice in the age group of 9.0 to 16.2 months, three were found to have lymph node metastasis. Six animals from the older age group (18.2-19.1 months) of nine animals displayed lymph node metastasis. The frequency of detection of lymph node involvement (Fig. 2C) was high, ranging from 50% to 66% with a trend of increased incidence with advanced age, although a study with a much larger number of animals would be required to determine its significance.

Characteristics of tumors found in the combined cPten+/−/F model. To define the cell of origin of the cancers, we examined the expression of different prostatic cell markers by immunohistochemistry. All lesions, hyperplasia, LGPIN, HGPIN, or cancer were positive for AR staining. However, there was heterogeneity in the intensity of the AR signal with a decline, in general, in cancer. Examples of staining in typical and mucinous adenocarcinoma lesions are shown in Fig. 3A. Basal cells expressing p63 were also commonly found inside tumors (Fig. 3B). It remains to be investigated whether these cells could be "reprogrammed" luminal cells or actually originating from prostatic basal/stem cells. The presence of the p63-positive cells in the tumor, however, was considered noteworthy because such cells might conceivably be the cells responsible for recurrence of the disease as androgen depletion–independent cancer (36) that invariably occurs in man after androgen ablation therapy. Obviously, the model would serve well in investigating this matter in the future. Cytokeratin 18, a marker for secretory epithelial cells, was detected in most of the cells in the tumor epithelium, and these cells did not stain for synaptophysin, a marker for neuroendocrine cells (Fig. 3C), indicating that there is no neuroendocrine differentiation in this model. These results were consistent with an epithelial origin for the cancer in the cPten+/−F line as in the cPten−/− model (11–13). Ki67 staining was used to identify proliferating cells in the model. FGFB8 was previously shown to directly stimulate proliferation of epithelial cells and indirectly stromal cells of the prostate (4, 37), and PTEN deficiency to increase proliferation of the prostate tumor cells (11, 13). Here in the compound mutants, we also detected proliferating cells in PIN lesions (data not shown) and in cancers (Fig. 3D). In several cases, this was accompanied by proliferation of stromal cells as well.

Analysis of downstream mediators, p-AKT and p27KIP1, in various types of lesions. AKT can be activated by PI3K that is induced and enhanced by both FGFB8 overexpression and Pten inactivation. In LGPINs, practically all cells showed plasma membrane staining of p-AKT (Fig. 4A). In HGPIN, however, the pattern became more heterogeneous (Fig. 4B). In areas of
adenocarcinoma, heterogeneity in p-AKT staining was even more pronounced. Figure 4C illustrates this observation in a typical adenocarcinoma, and Fig. 4D illustrates that of a mucinous adenocarcinoma. Although these findings are consistent with the report of the development of only PIN lesions with activated p-AKT in transgenic mice (28), it also indicates that other pathways must be integrated for further disease progression besides AKT.

Considering the findings of down-regulation of the cyclin-dependent kinase inhibitor p27KIP1 in prostate cancer as well as its regulation by both PI3K and MAPK pathways, we examined p27KIP1 expression by immunohistochemistry in prostatic lesions, ranging from hyperplasia to PIN to adenocarcinoma in our model. The results are shown in Fig. 5. Compared with the strong and fairly uniform nuclear staining in normal epithelium, hyperplasia, LGPIN, or HGPIN, as illustrated for the normal (Fig. 5A) and HGPIN from cPten+/−/C0 (Fig. 5B), an altered pattern was clearly evident with adenocarcinomas. In all the tumors we examined, whether obtained from the single cPten+/−/C0 mouse or cPten+/−/F mice, and regardless of whether the tumor was typical adenocarcinoma or mucinous adenocarcinoma, there was notable heterogeneity in staining of p27KIP1 (Fig. 5C and D) with a variable decrease in p27KIP1 expression.

Loss of heterozygosity of Pten in adenocarcinomas. Because cPten+/−/F mice presented a very high incidence of adenocarcinoma at late age (18-19 months), similar in frequency to cPten−/−/F animals of younger age (11, 12), it was of interest to determine the Pten gene status in the cancer cells of the compound mutants. We were fortunate to have a focal cancer originating from a single aged cPten+/−/F mouse for comparison. The question asked was whether loss of heterozygosity (LOH) of the Pten gene played a role in the late development of the cancer in both the cases of cPten−/−/F and cPten+/−/F genotypes. To this end, we did real-time PCR using microdissected cancer cells collected from tumor tissues of four arbitrarily selected cPten+/−/F mice and the single tumor from the cPten+/−/F mouse. To detect the WT allele, the reverse primer was designed based on the sequence that was disrupted in the floxed allele of Pten (Fig. 6A). The primer set used was considered to be of higher specificity than what was based on the polymorphic repeats and previously employed for a similar investigation (38). Tubulin served as internal control. One representative graph of a real-time PCR result is shown in Fig. 6B. We found LOH of Pten in the cancer cells of each of the five different adenocarcinomas examined, regardless of their origin and whether the phenotype was typical prostatic adenocarcinoma or the rare mucinous adenocarcinoma.
The results provided a strong evidence for the importance of inactivation of both alleles of \textit{Pten} in prostate tumorigenesis in these models. Generation of single major product in both types of reactions of real-time PCR was confirmed by dissociation analysis (Fig. 6C). The expected PCR products were ~100 bp for the WT allele and 250 bp for tubulin.

**Discussion**

Of the four possible isoforms of human FGF8, isoform b has been shown to possess the most transforming and tumorigenic potential (39–41). This isoform is the primary species in prostate epithelial cell lines and malignant epithelium in tissue (40–42). There is also evidence that FGF8b expression is increased in human prostatic lesions beginning from PIN to adenocarcinoma and remains elevated in androgen depletion–independent cancer as well (42, 43). By engineering FGF8b overexpression in the mouse prostate epithelium, we have previously shown that early effect of FGF8b-induced proliferation is followed by development of histopathologically identifiable preneoplastic lesions with variable severity up to HGPIN (4). The other gene of interest pertinent to this report is \textit{PTEN} whose activity is either suppressed or lost in human prostate cancer (44–46). Similar to the effect of FGF8b overexpression in the mouse prostate, loss in the level of expression of PTEN, such as in \textit{Pten} heterozygous (\textit{Pten}+/−) mice, generally results in PIN lesions up to HGPIN, mostly after a long latency, and the lesions do not progress to adenocarcinoma (11–13). The intent of the current work was to test whether prostate epithelium–specific manifestation of these two aberrations (FGF8b overexpression and PTEN deficiency), which are naturally and commonly associated with human prostate cancer, could lead to progression of the preneoplastic lesions encountered in mice with each of the single defects to invasive and metastatic adenocarcinoma in the combined mutants.

The results obtained underscored a strong synergy between FGF8b growth factor– and PTEN-regulated pathways in prostate tumorigenesis. By 19 months of age, the incidence of adenocarcinoma of the prostate is very high, approaching 100% in the combined mutants. The subsequent age-related and increasing incidence of readily detectable lymph node metastases in these animals is also noteworthy. In contrast to biallelic knockout of \textit{Pten} in the prostate as in \textit{cPten}+/− system, in which adenocarcinoma is produced at an age as young as 9 weeks (11, 12), the \textit{cPten}+/− animals depict a protracted pattern of cancer development that is more representative of the generally late-onset disease in humans. Because each of the two events, FGF8b overproduction and \textit{Pten} haploinsufficiency, contributes to proliferation, a synergy that increases proliferation is anticipated. Increased proliferation is likely to enhance the probability to

**References**

(39–41)
Another interesting observation in this report concerns the level of p27KIP1 expression in the prostatic lesions of our combined mouse model. A previous modeling study with Nkx3.1 inactivation and Pten deficiency indicated that p27KIP1 might possess a dose-sensitive, positive as well as negative roles in prostate cancer progression (47). In hypomorphic Pten and cPten+/− mice, a progressive down-regulation in p27KIP1 has been described (12). Similar to the cPten+/− mice, we also find decreased expression of p27KIP1 in the tumors of the cPten+/−F mice. Although p-akt is known to be a major factor in the down-regulation of p27KIP1 (48–50), there is, however, somewhat of a disconnect with p-akt immunostaining in tumor progression. Although p-akt staining is strong and fairly uniform in LGPIN in the combined model, heterogeneity is presented in HGPIN that further increases in adenocarcinoma. Similarly, a transgenic mouse line with prostate-specific expression of activated AKT (28) induces p27KIP1 overexpression in PIN lesions, which do not further progress, but in compound mutants with activated AKT and lacking one or both copies of p27kip1 there is increased proliferation and progression of PIN to invasive cancer.4

Because conditional homozygous inactivation of Pten in reported studies (11–13) do not display mucinous adenocarcinoma, and because tumors in cPten+/−F series are associated with LOH of the Pten allele, it seems that FGFB activation overexpression is also a contributory factor in the generation of mucinous variant in the background of Pten inactivation. Not much is known about differences in prognosis between human mucinous and typical adenocarcinomas. One report describes detection of high frequency microsatellite instability in this rare form of prostate cancer (51), and it is anticipated that our cPten+/−F could serve as a useful source of materials for examination of this and other related matters of the mucinous variant cancer compared with the typical adenocarcinomas of the littersmates.

In summary, we have shown that activation of the FGFB oncogene and homozygous loss of PTEN tumor suppressor function in the mouse prostate cooperate in the late-onset induction of metastatic prostate cancer with high incidence. Although it is clearly evident that the combined model may be eventually somewhat parallel to the disease formation in the homozygous Pten deletion models because of LOH at the Pten allele in the tumors of the combined mutants, the detection of rare mucinous variant adenocarcinoma only in the combination is unique and is likely to be associated with functions of FGFB, which are perhaps, different from its known proliferation and transformation activities.

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