**Nkx3.1 Mutant Mice Recapitulate Early Stages of Prostate Carcinogenesis**

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**Abstract**

Recent studies of human cancers and mutant mouse models have implicated the Nkx3.1 homeobox gene as having a key role in prostate carcinogenesis. Consistent with such a role, here we show that Nkx3.1 displays growth-suppressing activities in cell culture, and that aged Nkx3.1 mutant mice display histopathological defects resembling prostatic intraepithelial neoplasia (PIN), the presumed precursor of human prostate cancer. Using a tissue recombination approach, we found that PIN-like lesions from Nkx3.1 mutants can undergo progressively severe histopathological alterations after serial transplantation in nude mice. Our findings indicate that Nkx3.1 loss-of-function is a critical event in prostate cancer initiation, and that Nkx3.1 mutant mice accurately model early stages of prostate carcinogenesis. More generally, our tissue recombination assay provides an empirical test to examine the relationship of PIN to prostate carcinoma.

**Introduction**

During prostate carcinogenesis in humans, clinically undetectable precursor lesions progress to locally confined carcinoma and ultimately to life-threatening metastatic disease (1, 2). At the histopathological level, it is generally presumed that the precursor of human prostate cancer is PIN, which can be readily distinguished from nonneoplastic abnormalities such as benign prostatic hyperplasia (3, 4). Indeed, PIN lesions display similar histopathological characteristics as prostate cancer and are often found in close proximity to overt carcinoma (3, 4). Despite compelling circumstantial evidence, however, it has been difficult to establish an experimental paradigm to examine the precursor-product relationship of PIN to prostate carcinoma.

The Nkx3.1 homeobox gene is a key regulator of prostatic epithelial differentiation, while its loss-of-function in mice has been implicated in prostate cancer initiation (5–7). In particular, Nkx3.1 null mutant mice display abnormal prostatic differentiation as well as epithelial hyperplasia and dysplasia prior to 1 year of age (6). Human NKX3.1 maps to chromosome 8p21 (8, 9), which frequently undergoes loss of heterozygosity at early stages of prostate carcinogenesis (10, 11). Although NKX3.1 is not mutated in prostate cancer (9), it undergoes epigenetic inactivation through loss of protein expression in human prostate cancer and in mouse models (7, 12). Here we demonstrate that Nkx3.1 mutant mice develop lesions that histopathologically resemble human PIN, consistent with a key role for Nkx3.1 inactivation in prostate cancer initiation. Furthermore, the susceptibility of Nkx3.1 mutant mice to PIN formation has facilitated our development of a tissue recombination assay to explore the relationship of PIN to prostate cancer.

**Materials and Methods**

**Retroviral Gene Transfer and Tumorigenicity Assays.** Sequences corresponding to the coding region of Nkx3.1 (5) were subcloned into pLZRSΔ-IRES-GFP, a derivative of LZRSpBMN-Z (13). The Nkx3.1(L140S) mutant contains a substitution of leucine 140 to serine (position 16 of the homeodomain). Production of replication-defective mammalian retroviruses and infection of target cells has been described (14). Expression of Nkx3.1 protein was verified at the initiation and termination of each assay by Western blot analysis (Fig. 1A and data not shown). After retroviral infection, PC3 (15) or AT6 (AT6.3; Ref. 16) cells were seeded in triplicate at a density of 5 × 10^4/cm^2 or 1 × 10^5/cm^2, respectively, in low-serum medium (0.5% or 0.25%, respectively); medium was replenished every second day. At the indicated days, cell number was determined by absorbance after staining with Naphthol blue black (Sigma). Anchorage-independent growth was monitored by seeding AT6 cells in triplicate at a density of 1 × 10^3/cm^2 in medium containing 0.35% agarose layered over 0.5% agar. After growth for 14 days, the number of GFP-expressing colonies was determined by counting under a fluorescence microscope. Tumor growth in nude mice (Taconic) was initiated by s.c. injection of AT6 (1 × 10^7 in serum-free medium) or PC3 (1 × 10^6 in 50% Matrigel) cells. Tumor size was measured once per week for 4 (AT6) or 6 (PC3) weeks by caliper measurement, followed by determination of tumor weights at necropsy. Statistical analyses were performed using a two-sample t test for independent samples with unequal variances (Satterthwaite’s method).

**Analysis of Nkx3.1 Mutant Mice.** Nkx3.1 mutant mice have been described (6). Unless otherwise indicated, analyses were performed on virgin male mice (birth to 24 months) in a hybrid 129SvImJ and C57Bl/6J strain background. Congenic C57Bl/6J and FVB/N Nkx3.1 strains were produced by 10 generations of sequential back-crosses; the resulting congenic mice were analyzed at 12 months. Histological analyses were performed on formalin-fixed paraffin tissues on a nonblinded basis by R. D. C.; M. M. S. independently reviewed the histological data on a blinded basis, reaching similar conclusions. Immunohistochemical analyses were performed on formalin-fixed paraffin sections after antigen retrieval. Monoclonal antibodies were: anti-smooth muscle actin (Sigma), anti-cytokeratin 14 (Biogenex), anti-p63 (Santa Cruz Biotechnology, Inc.), and anti-E-cadherin (Transduction Laboratories). Immunodetection was performed using the Vector M.O.M. (Mouse on Mouse) kit with Vector NovoRED for substrate detection (Vector Laboratories).
Tissue Recombination Assays. Tissue recombination assays were performed using prostatic epithelium from Nkx3.1 homozygous mutant mice or wild-type littermates from ages 10 to 15 months. Regions of PIN-like lesions were identified by bright-field illumination under a dissecting microscope as hyperplastic ductal densities; ~300-μm segments of the hyperplastic prostatic ducts were isolated by microdissection. We confirmed that these hyperplastic ducts were enriched for PIN-like lesions by histological analyses (data not shown). These ducts (or normal ducts from wild-type littermates) were recombined with fetal rat or mouse urogenital sinus mesenchyme essentially as described (17). The tissue recombintants were incubated overnight at 37°C, followed by surgical implantation under the kidney capsule of male nude mice hosts. After growth for 1–2 months, the tissue recombintants were processed for histological and immunohistochemical analyses. For serial transplantation, an ~300-μm segment of the dissected tissue graft was recombined with fresh urogenital sinus mesenchyme and grafted in nude mice as before. Rat urogenital sinus mesenchyme was used for all recombinants used to calculate wet weights. In Fig. 3, the recombinants shown in panels N, D, H, L, P, and T were made with rat urogenital sinus mesenchyme and the others with mouse; similar findings were obtained regardless of whether mouse or rat urogenital sinus mesenchyme was used (data not shown).

Results

Growth-suppressing Activities of Nkx3.1. Because several lines of evidence have implicated Nkx3.1 loss-of-function as a contributing factor for prostate carcinogenesis, we examined whether its overexpression would suppress growth and tumorigenicity of prostate carcinoma cells. We expressed exogenous Nkx3.1 by retroviral gene transfer using a vector containing IRES-GFP sequences, so that the infected GFP-expressing cells could be enriched by flow cytometry. After cell sorting, >95% of the recovered cells expressed GFP and high levels of Nkx3.1 protein (Fig. 1A and data not shown). As a control, we compared the activity of Nkx3.1 to that of a mutated derivative [Nkx3.1(L140S)] that contains a substitution of a conserved homeodomain residue, which renders the protein inactive in DNA-binding and transcription assays.

For these assays, we used a human (PC3) and rodent (AT6) prostate carcinoma cell line, neither of which expresses the endogenous Nkx3.1 gene (Fig. 1A). We found that overexpression of exogenous Nkx3.1, but not the mutated gene [Nkx3.1(L140S)], resulted in an ~70% reduction in cellular proliferation in AT6 cells and 60% reduction in PC3 cells (Fig. 1A). In addition, Nkx3.1-expressing AT6 cells displayed an ~60% reduction in anchorage-independent growth compared with those expressing the vector control, whereas the Nkx3.1(L140S)-expressing cells produced a modest increase (~25%) in anchorage-independent growth (Fig. 1A). Moreover, Nkx3.1-expressing AT6 and PC3 cells displayed an approximately 50 or 60% decrease, respectively, in tumor growth in nude mice relative to the control vector and/or Nkx3.1(L140S) (Fig. 1C). Similar results were obtained in all assays using a human Nkx3.3 retrovirus, as well as stable tetracycline-inducible cell lines expressing mouse or human Nkx3.1 (data not shown). Notably, these growth-suppressive activities of Nkx3.1 in cell culture and nude mice are consistent with the increased proliferation of prostatic epithelium observed for Nkx3.1 mutant mice (6).

Formation of PIN-like Lesions in Nkx3.1 Mutant Mice. Following an initial observation that homozygous and heterozygous Nkx3.1 mutant mice develop prostatic epithelial hyperplasia and dysplasia prior to 1 year of age (6), we have now found that prostate glands from older Nkx3.1 mutants display successively more severe histopathological alterations in both the epithelium and the stroma (Fig. 2). These morphological alterations represent a continuum of changes ranging from an increase in the number of luminal epithelial cells with crowded papillae to more complex and severe forms, such as the formation of crowded, multilayered epithelium (Fig. 2, B, D, F, and H). The epithelial nuclei of Nkx3.1 mutants are larger with a more open chromatin and prominent nucleoli but lack extensive pleomorphism (Fig. 2, D, H, and insets). Accompanying these changes in the epithelium, the smooth muscle of the fibromuscular sheath becomes attenuated and is occasionally discontinuous (Fig. 2, M and N). Despite these extensive histopathological alterations, however, no cases of overt carcinoma have been observed to date, even in the oldest Nkx3.1 mutant mice (data not shown).

Notably, a majority (70%; n = 16) of homozygous mutants between 1 and 2 years of age developed histological features resembling human PIN, including cribriform or papillary architecture of epithelial cells, accompanied by atypical nuclei, enlarged nucleoli, and frequent mitotic figures (Fig. 2, A–H; Table 1A). Consistent with our previous findings indicating Nkx3.1 haploinsufficiency (6), an intermediate number of Nkx3.1 heterozygotes (36%; n = 11) developed PIN-like lesions with histopathological features indistinguishable from those in homozygotes (Table 1A, data not shown). In addition to these findings for mice in a mixed C57Bl/6J-129/SvImJ strain background, we found that the Nkx3.1 prostatic phenotype was similar in three inbred backgrounds (C57Bl/6J, 129/SvImJ, and FVB/N); however, the occurrence of PIN-like lesions was more frequent in C57Bl/6J and FVB/N congenic mice but less frequent in the 129/SvImJ mice (Table 1B).

Immunohistochemical analyses demonstrated that the PIN-like lesions of Nkx3.1 mutant prostates displayed several features consistent with human PIN (Fig. 2, I–P):

(a) Using two distinct basal cell markers, cytokeratin 14 and p63, we observed an increased ratio of luminal to basal cells in PIN-like lesions; the multilayered epithelium in the interior of these lesions lacked staining for these markers (Fig. 2, I–L). These findings are

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consistent with the nonuniform basal cell layer that is a cardinal feature of human PIN (3, 4).

(b) We observed that the stromal layer was significantly attenuated in Nkx3.1 mutants, as visualized by staining with anti-smooth muscle actin (Fig. 2, M and N), indicating an increased epithelial:stromal ratio that presumably reflects a decreased dependence of epithelial cells on the stromal layer.

(c) Expression of the luminal epithelial cell marker E-cadherin was increasingly heterogeneous in the PIN-like regions of Nkx3.1 mutants (Fig. 2, O and P), consistent with its altered expression in human prostate cancers (18, 19).

(d) We noted that Nkx3.1 mutant mice displayed no increase in neuroendocrine cells, as assessed by immunostaining with anti-chromogranin A or synaptophysin (data not shown); generally, these cells are not up-regulated in PIN (20).

Taken together, these findings demonstrate that Nkx3.1 mutant mice develop lesions that strongly resemble human PIN but do not display invasive carcinoma.

A Tissue Recombination Assay for Neoplastic Progression. The susceptibility of Nkx3.1 mutant mice to develop PIN-like lesions, but not carcinoma, prompted us to establish an experimental model system to test the capability of PIN to undergo neoplastic progression. For this purpose, we used a tissue recombination approach that circumvents the normal growth control of the prostatic epithelium. The rationale for this approach is based on our observations that although the prostatic epithelium is relatively growth quiescent in the context of the intact gland (even in Nkx3.1 mutants), such epithelium displays robust proliferation in the presence of embryonic urogenital sinus mesenchyme (17). Specifically, we have found that embryonic mesenchyme can induce a relatively small (~300 μm) segment of an adult prostatic duct, composed of ~5000 epithelial cells (~1 mg), to generate several million epithelial cells in 1 month after grafting in male nude mouse hosts (wet weight of recovered recombinant at round 1, 39.5 ± 11.7 mg, n = 8). When wild-type prostatic tissue is used, the resulting tissue recombinants have prostatic ducts that are histologically normal in appearance and produce secretory proteins (Ref. 17 and Fig. 3, A and E). Serial transplantation of such tissue recombinants leads to their continued expansion and proliferation for two additional transplant generations, but not further (wet weight at round 2, 19.3 ± 5.3 mg, n = 8; at round 3, 18.5 ± 5.5 mg, n = 8; round 4, none recovered, n = 8). Thus, we reasoned that the robust stimulation of epithelial proliferation after serial transplantation of tissue recombinants would provide an ideal model system to test whether the PIN-like lesions of Nkx3.1 mutants could undergo increasingly severe histopathological alterations.

Using this approach, we found that the initial round and serially
transplanted tissue recombinants of prostatic epithelium from wild-type (Nkx3.1^+/+) mice retained a recognizable papillary architecture surrounded by an organized fibromuscular stroma (n = 13; Fig. 3, A and E, and data not shown). The papillary fronds were lined by a single layer of columnar epithelium with a normal distribution of basal cells, uniform expression of markers such as E-cadherin, and copious production of secretory fluids (Fig. 3, A, E, I, M, and Q). Notably, the pattern of the round 1 (initial) transplant did not change upon serial transplantation, although the size of the transplants diminished in serial passages (compare wet weights from initial with rounds 2 and 3 above).

In contrast, serial passage of prostatic tissue recombinants from Nkx3.1 mutant mice resulted in a progressive loss of organized ductal architecture that is far more severe than the histopathological defects observed in mutant mice (compare Figs. 2 and 3). Although round 1 transplants retained a papillary architecture with columnar epithelium and a relatively unaltered relationship between the epithelium and stroma (n = 14; Fig. 3, B and F), similar to the intact mutant prostate (Fig. 2), round 2 and 3 recombinants displayed a dramatically increased disorganization of the prostatic ducts and the surrounding stroma (n = 14; Fig. 3, C, D, G, and H). These serially transplanted recombinants contained glandular epithelium that lost the papillary pattern and became subdivided into smaller glands with a variety of abnormal growth patterns, including solid masses, cribriform, and poorly differentiated (basal or embryoid). This severely disorganized growth was also reflected by alterations in the basal cell layer (Fig. 3, O and P) and by the heterogeneous expression of E-cadherin (Fig. 3, S and T). Furthermore, the relationship between the epithelium and stroma was drastically altered, such that the fibromuscular stroma did not form a uniform sheath around the glands, leaving gaps in the smooth muscle layer (Fig. 3, K and L). Notably, masses of smooth muscle appear in some transplants that had no apparent relationship with epithelium (Fig. 3, K and L), with frequent extensions into the renal parenchyma as "tongues" of smooth muscle (Fig. 3, K, inset); this invasive behavior of the stroma was characteristic of the round 2 and 3 recombinants from the Nkx3.1 mutants but was never observed in wild-type recombinants. Taken together, these findings provide experimental evidence supporting the potential for mouse PIN-like lesions to undergo successive stages of neoplastic progression.

**Discussion**

Our study demonstrates the relevance of Nkx3.1 mutant mice for studying early stages of prostate carcinogenesis, which are otherwise nearly inaccessible in humans. On the basis of the resemblance of the PIN-like lesions in Nkx3.1 mutant mice to human PIN, we conclude that the lesions in this mouse model are indeed representative of the disease in humans. Furthermore, these PIN lesions display increasingly severe histopathological alterations after serial transplantation of tissue recombinants, consistent with their potential precursor relationship to prostate carcinoma.

Our findings that Nkx3.1 suppresses growth and tumorigenicity complement our previous observations that Nkx3.1 mutant prostates exhibit increased cellular proliferation (6). Despite these activities, Nkx3.1 differs from classical tumor suppressor genes such as RB, p53, or Pten, because it does not undergo mutational inactivation but instead is inactivated through loss of protein expression (7, 12). Moreover, unlike classical tumor suppressors, which have broad functions in many types of cancers, the consequences of Nkx3.1 loss-of-function are largely restricted to the prostate, in accordance with its tissue-specific expression pattern (6). Instead, Nkx3.1 shares features in common with another homeobox gene, Cdx2, which displays gastrointestinal-specific expression in adults and whose loss-of-function results in epithelial dysplasia in mutant mice (21). Similar to Nkx3.1, Cdx2 displays haploinsufficiency in mice, is inactivated by down-regulation of protein expression (21), and displays growth-suppressing activities in cell culture (22). These striking parallels suggest that Nkx3.1 and Cdx2 may represent a new class of growth-regulatory genes that display some features of classical tumor suppressors in experimental assays but are essentially mediators of tissue-specific differentiation in vivo. We propose that such growth-regulatory genes normally function to establish and/or maintain tissue-specific differentiation states but result in epithelial dedifferentiation and consequent predisposition to neoplasia when inactivated.

In recent years, the generation and analysis of mutant mouse models has demonstrated the validity of the mouse for studying human prostate carcinogenesis, as shown by the fundamental similarities in their histopathological characteristics as well as underlying molecular pathways (this work and reviewed in Refs. 1, 23, 24). However, unlike SV40-based gain-of-function transgenic mice (23, 25), loss-of-function mouse models are prone to develop PIN but rarely develop carcinoma (24). Nonetheless, in the tissue recombinant assay, we found that PIN lesions from Nkx3.1 mutants undergo increasingly severe histopathological changes consistent with neoplastic progression, although these recombinants have yet to display overt invasive carcinoma. These findings are consistent with our interpretation that Nkx3.1 is a regulator of terminal differentiation of luminal epithelial cells, and that loss of Nkx3.1 function requires additional cooperating events to result in overt carcinoma. Indeed, we have shown that mutant mice lacking both Nkx3.1 and the Pten tumor suppressor develop high-grade PIN/carcinoma in situ lesions (7); thus, we anticipate that tissue recombinants from Nkx3.1; Pten compound

### Table 1 Summary of prostatic epithelial defects in Nkx3.1 mutant mice

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<th>Strain and genotype</th>
<th>Total</th>
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<th>Hyperplasia</th>
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* Data for the mice at 1–12 months include data reported previously by Bhatia-Gaur et al. (6).
stroma and frequent extensions into the kidney parenchyma (arrow). Recombinants have relatively normal association of stromal and epithelial layers. Tissue recombinants from anterior or dorsolateral prostate.

Fig. 3. Histopathology of Nkx3.1 tissue recombinants. Sections of tissue recombinants grown in male nude mice from anterior (AP) or dorsolateral (DLP) prostates of wild-type (Nkx3.1+/+) and homozygous (Nkx3.1−/−) mice. Shown are examples from the initial round of transplants (I; round 1) or after serial transplantation (S) for one or two additional generations (rounds 2 or 3). A–H, low and high power views of H&E-stained paraffin sections. A, B, E, and F, recombinants of serially transplanted wild-type (A and E) and initial round (B and F) Nkx3.1 mutants display recognizable papillary architecture. C, D, G, and H, prostatic ducts from serially transplanted Nkx3.1 mutant recombinants display cribriform architecture (G) or solid masses of epithelial cells (H); nuclei display multiple prominent nucleoli but not extreme pleomorphism (inset in H). I–T, immunohistochemical analysis of tissue recombinants from anterior or dorsolateral prostate. J and L, anti-smooth muscle actin staining shows that serially transplanted wild-type and initial round Nkx3.1 mutant recombinants have relatively normal association of stromal and epithelial layers. K and L, serially transplanted Nkx3.1 mutant recombinants display abundant masses of smooth muscle stroma and frequent extensions into the kidney parenchyma (arrow and inset in K). M and N, basal cells detected by anti-p63 staining are distributed in normal fashion along the basement membrane and in papillary tufts of serially transplanted wild-type and initial round Nkx3.1 mutant recombinants. O and P, basal cells in serially transplanted Nkx3.1 mutant recombinants are distributed aberrantly or are limited to margins of large masses of epithelial cells. Q–T, E-cadherin localization in luminal cells is increasingly heterogeneous in serially transplanted mutant recombinants (inset in S). kid, kidney. Scale bars, 100 μm.

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References


