Loss-of-Function of \textit{Nkx3.1} Promotes Increased Oxidative Damage in Prostate Carcinogenesis

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Abstract

Despite the significance of oxidative damage for carcinogenesis, the molecular mechanisms that lead to increased susceptibility of tissues to oxidative stress are not well-understood. We now report a link between loss of protection against oxidative damage and loss-of-function of \textit{Nkx3.1}, a homeobox gene that is known to be required for prostatic epithelial differentiation and suppression of prostate cancer. Using gene expression profiling, we find that \textit{Nkx3.1} mutant mice display deregulated expression of several antioxidant and prooxidant enzymes, including glutathione peroxidase 2 and 3 (\textit{Gpx2} and \textit{Gpx3}), peroxiredoxin 6 (\textit{Prdx6}), and sulfhydryl oxidase Q6 (\textit{Qscn6}). Moreover, the formation of prostatic intraepithelial neoplasia in these mutant mice is associated with increased oxidative damage of DNA, as evident by increased levels of 8-hydroxy-2'-deoxyguanosine. We further show that progression to prostate adenocarcinoma, as occurs in compound mutant mice lacking \textit{Nkx3.1} as well as the \textit{Pten} tumor suppressor, is correlated with a further deregulation of antioxidant enzymes, including superoxide dismutase, and more profound accumulations of oxidative damage to DNA and protein, the latter manifested by increased levels of 4-hydroxy-2'-deoxyguanosine. We propose that the essential role of \textit{Nkx3.1} in maintaining the terminally differentiated state of the prostate epithelium provides protection against oxidative damage and, thereby, suppression of prostate cancer. Thus, our findings provide a molecular link between a gene whose inactivation is known to be involved in prostate carcinogenesis, namely \textit{Nkx3.1}, and oxidative damage of the prostatic epithelium. (Cancer Res 2005; 65(15): 6773-9)

Introduction

The persistent generation of reactive oxygen species (ROS) in cells, which is a by-product of mitochondrial respiration (and other sources), is an inevitable consequence of aging in aerobic organisms (1–3). At low or moderate intracellular levels, ROS play important roles in signal transduction and regulation of redox-sensitive transcription factors (4, 5). However, elevated levels of ROS, which accrue with aging and/or exposure to dietary or environmental carcinogens, are coincident with an accumulation of genetic alterations that are associated with carcinogenesis (2, 6–8). Among the major risk factors for prostate cancer, for example, are aging and exposure to carcinogens (9). Indeed, age-related increases in free radicals have been associated with increased risk for prostate cancer (10), whereas sequence variants of a gene encoding a repair enzyme (hOGG1) that protects against oxidative damage of DNA leads to increased prostate cancer susceptibility (11). Conversely, antioxidants, such as vitamin E, selenium, and lycopene, have been implicated in reducing the risk of prostate cancer (9). However, despite a great deal of attention regarding the relationship between oxidative damage and carcinogenesis, the mechanisms that contribute to increased susceptibility of tissues to oxidative stress are not well-understood.

Among the cellular defenses against elevated levels of ROS and accumulation of oxidative damage are antioxidant enzymes, which act in concert to provide a coordinated network of protection against ROS accumulation and oxidative damage (4, 8, 12–15). For example, superoxide dismutase (SOD) enzymes in mitochondria convert superoxide to hydrogen peroxide, which is then further reduced by the selenium-containing GPx enzymes located in the mitochondria (\textit{Gpx1}), cytosol (\textit{Gpx2}), or plasma membrane (\textit{Gpx3}). Other antioxidants include the peroxiredoxins (\textit{Prdx}), a ubiquitous family of thiol-containing enzymes which are also major reductants of endogenously produced peroxides. Opposing the actions of these antioxidant enzymes are prooxidants whose cellular activities result in increased accumulation of ROS. These include the sulfhydryl oxidase Q6 (\textit{Qscn6}), which generates hydrogen peroxide as a by-product of the oxidation of sulfhydryl groups in the course of generating disulfide-containing secreted peptides (16).

Thus, perturbations of the balance in expression and/or function of antioxidant and prooxidant enzymes combined with exogenous factors (i.e., aging, etc.) that lead to accumulation of ROS may have profound consequences for oxidative damage. Indeed, the relationship between altered protection against ROS and carcinogenesis is highlighted by numerous examples in which antioxidant enzymes have been shown to be deregulated expression in cancer (e.g., refs. 6, 14, 17–19), as well as the consequences of the loss-of-function of antioxidant activities for carcinogenesis in mutant mouse models. For example, loss-of-function of \textit{Gpx1} and \textit{Gpx2} in mutant mice results in increased susceptibility to inflammation and cancer in the intestine (20), whereas mice lacking either \textit{Prdx1} or \textit{Prdx6} display increased levels of ROS, elevated oxidation damage, and increased propensity for tumor formation (21, 22).

We have been investigating the mechanisms underlying prostate cancer initiation through our analyses of the \textit{Nkx3.1} homeobox gene (23). In previous studies, we have shown that \textit{Nkx3.1} displays restricted expression to the prostatic epithelium and that its inactivation leads to inappropriate prostate epithelial differentiation (24). Moreover, \textit{Nkx3.1} mutant mice develop prostatic...
intraepithelial neoplasia (PIN) as a consequence of aging (24–26), which progresses to metastatic adenocarcinoma in compound mutant mice lacking Nkx3.1 as well as the Pten tumor suppressor (27, 28). In humans, NKK3.1 is located on a region of chromosome 8p21, which frequently undergoes allelic imbalance in PIN as well as prostate cancer (29–32). Although NKK3.1 is not mutated in prostate cancer (23, 33, 34), its inactivation by loss of protein expression is a hallmark of cancer progression in humans and mouse models (25, 26, 35–37). These and other lines of evidence support the idea that Nkx3.1 loss-of-function plays an important role in prostate-specific cancer initiation.

In our gene expression profiling analyses, we have now found that Nkx3.1 mutant mice display deregulated expression of several antioxidant and prooxidant enzymes. These gene expression changes occur in young mice and persist with aging; ultimately, they are accompanied by increased oxidative damage of DNA in the aged mice. Furthermore, cancer progression in Nkx3.1;Pten compound mutant mice is associated with additional perturbations of antioxidant enzymes as well as further accumulations of oxidative damage of DNA and protein. Thus, our findings show that Nkx3.1 loss-of-function leads to reduced protection against ROS and increased oxidative damage, which thereby provide a molecular link between a gene that predisposes to prostate cancer and oxidative damage. We propose that the Nkx3.1 mutant mice will be valuable for studying the relationship between oxidative damage response and carcinogenesis, as well as for preclinical studies to test the efficacy of potential antioxidants for prostate cancer prevention.

Materials and Methods

Generation and analysis of mutant mice harboring null alleles of Nkx3.1 and/or Pten have been described previously (24, 26, 27, 38). To avoid variability due to differences in strain background, gene expression profiling was done using whole anterior prostate tissue from age-matched (i.e., 15 months) cohorts of Nkx3.1 homozygous mutants (Nkx3.1<sup>−/−</sup>) or wild-type littermate controls (Nkx3.1<sup>+/+</sup>) on an inbred C57Bl/6J strain background (26). To further minimize variability from individual specimens, prostate tissues from three independent animals were pooled to generate RNA for each array and a minimum of three arrays were probed for the wild-type and mutant mice (thus allowing comparison of a total of nine mice for each).

RNA was extracted using TriZol (Invitrogen, Carlsbad, CA) and purified by sonication in buffer containing 10 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 1% deoxycytosine (sodium salt), 1% Triton X-100, with freshly added protease inhibitor and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). For in situ hybridization, sequence–verified expressed sequence tag clones were purchased from Invitrogen. Antibodies for immunostaining or Western blotting were as follows: Akt and p-Akt (Ser<sup>473</sup>), Cell Signaling, Beverly, MA; 1:200 and 1:50, respectively); Cu/Zn SOD (Upstate, Charlotte, NC; 1:1,000); Mn SOD (Upstate, 1:250 for immunohistochemistry; 1:1,000 for Western blotting); tubulin (Sigma, 1:1,000); Pten (Neomarkers, Fremont, CA; 1:200); 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (HNE; Japan Institute for the Control of Aging, Shizuoka, Japan; 1:50 and 1:80, respectively). Antibodies against Qscn6 and Nkx3.1 were published previously (16, 27), respectively.

8-OHdG levels were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described (39). Briefly, genomic DNA from the whole anterior prostate of Nkx3.1 wild-type or mutant mice was hydrolyzed to individual nucleosides and a PE Biosystems high-performance liquid chromatography was used to separate hydrolyzed DNA bases. 5-N15-8-oxoG from N15 algal genomic DNA (Spectra Stable Isotopes, Columbia, MD) was used as an internal standard. The detection limit for 8-OHdG with this method is 5 fmol with a small SD due to coelution with the N15-8-oxoG internal standard. For each sample, at least three independent measurements of dG and 8-OHdG, each with independent calibrations, were done. All analyses were done in a blinded fashion.

Results

Loss-of-function of Nkx3.1 leads to deregulated expression of anti- and prooxidative enzymes and increased oxidative damage of DNA. As a strategy to investigate the mechanisms by which loss-of-function of Nkx3 contributes to formation of PIN, we did gene expression profiling comparing Nkx3.1 homozygous null mutants with wild-type littermates as controls. We did these analyses using aged mice (>12 months), by which time a majority of Nkx3.1 mutants display PIN (24, 27). Prior to inclusion in the study, we examined the histologic phenotype of each experimental mouse to verify the occurrence of PIN (data not shown). We focused on the anterior prostate, which displays the most severe PIN phenotype relative to the other prostatic lobes (24, 27). Gene expression profiling was done using RNA obtained from the whole anterior prostate; however, the results were validated using RNA obtained by laser capture microdissection of epithelial cells (see below) to verify that the genes of interest were altered in the prostatic epithelial cells, as opposed to stromal or other cellular components present in the whole prostate tissue.

Our analyses revealed a total of 638 genes that were differentially expressed in anterior prostate of the Nkx3.1 mutants compared with the wild-type mice (see Supplementary Table S1). These included 299 genes that were up-regulated in the mutants and 339 that were down-regulated. Interestingly, many of these genes had been reported previously in an analysis of gene expression changes during castration regeneration of the prostate in Nkx3.1 mutants, although this previous study was done with a distinct Nkx3.1 mutant allele and by combining all of the prostatic lobes (40).

We noticed that among the deregulated genes in our analyses of Nkx3.1 mutants was a signature of those known to protect against or to promote oxidative damage (Fig. 1A). Specifically, Nkx3.1 mutant mice displayed a significant reduction in expression of Gpx2 and Prdx6 encoding antioxidant enzymes and up-regulation of the GPrx3 antioxidant and the Qscn6 prooxidant (Fig. 1A). We confirmed these results by real-time reverse transcription-PCR using RNA obtained by laser capture microdissection of prostatic epithelium from the anterior prostate of Nkx3.1 mutant or wild-type mice, as well as from the dorsolateral prostate (Fig. 1B, and data not shown). Although our gene expression profiling analyses were done using RNA from aged mice (>12 months), we found that these gene expression changes occurred in mice as early as 4 months of age (Fig. 1B), which is well before the occurrence of PIN phenotypes in these mutant mice (24, 27).

To investigate whether the deregulated expression of pro- and antioxidant enzymes in the prostatic epithelium of the Nkx3.1 mutants was correlated with oxidative damage in this tissue, we used a quantitative approach to measure levels of 8-OHdG, which
is a marker of DNA damage (12, 39). These analyses revealed that Nkx3.1 mutant prostates displayed a significant (~5-fold) increase in the levels of 8-OHdG by 12 months (Fig. 1C). Because the deregulated expression of antioxidant and prooxidant enzymes in the Nkx3.1 mutants precedes the increased accumulation of 8-OHdG, we infer that over time, this loss of protection leads to an aberrant accumulation of ROS and ultimately increased oxidative damage. Notably, the observed increase in DNA damage in the Nkx3.1 mutants is coincident with the onset of PIN phenotypes in these mice (26).

Deregulated expression of prooxidant and antioxidant enzymes in the prostatic epithelium of Nkx3.1 mutant mice. To further investigate the relationship between loss-of-function of Nkx3.1 and deregulated expression of genes that control ROS levels, we examined the expression of these genes in prostate tissues from Nkx3.1 wild-type or mutant mice by in situ hybridization, or immunohistochemistry if suitable antisera were available. We focused on the anterior prostate of mice from 4 to 12 months of age (Fig. 2; Supplementary Fig. S1). Note that the wild-type mice (Fig. 2A) display normal prostate histology, whereas Nkx3.1 mutant mice display hyperplasia and/or dysplasia prior to 8 months and are prone to develop PIN by 12 months (Fig. 2B; refs. 24, 27), as defined by histologic criteria previously described (41).

We were puzzled by our observation that Gpx2 and Prdx6 were down-regulated in the Nkx3.1 mutant prostates, whereas Gpx3 (which also encodes an antioxidant enzyme) was up-regulated (see Fig. 1B). In situ hybridization analyses revealed that Gpx2 and Prdx6 were expressed throughout the prostatic epithelium of the wild-type mice, whereas their expression was uniformly down-regulated in the prostatic epithelium of Nkx3.1 mutants (Fig. 2D, E, J, K; Supplementary Fig. S1A-D, F-L). In contrast, expression of Gpx3 was barely detectable in the wild-type prostate, whereas it displayed patchy expression in PIN lesions of the Nkx3.1 mutants but was not uniformly expressed throughout the epithelium (Fig. 2G and H; Supplementary Fig. S1E-H). Our interpretation of these findings is that up-regulation of Gpx3 may occur in PIN lesions to compensate for the loss of protection by Gpx2 and/or Prdx6.

Notably, the down-regulation of Gpx2 and Prdx6 expression occurred in Nkx3.1 mutant mice as young as 4 months of age (Fig. 1B; Supplementary Fig. S1), which is well before the occurrence of PIN phenotypes in these mice (>8 months; refs. 24, 26). Moreover, because Gpx1 expression was not altered in the Nkx3.1 mutant mice (Fig. 1A), it is unlikely that deregulated expression of Gpx2 is secondary to other effects of Nkx3.1, such as an effect on production of selenium. Thus, our findings suggest that the deregulated expression of Gpx2 and Prdx6 in the mutant mice is likely to be a primary consequence of loss-of-function of Nkx3.1 rather than

Figure 1. Loss-of-function of Nkx3.1 results in deregulation of protection against oxidative damage. A, hierarchical clustering of selected gene expression differences between the anterior prostates of wild-type (Nkx3.1+/+) and Nkx3.1 mutant (Nkx3.1−/−) mice. Selected genes involved in protection against or promotion of oxidative damage are shown. A complete list of gene expression differences is provided in the supplementary data. Red, up-regulated genes; green, down-regulated genes. B, real-time reverse transcription-PCR analysis showing the relative expression of Gpx2, Gpx3, Prdx6, and Gscr6 (as indicated) in RNA made from anterior prostates from wild-type (Nkx3.1+/+) and Nkx3.1 mutant (Nkx3.1−/−) mice (n = 6 per group) at the ages indicated. Expression levels are relative to expression of GADPH. Data represents the average of six independent RNA samples; the SD is indicated. C, relative levels of 8-OHdG in genomic DNA from age-matched wild-type (Nkx3.1+/+) and Nkx3.1 mutant (Nkx3.1−/−) mice. The data are expressed as the value of 8-OHdG measured by LC-MS/MS. Three independent experiments were done. One representative experiment done using three DNA samples for each group is shown; each of the DNA samples were measured in triplicate in blinded fashion. SEs for each sample are indicated.
secondary to the PIN phenotype. However, at present, we do not know whether these genes are direct targets for Nkx3.1 transcriptional control.

In addition to the deregulated expression of genes encoding antioxidant enzymes, our gene expression profiling data also revealed the up-regulation of a gene encoding the prooxidant enzyme, Qscn6, in the Nkx3.1 mutants. Although not normally expressed at high levels in the wild-type prostate (Fig. 2M; Supplementary Fig. S1M, N), expression of Qscn6 in other tissues is correlated with the demand for disulfide-containing secreted peptides (16). Immunohistochemical staining for Qscn6 revealed its uniform up-regulation throughout the prostatic epithelium of the Nkx3.1 mutants (Fig. 2N; Supplementary Fig. S1O, P), which was further confirmed by Western blot analyses (Fig. 3). The deregulated expression of Qscn6 is of particular interest given the known requirement of Nkx3.1 for appropriate production of prostatic secretory proteins (24). Interestingly, similar to the down-regulation of Gpx2 and Prdx6, up-regulation of Qscn6
occurred in mutant mice as young as 4 months, well before the onset of PIN (Fig. 1B; Supplementary Fig. S1M–P), suggesting that it is also a primary consequence of Nkx3.1 loss-of-function of rather than secondary to the PIN phenotype.

Taken together, these findings suggest that loss-of-function of Nkx3.1 perturbs the normal balance of antioxidant to prooxidant activities in the prostatic epithelium. We infer that this leads to the aberrant accumulation of ROS, which over time, leads to increased oxidative damage of DNA and ultimately contributes to the formation of PIN.

Prostate cancer progression is associated with further deficiencies in antioxidant protection. To further investigate the relationship of deregulated expression of antioxidant and prooxidant enzymes for prostate cancer progression, we employed compound mutant mice lacking Nkx3.1 as well as the Pten tumor suppressor gene, which develop high-grade PIN/carcinoma in situ by 6 months and adenocarcinoma by 12 months (27, 28). Figure 2C shows an example of the adenocarcinoma phenotype in mice lacking both alleles of Nkx3.1 and one allele of Pten (Nkx3.1<sup>WT</sup>/Pten<sup>KO</sup>). Cancer progression in these mice is coincident with loss of the wild-type allele of Pten and up-regulated expression of activated Akt, its major downstream effector (Fig. 3; ref. 27).

As expected, expression of Gpx2 and Prdx6, which were uniformly down-regulated in the prostatic epithelium of Nkx3.1 single mutants, were also barely detectable in the Nkx3.1:Pten compound mutants, whereas expression of Qscn6 continued to be elevated in the Nkx3.1:Pten mutants (Figs. 2F, L, O and 3I). However, Gpx3, which had been up-regulated in the PIN lesions of the Nkx3.1 mutants, was not expressed in the Nkx3.1:Pten compound mutants (Fig. 2F), suggesting that the compensation provided by Gpx3 in PIN may be lost during progression to adenocarcinoma.

We also observed additional losses of antioxidant enzymes in the Nkx3.1:Pten compound mutants, including reduced expression of Mn SOD and Cu/Zn SOD (Figs. 2P–R and 3I). Unlike Gpx2, Prdx6, and Qscn6, Mn SOD and Cu/Zn SOD were not deregulated in younger mice but were instead altered in expression only in older mice with more progressed PIN or cancer phenotypes (Figs. 2P–R and 3D; data not shown). Interestingly, we did not detect reduced levels of RNA for the genes encoding these enzymes in either the Nkx3.1 single or the Nkx3.1:Pten compound mutants, suggesting that the altered expression of these enzymes may occur posttranscriptionally (Fig. 1A; data not shown). Taken together, these findings suggest that progression to adenocarcinoma in these mutant mice is coincident with additional perturbations of antioxidant protection, which are unlikely to be a primary consequence of Nkx3 inactivation and more likely to be a consequence of cancer progression.

Accordingly, we asked whether these additional perturbations in protection against oxidative stress observed in adenocarcinoma in the mutant mice were coincident with further increased oxidative damage. Indeed, immunostaining revealed increased levels of 8-OHdG in the cancer lesions of the Nkx3.1:Pten compound mutants, relative to the PIN lesions of the Nkx3.1 single mutants (compare Fig. 2T and U). Moreover, the cancer lesions, but not the PIN lesions, also displayed damage of proteins, as evident by 4-HNE, a marker of protein oxidation (compare Figs. 2W, X and 3J). Therefore, the perturbation in the expression of oxidant protection is correlated with increased oxidative damage in cancer progression.

**Discussion**

Our findings provide new insights regarding the relationship between loss of protection against oxidative stress and prostate carcinogenesis, as well as the mechanisms by which Nkx3.1 suppresses prostate cancer (Fig. 4). We have shown that loss-of-function of Nkx3.1 in mutant mice leads to deregulated expression of several antioxidant and prooxidant enzymes (i.e., Gpx2, Gpx3, Prdx6, and Qscn6). These expression changes occur in young mice but persist in aged mice. Ultimately, they are associated with increased oxidative damage of DNA, which is correlated with the formation of PIN in the aged mice. We further show that progression to adenocarcinoma in Nkx3.1:Pten compound mutants

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Model to explain the relationship of loss of protection against oxidative damage and prostate cancer progression. The model is discussed in the text.
is associated with additional perturbations of the protective response (i.e., loss of SOD enzymes) and more profound consequences for oxidative damage of protein as well as DNA.

Thus, we propose that one of the principal roles of Nkx3.1 in prostate cancer suppression is to maintain the integrity of the prostatic epithelium by regulating the expression of genes that provide protection against oxidative damage. Notably, these changes in antioxidant protection are unique to Nkx3.1 mutant mice because they do not occur in other mouse models of PIN, including Myc, TRAMP, and Lady transgenic mice (37, 42, 43). We infer that Nkx3.1 inactivation perturbs the balance of antioxidant protection, which sets up a cascade of events that over time, leads to an accumulation of ROS, the promotion of oxidative damage of DNA, and ultimately PIN formation (Fig. 4). Intriguingly, the consequences of loss-of-function of Nkx3.1 for the oxidative damage response overlap with perturbations that are known to occur upon aging in the rodent prostate (44), which further emphasizes the link between aging and oxidative damage (e.g., ref. 1) and raises the possibility that Nkx3.1 inactivation may accelerate aging of the prostatic epithelium. Although loss-of-function of Nkx3.1 is not sufficient for progression to adenocarcinoma (24–26), it acts in collaboration with other genetic factors such as loss-of-function of Pten to promote cancer progression (27, 28). It is possible that the observed cooperativity of loss-of-function of Nkx3.1 and Pten for prostate carcinogenesis (27) is reflected, at least in part, through attenuation of protection against oxidative stress, which leads to an acceleration of oxidative damage of DNA and protein (Fig. 4).

Our findings provide support for the idea that defects in the oxidative response pathway occur early in prostate carcinogenesis, a notion that is supported by analyses of the expression of antioxidant enzymes in PIN versus cancer in the human prostate (e.g., refs. 18, 19). Furthermore, it has been suggested that perturbations of the oxidative response pathway in human cells as well as rodent models reflect alterations in androgen-signaling (45, 46), which is noteworthy considering that Nkx3.1 expression is known to be dependent on androgens in both the human and mouse prostate (47–49).

Given that the functions of NKX3.1 are highly restricted to the prostate and that its loss-of-function occurs with high frequency at early stages of prostate cancer (reviewed in ref. 23), it seems plausible that the sensitivity of the prostatic epithelium to oxidative stress (9) reflects, at least in part, a requirement for NKX3.1. In other words, as a consequence of defective protection against oxidative stress that occurs following NKX3.1 inactivation, the prostatic epithelium would tend to be more vulnerable to ROS that accumulate upon aging and/or exposure to carcinogens. This model is consistent with many of the known properties of NKX3.1, including the frequent deletion of at least one allele in prostate cancer, its known function for maintaining prostatic epithelial differentiation, and the age-dependent consequences of its loss-of-function for prostate carcinogenesis (reviewed in ref. 23).

In conclusion, our findings implicate Nkx3.1 mutant mice as a valuable and unique model to study the relationship between oxidative damage and cancer progression, as well as the potential role of Nkx3.1 as a key mediator of these events in the prostatic epithelium. One implication of our study is that men with loss-of-function of NKX3.1 would be more likely to benefit from dietary or pharmacologic interventions with antioxidants and that such treatments would be optimal if administered early in neoplastic transformation. This idea, and others, can be tested in the Nkx3.1 mutant mice, which are likely to be valuable for preclinical studies aimed at investigating the benefits of antioxidant treatments and the mechanisms by which they are effective.

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