Down-regulation of p57Kip2 Induces Prostate Cancer in the Mouse

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Abstract

p57Kip2 has been considered a candidate tumor suppressor gene because of its location in the genome, biochemical activities, and imprinting status. However, little is known about the role of p57Kip2 in tumorigenesis and cancer progression. Here, we show that the expression of p57Kip2 is significantly decreased in human prostate cancer, and the overexpression of p57Kip2 in prostate cancer cells significantly suppressed cell proliferation and reduced invasive activity. In addition, overexpression of p57Kip2 in LNCaP cells inhibited tumor formation in nude mice, resulting in well-differentiated squamous tumors rather than adenocarcinoma. Furthermore, the prostates of p57Kip2 knockout mice developed prostatic intraepithelial neoplasia and adenocarcinoma. Remarkably, this mouse prostate cancer is pathologically identical to human prostate adenocarcinoma. Therefore, these results strongly suggest that p57Kip2 is an important gene in prostate cancer tumorigenesis, and the p57Kip2 pathway may be a potential target for prostate cancer prevention and therapy.

Introduction

Aberrations in the normal cycling of a cell lead to uncontrolled proliferation that can result in the development of cancer. The cell cycle is a synchronized sequence of events set off in response to mitogenic stimuli, leading to cell duplication. The eukaryotic cell cycle is divided into four phases: the first growth phase (G1), DNA synthesis phase (S), the second growth phase (G2), and mitosis (M; ref. 1). Numerous mitogenic and quiescence-inducing stimuli must converge at certain points to allow a coordinated cellular response that typifies the normal cell cycle. The outcome of these opposing signals and the ensuing cell quiescence or cycling is response that typifies the normal cell cycle. Mitogenic stimuli for passage through G1, progression beyond the restriction point (R1) results in autonomy of the cell cycle, suggesting it is the cyclins, with their phase-dependent activities, and imprinting status. However, little is known about the biological function of p57Kip2. Specifically, the role of p57Kip2 in tumorigenesis and cancer progression is poorly understood. In this study, we investigated the functional role of p57Kip2 in prostate cancer tumorigenesis. Our results show that decreased expression of p57Kip2 occurs frequently in human prostate cancer, even in the precursor PIN stage. Additionally, we show that overexpression of p57Kip2 decreases cell proliferation and tumorigenesis. Furthermore, down-regulation of p57Kip2 induced...
cancerogenesis in the mouse prostate. Thus, p57Kip2 is an important gene in prostate cancer tumorigenesis and progression, and p57Kip2 may be a potential target gene for prostate cancer prevention and therapy.

Materials and Methods

Histology and immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin and paraffin embedded, and 4-μm sections were prepared. Routine H&E staining and immunohistochemistry were performed essentially as described previously (10). For signal detection for immunohistochemistry, primary antibodies used were antibodies against p57Kip2 (clone C20, Santa Cruz), p63 (M-18, Santa Cruz), and Ki67 (clone TEC-3, DAKO). The primary antibody was incubated at the appropriate concentration (p57Kip2, 1:3,000; p63, 1:1,000; Ki67, 1:1,000). The secondary antibodies were used either horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse antibodies. The signals were detected using enhanced chemiluminescence system (Amersham Biosciences).

Transwell migration assay. To test cellular invasive ability, Transwell migration assay was used. A Boyden chamber system was used for Transwell migration assay. Polypropylene inserts with 80-μm pore size (Becton Dickinson Labware) were coated with 500 μL of 250 μg/mL collagen I. This was then dried at room temperature, and kept sterile at 4°C before use. One hour before an experiment, the inserts were blocked using 1% bovine serum albumin in PBS at 37°C. A 100-μL suspension containing 1 × 10^4 cells in 2% FBS culture medium was loaded into each insert. FBS (10%) in 500 μL RPMI 1640 was added to the lower chamber. After incubation for 12 h, cells remaining in the top of the inserts were removed using a cotton swab. The cells that had migrated through the collagen and the filter were fixed with 11% glutaraldehyde (Sigma) for 20 min followed by 0.1% crystal violet staining and counted in five random fields. The mean of the number was used to quantitate the migration. The experiments were done in triplicate wells.

Genetic modification of cell lines. The full-length human p57Kip2 cDNA was inserted into a LZB5sEGFP backbone (Nolan Laboratory) as described previously (11). Viral particles were generated using the amphotropic PHNXA packaging cells, which were obtained from American Type Culture Collection. The viral supernatant from the transfected cells was centrifuged at 3,000 rpm and passed through a 0.45-μm filter. Successive rounds of infection over 5 d were used. The infected LNCaP cells (LNCaPp57Kip2) were selected based on expression of a bicistronic fluorescent tag. The empty vector was used to generate negative controls. Western blotting assay was used to confirm that p57Kip2 level is elevated in LNCAp57Kip2 cells.

Tissue recombination. Pregnant rats were obtained, and rat urogenital mesenchyme (RUM) was prepared from 18-d embryonic fetuses (plug date denoted as day 0). Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by trypsin digestion, as described previously (12). RUM was then additionally reduced to single cells by a 90-min digestion at 37°C with 187 units/mL collagenase (Life Technologies, Inc.). After digestion, the mesenchymal cells were washed extensively with RPMI 1640 tissue culture medium. Viable cells were then counted using a hemacytometer, with viability determined by trypan blue exclusion. LNCAp57Kip2 cells were released from tissue culture plastic with trypsin and washed in growth medium containing 20% FBS, and viable cells were counted using trypan blue exclusion and a hemacytometer. Cell recombinants were prepared by mixing 1 × 10^8 epithelial (LNCAp57Kip2) cells with 3 × 10^5 mesenchymal cells in suspension. Cells were pelleted and resuspended in 50 μL of neutralized type 1 rat tail collagen prepared as described previously (13, 14). The recombinants were allowed to set at 37°C for 15 min, then covered with growth medium (RPMI 1640 + 5% FBS), and cultured overnight. Recombinants were then grafted beneath the renal capsule of adult male athymic mice. All of the animals were housed in Vanderbilt University Laboratory Animal Resource Center with food and drinking water under controlled conditions (12 h light/12 h dark and 20 ± 2°C). The grafts were harvested at 2 mo and then processed for histology and immunohistochemistry. LNCAp cells infected with empty vector were used as controls. Each experiment group has at least four grafts.

Prostatic rescue model. Prostatic rescue is achieved from knockout (KO) mice that are either embryonic lethal or die shortly after birth by grafting the prostate under the kidney capsule of male athymic nude mice. The p57Kip2 KO mice die by 2 wk after birth (7, 8). Therefore, to examine a mature KO prostate requires rescuing the prostate from a newborn or embryo. Prostates from E15.5 to E18.5 days of p57Kip2 KO mice were grafted underneath the renal capsules of 6-week-old 7-wk-old male athymic immunodeficient mice. The prostates were harvested at 2, 4, and 6 mo after rescue. The prostates were dissected, fixed in 10% neutral formalin for 24 h at room temperature, dehydrated in ethanol, cleared in xylene, and embedded in paraffin for histology and immunohistochemistry. Prostates from wild type were used as controls. Each experiment group grafted at least four prostates.

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Statistical analysis. Where appropriate, experimental groups were compared using Student’s two-tailed t test and Newman-Keuls test (q test), with significance defined as P < 0.05. All numerical data are reported as the mean ± SEM.

Results

p57^Kip2 expression is decreased in human prostate cancer.

To understand p57^Kip2 expression in human prostate cancer, we performed immunohistochemical staining in human prostate tissue specimens. Prostate cancer tissue specimens (n = 42) were obtained from radical prostatectomies done at the Vanderbilt University Medical Center. Final Gleason scores varied from 5 to 9 (Gleason scores of 5 for 4 patients, 6 for 19 patients, 7 for 17 patients, 8 for 1 patient, and 9 for 1 patient). Two pathologists graded and counted all specimens in a blinded fashion. Cells were counted as positive for p57^Kip2 when immunoreactivity was clearly observed in nuclei of luminal epithelial cells. The positive areas for p57^Kip2 were counted by monitoring at least 200 luminal epithelial cells for normal (noncancerous), PIN, and carcinoma lesions in multiple regions of the same sample (Fig. 1A). Our results showed that p57^Kip2 expression (percentage of stained cells) was significantly (P < 0.01) decreased in adenocarcinoma cells (2.85%), compared with that of noncancerous cells (47.47%). This decreased expression of p57^Kip2 occurred early during disease progression, wherein levels were low even in PIN lesions (10.21%; Fig. 1B). The expression pattern of p57^Kip2 in human prostate cancer was further confirmed by RT-PCR using human prostate cancer tissues (Fig. 1C). These results suggested that decreased expression of p57^Kip2 occurs frequently in human prostate cancer, even in PIN lesions, a precursor for prostate cancer.

Overexpression of p57^Kip2 inhibited the proliferation of LNCaP cells and arrested the cell cycle at G0-G1 stage in vitro.

To understand the functional role of p57Kip2 in prostate cancer cellular processes, we generated a Tet-inducible system. In this system, transgenes are driven by a modified promoter containing transcription activation sites within the modified promoter, interfering with the formation of the transcription initiation complex and therefore silencing transcription. In the presence of Tet, the binding of TetR is inhibited, allowing the formation of the initiation complex and activation of transcription (Fig. 2A).

To investigate the affect of p57^Kip2 on prostate cancer cellular processes, MTT assay was performed. When maintained in culture in the presence of doxycycline, these cells proliferated significantly (P < 0.01) slower than those cultures without doxycycline (Fig. 2B). The flow cytometric assay was performed 24 hours after p57^Kip2 induction. Our results showed that overexpression of p57^Kip2 arrested the LNCaP cell cycle at G0-G1 stage (Fig. 2C), which is consistent with our results from the MTT assays. These results indicate that overexpression of p57^Kip2 inhibited the proliferation of LNCaP cells by arresting the cycle cell at G0-G1 stage in vitro.

p57^Kip2 regulates CDK2, CDK4, cyclin D1, and RB gene expression. To elucidate the mechanism by which p57^Kip2 affects the prostate cancer cell proliferation and cell cycles, Western Blot assay was performed 24 hours after p57^Kip2 induction. Our results showed that overexpression of p57^Kip2 in LNCaP prostate cancer cells increased RB protein expression and decreased CDK2, CDK4, and cyclin D1 protein expression (Fig. 3). These results suggest that p57^Kip2 inhibited proliferation of LNCaP prostate cancer cells and arrested the cell cycle at G0-G1 stage (see Fig. 2B and C) might be by affecting the RB pathway through CDK4/cyclin D1 and CDK2 complexes.

Overexpression of p57^Kip2 decreased the invasive ability in LNCaP cells. To further understand the functional role of p57^Kip2 on prostate cancer cellular processes, we performed Boyden chamber assay. The ability of cells to invade collagen and migrate to the underside of the inserts was determined by a 12-hour response to conditioned medium containing different concentrations of FBS in the lower chamber (Fig. 4A and B). In the presence of doxycycline, significantly fewer cells migrated to the underside of the inserts. These results indicate that overexpression of p57^Kip2 inhibited invasion of LNCaP prostate cancer cells.
underside of the filter compared with the absence of doxycycline (Student’s t test, P < 0.01). These results indicated that overexpression of p57Kip2 not only reduced the prostate cancer cells proliferation, but reduced the migration and invasive ability of prostate cancer cells.

Overexpression of p57Kip2 induced LNCaP tumors to become well-differentiated squamous phenotype and increased keratin expression. To determine the effects of p57Kip2 on cell differentiation and cancer progression, we established LNCaP(p57Kip2) cell lines which contain an integrated p57Kip2 expression vector. The LNCaP(p57Kip2) cells (p57Kip2 expression was about 3-fold over wild-type LNCaP) were recombined with rUGM and grafted under the renal capsule of a male athymic mouse using tissue recombination technique. After 2 months, our results showed that expression of p57Kip2 caused LNCaP tumors to become well-differentiated squamous lesions (p63 positive) and showed increased keratin expression (Fig. 5). These results suggest that p57Kip2 is not only involved in proliferation, but also involved in differentiation of LNCaP cells. However, when the expression level of p57Kip2 was increased to a higher concentration (another LNCaP cell line with a stably integrated p57Kip2 gene; p57Kip2 expression was ~5-fold over wild-type LNCaP) and a tissue recombination was performed with rUGM and grafted under the renal capsules of the male nude mice, there were no detectable LNCaP tumors formed even 2 months after grafting (0 of 5). This indicated that LNCaP(p57Kip2), when expression of p57Kip2 was very high, tumor growth was totally suppressed. These results suggest that p57Kip2 is an important tumor suppressor gene in prostate cancer.

Down-regulation of p57Kip2 induced carcinogenesis in the mouse prostate. p57Kip2 null mice exhibit growth retardation and early death (7, 8). To investigate the role of p57Kip2 in prostate development and cancer progression in vivo, the urogenital tissues were microdissected from E15.5 to E18.5 days of p57Kip2 KO mice and grafted underneath the renal capsules of 6-week-old to 7-week-old male athymic immunodeficient mice. This approach to rescue tissue from an embryonic lethal has been successfully applied before (16, 17). The prostates were harvested at 2, 4, and 6 months after grafting. Histologic analysis of p57Kip2 KO prostates showed significant hyperproliferation and hyperplasia at 2 mo (Fig. 6A, a and b) and 4 mo (Fig. 6A, c and d) after rescue. Although no typical PIN or carcinoma lesions were found at 4 months after rescue, we could still see multiple epithelial layers, elongated nuclei, and nonuniformly stained epithelial cells in some areas, all of which are features of early PIN lesions in the mouse prostate (see arrows in Fig. 6A, d).

However, the prostates of p57Kip2 KO mice developed typical PIN (two of six p57Kip2 KO prostates) and carcinoma (four of six p57Kip2 KO prostates) lesions at 6 months postgrafting (Fig. 6B, M1–M4). At low power (Fig. 6B, M1 and M3; original magnification, 200×), the prostates show architecture of simple glands and cribriforming glands with associated luminal secretions. At higher power (Fig. 6B, M2 and M4; original magnification, 400×), mouse tumors are composed of hyperchromatic small glands with enlarged nuclei and prominent macronucleoli. The chromatin seems evenly dispersed within the nucleus. In some areas, the glands are forming a cribriform pattern with a surrounding delicate interlacing...
fibrovascular network. Focal apoptotic bodies are present with rare mitotic figures. This tumor has many histologic similarities to human prostatic adenocarcinoma (Fig. 6B, H1–H8), including nuclear morphology with the enlarged, round nuclei and prominent macronucleoli. When comparing the mouse adenocarcinoma to the human needle core biopsy (Fig. 6B, H1–H4) and human radical prostatectomy (Fig. 6B, H1–H4) specimens, the glandular formation with Gleason patterns 3 and 4 is represented and similar. Mouse and human Gleason pattern 3 represent the simple glandular profiles typical of this pattern. The mouse and human Gleason pattern 4 has cribriforming and glandular luminal loss. The glandular morphology is virtually identical with the nonoverlapping cells, nuclear hyperchromasia, prominent nucleoli, nuclear enlargement, and paucity of inflammation. The mouse has the pink intraglandular debris, as areas of the human cancers also exhibit that, yet are not captured on these images.

Discussion

Evaluation of the biological characteristics of carcinomas requires understanding of cell cycle regulators. In this study, we provide compelling evidence that p57Kip2, a CDK inhibitor, is an important tumor suppressor gene in prostate cancer. In our study, we show that decreased expression of p57Kip2 occurs early in PIN lesions and remains in low levels in human prostate cancer. Reduced expression in PIN lesions, precursors to prostate cancer, indicates an important role for p57Kip2 in early stages of cancer development.

Loss of expression of p57Kip2 is not thought to occur due to mutations in the gene (18, 19). However, despite the potentially critical role that Kip/Cip proteins play in controlling cell cycle checkpoints, their deletion alone does not give rise to tumors in mice, and for that matter, mutations in p21CIP1/WAF1, p27Kip1, and p57Kip2 are not frequently encountered in human tumors (19–24). Cordon-Cardo and colleagues reported that more aggressive human prostatic cancers have decreased levels of p27Kip1 and that loss of p27Kip1 may be causally linked to benign prostatic hyperplasia (25). Loss of p27Kip1 in the mouse prostate results in increased prostatic epithelial hyperplasia and altered differentiation, but it does not result in PIN or prostate cancer (25, 26). Several studies have characterized p57Kip2 as an independent prognostic factor in a variety of human malignancies, including breast, lung, bladder, and pancreatic cancers (24, 27–33). The absence of p57Kip2 mutations within a variety of human cancer types suggests that other mechanisms of transcriptional or posttranslational silencing must be involved in the loss of p57Kip2 protein expression in cancer. Several possible mechanisms of gene inactivation have been proposed, including methylation in the promoter region of the p57Kip2 gene, histone deacetylation, lysine methylation, and microRNA (19, 34, 35). Shin and colleagues (19) showed that formation of inactive chromatin through histone deacetylation is a general mechanism for inactivation of both p21CIP1/WAF1 and p57Kip2 genes in gastric cancer cells and that methylation of the promoter region of the p57Kip2 gene occurred in five of eight gastric cancer cell lines. A recent study showed that transforming growth factor-β (TGF-β) causes p57Kip2 degradation in osteoblasts, suggesting a new system for regulating p57Kip2 (36). Because TGF-β plays an important role in tumorigenesis, determining whether p57Kip2 is regulated by TGF-β is important.

p57Kip2 has important functions, such as binding to proliferating cell nuclear antigen to prevent DNA replication and inhibit cell transformation (37).

Figure 3. p57Kip2 regulates expression of CDK2, CDK4, and cyclin D1 to induce cell cycle arrest. Western blot was performed at 24 h after p57Kip2 induction (doxycycline treatment) in LNCaP cells. Anti-actin blots are shown as a loading control. Overexpression of p57Kip2 in LNCaP prostate cancer cells increased phosphorylated Rb protein level and decreased CDK2, CDK4, and cyclin D1 protein level.

Figure 4. Overexpression of p57Kip2 reduced the invasive ability in LNCaP cells in vitro. Transwell migration assay was performed using a Boyden chamber system. The cells which had penetrated the filters (A) were counted in five microscopic fields (>200) per filter (6 mm²) and are reported as the mean of triplicate assays (B). Columns, mean; bars, SEM. **, P < 0.01 by Student’s t test.
antiproliferation (38). It also stabilizes MyoD during muscle differentiation (39, 40). Recently, overexpression of p57Kip2 has been shown to cause a cell growth arrest and senescent phenotype in many cell types (41, 42). In addition, expression of p57Kip2 is involved in inhibiting the conversion of conditionally immortal human mammary epithelial cells to the fully immortal phenotype, suggesting that p57Kip2 provides an important barrier against indefinite proliferation (43). Consistent with these results, our study showed that overexpression of p57Kip2 in prostate cancer cells significantly suppressed the cell proliferation and arrested the cell cycles at G0-G1 stage. In addition, our results indicated that p57Kip2 inhibit cell proliferation; this might be by affecting the Rb pathway through CDK4/cyclin D and CDK2 complexes. These results suggest that p57Kip2 can serve as a tumor suppressor to induce senescence and block immortalization in cancer. Matsuura and colleagues reported (44) that placentas of p57Kip2 null mice showed higher vascular endothelial growth factor mRNA and protein levels than wild-type mouse placenta, suggesting that p57Kip2 is also involved in angiogenesis, which is an important step in cancer metastasis.

Animal studies have shown that p57Kip2 KO mice have altered cell proliferation and differentiation and have a variety of other abnormalities, including muscle defects, bone defects, a cleft palate, adrenal cortical hyperplasia and cytomegaly, and gastrointestinal tract defects (7, 8). Many of these defective phenotypes are shared with patients with Beckwith-Wiedemann syndrome (BWS), a childhood overgrowth syndrome, suggesting that loss of p57Kip2 plays a role in BWS (7, 8). p57Kip2 null mice will die by 2 weeks of age (7, 8). However, KO prostatic embryonic or newborn tissue can be rescued by grafting the tissue under the kidney capsule of male athymic nude mice. In this study, the urogenital tissues were microdissected from E15.5 to E18.5 days of p57Kip2 KO mice, grafted, and allowed to mature for 2, 4, and 6 months in the male host. Our results show that the prostates of p57Kip2 KO mice develop PIN and adenocarcinoma at 6 months postgrafting. Noticeably, these tumors have many histologic similarities that make them indistinguishable from human prostatic adenocarcinoma, including nuclear morphology with the enlarged, round nuclei and prominent macronucleoli. The nuclear chromatin pattern with hyperchromasia and fine stippling shows strong similarities. The cytoplasm is amphophilic, is similar to human prostate cancer, and has a very strong diagnostic criteria used by pathologist. The architecture, both at low and higher power, is also comparable with human prostate cancer with the ability for gland formation, cribriforming pattern, and loss of gland formation focally. The luminal secretions are reminiscent of the pink secretions in human prostate cancer, one of the criteria used to diagnose limited cancers. The presence of focal apoptotic bodies are rarely seen in human prostatic adenocarcinoma, increasing in number with increasing Gleason grade. Mitotic figures are helpful.
in the diagnosis of human prostate cancer, but not a diagnostic criterion, and usually not a feature of benign prostate tissue. Previous mouse models for prostate have not displayed the same histology as human prostate cancer. Because the rodent has multiple lobes, each with distinct histology, compared to two major zones in the human prostate; it has generally been accepted that these differences account for the inability of mouse prostate models to reflect the same pathology as human prostate cancer. The of p57Kip2 KO is remarkable because of all the mouse models for prostate cancer, only the combination of a PTEN KO and Nkx3.1 KO have some similar pathology features but is still not identical to human prostate cancer (45). These results indicate that p57Kip2 is an important gene in early PIN development and prostate cancer tumorigenesis resulting in a mouse model that is pathologically identical to the features of human prostate cancer.

In summary, our studies show that decreased expression of p57Kip2 occurs frequently in human prostate cancer even in PIN lesions. Overexpression of p57Kip2 significantly suppressed cell proliferation and arrested the cell cycle by affecting the Rb pathway through CDK4/cyclin D1 and CDK2 complexes. In addition, overexpression of p57Kip2 induced LNCaP prostate tumors to become well-differentiated squamous tumors with increased keratin expression, whereas down-regulation of p57Kip2 induced carcinogenesis in the mouse prostate. Taken together, our results provide compelling evidence that p57Kip2 is an important gene in prostate cancer tumorigenesis and that the p57Kip2 pathway may be a potential target for prostate cancer prevention and therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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