Expression of the F-Box Protein SKP2 Induces Hyperplasia, Dysplasia, and Low-Grade Carcinoma in the Mouse Prostate

Eun-Hee Shim, Linda Johnson, Hye-Lim Noh, Yoon-Jung Kim, Hong Sun, Caroline Zeiss, and Hui Zhang

Departments of Genetics [E-H.S., H. S., H. Z.], Internal Medicine [H-L. N., Y.-J. K.], and Comparative Medicine [L.J., C. Z.], Yale University School of Medicine, New Haven, Connecticut 06520

ABSTRACT

Low or absent expression of the cyclin-dependent kinase inhibitor p27Kip1 serves as an excellent malignant marker for prostate and other human cancers. The level of p27Kip1 is regulated primarily by the ubiquitin ligase SCF-Skp2 through ubiquitin-dependent proteolysis. Expression of the F-box protein SKP2 is inversely correlated with p27 in many cancers. To determine the role of SCF-Skp2 in proliferation and tumorigenesis, we established transgenic mouse lines that specifically expressed SKP2 in the prostate gland. Unscheduled expression of SKP2 promoted marked overproliferation, resulting in hyperplasia, dysplasia, and low-grade carcinoma in the prostate gland. Consistent with its critical role in p27 proteolysis, SKP2 expression caused significant down-regulation of p27 in prostate glands from transgenic animals. Immunohistological staining indicated that SKP2 expression is restricted to the hyperplastic/dysplastic regions and that there is an inverse relationship between SKP2 and p27 expression in the ductal epithelium in transgenic animals. The prostate glands from transgenic mice also exhibited high levels of proliferative and mitotic markers such as Ki67 and cyclin B1. Our data suggest that SKP2 acts as an oncoprotein in the mouse prostate gland, probably through its function as a limiting factor for ubiquitin-dependent degradation of p27.

INTRODUCTION

Low or absent expression of the CDK inhibitor p27Kip1 has been observed in malignant human cancers (1–5) and serves as an excellent prognostic marker associated with poor patient survival (3, 6). It has been reported that proteolytic degradation of p27 is enhanced in malignant colorectal carcinomas and other cancers and may serve as a mechanism to down-regulate p27 (7, 8). Alteration of the post-transcriptional regulation is also implicated in the low or absent expression of p27 in advanced prostate cancer (9).

In the cell cycle, p27 is regulated primarily through ubiquitin-dependent degradation (10). The degradation of p27 at late G1 phase allows the activation of cyclin E/CDK2, promoting S-phase entry. Recently, it has been shown that a ubiquitin ligase, the SCF-Skp2 complex (SKP1, CUL-1/CDC53, and the F-box protein SKP2), mediates the polyubiquitination of p27 (11–14). SKP2, the substrate-targeting subunit of the SCF complex, binds to the phosphorylated p27 and targets p27 for polyubiquitination and subsequent proteolysis (11, 12). SKP2 was originally isolated as a protein that is highly expressed in many tumor cell lines (15). The level of SKP2 is elevated in malignant human cancers (16–20) and is inversely correlated with low expression of p27 in advanced prostate cancer (9).

To determine the exact effect of SKP2 expression in tumorigenesis, we sought to establish a mouse model that expresses SKP2. Because low or absent expression of p27 is frequently associated with advanced human prostate cancers, we developed a model that specifically expressed SKP2 in the mouse prostate gland. Our studies suggest that expression of SKP2 alone is sufficient to reduce p27 levels and induce hyperplasia, dysplasia, and low-grade carcinomas in the mouse prostate gland.

MATERIALS AND METHODS

Generation of SKP2 Transgenic Mice. The ARR-PB promoter was a kind gift from Robert Matusik (Department of Urology, Vanderbilt Medical Center, Nashville, TN; Ref. 22). The human SKP2 cDNA (15) was cloned into the vector under control of the ARR-PB promoter. The poly(A) signal sequence was derived from pcDNA3 (Invitrogen) and cloned downstream of the human SKP2 DNA sequence. The transgenic mice were developed in the Yale Medical School Transgenic Mouse and Targeted Mutagenesis Resources. The CD-1 mouse strain was used for initial injection and subsequent outbreeding to establish the transgenic lines. Genotypes were determined by PCR of genomic DNA isolated from mouse tails, using the primers derived from the probasin promoter (5′-GCAAGACACTGGCCATGGCAAATCA-3′; bp −585 to −562) and hSKP2 (5′-CGATTTAGCTCAGTTGCGGCAAA-3′; bp −46 to −22). All procedure were conducted in accordance with institutional protocols. In all of the experiments, 3–7-month-old mice were used.

RT-PCR Analysis. Total RNA was prepared using Trizol (Invitrogen) from the isolated prostate glands. The reverse transcription was conducted with 1 μg of RNA and an oligodeoxynucleotidime primer (Roche). hSKP2 expression in transgenic animals was confirmed by PCR with the following hSKP2 primers: forward, 5′-CTGAGCTGCTAAAGGTCTCTGGTG-3′ (bp 52–60); reverse, 5′-GCAAGACACTGGCCATGGCAAATCA-3′ (bp 950–971). For GAPDH primers, the following primers were used as the internal control: forward primer, 5′-CTGAGGATCTGCTCTTCCGC-3′; reverse primer, 5′-GATGGCATGGATCTGCTCTTC-3′.

Western Blot Analysis. The anti-CUL-1 and -SKP2 rabbit polyclonal antibodies have been described previously (11). We also used anti-SKP2 (H-435, Santa Cruz Biotechnology), anti-p27 (Transduction Laboratories), and anti-Cyclin B1 (PharMingen) antibodies in our studies.

Histology. Prostates for histology were microdissected from wild-type and transgenic mice. Prostates were fixed by immersion in 10% formaldehyde in PBS solution, dehydrated through ethanol, and embedded in paraffin. Blocks were sectioned (5 μm) and stained with H&E (Sigma).

Immunohistochemistry. Sections were deparaffinized and rehydrated in ethanol solutions. After antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 10 min, the sections were treated with 3% peroxide in methanol at −20°C to quench endogenous peroxidase and then blocked in 10% BSA. The sections were incubated with anti-SKP2 (1:100 dilution; Zymed), anti-p27 (C-19; 1:100 dilution; Santa Cruz Biotechnology), and anti-Ki67 (NCL-Ki67; 1:1500 dilution; Novocastra) rabbit polyclonal antibodies for 1 h at room temperature and incubated with biotinylated secondary antibody (BA-100, 1:200 dilution; Vector). Antibodies were visualized with streptavidin-biotin complex (Zymed) with diaminobenzidine as a chromogen. Nuclei were counterstained with hematoxylin.

Brdu Incorporation in Prostate. Mice received i.p. injections of BrdUrd (Sigma; 50 μg/g body weight). After being labeled with BrdUrd for 1 h, the prostates were dissected, fixed, and prepared for paraffin sections. BrdUrd staining was performed with anti-BrdUrd (1:5 dilution; Becton Dickinson) monoclonal antibody and visualized with the Zymed mouse kit (Zymed).
RESULTS

Establishment of Prostate-specific SKP2 Transgenic Lines. To specifically express SKP2 in mouse prostate gland, we cloned the cDNA encoding human SKP2 (15) under control of the rat probasin promoter (Fig. 1A; Ref. 22). The probasin promoter is prostate-specific and androgen-regulated. Expression is restricted primarily to the epithelial cells in the ventral, lateral, and dorsal lobes of the prostate, with only very low expression in the anterior prostate and seminal vesicles. After injection of the SKP2 construct, five transgenic founder mice were genotyped positive (Fig. 1B). Among them, three transgenic lines, 31, 33, and 79, were found to transcribe human SKP2 in the prostate gland (Fig. 1C).

The prostate gland and other tissues from wild-type and transgenic animals were microdissected, and the expression of human SKP2 protein was further determined. In both transgenic lines 33 and 79, there was substantial expression of SKP2 in the prostate gland but not in the testes, spleen, kidney, liver, and thymus (Fig. 1D), whereas line 31 produced only very low levels of SKP2 (Fig. 1C, and data not shown). In addition, SKP2 was expressed in the ventral and lateral lobes of the prostate glands in transgenic lines 33 and 79, with lower expression in the dorsal lobe (Fig. 1E). These analyses suggested that the transgenic mice specifically expressed SKP2 in the prostate gland.

SKP2 Induces Enlargement and Hyperplasia of the Ventral Prostate Gland. To determine potential pathological changes associated with SKP2, prostate glands from wild-type and transgenic mice were dissected and evaluated by histological analysis. Visual examination of the dissected prostate glands suggested that the glands of SKP2 transgenic mice were noticeably larger than those from the wild-type animals. Histopathological analyses of the ventral glands showed that SKP2 expression caused a spectrum of hyperplastic and dysplastic changes (PIN I–III) and low-grade carcinomas (PIN IV). In the wild-type animals, the duct size within the ventral prostate gland tended to be relatively consistent and the epithelium within the duct was usually composed of a single layer of cells with relatively even height (Fig. 2A). However, in the transgenic animals, low-grade hyperplastic changes (PIN I–II) caused marked enlargement and variation in the duct size and the epithelium height (Fig. 2A). The areas of tall columnar epithelium alternated with regions of low cuboidal epithelium within the same duct.

Dysplastic changes and low-grade carcinoma were also found in the ventral prostate glands in the transgenic but not in the control wild-type animals (Table 1). In the transgenic lines, the dysplastic ventral gland displayed primitive cribriform patterns, tufts that nearly filled the duct lumen, or disoriented epithelial cells, often with vacuolated cytoplasm containing prominent round to oval nuclei with one or more nucleoli (Fig. 2B). Multiple mitotic figures were observed. Immunostaining indicated that SKP2 was specifically expressed in these hyperplastic or dysplastic regions. However, no SKP2 staining

Table 1. SKP2 expression induces hyperplasia, dysplasia, and low-grade carcinomas in the mouse prostate gland

<table>
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<tr>
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*DLP, dorsolateral prostate; VP, ventral glands of prostate.
was observed in the ventral prostate glands from the wild-type animals.

In the dorsal lateral prostate glands from the transgenic lines, we also found a wide range of hyperplastic or dysplastic changes and low-grade carcinoma (Fig. 2C; Table 1). Dysplastic changes included aggregates of epithelial cells that accumulated beneath and between the simple epithelium typical of a normal duct (Fig. 2C). A dysplastic alteration (PIN III) from transgenic line 33 was characterized by...
nudular proliferation of disorganized epithelial cells with large euchromatic nuclei and prominent mitotic figures (Fig. 2C). The lumen of the duct was largely filled by the proliferative epithelial cells. Mild hyperplasia was noted in adjacent ducts.

A low-grade adenocarcinoma (PIN IV) in the dorsolateral prostate gland from transgenic line 79 was noted (Fig. 2C). In this neoplasm, enlarged ducts, including adjacent ones, were nearly completely filled by cuboidal to columnar epithelial cells arranged in a primitive cribriform pattern. Mitoses were prominent. The surrounding fibromuscular stroma was attenuated and nearly absent in places. These observations suggested that SKP2 expression promotes hyperplastic and dysplastic changes or even low-grade carcinomas in the ventral and dorsolateral prostate gland in the transgenic animals.

**SKP2 Expression Is Sufficient to Promote Down-Regulation of p27 in the Prostate Gland.** In many human cancers, elevated SKP2 levels are inversely associated with low or absent expression of p27. To determine the effect of SKP2 expression, the prostate glands were dissected, and proteins were then examined by Western blot analysis. In the wild-type mouse prostate gland or other tissues, such as thymus or spleen, p27 levels were high and readily detectable (Fig. 1F). In 3–6-month-old mice from SKP2 transgenic lines 33 and 79, we observed a marked down-regulation of p27 in the mouse prostate gland but not in other tissues (Fig. 1F); p27 was also down-regulated in line 31, although SKP2 expression was lower. These data suggest that unscheduled expression of SKP2 causes a persistent down-regulation of p27 in mature prostate glands.

**Immunological Staining of SKP2 and p27 in the Prostate Glands of Transgenic Mice.** We used immunohistological staining to determine the effect of SKP2 expression on the level and distribution of p27. SKP2 was expressed in many epithelial cells in the ducts from the transgenic prostate glands but was absent in the wild-type animals (Fig. 3A). On the other hand, whereas p27 was consistently and evenly expressed in the ductular epithelium in the wild-type prostate gland, p27 expression in the transgenic animals was patchy.

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**Fig. 3. Down-regulation of p27 in the prostate gland of SKP2 transgenic mice.** A. H&E-stained sections of ventral prostates derived from 3-month-old control wild-type and transgenic mice (line 33). No SKP2 staining is observed in control wild-type mice (upper left panel), and p27 expression is consistent and evenly distributed in the epithelial cells (lower left panel). In transgenic line 33, SKP2 is highly and specifically expressed in hyperplastic epithelium (upper right panel). In the hyperplastic regions, cells highly stained for SKP2 show reduced p27 expression (lower right panel, indicated by arrowheads). Magnification, ×100. B. Co-immunostaining of SKP2 and p27 in the ventral prostate glands of 4-month-old control and transgenic line 33 mice. In the wild-type ventral prostate gland (left), only evenly distributed p27 staining, indicated by the alkaline phosphatase dark-blue staining, is observed in the epithelial cells in ducts. In the transgenic animals (right), p27 staining is lower and displays patchy distribution. In SKP2-positive cells (the peroxide brown staining), p27 levels are reduced. SKP2 expression is restricted to the hyperplastic regions and inversely correlates with p27 expression. Magnification: top, ×200; bottom, ×400.
and unevenly distributed in both cuboidal and tall columnar epithelium (Fig. 3A). Immunostaining of consecutive sections of the ventral prostate revealed an inverse relationship between SKP2 and p27 expression in many epithelial cells (Fig. 3A). In SKP2 transgenic animals, SKP2-positive cells usually contained less p27, and cells with low p27 staining corresponded to regions with SKP2 expression. Co-immunostaining of SKP2 and p27 confirmed such an inverse relationship at the cellular level (Fig. 3B).

**SKP2 Expression Promotes Proliferation of Prostate Cells.** Our data suggest that expression of SKP2 causes hyperplasia, dysplasia, and down-regulation of p27 in the mouse prostate gland, as summarized in Table 1. To further determine the effect of SKP2, we examined the expression of some well-characterized cell cycle markers. The expression of mitotic cyclin B1 was usually very low in the ventral prostate glands from the wild-type animals (Fig. 4A). In contrast, in both transgenic lines 33 and 79, expression of cyclin B1 was significantly increased. This increase in cyclin B1 was accompanied by SKP2 expression and the down-regulation of p27. In the dorsolateral prostate, cyclin B1 levels were also induced in the SKP2-expressing gland in the transgenic animals (Fig. 4B). The level of Ki67, a widely used mitotic marker (23), was also significantly higher in the transgenic prostate glands (Fig. 4, C and E). In addition, the incorporation of BrdUrd was also increased in the prostate gland of the SKP2 transgenic animals (Fig. 4D). These results indicate that SKP2 expression is sufficient to promote proliferation of epithelial cells in the prostate gland.

**DISCUSSION**

In this study, we generated mouse transgenic lines that specifically expressed SKP2 in the prostate gland. We found that SKP2 expression is sufficient to induce enlargement of the ventral prostate gland and to promote hyperplasia, dysplasia, and low-grade carcinomas in the prostate glands. The expression of SKP2 was associated with the down-regulation of p27 and was restricted to the hyperplastic and dysplastic regions. These proliferative changes correlated with the increased expression of mitotic markers such as cyclin B1 and Ki67 and the enhanced incorporation of BrdUrd in the hyperplastic/dysplastic regions in the transgenic animals.

Our current studies suggest that SKP2 is rate limiting for p27 degradation in the mouse prostate gland. Previous studies indicated that ubiquitin-dependent proteolysis of p27 requires prior phosphorylation on threonine 187 in p27 (11, 12, 24). A recent report suggested that there are other phosphorylation sites in p27 that mediate SKP2 and p27 interaction (25). Although cyclin E/CDK2 kinase can phosphorylate p27 on threonine 187, several recent in vivo studies have suggested that under conditions in which cyclin E/CDK2 activity is greatly inhibited, p27 phosphorylation on threonine 187 and its sensitivity toward SKP2 are not significantly changed (26, 27). These observations suggest that either cyclin E/CDK2 is not limiting or that there might be additional kinases that can phosphorylate p27. The phosphorylation of p27, therefore, is not rate limiting under these conditions.

In human cancers, low or absent expression of p27 is often accompanied by elevated levels of SKP2. In the prostate glands of transgenic SKP2 mice, we also observed that there was an inverse relationship between the expression of SKP2 and p27, suggesting that p27 is a critical target of SKP2. These observations are consistent with previous studies showing that p27-deficient mice display enlarged prostate glands and development of hypercellular epithelium cells (9). However, there is a marked difference between SKP2 transgenic and p27-deficient mice in the latency period. In our study, hyperplastic and dysplastic changes in both the ventral and the dorsolateral prostate
d约束了在3–7-month-old SKP2转genic小鼠。与之相反，p27—deficient小鼠，hyperplasia在前列腺小鼠中出现得较早，尤在14个月（9, 23）。在这一研究中，SKP2转genic小鼠出现严重proliferation病变，包括dysplasia和low-grade carcinoma在前列腺小鼠中，但此类病变未在p27—deficient小鼠中出现。这并不表示p27基因在小鼠中的作用 negligibly。

0. It has previously been shown that transgenic expression of SKP2 in mouse thymus exhibited a cooperative effect in inducing lymphoma with N-Ras oncprotein in double transgenic mice (20). Such a tumorigenic activity of SKP2 is consistent with its function to target p27 for ubiquitination and proteolysis, and with the results in our current study. However, in the study on thymus-specific SKP2 expression, protein blot analysis suggested that there is no detectable change in the phosphorylated p27 or total p27 levels in the thymus of transgenic animals (20). It is possible that the transgenic expression of SKP2 may be restricted to a small fraction of cells in thymus that may be detectable by immunostaining. Alternatively, it remains possible that in thymus, SKP2 may not be the only rate-limiting factor for the degradation of p27.

Low or absent expression of p27 is often associated with malignant prostate cancer and other human cancers (1, 2, 18, 21), suggesting that SKP2 expression might be a causative factor in p27 down-regulation. Our studies suggest that unexpressed expression of SKP2 in the mouse prostate gland induces the down-regulation of p27 and consequently promotes hyperplasia, dysplasia, and low-grade carcinoma. Our results thus support the notion that SKP2 expression is a critical factor that induces p27 down-regulation and promotes growth advances in cancers. These studies suggest that SKP2 is an oncprotein that could serve as an excellent therapeutic target for the treatment of many human cancers.

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