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

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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 E3 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 CDK3 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 ubiquitin E3 ligase 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 many human cancers (16–20) and is inversely correlated with low expression of p27 (16, 18, 21). These studies suggest that overexpression of SKP2 in these cancers might serve as a primary cause for the down-regulation of p27.

MATERIALS AND METHODS

Generation of SKP2 Transgenic Mice. The ARR Pb promoter was a kind gift from Robert Matsuk (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 CDNA 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'-GCAAGACACTGGCCATGCAAATCA-3'; bp = 585 to 562) and hSKP2 (5'-GAGATTTAGCTTACGCTTGCGTCCGAA-3'; bp = 46 to 22). All procedures 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 purified using Trizol (Invitrogen) from the isolated prostate glands. The reverse transcription was conducted with 1 μg of RNA and an oligodeoxynucleotide primer (Roche), hSKP2 expression in transgenic animals was confirmed by PCR with the following hSKP2 primers: forward, 5'-CTGAGGTCTAAAGGTGCTCTGGTG-3' (bp 52–60); reverse, 5'-GACAGTGTCAGTCGCGATTG-3' (bp 950–971). For GAPDH primers, the following primers were used as the internal control: forward primer, 5'-CTGAGGACTCGTTGTCGTCTACCC-3'; reverse primer, 5'-GATGGCAATGACGTGGTCCGTCGCC-3'.

Western Blot Analysis. Total RNA (10–25 g) was labeled with [3H]uridine by run-on transcription. After treatment with 0.5 μg/ml BrdU for 1 h, the BrdUrd was fixed, dehydrated through ethanol, and embedded in paraffin. Blocks were sectioned (5 μm) and stained with H&E (Sigma). The ARR 2 PB promoter was a kind gift from Robert Matsuk (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 CDNA 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'-GCAAGACACTGGCCATGCAAATCA-3'; bp = 585 to 562) and hSKP2 (5'-GAGATTTAGCTTACGCTTGCGTCCGAA-3'; bp = 46 to 22). All procedures were conducted in accordance with institutional protocols. In all of the experiments, 3–7-month-old mice were used.

Histology. Prostata for histology were microdissected from wild-type and transgenic mice. Prostata 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 m M citrate buffer (pH 6.0) for 10 min, the sections were treated with 3% H2O2 to quench endogenous peroxidase and then blocked in 10% BSA. The sections were incubated with anti-SKP2 (1:100 dilution; Zymed), 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-CyclinB1 (PharMingen) antibodies in our studies.

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.
prostate (VP), and ventral, the isolated prostate glands were further dissected into separate parts of the gland: coagulating gland (CG), seminal vesicle (EV), dorsal prostate (DP), and ventral prostate (VP). The ventral prostates and thymus were dissected and used for examination of p27 and hSKP2 expression by Western blot.

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 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 testis, 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...
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...
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
glands were detected in 3–7-month-old SKP2 transgenic mice. In contrast, in p27-deficient mice, hyperplasia, dysplasia, and low-grade carcinoma in the prostate glands occurred much later, at the advanced age of 14 months (9, 23). In addition to this shortening of the latency period, SKP2 transgenic mice displayed severe proliferation alterations, including dysplasia and low-grade carcinoma in the prostate glands, whereas such abnormalities were not reported in p27-deficient mice. It is not clear whether these discrepancies are attributable to the difference in mouse strains or other causes. It has been shown that although p27 acts as a CDK inhibitor, it is also required for the assembly of the cyclin D/CDK4 or/CDK6 complex (28). It is possible that SKP2 expression may not completely down-regulate p27 and that the residual p27 may be sufficient to perform its assembly function. Alternatively, it is possible that, in vivo, SKP2 may promote proliferation by targeting other cell cycle regulators for proteolysis. Further studies are needed to clarify these issues.

It has previously been shown that transgenic expression of SKP2 in mouse thymus exhibited a cooperativity in inducing lymphoma with N-Ras oncoprotein in double transgenic mice (20). Such a tumorigenic activity of SKP2 is consistent with its function to target p27 for ubiquitin-dependent 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, 5, 9). Recent studies suggest that there is an inverse relationship between the levels of p27 and SKP2 in many malignant cancers (16, 18, 21), suggesting that SKP2 expression might be a causative factor in p27 down-regulation. Our studies suggest that unscheduled 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 advantages in cancers. These studies suggest that SKP2 is an oncoprotein that could serve as an excellent therapeutic target for the treatment of many human cancers.

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