Spatial repression of PCNA by p53 during kidney development

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Saifudeen, Zubaida, Jessica Marks, Hong Du, and Samir S. El-Dahr. Spatial repression of PCNA by p53 during kidney development. Am J Physiol Renal Physiol 283: F727–F733, 2002. First published May 22, 2002; 10.1152/ajprenal.00114.2002.—Transcriptional repression is a key mechanism for the spatial specification of gene expression and cell fate determination. During kidney development, proliferating cell nuclear antigen (PCNA) is expressed in the nephrogenic zone and is downregulated rapidly as renal epithelial cells enter terminal differentiation and acquire functional characteristics. Our laboratory reported that the transcription factor p53 stimulates the terminal differentiation of renal epithelial cells by means of transcriptional activation of renal function genes (Saifudeen Z, Dipp S, and El-Dahr SS. J Clin Invest 108: 1021–1030, 2002). Because p53-induced growth arrest correlates with downregulation of PCNA gene expression, we examined the impact of p53 inactivation on PCNA expression in mice and evaluated the effect of p53 on PCNA transcription. Immunohistochemistry revealed that the transition from nephrogenesis to terminal epithelial cell differentiation correlates with accumulation of the transcription factor p53. Importantly, the spatially restricted pattern of PCNA expression is disrupted in kidneys of p53-deficient pups, in which there was a redistribution of PCNA expression into the differentiation zone (without a change in total kidney PCNA content) and distortion of the tubular architecture. Electrophoretic mobility shift assays revealed that the binding of kidney nuclear extracts to the p53 response elements in human and rat PCNA promoters is developmentally regulated. Transient transfection assays performed in p53-deficient HeLa cells revealed that exogenous p53 strongly represses transcription from human PCNA promoter-reporter constructs. Interestingly, deletion of the p53-binding site confers enhanced responsiveness to p53-mediated repression, suggesting that transcriptional repression of PCNA by p53 is achieved by a mechanism other than direct DNA binding. On the basis of these results, we propose the hypothesis that p53-mediated transcriptional repression plays a role in the spatial restriction of PCNA gene expression during normal renal development.

THE TUMOR SUPPRESSOR PROTEIN p53 regulates cell cycle, programmed cell death, differentiation, senescence, and DNA repair (21, 31, 44). As a sequence-specific DNA-binding protein, p53 activates transcription of its target genes through binding to a consensus DNA element that consists of two copies of the inverted repeat sequence [RRRC(AT)(T/A)GYYY] separated by 0–13 nucleotides (10, 12). Several reports also indicate that p53 represses gene transcription. The mechanisms of p53-mediated repression are complex and may involve direct DNA binding (8, 20, 24), interaction with the basal transcription machinery (5, 11, 32), or recruitment of corepressors, such as the Sin3-histone deacetylase complex (29).

Among the well-characterized p53 target genes are p21(Cip1), GADD45, IGF-BP3, Bak, cyclin G, and PCNA (21, 23, 31, 36, 44). PCNA is a processivity factor for DNA polymerase-6 (4) and is expressed in a cell cycle-dependent manner. PCNA is expressed at high levels in the S phase of the cell cycle but decreases dramatically during terminal differentiation (22, 27). PCNA is required for cell cycle progression in vivo, because antisense targeting of PCNA mRNA inhibits growth factor-stimulated proliferation in cultured cells (19) as well as in the intact animal (42).

In the developing kidney, PCNA expression is restricted to the nephrogenic zone, a subcapsular region containing primitive nephrons and blastema. PCNA expression is downregulated rapidly as renal epithelial cells differentiate and acquire functional characteristics. Previous studies have shown that p53-mediated inhibition of cellular growth is accompanied by selective downregulation of PCNA gene expression (25). These findings suggest that elevated levels of cellular p53 may restrict PCNA expression through transcriptional repression. In this study, we examined the regulation of the PCNA promoter by p53 and determined the consequences of p53 gene inactivation in mice on the spatial expression of PCNA during nephrogenesis.

EXPERIMENTAL PROCEDURES

Animals and tissues. Rat kidneys were harvested from newborn (days 1–5) and adult (day 90) Sprague-Dawley rats (Charles Rivers, Wilmington, MA). Breeding pairs of p53+/− mice on C57Bl/6J genetic background (17) were obtained from Jackson Laboratories. Genotyping of the progeny was performed on liver genomic DNA by multiplex PCR according to the supplier's protocol.

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Immunohistochemistry. After fixation in 10% buffered formalin or Bouin’s solution, kidneys were dehydrated in serial alcohol solutions and embedded in paraffin blocks. Five-micrometer serial sections were immunostained by an immunoperoxidase technique using the ABC Elite Vectastain kit (Vector Laboratories, Burlington, CA) as previously described (9). The primary antibodies consisted of a monoclonal PCNA PC10 antibody (DAKO) diluted 1:500–1:1,000 and a polyclonal p53 antibody (FL-393, Santa Cruz) diluted 1:100. In negative controls, the primary antibody was omitted or replaced by nonimmune serum.

Nuclear extracts and EMSA. Kidney nuclear extract preparation was performed as previously described (34). Freshly harvested kidneys were rinsed in ice-cold PBS and homogenized (×20 strokes) with a type A Dounce homogenizer in ice-cold NB1 (10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, 0.1 M sucrose). The homogenate was gently mixed with an equal volume of chilled NB2 (NB1 with 0.25 M sucrose). Approximately 2–3 ml NB3 (10 mM Tris, pH 8.0, 5 mM MgCl₂, 0.5 mM DTT, 0.33 M sucrose) was layered under the cell suspension, and nuclei were pelleted at 1,000 g at 4°C for 10 min. The nuclear pellet was washed in chilled NB4 (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM DTT) and then resuspended in NB5 (NB4 with 0.42 M NaCl) and slowly rotated at 4°C for 1 h. The supernatant was retained after centrifugation at 12,000 g at 4°C for 1 h. Double-stranded oligonucleotides were 5’-end labeled with [γ-³²P]dATP with T4 kinase for use as probes in EMSA. Reaction mixtures including nuclear extracts or recombinant p53 and the binding buffer [5 mM HEPES, pH 7.9, 5% glycerol, 0.2 mM DTT, 5 mM spermidine, 1.5 μg poly(dI-dC)] were preincubated on ice for 10 min. Specific competitor oligonucleotides, in 100-fold molar excess of radiolabeled probe, were added to the mixtures and incubated for 15 min on ice. The radiolabeled probe was then added to the reaction and incubated for another 20 min at room temperature. The binding reaction mixtures were

Fig. 1. Redistribution of proliferating cell nuclear antigen (PCNA) expression in kidneys of p53-deficient mice. A–C: normal PCNA expression in the nephrogenic zone in p53 +/+ newborn mice. D–F: PCNA is ectopically expressed in the tubules of the differentiated zone of newborn p53 −/− mice. Note the aberrant morphology of the renal tubular epithelium. Magnification: A, D, ×40; B, E, ×100; C, F, ×200.
resolved in a nondenaturing 5% polyacrylamide gel containing 22.5 mM Tris-borate at 200 V for 1.5 h. Subsequently, the gel was placed on 3MM Whatman paper, dried in a vacuum, and exposed to X-ray film. The oligonucleotide sequences used in the gel shift assays were as follows (double stranded): consensus p53-binding sequence, 5’-TCCAACACCGGCGGTGACGA-3’ (10); p53-binding sequence in the human PCNA (hPCNA) promoter, 5’-GAACAACTCCGGGGCATATGT-3’ (positions −217 to −236) (26); and p53-binding motif in the rat PCNA (rPCNA) promoter, 5’-TCCAACACCGGCGGTGACGA-3’ (positions −189 to −208) (30).

Plasmids. The hPCNA-chloramphenicol acetyl transferase (CAT) reporter constructs containing the hPCNA promoter fragments −1,265 to +62, −249 to +62, or −213 to +62 relative to the transcription initiation site (+1) fused to the CAT reporter gene in pBACAT were described previously (28). Deletion of the p53 site (nucleotide positions −217 to −236) was performed by the QuickChange site-directed mutagenesis system (Stratagene, La Jolla, CA) following the manufacturer's recommendations. Plasmid pCMV-p53 expresses a wild-type p53 protein from the cytomegalovirus promoter enhancer in the pCGL expression vector. All constructs were sequenced to verify the sequence by automated DNA sequencing (model 373A, Applied Biosystems).

Cell culture and transfections. p53-deficient human cervical carcinoma (HeLa) cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml; Life Technologies) at 37°C in a humidified incubator with 5% CO2. Cells were plated in duplicates in six-well plates at 4 × 10⁶ cells/well in the culture medium 1 day before transfection using a reporter lysis reagent (Promega). CAT activity was normalized to protein concentration.

RESULTS

Redistribution of PCNA expression in kidneys of p53-deficient mice. To determine whether p53 contributes to the spatial regulation of PCNA in the developing kidney, we examined its distribution in p53-deficient mice (Fig. 1, A–C). In p53 +/+ mice, primitive epithelial nephrons and undifferentiated blastema in the nephrogenic zone express high levels of PCNA. PCNA expression declines markedly during the process of terminal differentiation. Compared with this normal pattern of gene expression, p53 −/− mice exhibit ectopic PCNA expression extending deep into the differentiation zone (Fig. 1, D–F). This abnormal pattern of PCNA expression is accompanied by aberrant tubular growth and morphology. Hydronephrosis was observed in ∼50% of affected mice (Fig. 1) and multicystic renal dysplasia in 25% (43). Western blot analysis indicated no significant differences in total kidney PCNA content, factored for β-actin, between p53 −/− and +/+ pups (Fig. 2).

![Figure 2](https://www.ajprenal.org/)

**Fig. 2.** Total kidney PCNA content is similar in p53 +/+ and −/− pups. A: Western blot analysis of PCNA (29 kDa) and β-actin (42 kDa) of whole kidney protein extracts. B: bar graph analysis of PCNA β-actin ratio (densitometric units) for p53 +/+ and −/−.}

![Figure 3](https://www.ajprenal.org/)

**Fig. 3.** Sequence-specific binding of p53 to the human PCNA (hPCNA) and rat PCNA (rPCNA) promoters. A: nucleotide sequence alignment of the putative p53-binding motifs in the hPCNA and rPCNA genes with the consensus p53-binding sequence. Underlined bases denote sequence variation from the consensus p53-binding motif. B: EMSA using 32P-labeled hPCNA p53 oligoduplex (5’-TG-TATACGGGCCTGAACAAG-3’) and recombinant p53. Lane 1: no competitor. Lane 2: 100-fold molar excess of unlabeled consensus p53-binding site. Lane 3: 100-fold molar excess of unlabeled rPCNA p53-binding site. C: EMSA using 32P-labeled rPCNA p53 oligoduplex (5’-TCCAACACCGGCGGTGACGA-3’). Lane 1: no competitor. Lane 2: 100-fold molar excess of unlabeled consensus p53-binding sequence. Lane 3: 100-fold molar excess of unlabeled hPCNA p53-binding site.
Binding of recombinant p53 to hPCNA and rPCNA promoters. The p53-response element consists of two copies of the inverted repeat sequence (RRRC/TAGYYY). A consensus p53-binding site is present in the hPCNA promoter at nucleotide positions −217 to −236 (relative to the transcription start site) (26). A transcription factor search also revealed the presence of a putative p53-binding site in the rPCNA promoter at nucleotide positions −189 to −208 (Fig. 3A). EMSAs demonstrated binding of recombinant p53 to its cognate site in the hPCNA promoter (Fig. 3B, lane 1). The DNA-protein complex is eliminated by 100-fold molar excess of unlabeled consensus p53 oligoduplex (Fig. 3B, lane 2) and is attenuated by 100-fold excess of unlabeled rPCNA p53 sequence (Fig. 3B, lane 3). p53 also binds to the putative site in the rPCNA promoter (Fig. 3C, lane 1), and this binding is eliminated by the addition of 100-fold molar excess of unlabeled consensus p53 (Fig. 3C, lane 2) or hPCNA p53 oligoduplexes (Fig. 3C, lane 3). These EMSA cross-competition experiments indicate that p53 binds in a sequence-specific manner to hPCNA and rPCNA in vitro. Furthermore, p53 exhibits a higher DNA binding activity to hPCNA than rPCNA promoters, which is consistent with the higher sequence homology of the hPCNA p53-binding site to the consensus p53 sequence (Fig. 3A).

Developmentally regulated binding of kidney nuclear proteins to the PCNA p53 response element. To examine the DNA binding activity of endogenous p53, we tested the binding of nuclear extracts derived from newborn and adult rat kidneys to hPCNA or rPCNA p53-response elements by EMSA. As shown in Fig. 4A, lane 1, a ^32P-labeled oligoduplex containing the p53-binding site in hPCNA promoter binds strongly to newborn kidney nuclear extracts, producing two major and one minor DNA-protein complex. These band shifts were eliminated by 100-fold molar excess of unlabeled consensus p53-binding sequence (Fig. 4A, lane 2) and partially competed by 100-fold molar excess of unlabeled rPCNA p53 site (Fig. 4A, lane 3). An irrelevant oligoduplex (Jun/AP-1) did not compete for binding (data not shown). In comparison, the binding of
32P-labeled hPCNA p53-binding site to adult kidney nuclear extracts was much weaker than that of the newborn (Fig. 4A, lane 4). A qualitatively similar developmental pattern was noted in the binding of rPCNA p53 DNA motif to newborn and adult kidney nuclear extracts (Fig. 4B). Integrity of the nuclear proteins was documented by Coomassie blue staining of SDS-PAGE gels. These results demonstrate that the interaction of kidney p53 with its cognate site in the PCNA promoter is developmentally regulated. Immunolocalization studies revealed reciprocal spatial expression of p53 and PCNA in the developing kidney (Fig. 4, C and D). PCNA expression in the nephrogenic zone is rapidly downregulated during terminal differentiation of the renal epithelium. This is accompanied by reciprocal upregulation of p53 in the differentiation zone.

High levels of p53 repress the PCNA promoter. Previous studies have examined p53-mediated regulation of the PCNA promoter in the context of DNA damage. Although most studies found that p53 represses PCNA transcription (6, 18, 46), others found that low levels of p53 activate the hPCNA promoter (26, 41), whereas high levels repress the promoter (41). Because p53 levels increase markedly during terminal differentiation of the renal epithelium, we reexamined the responses of the hPCNA promoter to increasing levels of p53. p53-Deficient HeLa cells were used to avoid the confounding effects of endogenous p53. Cells were cotransfected with pCMV-p53 and PCNA-CAT constructs. Figure 5 shows that baseline PCNA promoter activity was higher in the −1,265 than the −249 or −213 constructs. In the −1,265 and −249 constructs, low levels of p53 (10 ng) stimulated PCNA transcription, whereas higher levels (50 ng or higher) decreased PCNA transcription. High levels of p53 (100 ng) inhibited PCNA transcription to ~60% of baseline in each of these constructs. In contrast, in the mutated −249 and −213 constructs, both of which lack the p53-binding site, PCNA transcription was not stimulated by low levels of p53; in fact, it was decreased to ~20% of baseline in the −213 construct by only 10 ng p53. Thus high levels of p53 repressed all the promoter constructs regardless of whether they contained the p53-binding site. Importantly, p53-mediated repression of PCNA transcription is selective because increasing levels of p53 caused progressive activation of the epidermal growth factor promoter (41) or the bradykinin B2 receptor promoter (Ref. 38 and Marks J and El-Dahr S, unpublished data).

**DISCUSSION**

Aberrant terminal differentiation is a hallmark feature of congenital renal dysplasia, polycystic kidney disease, and renal cell cancer. Accordingly, identification of the genetic programs that control the biochemical and morphological differentiation of the renal epithelium is of great clinical significance. The present study demonstrates that the spatial transition from nephrogenesis to differentiation correlates with induction of p53 and reciprocal downregulation of PCNA. Progeny of p53 mutant mice lacked the spatially restricted pattern of PCNA expression and exhibited defects in terminal renal epithelial differentiation. These findings, combined with the functional evidence that p53 represses the PCNA promoter, provide compelling evidence that p53-mediated repression of PCNA is a physiological process that occurs in vivo during renal development.

The complementary distribution patterns of p53 and PCNA are reminiscent of the localization of transcription factors WT-1 and Pax-2 during nephrogenesis. Ryan et al. (35) demonstrated that Pax-2 expression is elevated in the nephrogenic zone but decreases as cells begin to differentiate and express WT-1. The increasing levels of WT-1 in differentiating tubules and glomeruli repress Pax-2 transcription. Transgenic overexpression of Pax-2 in mice impairs renal development (33, 45), indicating that proper spatial regulation of gene expression is required for normal differentiation of the renal epithelium.

The tumor suppressor protein p53 plays a key role in the cellular response to DNA damage (1, 3). Activation of p53 is accomplished by posttranslational modifications such as phosphorylation and acetylation, leading to stabilization and enhanced transactivation. p53 is a sequence-specific transcription factor, and its transcriptional regulatory functions are essential for its
biological roles in tumor surveillance through apoptosis, cell cycle arrest, and DNA repair (21, 31, 44). In addition to its classical role in tumor surveillance, p53 has important roles in development (reviewed in Refs. 2 and 7). During organ differentiation, p53 expression shifts from a widespread to a more restricted distribution pattern (7, 39). Moreover, introduction of p53 into undifferentiated cells results in progression from an undifferentiated to a more differentiated state, whereas the normal differentiation pathways can be blocked by addition of dominant-negative forms of p53 (43). These effects have been demonstrated in a variety of cell lineages, such as skeletal myoblasts, osteoblasts, keratinocytes, neurons, thyroid, and renal epithelial cells. In addition, the p53 protein is stabilized and its DNA binding and transcriptional activities are induced during terminal differentiation (15). p53 is essential for normal development. For example, introduction of mutant p53 in *Xenopus laevis* embryos is associated with severe defects in terminal tissue differentiation (16). Studies from our laboratory have demonstrated that p53 binds to and activates the promoters of renal function genes, such as vasoactive hormone receptors and water channels (37). Interestingly, excess or unopposed p53 activity is detrimental to embryonic development because transgenic p53 overexpression in mice interferes with ureteric bud morphogenesis, leading to renal hypoplasia and cyst formation (13). Thus p53 levels and activity must be tightly controlled to complete normal renal development.

Transcriptional repression is a key mechanism for the spatial specification of gene expression and cell fate determination during development (14, 40). The present study demonstrates that progressive rises in cellular p53 levels exert a powerful dose-dependent inhibitory effect on the hPCNA promoter. The rPCNA and mouse PCNA promoters are similarly repressed by p53 (6, 46). Our finding of ectopic expression of PCNA in p53-deficient mice is consistent with the available functional data and strongly suggests that high levels of p53 repress PCNA gene expression in vivo. Furthermore, lack of significant differences in total kidney PCNA contents between p53−/− and +/+ mice suggests that the spatial redistribution of PCNA in the renal cortex of p53 mutant mice has occurred in the absence of a change in the total number of proliferating cells. To help resolve this issue, we attempted to estimate the relative quantities of p53 and PCNA in the nephrogenic vs. differentiation zones after separating the two zones by microdissection. Unfortunately, these attempts failed because of significant cross-contamination. In future studies, we plan to utilize laser capture microdissection as well as transgenic approaches to further test this hypothesis. Dual label confocal microscopy might also be useful to better define cells on the edge of each zone with expression of both proteins. It is anticipated that this occurs only transiently if p53 indeed causes the suppression of PCNA.

p53-Mediated transcriptional repression involves both sequence-dependent and -independent mechanisms (e.g., squelching of basal transcription factors) (8, 11, 20, 29). Our results indicate that p53-mediated repression is not dependent on DNA binding. Whether the mechanism represents interference with basal transcription or recruitment of histone deacetylases to the proximal promoter remains to be established. It is also interesting that the promoter constructs containing the p53-binding site (−1,265/+62 and −249/+62) were more resistant to p53-mediated repression, confirming previous reports that indicate a role for this binding site in the regulation of PCNA promoter function at relatively lower levels of p53 (26, 41).

In summary, this study suggests that p53 represses PCNA gene expression during normal nephrogenesis. We propose that p53 plays an important role in the spatial restriction of PCNA during terminal renal epithelial cell differentiation.

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