Spatiotemporal Switch from ΔNp73 to TAp73 Isoforms during Nephrogenesis

IMPACT ON DIFFERENTIATION GENE EXPRESSION*

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*p is a member of the p53 gene family, which also includes p53 and p63. These proteins share sequence similarity and target genes but also have divergent roles in cancer and development. Unlike p53, transcription of the p73 gene yields multiple full-length (transactivation (TA) domain) and amino terminus-truncated (ΔN) isoforms. ΔNp73 acts in a dominant negative fashion to inhibit the actions of TAp73 and p53 on their target genes, promoting cell survival and proliferation and suppressing apoptosis. The balance between TAp73 and its negative regulator, ΔNp73, may therefore represent an important determinant of developmental cell fate. There is little if anything known regarding the developmental regulation of the p73 gene. In this study, we showed that TAp73 and ΔNp73 exhibit reciprocal spatiotemporal expression and functions during nephrogenesis. TAp73 was predominantly expressed in the differentiation domain of the renal cortex in an overlapping manner with the vasopressin-sensitive water channel aquaporin-2 (AQP-2). Chromatin immunoprecipitation assays demonstrated that the endogenous AQP-2 promoter was occupied by TAp73 in a developmentally regulated manner. Furthermore TAp73 stimulated AQP-2 promoter-driven reporter expression. TAp73 also activated the bradykinin B2 receptor (B2R) promoter, a developmentally regulated gene involved in regulation of sodium excretion. The transcriptional effects of TAp73 on AQP-2 and B2R were independent of p53. In marked contrast to TAp73, ΔNp73 isoforms were induced early in development and were preferentially expressed in proliferating nephron precursors. Moreover ΔNp73 was a potent repressor of B2R gene transcription. We conclude that the p73 gene is developmentally regulated during kidney organogenesis. The spatiotemporal switch from ΔNp73 to TAp73 may play an important role in the terminal differentiation program of the developing nephron.

Terminal differentiation is a crucial developmental process in which cell cycle arrest is temporally and spatially coordinated with expression of specialized cellular functions. In the kidney, aberrant terminal epithelial cell differentiation leads to renal dysplasia, polycystic kidney disease, and renal cell carcinoma (1–3). Despite a wealth of knowledge regarding the early steps of nephron induction and patterning, little is known regarding the intracellular signaling pathways and downstream transcription factors that regulate renal epithelial cell terminal differentiation. p53 is a sequence-specific DNA-binding protein that maintains genomic integrity via its ability to provoke cell cycle arrest or apoptosis genes, depending on the type and magnitude of the stress and the cell type (4, 5). Previous studies have demonstrated a developmental role for p53 in several organisms including Xenopus (6–10), zebrafish (11, 12), and mouse (13–19). In the mouse, p53 is important for neurogenesis (16, 17, 20–23) but is dispensable for muscle differentiation (19). We recently reported that terminal nephron differentiation is accompanied by p53 phosphorylation and acetylation, protein stabilization, and enhanced DNA binding activity. Moreover we identified several terminal differentiation genes, including the bradykinin B2 receptor (B2R), aquaporin-2 (AQP-2), Na-K-ATPase α1, and angiotensin type 1 receptor (Agtr1), as direct p53 target genes (17, 24). In keeping with these findings, we found that p53-deficient newborn mice exhibit persistent renal cell proliferation, impaired cell cycle control, and disorganized spatial expression of nephron differentiation markers (17, 24). Interestingly expression of terminal differentiation genes is attenuated but not abrogated in p53-null kidneys (17), raising the possibility of compensatory regulation by other developmentally regulated transcription factors with overlapping functions.

Recent studies have identified two homologues of p53, p63 and p73 (for reviews, see Refs. 25 and 26). Both genes encode transcription factors with significant sequence homology to p53. Highest homology lies in the DNA-binding domain allowing p63 and p73 to bind and activate transcription of p53 target genes and to induce apoptosis and/or growth arrest. However, unlike the p53 gene, p73 and p63 are not induced by most DNA-damaging agents and are rarely mutated in tumors (27–29). On the other hand, there is strong genetic evidence supporting an important role for p63 and p73 in embryonic development. p63-null mice exhibit severe defects in limb,
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craniofacial, and epithelial development (30), whereas p73-null mice have central nervous system defects and phenomeral abnormalities (31).

The p73 gene comprises 65 kb of genomic DNA and consists of 14 exons of which exons 10–14 are differentially spliced, giving rise to proteins that differ at the carboxyl terminus. In addition, alternate promoter usage results in isoforms that either contain the amino terminus transactivation domain (P1 5′ promoter; transactivation (TA) domain isoforms) or lack this domain (P2 intronic promoter; amino terminus-truncated (ΔN) isoforms) (27, 28, 32, 33). The ΔN isoforms act in a dominant negative fashion to inhibit TaP73- and p53-mediated transactivation. Importantly, although the TaP73 isoforms induce cell cycle arrest or apoptosis, the ΔN isoforms are prosurvival and antiapoptotic (34–37). Whether the p53 family members share physiological target genes in specific tissues during development remains to be determined. The developing kidney is an excellent model system to study terminal differentiation because the renal cortex contains nephrons in various stages of development, cell cycle regulation, and functional differentiation. Accordingly in the present study we examined the ontogeny the p73 gene in the kidney and compared the transcriptional activity of p73 isoforms on the promoters of renal function genes.

MATERIALS AND METHODS

Reverse Transcription-Polymerase Chain Reaction of p73 Isoforms—
Total kidney RNA was extracted from newborn (4-day-old) and adult (90-day-old) rats (kidney tissue weight = 150–200 mg) using TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. Integrity of the RNA was verified by gel electrophoresis on a 1% agarose gel and observing intact 28 and 18 S ribosomal RNA. Three micrograms of RNA were treated with DNase I (1 unit/μl) for 15 min followed by enzymatic inactivation by adding 1 μl of 25 mM EDTA solution. The RNA samples were subsequently subjected to reverse transcription (RT) and first strand cDNA synthesis using SuperScript II (200 units) used in the binding reactions.

Cell Culture, Transient Transfection, and Reporter Assays—The rat B2R (pBK-Z/4′-5′-CAT) and mouse AQP-2 (p120B5) promoters were subcloned into the pCMV reporter vector (Vector Laboratories, CA) as described previously (38). The following primary antibodies were used: 1) Rabbit polyclonal p73 antibody (H79, diluted 1:5000) was from Santa Cruz Biotechnology. Its epitope maps within amino acids 1–295, representing full-length p53 of human origin. This antibody does not cross-react with p73 or p63. 2) Mouse monoclonal αNp73 antibody (IMG 313, diluted 1:2000) was from Imgenex. Mouse and human se-

ances are more than 95% identical at these amino acid residues. This antibody does not cross-react with TaP73. 3) Rabbit polyclonal p53 antibody (FL-393, diluted 1:100) was from Santa Cruz Biotechnology. Its epitope maps within amino acids 1–295, representing full-length p53 of human origin. This antibody does not cross-react with p73 or p63. 4) Monoclonal bradykinin B2R antibody (clone 20, diluted 1:5000) was from BD Biosciences-PharMingen. Its epitope maps to the carboxyl terminus (amino acids 350–364 of B2R). 5) Rabbit polyclonal AQP-2 antibody (diluted 1:300) was a gift from M. Knepper. In negative controls, the primary antibody was omitted or replaced by non-immune serum.

For the detection of AQP-3 and TaP73 proteins on the same tissue section, we adapted a double immunofluorescence technique that allows the use of two polyclonal primary antibodies. Initially the sections were subjected to antigen retrieval (microwave heating in 10 mM citrate buffer for 20 min). After incubation with blocking serum (20% normal goat serum in PBS) for 90 min, the sections were incubated with the first primary antibody overnight (AQP-2, diluted 1:300) followed by extensive washings in PBS with 0.1% Tween (PBS-T). The sections were subsequently incubated with the secondary antibody (Alexa Fluor 594 goat anti-rabbit Fab, diluted 1:200; Molecular Probes) for 2 h and subjected to extensive washings thereafter. Before addition of the secondary primary antibody (TaP73, H-79, diluted 1:50), the sections were incubated in blocking serum for 90 min. This was followed by incubation with a secondary antibody (Alexa Fluor 488 goat anti-rabbit Fab, diluted 1:2000) for 90 min. Note that the concentrations of the second secondary antibody are 10-fold lower than the first secondary antibody to reduce background and nonspecific staining. Following washes in PBS-T for 30 min, the sections were covered with fluorescent mounting solution and coverslip

Western Blot Analysis—Western blot analysis was performed on newborn extracts from newborn and adult rat kidneys or cells transfected with p73 expression vectors as described previously (39). Band intensities was normalized to β-actin.

Nuclear Extracts and Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay was performed as described previously (40). The oligonucleotide used in the gel shift assays consisted of the high affinity P1-p53-binding site in the rat bradykinin B2R promoter: 5′-CTGGGAGGA-TGGCAGGACGTTG-3′ (the p53-binding site is underlined) (40). Purified full-length p53 (Santa Cruz Biotechnology, 500 ng) or bacterially produced p73a (500 ng) were used in the binding reactions.

Chromatin Immunoprecipitation (ChiP) Assays—Tissue ChiP was performed using reagents and protocols from the Upstate Biotechnology ChiP kit with modifications. Freshly isolated or snap frozen kidneys were minced into fine pieces and immediately immersed in 1% formaldehyde solution in PBS for cross-linking for 15 min at room temperature with rotation. The reaction was quenched by addition of 0.125 M glycine. Tissue was rinsed two times in ice-cold 1× PBS and homogenized with a Dounce A homogenizer (~20 strokes), and the homogenate was lysed in SDS lysis buffer. DNA was sheared by sonication and diluted 10-fold in ChIP dilution buffer. Immunoprecipitation was performed with antibodies to p53 (FL-393, 2 μg), TaP73 (H79 or C-20, 2 μg), ΔNp73 (Imgenex, IMG 313, 5 μg), or control normal immunoglobulin (IgG) antibodies overnight at 4 °C. DNA-protein-antibody complexes were captured on Protein A/G-conjugated agarose beads. After washing and elution of the complexes from the beads, DNA-protein cross-links were reversed at 65 °C overnight. Immunoprecipitated DNA was ethanol-precipitated after proteins were removed by phenol-chloroform-isomyl alcohol extraction following proteinase K treatment and used for PCR. Sequences of the primers used for PCR of rat/mouse B2R gene are as follows: forward primer, 5′-GACCTGACTGACTATTGAG-3′; reverse primer, 5′-GGGGGATCCAGGTCGCTCC-3′. The amplicon size is 555 bp. Sequences of primers used to amplify the AQP-2 promoter were as follows: forward primer, 5′-CATGGTTCATCTCATCG-3′; reverse primer, 5′-AGGGGATCCAGGTCGCTCC-3′. The amplon size is 311 bp. Sequences of primers used to amplify the AQP-2 promoter were as follows: forward primer, 5′-TGGTTGCTTGGTTGCGGTCGTCC-3′. The amplicon size is 311 bp. Sequences of primers used to amplify the AQP-2 promoter were as follows: forward primer, 5′-GACCTGACTGACTATTGAG-3′; reverse primer, 5′-GGGGGATCCAGGTCGCTCC-3′; The amplicon size is 555 bp.

Immunohistochemistry—Rat kidneys were harvested from embryonic day 15 (E15), newborn (postnatal day 5), and adult male Sprague-Dawley rats (Charles Rivers, Wilmington, MA). Following fixation in 10% buffered formalin, kidneys were dehydrated in serial alcohol solutions and embedded in paraffin blocks. Five-micrometer serial sections and random hexamers (100 ng) in a 20-μl reaction volume. Control samples were treated in an identical manner except that SuperScript II enzymatic inactivation by adding 1 μl of 25 mM EDTA solution. The reaction was quenched by addition of 1 μl of 25 mM EDTA solution. The reaction was quenched by addition of 1 μl of 25 mM EDTA solution. The reaction was quenched by addition of 1 μl of 25 mM EDTA solution.
1 day prior to transfection. Cells were transfected with 1.0 g of DNA/well promoter-reporter vectors along with pCMV-p53 (wild type) or pCMV-p73 isoform expression plasmids (0–250 ng). A control β-galactosidase-encoding vector, pSVZ (Promega, 0.4 g of DNA/well), was co-transfected to correct for transfection efficiency. Additional controls included transfections with pCMV-CAT (pCAT3 Basic) or pCMV-empty (pCDNA3.1) vectors. Transfection was performed using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s recommendations. Four hours after transfection, fresh medium was replaced, and cell extracts were prepared 24–48 h later using a reporter lysis reagent (Promega). Aliquots of cell lysate were analyzed for CAT activity after normalization for protein content or β-galactosidase activity as described previously (40).

RESULTS

Spatiotemporal Expression of TAp73 in the Developing Kidney—Histologically the developing kidney can be divided into two zones: a nephrogenic zone (NZ), which contains proliferating nephron precursors, and a differentiation zone (DZ), which contains nephrons entering terminal differentiation to express renal function genes. In rodents, nephrogenesis begins on E11.5–E12.5 and continues until the end of the 1st week after birth. We examined the spatial distribution of TAp73 in the rat kidney on E15, postnatal day 5 (PN5) and PN23 by immunohistochemistry utilizing a TAp73-specific antibody (H79), which does not recognize ΔNp73. However, like most other TAp73 antibodies, this antibody cannot distinguish among the various TAp73 isoforms. Fig. 1A depicts a section from E15 embryo showing strong TAp73 immunoreactivity in the adrenal cortex. In the kidney, TAp73 protein was expressed at higher levels in the inner, more differentiated cortex than the outer NZ (Fig. 1B). Capillary loop stage glomeruli and nephron precursors (vesicles and comma- and S-shaped bodies) expressed low levels of TAp73 (Fig. 1B). On PN5, TAp73 was expressed exclusively in the maturing tubules located in the DZ, and the transition from the NZ to DZ was accompanied by a surge in TAp73 expression and enhanced nuclear staining (Fig. 1C). By the time of weaning (PN23), distinct TAp73 expression was most abundant in the cortical distal nephron (connecting tubules and collecting ducts) in nuclear or perinuclear distribution patterns (Fig. 1D).

We next asked whether the p73 gene is expressed in the kidney and if so whether p73 isoforms are differentially expressed. As shown in Fig. 2A, the p73 gene gives rise to several mRNA species, which are translated into several different proteins. The different mRNAs arise by both alternate splicing and by the use of alternative promoters. The P1 and P2 promoters control the transcription of TAp73 and ΔNp73; the former contains a TA domain, which is encoded by exons 2 and 3, and the latter has a shorter amino terminus without the TA domain. Splicing takes place in the 3’-end of the p73 mRNA creating proteins that have different carboxyl termini. At least seven different p73 proteins (α to γ) are generated in normal cells. The carboxyl-terminal region also contains a sterile α motif, a protein-protein interaction domain (26, 29, 32, 41–43).

To amplify all p73 isoforms, we adapted an RT-PCR strategy in which the PCR primers flank the last four exons of p73 (see primers positions in Fig. 2A). As a negative control, we omitted the RT step from the RT-PCR, thus allowing us to detect any genomic DNA contamination. As shown in Fig. 2B, the adult
kidney expresses several p73 mRNA isoforms in the following order of abundance: \( \beta > \gamma > \delta > \zeta > \alpha > \epsilon \). Some of these p73 mRNA isoforms are developmentally regulated and increase with age. No differences were seen in glyceraldehyde-6-phosphate dehydrogenase mRNA expression between newborn and adult kidneys. Fig. 2C depicts a quantitative assessment of the RT-PCR results, which show a modest increase in TAp73 \( \alpha \) and \( \beta \) mRNA levels during postnatal maturation as compared with a significant up-regulation in the \( \gamma \) and \( \delta \) isoforms. In addition, whereas the adult kidney expressed abundant levels of \( \alpha \), \( \gamma \), \( \zeta \), and \( \delta \) mRNAs, the newborn kidney expressed predominantly the \( \alpha \) isoform.

We also determined the relative abundance of TAp73 proteins in nuclear extracts obtained from newborn and adult kidneys by Western blot analysis. Fig. 2D, lanes A, shows that the kidney expresses three major TAp73 proteins, which based on their molecular weights correspond to the TAp73 \( \alpha \), \( \gamma \), and \( \delta \) isoforms. The abundance of these TAp73 isoforms increases dramatically during postnatal maturation.

**TAp73 Binds the Endogenous AQP-2 Gene and Activates Its Transcription**—AQP-2 is water channel that is expressed in principal cells of the collecting duct. The AQP-2 gene is expressed relatively late in fetal development marking the functional differentiation of the collecting duct and is up-regulated postnata tally (44). We previously demonstrated that the mouse and rat AQP-2 promoters harbor functional p53-responsive elements (17). To determine whether AQP-2 is a physiological target of p73, we first determined whether TAp73-expressing cells co-express AQP-2 (immunohistochemistry). Fig. 3, A–C, represents a kidney tissue section incubated with antibodies against TAp73 and AQP-2 and developed using fluorescently labeled secondary antibodies (TAp73 in green (A) and AQP-2 in red (B)). As shown in Fig. 3C, TAp73 and AQP-2 are co-expressed in collecting duct cells (arrowheads). In addition, lower levels of TAp73 immunoreactivity were observed in some proximal tubules (AQP2-negative) (double arrows). Thus, TAp73 and AQP-2 have overlapping distribution in the collecting ducts.

We next determined whether endogenous TAp73 is bound to the kidney AQP-2 promoter in vivo (ChIP assay). The results revealed that TAp73 and p53 were bound to the AQP-2 promoter in the kidney (Fig. 3D). Interestingly, although the occupancy of AQP-2 promoter by p53 and TAp73 was equivalent in the newborn kidney, p53 binding to AQP-2 gene declined more dramatically than TAp73 postnatally (Fig. 3D). These results were confirmed using a different TAp73 antibody directed against the carboxyl terminus of p73, demonstrating the reproducibility of the ChIP assay (Fig. 3E). Control samples incubated without primary antibody or nonspecific IgG showed no specific bands (data not shown).
To determine the functional significance of the interaction between TAp73 and AQP-2 promoter, we performed transient transfection assays to test whether the AQP-2 promoter is activated by TAp73. Collecting duct cells (IMCD-3) were co-transfected with AQP-2/CAT promoter-reporter construct and expression vectors for either p53 or various TAp73 isoforms (Fig. 3F). The amounts of p53 and p73 expression vectors were chosen based on pilot experiments showing that p53 is more potent in AQP-2 promoter transactivation than p73. The cell lysates were harvested 24 h after transfection and assayed for CAT activity. At the maximal dose used (250 ng of expression plasmid), TAp73 isoforms elicited variable increases in AQP-2 promoter activity by up to 40-fold. In comparison, p53 strongly and dose dependently activated the AQP-2 promoter (up to 15-fold). TAp73 also activated the AQP-2 promoter, although the activation by TAp73 isoforms was more modest than that induced by p53 (range, 2–3.5-fold) (Fig. 3F).

In these assays, the developmentally regulated TAp73 (A, green) and AQP-2 (B, red) are co-localized (orange, merged in C) in renal collecting ducts revealed by double immunofluorescence (arrowheads). Also note that some tubules express TAp73 (green, double arrows) but not AQP-2. D, ChIP assays performed on kidney tissue of newborn and adult rats (see “Materials and Methods”). Immunoprecipitation was performed with p53 antibody (FL-393, 2 μg) or TAp73 antibody directed against the amino terminus (H79, 2 μg) (D) or carboxyl terminus of TAp73a (C-20, 2 μg) (E) overnight at 4 °C. PCR was performed using primers flanking the p53-binding site in the rat AQP-2 promoter (17). Input PCR was performed on 1/10 amount of input DNA before immunoprecipitation. F, IMCD-3 cells were co-transfected with AQP-2/CAT promoter-reporter construct (1.2 μg) and the indicated amounts of p53 or TAp73 expression vectors. pSV-lacZ (0.4 μg) plasmid was transfected for normalization of transfection efficiency. Cell lysates were harvested 24 h later and assayed for CAT and β-galactosidase activity. The 250-ng dose of p53 plasmid was omitted because non-physiological levels of p53 tend to repress gene transcription nonspecifically presumably by squelching of basal transcription factors and/or cofactors.

To determine the functional significance of the interaction between TAp73 and AQP-2 promoter, we performed transient transfection assays to test whether the AQP-2 promoter is activated by TAp73. Collecting duct cells (IMCD-3) were co-transfected with the 10.0-kb mAQP-2/CAT promoter-reporter construct and expression vectors for either p53 or various TAp73 isoforms (Fig. 3F). The amounts of p53 and p73 expression vectors were chosen based on pilot experiments showing that p53 is more potent in AQP-2 promoter transactivation than p73. The cell lysates were harvested 24 h after transfection and assayed for CAT activity. As reported previously (17), p53 strongly and dose dependently activated the AQP-2 promoter (up to 15-fold). TAp73 also activated the AQP-2 promoter, although the activation by TAp73 isoforms was more modest than that induced by p53 (range, 2–3.5-fold) (Fig. 3F). In these assays, the developmentally regulated TAp73γ isoform was most potent in activation of AQP-2 promoter. Promoter truncation indicated that the p53/p73-response element is located in the fragment −1.2 kb relative to the transcription start site (data not shown and Ref. 17). TAp73-mediated activation of the AQP-2 promoter was also observed in p53-deficient H1299 cells, indicating that activation of AQP2 promoter by TAp73 is p53-independent (data not shown).

Previously we reported the spatial co-expression of p53 and B2R in differentiating renal epithelial cells (17) and that the B2R promoter has a conserved and functional p53-binding site located at position −70 from the transcription start site (40, 45). It has been shown that some (but not all) p53 target genes are regulated by TAp73 via direct binding to the p53-binding site (46–50). Therefore, we examined whether TAp73 fulfills functions similar to p53 in the regulation of B2R gene expression. Electrophoretic mobility shift assays using a radiolabeled oligoduplex corresponding to the p53-binding site in the B2R promoter (40) and equal amounts of recombinant p53 or TAp73a demonstrated that TAp73 binds the B2R promoter but at lower affinity than the binding of p53 to the same site (Fig. 4A).
strate that the renal function genes AQP-2 and B2R are p53 and p73 target genes.

Spatiotemporal Expression of ΔNp73 in the Kidney—ΔNp73 was detected utilizing a monoclonal antibody raised against amino acid residues 2–13 (LYVGDPARHLAT) of human ΔNp73 protein (the sequence is >95% identical in mouse and human). Interestingly immunoblotting with the ΔNp73 antibody showed a reciprocal developmental pattern relative to that seen with the TAp73 antibody. Thus, the ΔNp73 isoforms were enriched in the developing kidney and declined during postnatal life (Fig. 5A). Immunolocalization of ΔNp73 in E15 embryos confirmed its abundant expression in the epidermis and neuroepithelium (51, 52) (Fig. 5, B and C). In the E15–E17 kidney, ΔNp73 expression was abundant and was observed in terminal ureteric bud branches, metanephrogenic mesenchyme, and metanephrogenic mesenchyme-derived epithelial nephrons (vesicles and S-shaped bodies) (Fig. 5, D and E). Intriguingly ΔNp73 was associated with the basolateral membranes of immature epithelial cells, consistent with the interaction of the carboxyl terminus sterile motif of p73 with plasma membrane lipids (53). The functional significance of this finding is not clear. The early mesenchymal condensate, the stroma, and the glomerular podocytes did not express ΔNp73 (Fig. 5, D and E).

ΔNp73 Represses Transcription from the B2R Promoter—On PNS, ΔNp73 was highly expressed in nuclei of nephron progenitors in the NZ in an overlapping distribution with the transcription factor Pax-2 (Fig. 6, A and B). Accordingly ΔNp73 and TAp73 exhibited non-overlapping cellular distribution in the developing kidney in proliferating and differentiating epithelial cells, respectively. Because B2R was predominantly expressed in the DZ (Fig. 6C) in a reciprocal manner to ΔNp73, we next tested the hypothesis that B2R gene transcription is negatively regulated by ΔNp73. The ΔNp73 protein lacks the amino terminus activation domain but retains the DNA-binding domain and is thus capable of competitive binding to p53-response elements and counteracting p53-mediated transactivation (37). Recent evidence also suggests that ΔNp73 possesses intrinsic ability to regulate gene transcription in a p53-independent manner (54, 55). We tested the effect of ΔNp73 on the activity of the proximal B2R promoter (nucleo-
tides -94 to +55, relative to the transcription start site). This promoter fragment contains the necessary cis-elements, including a functional p53-binding site (40), required for expression in IMCD-3 cells (56). IMCD-3 cells were co-transfected with the B2R promoter-reporter construct and increasing amounts of hemagglutinin-tagged ΔNp73α and -β expression plasmids or empty pCDNA3.1 (0, 100, and 500 ng). pSV-lacZ (0.3 μg) was co-transfected to assess transfection efficiency. In some samples, p53 expression vector (50 ng) was co-transfected. CAT activity in the cell lysates was measured 24 h after transfection. Expression of ΔNp73 in lysates was assessed by Western blotting using anti-hemagglutinin (HA) antibody. I.B., immunoblot.

**DISCUSSION**

The murine metanephric kidney represents an excellent model system to investigate the mechanisms governing terminal epithelial cell differentiation. Unlike humans in whom nephrogenesis ceases around the 34th week of gestation, in the rat and mouse, nephrogenesis continues postnatally, thus allowing easy access to various tissues postpartum without the need to interfere with pregnancy. Furthermore terminal differentiation in the kidney is marked histologically by abrupt cessation of proliferation and simultaneous expression of differentiation markers (i.e. renal function genes). As a result, it is possible to readily distinguish the outer nephrogenic zone and inner differentiation zone using routine histological and immunohistochemical stains (17, 24).

The molecular pathways of terminal nephron differentiation are largely unknown. We previously reported that p53 expression in the developing kidney coincides spatiotemporally with terminal differentiation and that p53 binds and transactivates the promoters of differentiation genes in the kidney (17). Morphological analysis indicated that although the early steps of nephron induction and mesenchymal-to-epithelial conversion are intact, p53-/- pups (bred on a uniform C57Bl6 background) exhibited signs of aberrant terminal differentiation manifested by persistent proliferation, ectopic expression of proliferation markers in differentiating tubular segments, and altered spatial expression of renal function genes such as AQP-2 and B2R (17, 24).
The present study demonstrated that TAp73 expression was highly induced in differentiating renal epithelial cells but was excluded from the nephrogenic zone. Furthermore TAp73 was capable of activating the promoters of two renal function genes, B2R and AQP-2, independently of p53. Importantly, as shown by the ChiP assays, TAp73 and p53 were recruited to the AQP-2 promoter in vivo, indicating that the regulation is physiologically relevant. The functional overlap between p53 and TAp73 shown here suggests that these two proteins may cooperate or compensate for each other during kidney development. Because TAp73 expression increased postnatally, it is conceivable that the ability of TAp73 to functionally compensate for p53 in the kidney is dependent on the stage of nephron maturation. It is interesting that whereas TAp73 was more potent in activation of the AQP-2 promoter, TAp73β was more effective in B2R activation. Because different intervariant combinations of TAp73 isoforms possess varying degrees of transcriptional activity, it is likely that the TAp73 isoforms have differential targets in the kidney. Future development of mutant mouse strains selectively lacking TAp73 or ΔNp73 isoforms will provide a valuable tool to address the developmental and isoform-specific functions of p73.

The present study provides evidence for differential mRNA splicing and promoter usage of the p73 gene during renal organogenesis. The differential mRNA splicing, which occurs in the carboxyl terminus of p73, was associated with postnatal up-regulation of four TA isoforms (α, γ, ζ, and δ). In contrast, the preferential utilization of the P2 promoter was negatively regulated during maturation resulting in higher ΔNp73 isoforms in developing than adult kidneys. Moreover TAp73 and ΔNp73 exhibited complementary distribution in the developing kidney, supporting the notion that they mediate differential roles during nephrogenesis. Recent evidence has implicated both p53 and TAp73 in thyroid hormone-induced neuronal cell differentiation in culture (22, 57). In addition, p73 may influence developmental cell fate decisions by activation of the Notch signaling pathway via up-regulation of the Notch receptor ligands Jagged 1 and 2 (58). Our finding that TAp73 can up-regulate the AQP-2 and B2R promoters in p53-deficient cells is consistent with other reports showing that p73 up-regulates differentiation genes independently from p53 as in the case of p57 (Kip2) (46). In other circumstances, p53 and TAp73 exert opposing effects; for example, p53 promotes β-catenin degradation and therefore may suppress Wnt signaling, whereas TAp73 activates T cell factor-mediated transcription (59).

Current evidence indicates that ΔNp73 tends to be expressed in immature cells and that it plays a role in promotion of cell survival and proliferation (34–36). Our findings support this notion because ΔNp73 was selectively expressed in proliferating precursors in the nephrogenic zone. ΔNp73 was not expressed in the differentiation zone of the developing kidney. Moreover overexpression of ΔNp73α and -β potently repressed the B2R promoter. These results suggest that expression of ΔNp73 in the nephrogenic zone may be required to maintain the undifferentiated state of the nephrogenic zone. As maturation proceeds, renal epithelial cells begin to accumulate TAp73 as ΔNp73 expression is turned down. Accordingly the spatial switch from ΔNp73 to TAp73 may be an important factor in cell fate determination during terminal nephron differentiation. Because TAp73-expressing cells in the developing kidney are healthy, we believe that TAp73 promotes nephron differentiation rather than apoptosis. During zebrafish development, TAp73 expression is restricted to the olfactory system, telenephalon, and pronephric kidney, and overexpression of TAp73 in developing zebrafish promotes differentiation rather than apoptosis (60).

What are the upstream signaling pathways governing the developmental expression of p53 and p73 genes during nephrogenesis? Our previous studies suggest that the accumulation of p53 during terminal nephron differentiation is mediated by amino terminus phosphorylation and protein stabilization (17, 45). Amino terminus phosphorylation of p53 stabilizes p53 by preventing the interaction of MDM-2 with p53 (61–64). MDM-2 is an E3 ubiquitin ligase that targets p53 to proteosomal degradation thus maintaining low p53 levels in normal cells. Recent studies have demonstrated that p53 stabilization by amino terminus p53 phosphorylation is not sufficient to induce apoptosis and that additional modifications (e.g. serine 46 phosphorylation and carboxyl terminus acetylation on lysine residues) may be required (65, 66). Similar to p53, MDM-2 interacts with the amino terminus of p73; however, unlike p53, MDM-2 does not target p73 to proteosomal degradation (67, 68). p73 is activated transcriptionally in response to oncogene activation (69). The co-expression of ΔNp73 and Pax-2 in proliferating renal epithelia is intriguing and raises the possibility that the P2 promoter may be a transcriptional target for Pax-2. It has been reported that TAp73α can stabilize and activate p53 independently from p73 transcriptional activity (70); this may partially account for the spatial co-expression of the two transcription factors during terminal nephron differentiation.

In summary, the present study demonstrated that the p73 gene is subject to considerable developmental control during kidney organogenesis. TAp73 is not only enriched in differentiating renal epithelial cells but also activates terminal differentiation gene transcription. In contrast, ΔNp73 is enriched in nephron progenitors and functionally inhibits differentiation gene expression. The emerging hypothesis from this work is that the balance between prodifferentiation (TAp73) and pro-survival/proliferation (ΔNp73) isoforms is an important determinant of the developmental cell fate in the maturing nephron.

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REFERENCES

1. Levine, E. M. (2004) Development 131, 2241–2246
2. Li, Z. R., Hromněk, R., Mulipala, A., and Bloch, A. (1998) Cancer Res. 58, 4282–4287
3. Nadasdy, T., Lajoie, G., Laszik, Z., Blick, K. E., Molnar-Nadasdy, G., and Silva, F. G. (1998) Pediatr. Dev. Pathol. 1, 49–55
4. Ko, J. L., and Prives, C. (1996) Genes Dev. 10, 1054–1072
5. Vossen, K. H., and Lu, X. (2002) Nat. Rev. Cancer 2, 594–604
6. Amargiò, F., Tchang, F., Pieirolo, M. N., Soussi, T., Cibert, C., and Mechali, M. (1997) Oncogene 15, 2191–2199
7. Hoever, M., Clement, J. H., Wedlich, D., Montenarh, M., and Knochel, W. (1994) Oncogene 9, 109–120
8. Tchang, F., Gasse, M., Soussi, T., and Mechali, M. (1993) Dev. Biol. 159, 163–172
9. Tchang, F., and Mechali, M. (1999) Exp. Cell Res. 251, 46–56
10. Wallingford, J. B., Seufert, D. W., Virta, V. C., and Vize, P. D. (1997) Curr. Biol. 7, 747–757
11. Langheinrich, U., Hennen, E., Stott, G., and Vacun, G. (2002) Curr. Biol. 12, 2023–2028
12. Thiese, C., Neel, H., Thiese, B., Danjat, S., and Piette, J. (2000) Differentiation 66, 61–70
13. Almg, N., and Rotter, V. (1997) Biochim. Biophys. Acta 1333, F1–F27
14. Choi, J., and Donehower, L. A. (1998) Cell. Mol. Life Sci. 55, 38–47
15. Rotter, V., Aloni-Grinstein, R., Schwartz, D., Elkind, N. B., Simons, A., Wolkowicz, R., Lavigne, M., Beserman, P., Kapon, A., and Goldfinger, N. (1996) Semin. Cancer Biol. 4, 229–236
16. Sah, V. P., Attardi, L. D., Mulligan, G. J., Williams, B. O., Bronson, R. T., and Jacks, T. (1995) Nat. Genet. 10, 175–180
17. Saito, Z., Dipp, S., and El-Dahr, S. (2002) J. Clin. Investig. 109, 1021–1030
18. Schmid, P., Lorenz, A., Hameister, H., and Montenarh, M. (1991) Development 113, 857–865
19. White, J. D., Rachel, C., Vermeulen, R., Davies, M., and Grouds, M. D. (2002) Int. J. Dev. Biol. 46, 577–582
20. Aloyo, R. S., Banjii, S. X., Pozniak, C. D., Tooma, J. G., Atwal, J., Kaplan, D. B., and Miller, F. F. (1998) J. Cell Biol. 143, 1691–1703
21. Armstrong, J. F., Kaufman, M. H., Harrison, D. J., and Clarke, A. R. (1995)
