The limited regenerative capacity of the glomerular podocyte following injury underlies the development of glomerulosclerosis and progressive renal failure in a diverse range of kidney diseases. We discovered that, in the kidney, cyclin I is uniquely expressed in the glomerular podocyte, and have constructed cyclin I knock-out mice to explore the biological function of cyclin I in these cells. Cyclin I knock-out (−/−) podocytes showed an increased susceptibility to apoptosis both in vitro and in vivo. Following induction of experimental glomerulonephritis, podocyte apoptosis was increased 4-fold in the cyclin I−/− mice, which was associated with dramatically decreased renal function. Our previous data showed that the Cdk inhibitor p21Cip1/Waf1 protects podocytes from certain apoptotic stimuli. In cultured cyclin I−/− podocytes, the level of p21Cip1/Waf1 was lower at base line, had a shorter half-life, and declined more rapidly in response to apoptotic stimuli than in wild-type cells. Enforced expression of p21Cip1/Waf1 reversed the susceptibility of cyclin I−/− podocytes to apoptosis. Cyclin I protects podocytes from apoptosis, and we provide preliminary data to suggest that this is mediated by stabilization of p21Cip1/Waf1.

Cyclins were originally discovered for their role in governing cell cycle progression and proliferation (1). More recently it has been appreciated that cyclins may influence a wide range of additional cellular functions, including apoptosis, hypertrophy, and differentiation. The increasingly diverse members of the cyclin protein family are all characterized by the presence of a conserved domain through which they bind to cyclin-dependent kinases, the cyclin box (2). Cyclin I, the focus of this manuscript, is most abundant in post-mitotic tissues. In contrast to the classical cyclins, its level does not fluctuate during the cell cycle (3, 4). Cyclin I shows highest sequence homology to cyclins G1 and G2, and these three proteins are considered to form a separate subgroup (5). However, the biological function of cyclin I is not known.

Our data show that within the kidney cyclin I is specifically expressed by glomerular podocytes. These are terminally differentiated, post-mitotic, highly specialized epithelial cells, which serve as the major barrier to prevent the excretion of serum proteins into the urine. The inability to replace podocytes lost by apoptosis is thought to underlie the subsequent development of glomerulosclerosis and progressive renal impairment, regardless of the initiating injury (6–14). Given their limited regenerative capacity, prevention of podocyte apoptosis is of critical importance for the maintenance of normal renal function.

The restricted expression of cyclin I to the renal podocyte suggested that it might play a specialized biological role in these cells. We describe here the characterization of cyclin I expression in the normal kidney and the first analysis of its function using cyclin I knock-out mice. We report that cyclin I regulates podocyte apoptosis, both in vitro and in a model of glomerular disease in vivo. Previous work has shown that p21Cip1/Waf1 has an important role in preventing podocyte apoptosis (15), and we show here that cyclin I may control p21Cip1/Waf1 abundance by regulating its stability. We propose a role for cyclin I in protecting terminally differentiated cells from apoptosis.

TARGETING PROCEDURES

Targeting Vector and Generation of Cyclin I Mutant Mice

An 11-kb NotI fragment of the cyclin I gene was obtained from a mouse 129/SvJ mouse genomic library and sequenced. The sequencing data were then assembled into contigs using Sequencher software. The knock-in vector was constructed by cloning a 1.3-kb Spel-Sacl fragment that encompasses the intron sequence immediately 5′ to the second exon of cyclin I as the upstream arm and a 4.5-kb BstEII-HindIII fragment that contained a portion of the coding region of the last exon of cyclin I and intron sequence cloned into the SA-β gal vector as the downstream arm. The vector was linearized with Scal and electroporated into XY AK7 ES cells. The ES cells were then selected in 400 μg/ml G418. ES cell colonies with homologous recombination were identified by PCR amplification of a 2-kb fragment with a primer from the SA-β gal gene (SARev, 5′-CATCAAGGAAACCGTGCGACTCTG-3′) and a primer from cyclin I genomic DNA just 5′ to the Spel site (1BR+, 5′-TGGAGACATGATGCAGCAAC-3′). PCR reactions were performed for 40 cycles (93 °C for 30 s, 57 °C for 30 s, 65 °C for 2 min). Proper recombination within the cyclin I locus was also confirmed by Southern hybridization using probes specific for cyclin I and intron sequence.

REFERENCES

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3. An established member of the American Heart Association.
4. The abbreviations used are: contig, group of overlapping clones; +/+ , wild-type; −/− , knock-out; ES, embryonic stem; MEF, mouse embryonic fibroblast; PAN, puromycin aminonucleoside; GFP, green fluorescent protein; UVO, unilateral ureteric obstruction; WT-1, Wilms’tumor protein 1; TUNEL, terminal deoxynucleotidyltranserase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline; RT, reverse transcription.
confirmed by Southern blot of PstI digested genomic DNA using a probe designed with cyclin I sequences not contained within the original knock-in vector. ES cells were introduced into 5 days post-coitus C57/B6J mouse embryos. Germ line transmission, as determined by PCR, was identified in chimeric males obtained from two independent clones that were used for subsequent experiments. The wild-type allele of cyclin I was amplified using the 2718 oligonucleotide (5′-GGTGTGACCT- 
TATGGTATTTT-3′) and the 1BR primer described above using the same PCR conditions.

**Staining of Embryos for β-Galactosidase Activity**

Day 13 embryos were washed twice in PBS and then fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS containing 0.1% sodium deoxycholate and 0.2% Nonidet P-40 (Nonidet P-40) for 2 h at 4 °C. Fixed embryos were washed for 15 min three times in PBS. Embryos were incubated for 6–8 h at room temperature in staining solution (2.5 mM ferrocyanide, 2.5 mM ferricyanide, 2 mM MgCl₂, 0.1% sodium deoxycholate, 0.2% Nonidet P-40 in PBS) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (diluted from a 40× stock solution in N,N-dimethylformamide). After staining, embryos were extensively washed in PBS and photographed.

**Cell Culture**

**Mouse Embryonic Fibroblasts**—Mouse embryonic fibroblasts (MEFs) were isolated from 13-day-old embryos and maintained using standard procedures. To induce quiescence, confluent MEFs were washed twice with phosphate-buffered saline (PBS) and cultured in Dulbecco’s modified Eagle’s medium with 0.1% fetal bovine serum for 24 h. Quiescent cells were trypsinized, replated at low density, and stimulated with complete medium containing 10% fetal bovine serum to enter the cell cycle. Entry into S phase was monitored by estimating the DNA content of propidium iodide-stained nuclei using flow cytomtery or by bromodeoxyuridine incorporation.

**Mouse Podocytes**—Female cyclin I −/− mice were crossed with a male H-2Kb-tsA58 transgenic mouse (ImmortoMouse; Jackson Laboratory, Bar Harbor, ME) and the F1 generation intercrossed. Conditionally immortalized mouse podocytes were derived from cyclin I +/+ and −/− littermates as described previously (16). Briefly, proliferating podocytes were grown on collagen I (BD Biosciences, Bedford, MA) at 33 °C in medium supplemented with recombinant mouse γ-interferon (10 units/ml; Coulter, Hialeah, FL) to promote expression of the thermosensitive SV40 large T antigen. To induce quiescence and the differentiated phenotype, cells were grown at 37 °C in the same medium with no γ-interferon for 14 days and characterized by their expression of podocyte specific proteins. A similar strategy was used to generate p21Waf1/Cip1−/− podocytes.

**RT-PCR**

The expression of cyclin I by cultured mouse podocytes was determined by RT-PCR. cDNA was amplified in a semiquantitative fashion using primer sets specific for the mouse cyclin I gene (forward primer, 5′-ATGAAGTTTCCAGGACCTTTG-
3′; reverse primer, 5′-CTACATGACAAACAGGCTG-3′). The PCR reaction was performed as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. PCR products were resolved on a 2% agarose gel and normalized to expression of glyceraldehyde-3-phosphate dehydrogenase.

**Western Blot Analysis**

Total cell protein was extracted using TG buffer (1% Triton, 10% glycerol, 20 mM HEPES, 100 mM NaCl) with protease inhibitors (Roche Applied Science). Protein concentration was determined by the BCA protein assay (Pierce).

For Western blot analysis, 15–40 μg of protein extracts were separated under reduced conditions on 15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated overnight at 4 °C with the following commercially available primary antibodies: mouse monoclonal anti-p21 (clone SX118, Pharmingen), anti-human p21 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 (BD Transduction Laboratories, Lexington, KY), anti-p53 (clone PAb421, Oncogene Research Products, San Diego, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, MA), anti-Grb2 (Santa Cruz Biotechnology), and anti-actin (Chemicon International Inc., Temecula, CA). The cyclin I antibody was developed in house. Cyclin I was subcloned into pET16b (Novagen, Madison, WI) as a full-length coding sequence or the sequence encoding the amino terminus (amino acids 1–52).

Protein inductions were performed in BL21 pLysS bacteria and purified under denaturing conditions with 8 M urea on nickel-nitrilotriacetic acid (Qiagen, Valencia, CA). Antibodies to the two cyclin I proteins were raised in New Zealand White rabbits and affinity-purified using antigen immobilized on nickel-nitrilotriacetic acid. Antibody was eluted from the column using 4 M MgCl₂ and dialyzed extensively against PBS at 4 °C.

Proteins were visualized using the chromagen 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) or ECL reagents (Pierce).

**Induction of Apoptosis in Vitro**

Apoptosis was induced in 80–90% confluent, differentiated cyclin I +/+ and −/−, and p21Cip1/Waf1−/− podocytes grown in 24-well plates (Primaria, VWR, West Chester, PA). Each experimental condition was carried out in quadruplicate, and experiments were performed at least three times. Apoptosis was detected as described below. Three apoptotic stimuli were used. (i) uv-C irradiation, 0–25 J/m² using a Hoefer cross-linker (Stratagene, La Jolla, CA). Cells were irradiated in the absence of media and apoptosis assessed after 6 h. Protein was harvested from similarly treated cells for Western blot analysis. (ii) Puromycin aminonucleoside (PAN) has previously been shown to induce podocyte apoptosis in culture (17, 18) and induces proteinuria and apoptosis in vivo (10, 19). Cells were cultured in the presence of 0–100 μg/ml PAN (Sigma) and apoptosis measured after 24 h. (iii) Anti-podocyte antibody induces podocyte injury in vitro and in vivo and as described below was also used to induce experimental glomerulonephritis in mice. Cells were exposed to media containing 0–5% nephrotoxic or normal
sheep serum for 30 min at 37 °C. The cells were then washed in HBSS and fresh media applied. Apoptosis was assessed after 16 h. In separate experiments, cells were fixed overnight in ice-cold methanol prior to immunofluorescence to confirm equal antibody binding.

Detection of Apoptosis

At the end of each experiment, Hoechst 33342 (Sigma) at a final concentration of 10 mM was added to each well. At least 400 cells were counted for each well, and the number of apoptotic nuclei expressed as a percentage of the total. Apoptosis was also assessed by a caspase 3 activity assay according to the manufacturer’s instructions (BD Biosciences).

Retroviral Transduction of Cyclin I −/− Podocytes

pBabe vectors encoding cyclin I, wild-type human p21Cip1/Waf1, lysineless (AK) human p21Cip1/Waf1, or GFP were transfected into Phoenix packaging cells to generate retrovirus. The retrovirus-containing media were harvested and filtered onto 50% confluent proliferating, undifferentiated cyclin I −/− podocytes. Following 48-h selection with puromycin (2.5 μg/ml), cells were passaged and transferred to growth restrictive conditions. Apoptotic susceptibility following uv-C irradiation was assessed as above.

Animal Models

Crescentic Glomerulonephritis—Glomerulonephritis was induced in 10–12-week-old male cyclin I +/+ and −/− matched control mice by the intraperitoneal injection of sheep anti-rabbit glomerular antibody (0.5 ml/20 g of body weight) on 2 consecutive days, as described previously (20, 21). We have previously characterized this model in detail and demonstrated that the observed pathological changes are not due to the presence of infiltrating cells.

Cyclin I +/+ and −/− mice (n > 6/group) were sacrificed on days 7 and 14 after the second injection of anti-glomerular antibody. Urine was collected from each animal before disease induction and just prior to sacrifice for quantification of proteinuria by the sulfosalicylic acid method. Blood was collected at sacrifice to measure serum creatinine (Sigma kit number 555-A). Renal tissue was embedded in OCT compound (Miles, Elkart, IN) and frozen at −70 °C or fixed in either 10% neutral buffered formalin or methyl-Carnoy’s solution (60% methanol, 30% chloroform, 10% acetic acid) for immunostaining (see below).

Unilateral Ureteric Obstruction (UUO)—Experimental UUO was performed on anesthetized 8–10-week-old cyclin I +/+ and −/− animals (n = 8/group) by ligation of the left ureter at the ureteropelvic junction (22). Mice were sacrificed at day 7 and kidneys fixed as above for histological assessment. The contralateral non-obstructed kidney served as control.

Immunostaining

Indirect immunoperoxidase staining was performed on formalin or methyl-Carnoy’s fixed tissue with antibodies against β-galactosidase (1:1000 dilution, Abcam), WT-1 (sc192, 1:1000 dilution, Santa Cruz Biotechnology), and fibronectin (1:500 dilution, Chemicon International Inc.). Measurement of apoptosis was by the TUNEL assay, performed as described previously (23). Apoptosis was also quantified in the thymus of unmanipulated cyclin I +/+ and −/− mice.

Frozen sections were rehydrated in PBS and stained with fluorescein isothiocyanate-conjugated antibodies to sheep IgG (Cappel, Durham, NC), to ensure comparable glomerular antibody deposition between the cyclin I +/+ and −/− mice. The autologous phase of the disease was similarly assessed by immunostaining with a fluorescein isothiocyanate-conjugated antibody to mouse IgG (Cappel). Double immunostaining for β-galactosidase (goat polyclonal antibody, 1:100 dilution, Biogenesis, Kingston, NH) and WT-1 (sc192 rabbit polyclonal antibody, 1:100 dilution, Santa Cruz Biotechnology) was performed. After washing, sections were incubated with a biotinylated goat anti-rabbit IgG (1:500 dilution, Vector laboratories, Burlingame, CA). Binding was detected using Alexa Fluor 488-conjugated donkey anti-goat IgG (1:100 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 594 streptavidin (Molecular Probes).

Assessment of Glomerulosclerosis

Glomerulosclerosis was determined on periodic acid Schiff-stained sections for a minimum of 50 glomeruli in each animal and was graded quantitatively based on the percentage of glomerular tuft area involvement as follows: grade 1 = <25%; grade 2 = 25–50%; grade 3 = 50–75%; grade 4 = 75–100%. Slides were viewed using a Leica confocal microscope (Leica, Deerfield, IL) using either bright-field or appropriate epifluorescent optics.

Statistical Analysis

All results are expressed as mean ± S.D. Statistical significance was evaluated using the Student’s t test.

RESULTS

Targeted Disruption of the Cyclin I Gene Creates a Null Mutation—We constructed a cyclin I knock-out (−/−) mouse in which the cyclin I coding exons were replaced with the bacterial β-galactosidase gene (Fig. 1A). Staining of day 13 embryos for β-galactosidase activity confirmed expression of the transgene (Fig. 1B). Viable cyclin I −/− mice were obtained from intercrosses of cyclin I +/+ heterozygotes at the normal Mendelian frequency of 25% (142/512) and showed no apparent developmental defects. The genotype of the offspring was confirmed by Southern blotting (Fig. 1C). Protein extracts from various tissues from one month old pups underwent Western blot analysis, and highest expression was seen in the brain, followed by testis (data not shown), although cyclin I −/− mice were fertile and displayed no behavioral abnormalities. The lack of cyclin I protein expression in the homozygous knock-out mice was confirmed by immunoprecipitation and Western blotting with an anti-cyclin I antibody (Fig. 1D).

To test whether cyclin I −/− cells had detectable defects in cell proliferation, we isolated MEFs from 13-day-old embryos (Fig. 1E). Cyclin I −/− MEFs responded to serum stimulation and progressed through the cell cycle at the same rate as control MEFs (Fig. 1F). Population doubling times were also unaffected by the absence of cyclin I (data not shown). Taken together,
these results suggested that cyclin I was not required for cell proliferation, and its abundant expression in some post-mitotic cells suggested that it may have a role distinct from the cell cycle.

**Cyclin I Is Expressed by Podocytes in Vitro and in Vivo**—We used immunohistochemical analysis of β-galactosidase to determine the pattern of cyclin I expression in the kidney, as this gene has been introduced in place of cyclin I. As expected, no β-galactosidase expression was detected in the cyclin I−/− mice (Fig. 2A). However, in the cyclin I−/− mice, distinct staining was seen in the glomerulus in a podocyte distribution (Fig. 2B). Weaker and variable expression was seen in tubular cells (data not shown). Wilm’s tumor protein 1 (WT-1) was used as a podocyte specific marker (24) and by immunofluorescence co-localized with β-galactosidase (Fig. 2, C–E), confirming within the glomerulus the exclusive expression of β-galactosidase (and therefore the cyclin I gene) by podocytes in vitro.

To confirm the podocyte-specific expression of the endogenous cyclin I protein, we used RT-PCR (Fig. 2F) and Western blot analysis (Fig. 2G) for cyclin I using RNA and protein from proliferating, undifferentiated and from post-mitotic, differentiated podocytes. Cyclin I expression was similar in proliferating and quiescent cells. Similarly to MEFs, cyclin I−/− podocytes showed no detectable defects in cell proliferation. For our further studies we focused on the role of cyclin I in renal podocytes.

**Cultured Cyclin I−/− Podocytes Are More Susceptible to Apoptosis**—The expression of cyclin I in post-mitotic cells suggested involvement in pathways other than the cell cycle. We therefore induced apoptosis in cultured cyclin I+/+ podocytes using uv-C irradiation. Western blot analysis demonstrated
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FIGURE 2. Cyclin I is expressed by mouse podocytes in vivo and in vitro. A, immunohistochemistry for bacterial β-galactosidase shows no expression in the cyclin I +/+ mice. B, in the cyclin I −/− mice, β-galactosidase is expressed as a surrogate for cyclin I, and distinct staining was seen in the glomerulus in a podocyte distribution. Weaker and variable expression was seen in tubular cells (data not shown); Immunofluorescence for WT-1 (C) and β-galactosidase (D) co-localize (E), confirming within the glomerulus the exclusive expression of β-galactosidase (and therefore cyclin I) by podocytes in vivo. F, RT-PCR for cyclin I in proliferating and differentiated mouse podocytes. G, Western blot for cyclin I in cyclin I +/+ and −/− podocytes. Original magnification ×400 (A and B) and ×600 (C–E). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Apoptosis Is Increased in Cyclin I −/− Nephritic Mice—We reasoned that a critical role for cyclin I might be revealed following injury, as suggested by the in vivo data. To test this hypothesis, experimental glomerulonephritis was induced in cyclin I +/+ and −/− mice with a sheep anti-podocyte antibody (20, 21). We have previously demonstrated that this model is not characterized by the presence of infiltrating leukocytes, and the observed rates of apoptosis are in resident glomerular cells (20). By immunofluorescence, equal deposition of sheep and mouse immunoglobulin was seen in both cyclin I +/+ and −/− mice at the same time points (data not shown), confirming comparable initiating injury.

Consistent with our in vitro data, glomerular cell apoptosis was increased 4-fold in the cyclin I −/− mice at day 7 of nephritis (0.07 + 0.03 versus 0.28 + 0.08 apoptotic cells per glomerular cross-section, p < 0.005), and these apoptotic cells were in a podocyte distribution (Fig. 4, A and B). To characterize the consequences of the early increased apoptosis, the number of podocytes was determined by counting WT-1-positive cells. There was no difference in podocyte number in unmanipulated mice (Fig. 5, A and B). However, by day 14 after disease induction, podocyte number was significantly less in the cyclin I −/− versus wild-type mice (1.46 + 1.24 versus 3.19 + 0.90 cells per glomerular cross-section; p < 0.01) (Fig. 5, E and F).

As a decline in podocyte number has been reported to underlie pathological extracellular matrix accumulation and progressive glomerulosclerosis in both experimental and human disease, these parameters were assessed for the cyclin I +/+ and −/− mice. There was scant fibronectin staining in the glomeruli of unmanipulated mice (Fig. 5, C and D). At day 14 of disease, there was markedly greater immunostaining for fibronectin in the cyclin I −/− mice (Fig. 5, G and H). Although glomerulosclerosis was initially similar in the two groups, by day 14 of disease this had progressed to extensive involvement in the cyclin −/− mice (score 1.1 + 0.5 versus 2.6 + 0.8; p < 0.005).

down-regulation of cyclin I protein levels in podocytes following irradiation (Fig. 3A). We then hypothesized that cyclin I might be important for determining the threshold at which podocytes undergo apoptosis following stimulation and explored this using cyclin I +/+ and −/− cultured podocytes. As determined by Hoechst 33342 staining, apoptosis occurred earlier, and was of a greater magnitude, in the cyclin I −/− podocytes following induction by three different stimuli: (i) uv-C (Fig. 3B), (ii) PAN (Fig. 3C), and (iii) anti-podocyte antibody (Fig. 3D). Apoptosis induced by uv-C activates both the intrinsic and extrinsic pathways of apoptotic signaling (25), whereas PAN principally activates the intrinsic pathway (26), and the mechanism by which the anti-podocyte antibody causes apoptosis is unknown. The increased susceptibility of the cyclin I −/− podocytes to apoptosis induced by all three stimuli suggests that cyclin I acts distally in the pathways converging to cause cell death. To further confirm the increased apoptosis in cyclin I −/− cells, we performed an activity assay for caspase 3 using uv-C-irradiated cyclin I +/+ and −/− podocytes (Fig. 3E). The increased caspase 3 activity in the irradiated cyclin I −/− podocytes validates the results using Hoechst 33342 staining, showing that apoptosis was significantly increased in cyclin I −/− cells compared with cyclin I +/+ cells receiving the same stimulus.

Apoptosis of Cyclin I −/− Podocytes Is Rescued by Reconstitution of Cyclin I—To verify that the lowered apoptotic threshold observed in cyclin I −/− podocytes is indeed due to a deficiency in cyclin I itself (rather than due to altered expression of an adjacent gene that might have been affected by the deletion of cyclin I) we stably reintroduced cyclin I into cyclin I −/− podocytes by retroviral transduction. GFP served as a negative control. Following cyclin I reconstitution in cyclin I −/− cells, apoptosis was measured 6 h following irradiation with 25 J/m² (Fig. 3F). The reconstitution of cyclin I completely reversed the apoptotic susceptibility of the cyclin I −/− cells.

Podocyte Number and Renal Function Are Normal in Unmanipulated Cyclin I −/− Mice—We next studied the role of cyclin I in vivo. The cyclin I −/− mouse is phenotypically normal under physiological conditions. Renal function and histology were evaluated in detail from 12-week-old male cyclin I +/+ and −/− mice (n = 6/group). There was no difference in serum creatinine (+/+ 0.23 + 0.06 mg/dl; −/− 0.28 + 0.08 mg/dl, p = not significant), urine protein:creatinine ratio (+/+, 8.2 + 4.6 mg/mg; −/−, 8.3 + 7.8 mg/mg, p = not significant) or podocyte number (+/+, 8.1 + 0.3; −/−, 7.9 + 1.0 per glomerular cross-section, p = not significant) between the two groups. These results indicate that cyclin I is not required for normal glomerular development nor for maintenance of normal renal function.
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(Fig. 5). There was a strong correlation between the decline in podocyte number and glomerulosclerosis for both groups of mice (Fig. 5K), supporting previous published reports of a causal relationship between podocyte loss and increased sclerosis. Similar results were seen following disease induction in cyclin I null mice in which the β-galactosidase gene had not been introduced. Thus, in this model of glomerulosclerosis cyclin I −/− mice suffered from an early increase in podocyte apoptosis, which culminated, at later stages, in a greater severity of glomerular disease.

Renal Function Is Worse in Nephritic Cyclin I −/− Mice—To determine the clinical consequences of the increased podocyte apoptosis, we assessed kidney function. Both groups of mice developed acute renal failure due to glomerular disease (Fig. 6A). The rise in serum creatinine was similar for the cyclin I +/+ and −/− mice at day 7 (1.11 ± 0.36 mg/dl versus 0.98 ± 0.37 mg/dl). However, at day 14, renal function was improving in the cyclin I +/+ mice (0.86 ± 0.32 mg/dl), but there was a marked continued rise in creatinine in the cyclin I −/− mice (2.30 ± 0.59 mg/dl; p < 0.005). Reflecting their increased podocyte injury, nephritic cyclin I −/− mice developed proteinuria earlier than the cyclin I +/+ mice (urine protein:creatinine ratio at day 7: 79.1 vs 30.6 mg/mg) (Fig. 6B). The rise in serum creatinine was similar for the cyclin I +/+ and −/− mice at day 7 (1.11 ± 0.36 mg/dl versus 0.98 ± 0.37 mg/dl). However, at day 14, renal function was improving in the cyclin I +/+ mice (0.86 ± 0.32 mg/dl), but there was a marked continued rise in creatinine in the cyclin I −/− mice (2.30 ± 0.59 mg/dl; p < 0.005). Reflecting their increased podocyte injury, nephritic cyclin I −/− mice developed proteinuria earlier than the cyclin I +/+ mice (urine protein:creatinine ratio at day 7: 79.1 vs 30.6 mg/mg) (Fig. 6B).

Reduced Levels of p21Cip1/Waf1 May Underlie the Susceptibility of Cyclin I −/− Podocytes to Apoptosis—There are close parallels between the disease susceptibility of the cyclin I −/− mice and p21Cip1/Waf1 −/− mice (15, 27). Indeed, we found that the decline in cyclin I levels following low dose
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UV-C irradiation was reminiscent of the previously reported decline in p21\(^{\text{Cip1/Waf1}}\) following irradiation (28). Moreover, as judged by Western blot analysis, the cyclin I \(+/−\) cells had less p21\(^{\text{Cip1/Waf1}}\) protein at baseline, and levels declined more rapidly following irradiation, in association with increasing apoptosis (Fig. 7A). This finding was confirmed in a second, independently derived podocyte cell line. The cyclin I \(+/+\) and \(-/−\) podocytes have comparable levels of p53, which are unaffected by UV-C at the doses used. No differences were detected in the expression of p27\(^{\text{Kip1}}\) or p53 (data not shown). Interestingly, the increased susceptibility of cyclin I \(-/-\) podocytes to UV-C-induced apoptosis was identical to that of p21\(^{\text{Cip1/Waf1}}\) \(-/-\) podocytes (Fig. 7B), suggesting their involvement in a common pathway. Indeed, we found that the half-life of p21\(^{\text{Cip1/Waf1}}\) was reduced from 250 min in control podocytes to 120 min in the cyclin I \(-/-\) cells (Fig. 7, C and D). The half-life of p21\(^{\text{Cip1/Waf1}}\) is variable in different cell types (28, 29) and may reflect the role of other proteins, like cyclin I, in the stabilization of p21\(^{\text{Cip1/Waf1}}\) (30).

The re-introduction of cyclin I into cyclin I \(-/-\) podocytes restored the expression of p21\(^{\text{Cip1/Waf1}}\) to normal levels, both at baseline and following UV-C irradiation whereas the transduction of GFP had no effect (Fig. 7E). Re-introduction of cyclin I was accompanied by rescue of the pro-apoptotic phenotype (Fig. 3F).

We further explored the role of p21\(^{\text{Cip1/Waf1}}\) instability in cyclin I \(-/-\) podocytes. We used retroviral transduction to enforce expression of either wild type or lysineless (ΔK) human p21\(^{\text{Cip1/Waf1}}\) in cyclin I \(-/-\) podocytes. The use of human constructs enables us to detect expression using a human-specific antibody, and ΔKp21\(^{\text{Cip1/Waf1}}\) was studied as this protein is not ubiquitinated and therefore does not undergo accelerated degradation following UV-C (28). Western blot analysis demonstrated that although the wild-type p21\(^{\text{Cip1/Waf1}}\) is successfully expressed in the cyclin I podocytes, it remains unstable, whereas levels of the ΔKp21\(^{\text{Cip1/Waf1}}\) persist following UV-C (Fig. 7G). The wild-type p21\(^{\text{Cip1/Waf1}}\) was ineffective at rescuing the cyclin I \(-/-\) podocytes from UV-C-induced apoptosis; however, protection was seen in the presence of ΔKp21\(^{\text{Cip1/Waf1}}\) (Fig. 7F). Taken together, these results suggest that the stabilization of p21\(^{\text{Cip1/Waf1}}\) by cyclin I was required to inhibit apoptosis following injury.

DISCUSSION

The experiments described in this manuscript show that cyclin I functions as a cell survival factor in limiting apoptotic cell death in podocytes following injury. We report constitutive expression of cyclin I by podocytes, terminally differentiated epithelial cells, and several lines of evidence implicating cyclin I in protecting podocytes from apoptosis. These include a decrease in cyclin I levels in...
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Apoptotic cells, earlier onset, and an increased magnitude of apoptosis in cyclin I /-/- cells, which is rescued by restoring cyclin I levels, and increased apoptosis in nephritic cyclin I /-/- mice compared with nephritic cyclin I +/- mice.

The complex architecture of the podocyte is essential for its normal functions, namely maintenance of the glomerular permselectivity barrier by the intercellular slit diaphragms, synthesis of glomerular basement membrane components, and counteraction of glomerular capillary distension (31–33). Although highly metabolically active, the podocyte is considered post-mitotic and unable to progress through the cell cycle and divide. However, the factors responsible for maintenance of the mature, differentiated podocyte phenotype while preventing apoptosis remain poorly understood. It is becoming increasingly apparent that podocytes lost by apoptosis during the course of glomerular disease cannot be effectively replaced (8, 13). This results in areas of denuded basement membrane, formation of synchiae with Bowman's capsule, and progressive glomerulosclerosis (34).

Several potent stimuli of podocyte apoptosis have recently been described, including TGFβ1 (13, 14), angiotensin II (35), mechanical stretch (36), glucose-induced reactive oxygen species (37), and cyclosporine A (38), which are likely to be important for the induction of podocyte apoptosis in disease states in vivo.

The model of glomerulonephritis used in this study has previously been reported in detail by our laboratory, and similar severity of disease was seen in the cyclin I +/- animals to that we have observed previously (15, 20, 21). The most significant histological finding was the 4-fold increase in apoptosis in the cyclin I /-/- mice at day 7, compared with the nephritic cyclin I +/- animals. The apoptotic cells were almost exclusively located in the periphery of the glomerulus. The location of the apoptotic cells, the initial restriction of cyclin I expression to podocytes, together with the loss of podocytes as determined by WT-1 staining, strongly suggest that the podocyte is the predominant cell type undergoing apoptosis. However, due to the morphological changes associated with apoptosis and the loss of cell type-specific proteins it was not possible to incontrovertibly confirm the origin of the apoptotic cells. There was a dramatic increase in glomerulosclerosis and extracellular matrix accumulation in the cyclin I /-/- mice at day 14, which
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correlated strongly with a greater decline in podocyte number in the cyclin I −/− compared with the cyclin I +/+ mice.

Interestingly, the role of cyclin I in controlling apoptosis is cell type-specific, as cyclin I deficiency had no effect in two other models of in vivo apoptosis: (i) spontaneous apoptosis in the thymus of unmanipulated mice and (ii) tubular and interstitial cell apoptosis following UUO. We observed no increase in apoptosis above wild type in either tissue (data not shown) and therefore conclude that the apoptotic susceptibility of the cyclin I −/− mice is indeed restricted to certain cell types and within the kidney is limited to the non-dividing podocyte.

How does cyclin I protect podocytes from apoptosis? In addition to activating their partner Cdks, cyclins have a binding site within the kidney is limited to the non-dividing podocyte. of p21Cip1/Waf1 is reduced in cyclin I

The mechanism by which p21Cip1/Waf1 per se inhibits apoptosis is not well understood. p53-dependent up-regulation of p21Cip1/Waf1 results in cell cycle arrest, which is proposed to allow repair of DNA damage. However, our results support a p53-independent role in the inhibition of apoptosis, as the uv-C dose used to initiate apoptosis was insufficient to induce p53. A proposed mechanism by which p21Cip1/Waf1 inhibits apoptosis is by its interaction with procaspase 3, preventing the cleavage and activation of caspase 3 (46–48). This mechanism is in accordance with our results, as increased caspase 3 activity was observed in the cyclin I −/− cells in which levels of p21Cip1/Waf1 were low.

In summary, we have demonstrated that cyclin I prevents apoptosis in the post-mitotic podocyte in vitro and in vivo. We also provide evidence that cyclin I is a regulator of p21Cip1/Waf1 and that by maintaining p21Cip1/Waf1 levels, cyclin I protects cells from apoptosis. We propose that cyclin I has a critical role in protecting podocytes from apoptosis, thus preventing glomerulosclerosis and progression of renal disease.

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