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Epidermal growth factor potentiates renal cell death in hydronephrotic neonatal mice, but cell survival in rats

SUSAN C. KILEY, BARBARA A. THORNHILL, BRIAN C. BELYEA, KAREN NEALE, MICHAEL S. FORBES, NOREEN C. LUETTEKE, DAVID C. LEE, and ROBERT L. CHEVALIER

Department of Pediatrics, University of Virginia, Charlottesville, Virginia; H. Lee Moffitt Cancer Center, Department of Interdisciplinary Oncology University of South Florida, Tampa, Florida; and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina

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Background. Epidermal growth factor (EGF) markedly attenuates tubular apoptosis induced by unilateral ureteral obstruction (UUO) in the neonatal rat, and reduces apoptosis induced by mechanical stretch of cultured rat tubular cells.

Methods. To investigate the role of EGF in modulating apoptosis resulting from UUO, neonatal wild type and mutant mice lacking EGF (knockout), or with diminished EGF receptor activity (waved-2 mutant) were compared to control mice for tubular apoptosis and atrophy. Rat and mouse kidneys were compared for localization of the EGF receptor. Apoptosis was also measured in cultured mouse tubular cells subjected to stretch and exposed to EGF.

Results. UUO reduced endogenous renal EGF expression in wild-type mice. Unlike the rat, exogenous EGF did not decrease tubular apoptosis or atrophy in the obstructed kidney, and significantly increased stretch-induced apoptosis of cultured mouse tubular cells. Tubular apoptosis was 50% lower in the obstructed kidney of EGF knockout and waved-2 mice relative to wild type and heterozygous animals. Exogenous EGF increased tubular apoptosis and doubled atrophy in the obstructed kidney of waved-2 mice. Species differences in EGF receptor localization were detected in 3-day-old kidneys.

Conclusion. EGF acts as a survival factor in the neonatal rat, but potentiates tubular cell death in the neonatal mouse. Species differences are maintained in cultured cells, suggesting that differences in EGF receptor signaling underlie these opposing effects.

There is increasing evidence that the initiation and progression of a variety of renal disorders involves alterations in tubular apoptosis [1, 2]. Congenital obstructive nephropathy constitutes a major cause of renal insufficiency in infants and children [3]. To elucidate the mechanisms underlying the renal response to urinary tract obstruction in the developing kidney, we have created models of unilateral ureteral obstruction (UUO) in neonatal rats and mice [4, 5]. In contrast to the human, in whom nephrogenesis is complete before birth, nephrogenesis in the rat is incomplete at birth and progresses during the first 2 postnatal weeks. As a consequence of UUO in the neonatal rat, there is a marked increase in tubular apoptosis, beginning within several days of obstruction, and continuing for several weeks, leading to tubular atrophy [6–8]. Factors regulating tubular apoptosis are therefore of significant interest, as inhibition of apoptosis may afford a therapeutic intervention to preserve functioning renal mass.

Epidermal growth factor (EGF) is a peptide that stimulates proliferation, and acts as a survival factor in the developing rat kidney [9]. EGF is normally synthesized by distal tubular cells, with increasing expression during maturation [4]. Chronic UUO suppresses renal EGF production in neonatal rats or in infants and children [4, 10]. Exogenous EGF reduces tubular apoptosis by 80% in the neonatal rat with chronic UUO, and enhances recovery after relief of obstruction [11, 12]. Exogenous EGF also inhibits tubular apoptosis in the adult rat subjected to UUO [13]. We have reported recently that in vitro stretching of rat tubular cells (a simulation of stretched cells lining dilated tubules in the hydronephrotic kidney) stimulates apoptosis. This is mediated through dephosphorylation of an oncoprotein, BAD, which binds to BclX on the mitochondria, leading to release of cytochrome c and initiation of the apoptotic cascade [14]. Incubation of stretched tubular cells with EGF reduces apoptosis by 50%, a response that is mediated through ERK1/2 (MAPK) signaling and maintenance of BAD phosphorylation [14].

Although their smaller size makes surgical models of neonatal UUO more difficult in the mouse, the availability of mutant murine strains makes them desirable

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in studying the cellular pathophysiology of obstructive nephropathy. Similar to the rat, nephrogenesis in the mouse is incomplete at birth and progresses throughout the first 2 postnatal weeks. We have recently reported a detailed study of the cellular consequences of chronic UUO in the neonatal mouse, showing that tubular apoptosis is directly proportional to tubular dilatation, and that in vitro stretching of mouse tubular cells induces apoptosis, as in the rat [5]. Using selectin null mutations, we have reported that tubular apoptosis in the neonatal mouse subjected to UUO is also mediated in part by infiltrating interstitial macrophages [15, 16].

To elucidate the role of EGF in tubular apoptosis following UUO, the present studies were performed in wild type and mutant neonatal mice lacking functional EGF or with a mutation of the EGF receptor, resulting in a marked reduction in its intrinsic tyrosine kinase activity (waved-2 mutant mice). The results were unanticipated. In neonatal wild-type mice, exogenous EGF promotes apoptosis instead of cell survival in the obstructed kidney. In the mutant mice, either a reduction in endogenous EGF or a reduction in EGF receptor activity promotes cell survival, rather than apoptosis in the hydronephrotic kidney. Different EGF/HER receptor expression patterns in the developing kidneys of rats and mice may contribute to this conundrum.

METHODS

Exogenous EGF treatment of wild-type mice subjected to UUO

Wild-type C57BL/6J mice were subjected to complete left ureteral obstruction or sham operation within the first 48 hours of life. Under general anesthesia with isoflurane and oxygen, the left ureter was exposed through an incision and was ligated (UUO). The incision was closed, and the animal was returned to its mother. One day after surgery, 6 mice were injected subcutaneously with EGF (Upstate Biotechnology, Lake Placid, NY, USA) for 7 days at a daily dose of 0.1 mg/kg body weight, and 6 mice were injected subcutaneously with saline vehicle to serve as controls. Mice were sacrificed 8 days after surgery, kidneys were removed, decapsulated, weighed, and fixed in 10% phosphate-buffered formalin (pH 7.1) for 24 to 48 hours before transfer to 70% ethanol. Kidneys were then dehydrated, embedded in paraffin, and sectioned at 4 μm.

Mice lacking functional EGF

Mice with the EGF gene disrupted by conventional gene targeting were obtained as heterozygote breeding pairs [17]. To identify homozygous wild type and homozygous EGF null animals for breeding, genomic DNA was extracted from mouse tail biopsies of progeny using the DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, CA, USA), and genotype was determined by polymerase chain reaction using the following primers: 5′ ATA AGA TGC TGA TGA TGC TGA TGC 3′ (forward primer, mI19-3 in mouse intron 19), 5′ AGC TGT CCT CTA TAG ACC TGG CTG 3′ (reverse primer, mI19-up in mouse intron 19, wild-type allele), and 5′ CTG CAC GAG ACT AGT GAG ACG TGC 3′ (reverse primer, PGK-2 in PGK neo of targeted allele). Genotyped homozygous EGF null male and female mice were bred to generate animals for knockout study; progeny were not genotyped. Homozygous wild-type littermates (a mixed background of 129 and C57BL/6J) were used as breeders for controls. Newborn male and female mice were subjected to complete left UUO within the first 48 hours of life [18]. Seven or 14 days after operation, animals were sacrificed by pentobarbital injection, and their kidneys were decapsulated, removed, weighed, and processed for histologic study as described above. $N = 12$ and 19 for 7-day knockout and wild type, respectively; $N = 4$ and 15 for 14-day knockout and wild type, respectively.

Waved-2 mice

The waved-2 mouse model is a spontaneous hypomorphic, or partial loss-of-function mutant. The waved-2 EGFR contains a single point mutation in the tyrosine kinase domain that severely impairs its activity in vitro, and partially impairs its activity in vivo, depending on the tissue context and endogenous or exogenous levels of ligands [19]. Because the waved-2 EGFR is full length and expressed at normal levels, its signaling capacity may be rescued via heterodimerization with other erbB/HER receptors because it can still bind ligand, be phosphorylated, and recruit adaptor or effector molecules.

Homozygous mutant waved-2 mice [19] obtained from Jackson Laboratory (Bar Harbor, ME, USA) were identified at 2 days of age by the presence of wavy vibrissae. Newborn male and female mice were subjected to complete left UUO or sham operation within the first 48 hours of life [18]. Seven or 14 days after operation, animals were sacrificed by pentobarbital injection, and their kidneys were decapsulated, removed, weighed, and processed for histologic study. $N = 12$ and 31 for 7-day waved-2 and combined heterozygote and wild type controls, respectively; $N = 5$ and 21 for 14-day waved-2 and combined heterozygote and wild type controls, respectively.

Administration of exogenous EGF to waved-2 mice subjected to UUO. One day after surgery (below), 6 waved-2 mice were injected subcutaneously with EGF (Upstate Biotechnology) for 12 days at a daily dose of 0.1 mg/kg body weight. Five waved-2 animals were injected daily with saline vehicle to serve as controls. Mice were

sacrificed 12 days after surgery on day 14, kidneys were removed, decapsulated, weighed, and processed for histologic study. $N = 11$ for waved-2 and combined heterozygote and wild-type controls, respectively.

Ribonucleic acid analysis by slot blot

Total RNA was extracted from frozen kidney tissue of wild-type C57BL/6J mice, and applied to nylon membranes for slot blot analysis, as previously described [4]. ^{32}P-labeled EGF and GAPDH cDNA probes, probe hybridization conditions, and radiograph analysis were also described [4].

Immunohistochemistry and histology

Kidney sections were deparaffinized in xylene, and rehydrated through graded ethanols. Proteinase K at 20 μg/mL (Roche, Indianapolis, IN, USA) was applied for 15 minutes, and endogenous peroxidase activity was suppressed by quenching the slides in 3% H_2O_2 in methanol for 5 minutes. The following staining and immunohistochemical techniques were used.

Identification of cellular apoptosis. Apoptotic cells were identified with the TUNEL technique, using the ApopTag® peroxidase in situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA). Positive nuclei were visualized by applying peroxidase-conjugated anti-digoxigenin antibody for 30 minutes, followed by a 0.05% solution of 3,3′-diaminobenzidine tetrahydrochloride for 4 minutes. For negative controls, TdT enzyme was omitted. Slides were counterstained with methylene blue. The number of apoptotic cells was counted in each of 10 randomly selected fields.

Identification of tubular atrophy by basement membrane thickening. The tubular basement membrane (TBM) was identified by periodic acid–Schiff (PAS) staining. Thickening and folding of the TBM has been shown to be a reliable indicator of tubular atrophy [20]. TBM thickening was identified in 100 cross-sectioned tubules. Results are expressed as the percentage of cross-sectioned tubules having thickened TBM relative to the total number of cross-sectioned tubular segments counted.

Identification of proximal tubules with lectins. Selected serial sections were treated for 30 minutes with biotinylated *Lotus tetragonobulus* lectin (1:50; Vector Laboratories, Burlingame, CA, USA) to identify proximal tubules in mouse kidneys or *Phaseolus vulgaris* erythroagglutinin (1:500; Vector Laboratories) to identify proximal tubules in rat kidneys.

Identification of the EGF receptor. Rabbit anti-EGF receptor antibody #2232 (Cell Signaling Technology, Beverly, MA, USA) was diluted 1:50 in phosphate-buffered saline (PBS) with 5% goat serum and used according to the manufacturer's recommended protocol, with no counterstain.

Table 1. Body weight of mice

	Wild type or heterozygote	Homozygous mutant
7-day-old EGF deletion	4.75 ± 0.14	4.41 ± 0.14
7-day-old wa-2	5.04 ± 0.12	4.39 ± 0.30[a]
14-day-old EGF deletion	8.10 ± 0.46	6.75 ± 0.21
14-day-old wa-2	8.06 ± 0.25	7.84 ± 0.56
14-day-old wa-2 + EGF	9.01 ± 0.30	7.43 ± 0.50[a]

[a] $P < 0.05$ vs. wild type or heterozygote.

In vitro mechanical stretch experiments. Proximal tubule epithelial cells (PKSV-PR) were provided by Dr. Alain Vandewalle (Paris, France) and were cultured as previously described [5, 21]. Cells were grown to confluence on collagen type I coated Bioflex plates (Flexcell International Corp., Hillsborough, NC, USA) for 7 days. Static stretch was applied to Bioflex plates with an FX-3000 Flexercell Strain Unit (Flexcell International Corp.), increasing the percent elongation (degree of stretch) from zero to 20% during the first hour, then holding at 20% elongation for 4 hours. Control plates were not subjected to stretch. At the beginning of stretch application or the control period, recombinant EGF (20 ng/mL) or vehicle was added to the medium. Cell monolayers were fixed with 3.7% paraformaldehyde/PBS and permeabilized with ethanol/acetic acid as described previously [5]. Cloning rings (10 mm) were used to isolate areas on the flexible membrane for TUNEL staining with ApopTag Red (Chemicon International), and staining was performed according to manufacturer's recommendations. Coverslips were mounted with Aqua Polymount (Polysciences, Inc., Warington, PA, USA). Positively stained nuclei were counted at 200× magnification in at least 10 nonoverlapping fields from triplicate wells to determine the mean number of apoptotic nuclei per field. Images were captured using a Leica DMIRE2 fluorescent microscope fitted with a Retiga Exi digital camera (Q-Imaging, Burnaby, BC, Canada) and Simple PCI software (Compix, Inc., Imaging Systems, Cranberry Township, PA, USA).

Statistical analysis

Data are presented as mean ± standard error. Student t test for unpaired variables was used to compare mutants with wild type and/or heterozygotes (Tables 1 and 2, Figs. 2 and 3), and EGF knockout groups in Figure 4. Student t test for paired variables was used to compare left and right kidneys in Table 2 and Figures 2 and 3. Two-way analysis of variance (ANOVA) was performed for waved-2 data in Figure 4B, and cell-stretch data in Figure 7B. Statistical significance was defined as $P < 0.05$.

Table 2. Kidney weight of mice

	Wild type or heterozygote		Homozygous mutant	
	UUO	Intact	UUO	Intact
7-day-old EGF deletion	27 ± 0.9[a]	31 ± 1.3	29 ± 1.6	32 ± 1.4
7-day-old wa-2	27 ± 0.7[a]	30 ± 1.6	23 ± 2.2[a]	29 ± 1.7
14-day-old EGF deletion	22 ± 1.9[a]	69 ± 1.7	21 ± 1.1[a]	56 ± 6.1
14-day-old wa-2	24 ± 1.0[a]	67 ± 2.4	29 ± 3.4[a]	65 ± 5.6
14-day-old wa-2 + EGF	17 ± 1.5[a]	69 ± 1.6	21 ± 3.0[a]	59 ± 4.4[b]

[a] $P < 0.05$, left kidney vs. right kidney (paired t test).
[b] $P < 0.05$ vs. wild type or heterozygote (unpaired t test).

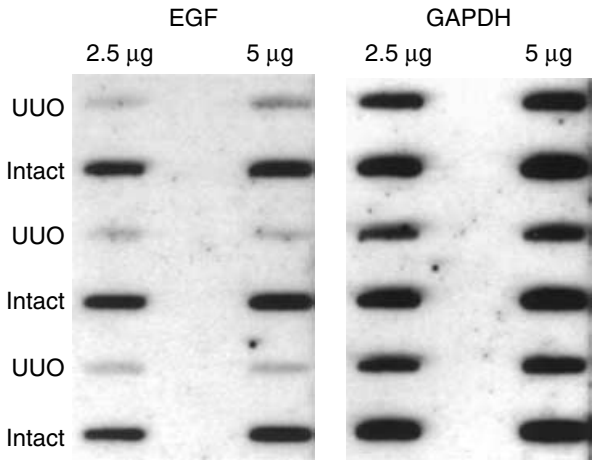

Fig. 1. Relative abundance of mRNA for EGF and GAPDH from obstructed (UUO) and intact kidneys of 14-day-old C57BL/6J wild-type mice. Either 2.5 µg or 5.0 µg RNA was loaded in each slot. Each pair of UUO and intact kidney RNA was obtained from a different animal ($N = 3$).

RESULTS

Compared to the intact kidney, or to that of sham-operated mice, the expression of endogenous EGF (factored for GAPDH) was reduced 78-fold after 14 days of UUO in wild-type mice (Fig. 1). Previous studies also showed suppression of EGF expression in the kidneys of neonatal rats with UUO [4]. This provided a rationale for administering exogenous EGF, which markedly attenuated renal tubular injury in neonatal rats with UUO [11]. The administration of exogenous EGF to neonatal mice with UUO reduced the kidney weight/body weight ratio relative to vehicle control animals (Fig. 2A). In contrast to rats, EGF tended to increase tubular apoptosis (Fig. 2B) and tubular atrophy (Fig. 2C) in neonatal mice relative to vehicle control. To further investigate this apparent species difference, and to evaluate the role of endogenous EGF, neonatal mutant mice that lack functional EGF (EGF knockout), and mice with functionally deficient EGF receptors (waved-2) were subjected to UUO.

As shown in Table 1, body weight for wild type or heterozygous mice tended to be greater than for homozygous mutants at both 7 and 14 days of age, and the differences were statistically significant for 7-day-old waved-2 mice, and for 14-day-old waved-2 mice receiving exogenous EGF. The absolute kidney weight data provided in Table 2 indicates that suppression of renal growth resulting from UUO is not significantly altered by EGF deletion or by the waved-2 mutation. In contrast, kidney weight/body weight ratio was 18% greater for the obstructed kidney of 7-day-old EGF knockout mice than for that of the control obstructed kidney (Fig. 3A), 30% greater than controls for the obstructed kidney of 14-day-old EGF knockout mice (Fig. 3C), 24% greater than controls for the obstructed kidney of 14-day-old waved-2 mice (Fig. 3D), and 53% greater than controls for 14-day-old waved-2 mice receiving EGF (Fig. 3E). There was also an increase in kidney weight/body weight ratio for the intact kidney of EGF knockout compared to control mice (Figs. 3A and C). Regardless of genotype, growth of the obstructed kidney at 14 days was significantly impaired in the obstructed compared to the contralateral kidney (Figs. 3C, D, and E).

Compared to wild-type animals, renal tubular apoptosis in 14-day-old mice with UUO was reduced by 50% in EGF knockout animals (Fig. 4A). A similar reduction in apoptosis was found in waved-2 homozygous mutant mice compared to combined wild type and heterozygous animals. However, the administration of exogenous EGF to waved-2 homozygous mice prevented the reduction in apoptosis, which was not different from the response of combined wild type and heterozygous mice (Fig. 4A). There was a trend to reduced tubular atrophy in the obstructed kidney of 14-day-old EGF knockout and waved-2 homozygotes compared to heterozygotes or wild-type mice, but differences were not significant (Fig. 4B). The administration of exogenous EGF doubled tubular atrophy in the obstructed kidney regardless of genotype ($P < 0.05$) (Fig. 4B).

Combined results from the wild type and mutant mouse studies indicate that detrimental effects of EGF are mediated by EGF binding to its receptor, even a receptor with diminished tyrosine kinase activity. This led us to question whether there might be species differences in

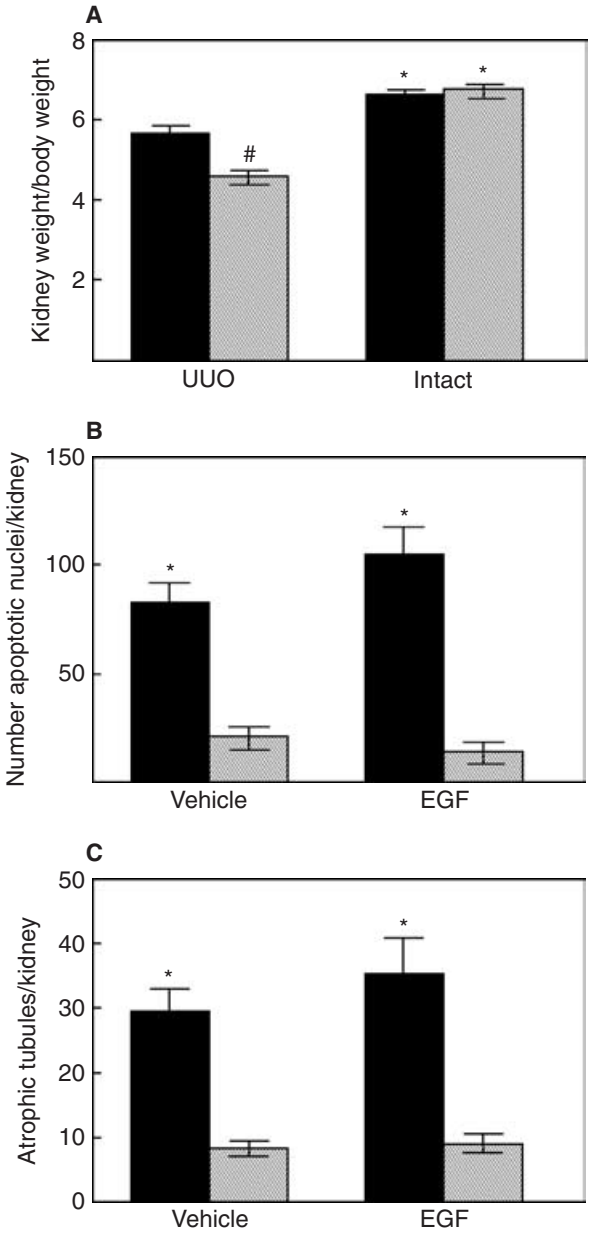

Fig. 2. Effects of exogenous EGF administered to wild-type C57BL/6J neonatal mice. (*A*) Kidney weight/body weight ratio (mg/g) for left obstructed (UUO) and right (intact) kidneys harvested from 10-day-old mice. Solid bars represent vehicle-treated animals and hatched bars represent EGF-treated animals. $^*P < 0.05$, intact vs. UUO, $\#P < 0.05$, vehicle vs. EGF-treated (UUO). (*B*) Number of apoptotic cells per field, measured by TUNEL assay in 10-day-old vehicle- or EGF-treated mice. Solid bars, left obstructed (UUO) kidneys; hatched bars, right (intact) kidneys. $^*P < 0.05$, UUO vs. intact. (*C*) Number of atrophic tubules per field, identified by thickened tubular basement membranes on PAS-stained sections of 10-day-old mice. Groups and legends as for (B). $^*P < 0.05$, UUO vs. intact.

EGF receptor expression or localization in the developing kidney. In sham-operated kidneys of 14-day-old wild-type C57BL/6J mice, the EGF receptor was abundantly expressed in the basal membrane folds of proximal tubules, as shown in serial sections (Fig. 5A and B). In contrast, the EGF receptor was detected in apical and lateral membranes of dilated tubules in 14-day-old obstructed mouse kidneys (Fig. 5C). The pattern of the EGF receptor expression was similar in 14-day-old rat kidneys: basal membranes of proximal tubules in sham-operated kidneys (Fig. 5D and E) and apical membranes of dilated tubules in UUO kidneys. Although no species differences were detected in 14-day-old kidneys, striking differences were apparent in 3-day-old sham kidneys. In 3-day-old vehicle control mouse kidneys, the EGF receptor was detected in membranes of glomerular cells and the apical membranes of some tubules (Fig. 6A). Twenty-four hours after exogenous EGF administration, expression of the EGF receptor in 3-day-old mouse kidneys increased, and was detected in the basal folds of proximal tubules (Fig. 6B). In contrast, abundant EGF receptor expression was already localized in the basal membranes of 3-day-old rat proximal tubules, and exogenous EGF treatment caused no apparent change in expression level or localization (Fig. 6C and D). This suggests that developmental timing of EGF receptor expression may be different in the 2 rodent species.

To determine if EGF-induced apoptosis is an intrinsic response of mouse tubular cells, we used an in vitro culture model. Axial strain applied to cultured mouse tubular cells increased apoptosis by more than 2-fold (Fig. 7A and B). The addition of exogenous EGF further increased apoptosis in both control and stretched cells ($P < 0.05$), and this increase was independent of mechanical stretch–induced apoptosis (Fig. 7B).

DISCUSSION

The present study demonstrates that tubular apoptosis was reduced in the obstructed kidneys of EGF null mutants or waved-2 mutant mice (EGF-receptor kinase mutants) relative to obstructed kidneys of wild type or heterozygote controls. Administration of exogenous EGF increased renal apoptosis in waved-2 mice, and increased tubular atrophy in wild type, heterozygous, or mutant waved-2 mice. The latter is consistent with tubular apoptosis, contributing to progressive tubular atrophy [22]. Moreover, enhancement of apoptosis by exogenous EGF in cultured mouse tubular cells subjected to axial strain contrasts with the suppression of apoptosis by EGF in stretched rat tubular cells [14]. This indicates that the contrasting species-specific renal tubular responses to EGF are maintained in immortalized cultured cells.

We have shown in both neonatal rats and mice subjected to complete UUO that the obstructed kidney fails to grow [4, 5]. We have also recently reported that severe partial UUO impairs renal growth in proportion to the severity of obstruction [23]. While lack of endogenous

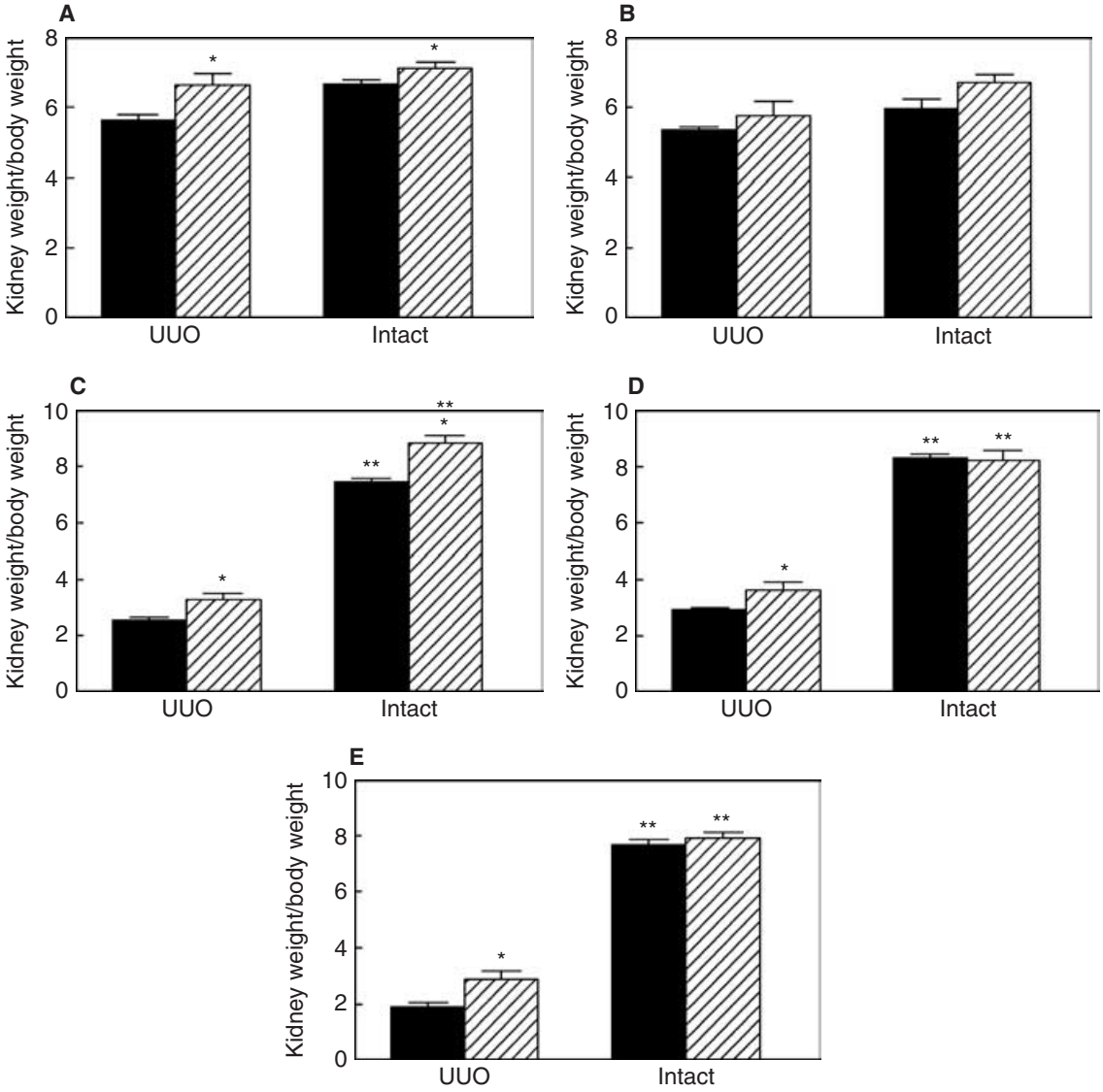

Fig. 3. Kidney weight/body weight ratio (mg/g) for left obstructed (UUO) and right (intact) kidneys of neonatal mice. Solid bars represent wild type or combined wild type and heterozygous mice; hatched bars represent homozygous mutant mice. (*A*) Seven-day-old EGF knockout mice; (*B*) 7-day-old waved-2 mice; (*C*) 14-day-old EGF knockout mice; (*D*) 14-day-old waved-2 mice; (*E*) 14-day-old waved-2 mice receiving exogenous EGF. $^{*}P < 0.05$ vs. wild-type or heterozygous mice, $^{**}P < 0.05$ vs. contralateral kidney (UUO).

EGF increases the relative growth of the obstructed or contralateral kidney (Figs. 3A and C), exogenous EGF impairs the growth of the obstructed mouse kidney (Fig. 2A). Somatic growth tended to be reduced in the murine mutants compared to wild type or heterozygous controls (Table 1). While the administration of exogenous EGF to adult rats increases kidney growth [24], EGF impairs renal and hepatic growth of neonatal rats, but does not affect growth of heart or liver [25]. Treatment of normal neonatal mice with EGF inhibits renal cellular proliferation [26], a finding that is consistent with the present study. Taken together, these results suggest that the effects of EGF on growth are organ-specific, and developmentally determined.

Both EGF mRNA and immunoreactive EGF are detectable in the distal nephron of the mouse by 3 to 5 days postnatally [27]. By the second postnatal week, EGF is expressed by medullary as well as cortical regions, and is localized to the thick ascending limb of Henle and distal convoluted tubule [27]. In the mouse, renal EGF content increases 20-fold from 1 to 3 weeks of life, and peaks at 5 to 7 weeks [28]. As shown in the present study, chronic UUO markedly suppresses the developmental increase in renal EGF expression. In the rat, EGF is expressed in proximal tubules by gestational day 19, and in distal tubules by postnatal day 7 [29]. As in the mouse, there is a progressive increase in renal EGF expression throughout the first month of life in the rat, which is suppressed

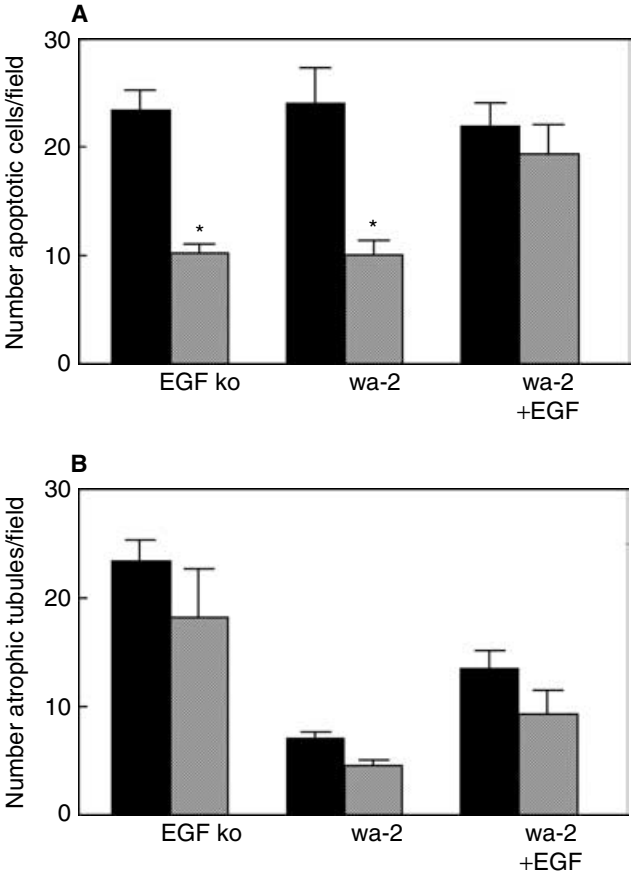

Fig. 4. Effect of mutations on markers of tubular cell injury. (*A*) Number of apoptotic cells per field, measured by the TUNEL assay in the obstructed kidney of 14-day-old mice. EGF ko, EGF knockout; wa-2, waved-2; wa-2 + EGF, waved-2 mice receiving exogenous EGF. Solid bars represent wild type or combined wild type and heterozygous mice; hatched bars represent homozygous mutant mice, $^*P < 0.05$ vs. wild-type or heterozygous mice. (*B*) Number of atrophic tubules per field, identified by thickened tubular basement membranes on PAS-stained sections in the obstructed kidney of 14-day-old mice. Groups and legends as in Figure 3. Exogenous EGF administration increased tubular atrophy in both waved-2 and wild type combined with heterozygous control mice ($P < 0.05$).

by chronic UUO in the neonatal period [4]. There is an increase in EGF receptor abundance and activation in late gestation in the rat, falling toward adult levels during the first postnatal week [30]. In man, EGF is localized in S-shaped mesonephric vesicles of 6-week embryos, as well as in the blastemic caps of the metanephros [31]. EGF receptor is localized to the ureteric bud branches of the metanephros of the 6-week embryo, and to the collecting ducts by 11 weeks and later [31]. There are relatively minor differences between the DNA sequences of preproEGF in mouse, rat, and man, suggesting that species-specific differences in the response to EGF are due to variations in receptor signaling [32].

Despite the similarities across mammalian species in the renal development of EGF and its receptors, the dramatic contrasts in the cellular responses to EGF between rats and mice clearly have important implications if growth factors are to be used as therapeutic agents in renal disease. In the developing rat kidney, normal mesenchymal cell apoptosis in the nephrogenic zone and medullary papilla can be inhibited by exogenous EGF administration [9]. In this circumstance, interference with apoptosis that is part of normal developmental remodeling may prove injurious, as postulated for the renal anomalies of rats lacking the AT2 angiotensin receptor [33]. Using an in vitro model of rat metanephric development, exogenous EGF was shown to rescue mesenchyme from apoptosis, but did not lead to differentiation [34]. In a model of ischemic renal failure in the rat, exogenous EGF was shown to enhance renal tubular regeneration and repair by increasing tubular proliferation (apoptosis was not measured) [35]. In contrast, renal injury following renal ablation or renal ischemia in the mouse is attenuated in animals expressing a dominant-negative EGF receptor in the kidney (apoptosis was not measured in this study) [36]. Exposure of cultured mouse kidney cells to EGF induces de novo expression of Fsp1, a gene encoding a fibroblast-specific protein associated with epithelial-mesenchymal transformation in vivo [37]. This process, in turn, is likely to play a major role in the evolution of glomerular and interstitial fibrosis, which figures prominently in all chronic progressive renal disease. In a preliminary study, waved-2 mice were found to have markedly less nephrosclerosis and albuminuria than wild-type littermates receiving angiotensin II infusion and high-salt diet [abstract; Ciroldi M et al, *J Am Soc Nephrol* 14:617A, 2003). This effect was postulated to be mediated by transforming growth factor-α, an alternate ligand for the EGF receptor.

Heparin binding EGF (HB-EGF) is another ligand for the EGF receptor. Following complete UUO in the adult mouse, there is a transient rise in renal HB-EGF production, peaking 6 hours after UUO, and returning to baseline 24 hours after UUO [38]. Mouse tubular cells subjected to axial strain also increase HB-EGF production, while tubular cells transfected with HB-EGF were protected from stretch-induced apoptosis [38]. These results demonstrate that other EGF receptor ligands may, in fact, play a protective role in attenuating the tubular injury resulting from UUO in the mouse.

There has been significant interest in manipulating the EGF receptor as a means of reducing renal injury and dysfunction in animal models of polycystic kidney disease. There are a number of parallels between the renal lesion of obstructive nephropathy and cystic kidney diseases, including focal cystic dilatation of the tubule in the former, and tubular obstruction in the latter [39]. Moreover, in both obstructive nephropathy and cystic kidney disease, renal tubular expression of EGF is suppressed, the renin-angiotensin system is activated, and dilated tubules

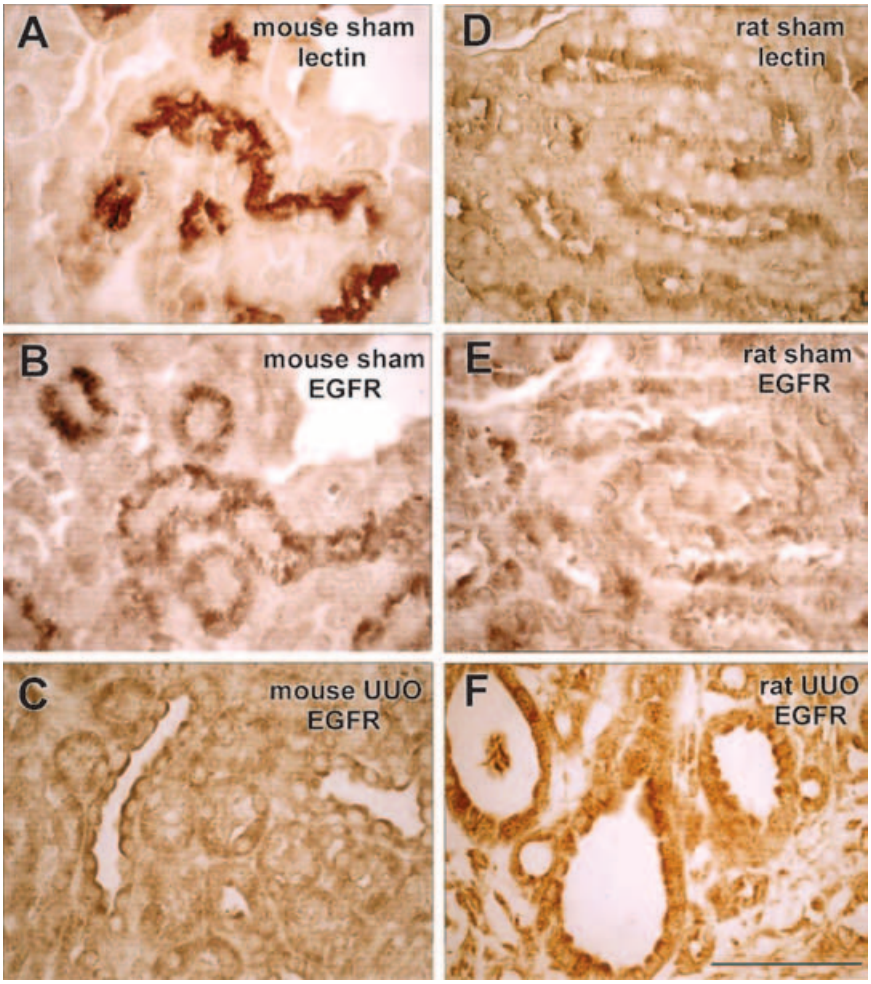

Fig. 5. Representative photomicrographs of 14-day-old neonatal mouse and rat kidneys. Left column: serial sections of sham-operated wild-type C57BL/6J mouse kidney stained with (*A*) *Lotus tetragonobulus* lectin, a marker for proximal tubule brush border in mouse, and (*B*) anti-EGF receptor (EGFR) antibody. (*C*) Obstructed (UUO) wild type mouse kidney stained with anti-EGFR antibody. Right column: serial sections of sham Sprague-Dawley rat kidney stained with (*D*) *Phaseolus vulgaris* erythroagglutinin lectin, a marker for proximal tubule brush border in rat, and (*E*) anti-EGFR antibody. (*F*) Obstructed rat kidney stained with anti-EGFR antibody. Bar inset, 50μm.

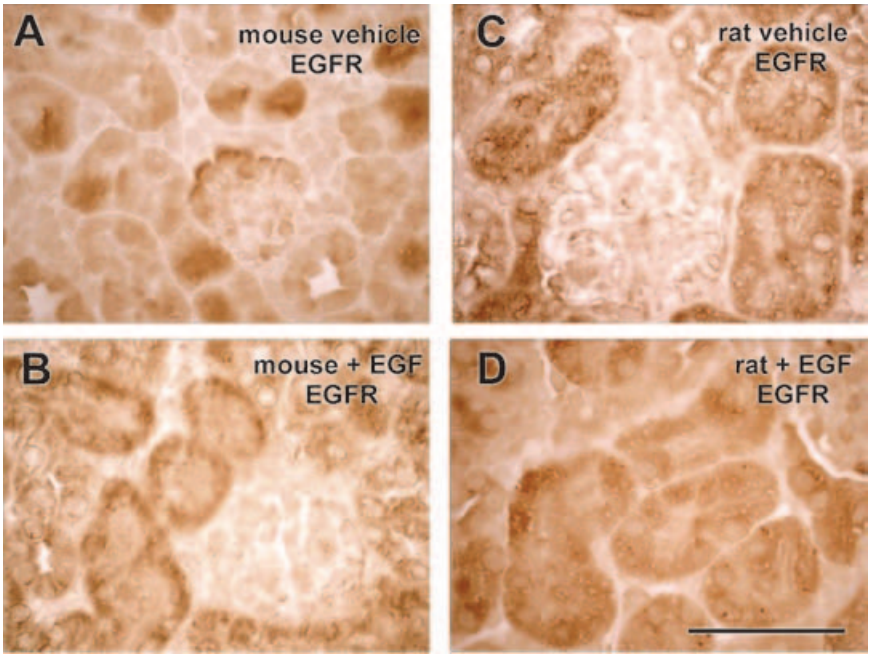

Fig. 6. Representative photomicrographs of EGF receptor localization in 3-day-old neonatal mouse and rat kidneys. Sham kidneys from neonatal wild-type C57BL/6J mice injected with saline vehicle (*A*) or EGF (*B*) 24 hours before harvest on day 3. Sham kidneys from neonatal rats injected with vehicle (*C*) or EGF (*D*) 24 hours prior to harvest. Bar inset, 50μm.

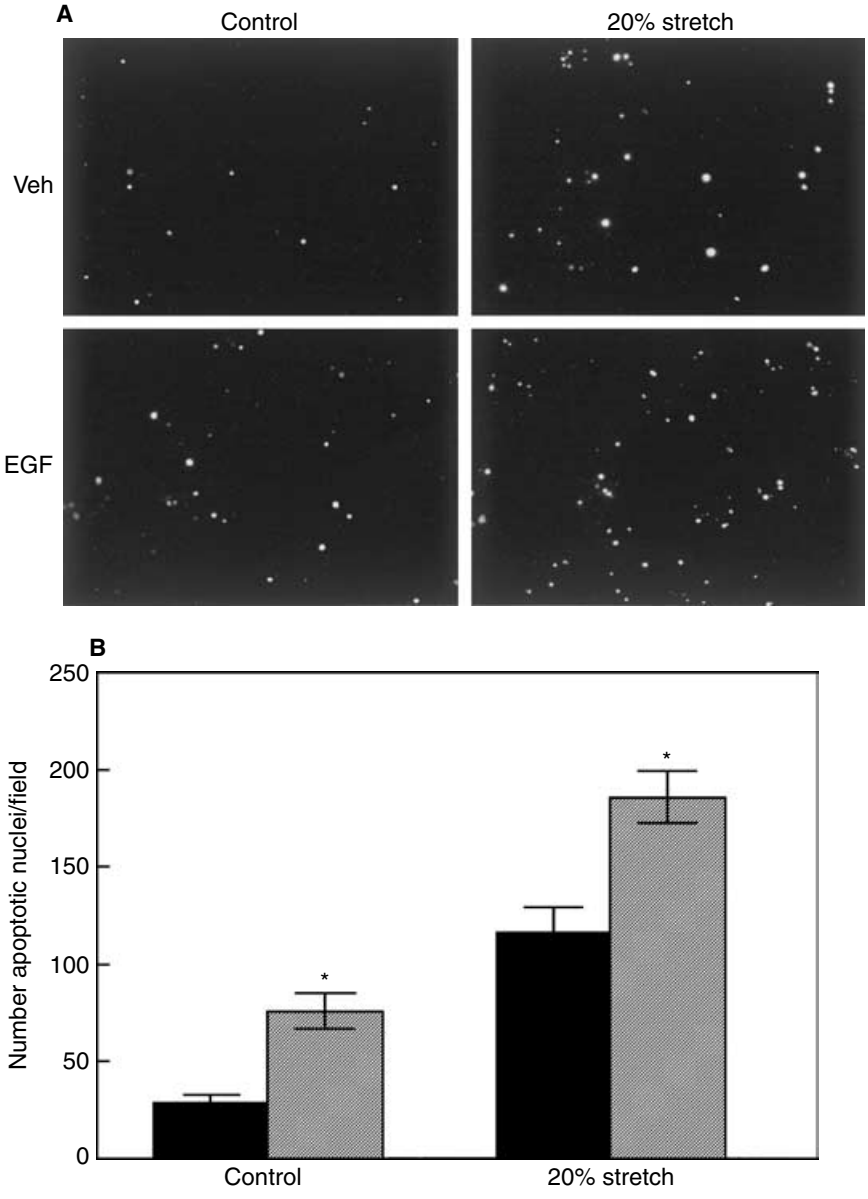

Fig. 7. Effect of EGF on control mouse PKSV-PR cell cultures versus cultures undergoing mechanical stretch. (*A*) Representative images of TUNEL staining for each experimental condition; control versus stretch and vehicle- versus EGF-treated cultures at 200× magnification. (*B*) Number of apoptotic nuclei per field for confluent mouse tubular epithelial cell monolayers subjected to 20% distention by continuous axial strain (right), or control nonstretched conditions (left). Solid bar, control cells; hatched bar, cells treated with exogenous EGF; $^{*}P < 0.05$ EGF-treated vs. control cells. Stretch increased apoptosis independent of EGF administration ($P < 0.05$).

undergo apoptosis [39]. Using a genetic approach, waved-2 mice were crossed with Oak Ridge polycystic kidney (orpk) mice with autosomal-recessive polycystic kidney disease [40]. The mice with cystic kidney disease carrying the waved-2 mutation had significant attenuation of collecting duct cysts and improved kidney function compared to homozygous orpk mice [40]. Interestingly, as demonstrated in the present study with chronic UUO, EGFR is localized to the apical surface of tubular cells in mice with cystic kidney disease [40]. Treatment of 2- to 3-week-old Balb/c polycystic kidney (bpk/bpk) mice with an EGF receptor tyrosine kinase inhibitor ameliorates this form of polycystic kidney disease [41]. These responses are consistent with our studies of UUO in waved-2 mice. However, treatment of 3- to 9-day-old bpk mice with EGF retards the development of azotemia, while extension of treatment beyond 9 days increases morbidity [42]. These reports suggest that, in addition to species differences, the maturational stage of the animal affects the tubular response to EGF.

In the present study, the administration of exogenous EGF to waved-2 mice increased apoptosis in the obstructed kidney similar to levels measured in wild type or heterozygous controls (Fig. 4A). EGF also increased tubular atrophy (Fig. 4B). Assuming that the waved-2 mutation markedly reduced the intrinsic tyrosine kinase activity of the EGF receptor, it is likely that coexpressed alternate receptors pair with the mutant receptor to rescue EGFR signaling. The epidermal growth factor receptor family consists of the EGF receptor (EGFR/HER1) and 3 alternate receptors, HER2/neu, HER3, and HER4 [43, 44]. HER2 has no ligand and is

the preferred heterodimerization partner for the other 3 family members. EGF binds primarily to EGFR, but at high concentrations can also bind with low affinity to the HER2/HER3 heterodimer [45, 46]. Heregulin binds to both HER3 and HER4. The EGF-induced high-affinity EGFR/HER2 heterodimer is relatively short-lived and EGFR is downmodulated upon dissociation from HER2 [43]. EGF-"activated" HER2 is then potentially free to couple with HER3 or HER4 to transmit EGF signaling laterally through these alternate receptors [43]. A recent study indicates that heregulin and its receptors are coexpressed in the developing mouse kidney: HER3 is abundant in ureteric bud epithelia of the fetal mouse kidney and disappears from proximal tubules between days 5 and 15 after birth [abstract; Polgar K et al, *J Am Soc Nephrol* 14:357A, 2003]. In neonatal rats, HER3 is detected only in the renal vasculature (M.S.F. and S.C.K., unpublished observation). Because EGF was administered on day 3 after birth and for 7 days afterward in our mouse studies, it is likely that HER3 was expressed on the proximal tubules during the EGF treatment period, and may have been activated by lateral signaling of HER2 or by direct binding of EGF to EGFR/HER3 or HER2/HER3 heterodimers [abstract; Kiley SC et al, *Mol Biol Cell* 15S:13a, 2004]. A constitutive src kinase activity in mouse, but not rat, kidney may activate HER2 in the absence of waved-2 EGFR kinase activity [abstract; Kiley SC et al, *J Am Soc Nephrol* 14:324A, 2003]. Once dissociated from the mutant EGFR, "activated" HER2 could potentially activate the heregulin/HER3 apoptosis pathway in neonatal mouse kidneys [44, 47].

CONCLUSION

In man, inhibition of EGF has been used to suppress tumor proliferation, or to induce apoptosis of malignant cells [48, 49]. On the other hand, exogenous EGF may be clinically therapeutic in promoting the repair of tissues damaged by bowel inflammation [50]. Based on studies in rats and mice, EGF-receptor tyrosine kinase inhibitors have been proposed as potential therapy for human polycystic kidney disease [41, 51]. The dramatic differences in the renal cellular response to EGF administered to neonatal mice compared to rats with chronic UUO highlight the importance of thoroughly understanding EGF-receptor signaling before embarking on clinical trials.

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Reprint requests to Robert L. Chevalier, M.D., Department of Pediatrics, Children's Medical Center University of Virginia, Box 800386, Charlottesville, VA 22908.
E-mail: RLC2M@virginia.edu

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