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Met and the epidermal growth factor receptor act cooperatively to regulate final nephron number and maintain collecting duct morphology

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Ureteric bud (UB) branching during kidney development determines the final number of nephrons. Although hepatocyte growth factor and its receptor Met have been shown to stimulate branching morphogenesis in explanted embryonic kidneys, loss of Met expression is lethal during early embryogenesis without obvious kidney abnormalities. *Met$^{fl/fl}$;HoxB7-Cre* mice, which lack Met expression selectively in the UB, were generated and found to have a reduction in final nephron number. These mice have increased Egf receptor expression in both the embryonic and adult kidney, and exogenous Egf can partially rescue the branching defect seen in kidney explants. *Met$^{fl/fl}$;HoxB7-Cre;wa-2/wa-2* mice, which lack normal Egfr and Met signaling, exhibit small kidneys with a marked decrease in UB branching at E14.5 as well as a reduction in final glomerular number. These mice developed progressive interstitial fibrosis surrounding collecting ducts with kidney failure and death by 3-4 weeks of age. Thus, in support of previous in vitro findings, Met and the Egf receptor can act cooperatively to regulate UB branching and mediate maintenance of the normal adult collecting duct.

KEY WORDS: Kidney, Met receptor, Ureteric bud, Branching, Mouse

INTRODUCTION

Kidney development occurs via a series of carefully orchestrated interactions between the ureteric bud and the adjacent metanephric mesenchyme. The ureteric bud branches to form the collecting system of the kidney (collecting duct through ureter), whereas the more proximal parts of the nephron (glomerulus through the connecting segment) develop via mesenchymal-epithelial transformation of induced mesenchyme at the tips of ureteric bud branches (Kobayashi et al., 2008; Saxen, 1987). The result of this interplay is that the final nephron number is determined by the signals that regulate ureteric bud branching. In vitro studies have demonstrated that a number of factors may play a role in either stimulating (Gdnf, Fgf2, Fgf7, Fgf10, Hgf, Egf and pleiotropin) or inhibiting (Bmp4, activin A and semiphorin) this process (Bates, 2007; Sainio et al., 1997; Sakurai et al., 2001; Tufro et al., 2007). However, in vivo studies using knockout technologies have, to date, supported only a few of these as key regulators of UB branching (reviewed by Costantini, 2006).

The process of UB branching requires the proliferation and migration of cells at the UB tip, as well as alterations in tissue morphology that create the branch point. This process has been partially modeled in vitro by first growing renal epithelial cells into cysts, and then stimulating them with factors that induce branching morphogenesis to form tubules sprouting from the cysts (Pollack et al., 1998). One of the factors that has been most heavily studied in these in vitro models of branching morphogenesis is Hgf (Montesano et al., 1991). Hgf binds to the Met tyrosine kinase receptor and activates downstream signaling via Erk, PI 3-kinase, Pkc, Plc, Src, Fak and Jak/Stat that are crucial for the cytoskeletal remodeling, focal adhesion turnover and cell-cell junction reshaping that is necessary for branching morphogenesis (Ishibe et al., 2006; Ishibe et al., 2004; Ishibe et al., 2003; Karihaloo et al., 2005; Liu et al., 2002; O'Brien et al., 2004; Ponzetto et al., 1994; Rosario and Birchmeier, 2004; Weidner et al., 1993).

In the developing kidney, Hgf is expressed by the metanephric mesenchyme whereas the Met receptor is present on both UB and mesenchymal cells (Woolf et al., 1995). Furthermore, in embryonic kidney explant studies, the addition of neutralizing antibodies that block endogenously produced Hgf inhibited explant growth and UB branching (Santos et al., 1994; Woolf et al., 1995). Based on these findings, it was predicted that Hgf signaling would play a significant role in the regulation of UB branching. However, loss of either Hgf or Met expression in the mouse led to death by embryonic day 12-13 due to liver and placental abnormalities (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). In these early embryos, limb bud development was significantly impaired but early kidney development, including initial UB branching, appeared to occur normally.

As UB branching continues through E18 (Saxen and Sariola, 1987), we used a conditional knockout approach to more accurately study the role of Hgf-Met signaling in the development of the kidney collecting system, and specifically in the regulation of final nephron number. Selective loss of Met receptor expression in the collecting system of the kidney was achieved using a Cre-loxP approach. The collecting system morphology was not demonstrably abnormal in these mice, but there was a 35% reduction in nephron number at 12 weeks of age and glomerular hypertrophy by 1 year of age. Examination of the collecting ducts revealed that there was sustained

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upregulation and activation of the Egf receptor, and addition of Egf to explanted kidneys from E12.5 *Met$^{fl/fl}$;HoxB7-Cre* mice rescued the decrease in ex vivo UB branching that was observed. *Met$^{fl/fl}$;HoxB7-Cre:wa-2/wa-2* mice lacking both Met and Egfr signaling in the collecting duct demonstrated a marked decrease in UB branching, small kidneys, renal failure and early death.

MATERIALS AND METHODS

Reagents

Antibodies to Met and Aqp2 were obtained from Santa Cruz Biotechnology (San Diego, CA, USA). Anti-Egf and E-cadherin antibodies were obtained from BD Biosciences (San Jose, CA, USA). Anti-pEgfr 992 and 1068 and pErk5 antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). *Dolichos biflorus* agglutinin (DBA) was obtained from Vector Laboratories (Burlingame, CA, USA).

Creation and genotyping of conditional Met knock-out mice

The *Met$^{fl/fl}$* mouse was developed on the 129SV/C57Bl/6 background as described (Huh et al., 2004). HoxB7-Cre mice on the C57Bl/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Tail genotyping was performed using the Met forward primer (F) 5′-tta ggc aat gag gtg tcc cac-3′ and reverse primer (R) 5′-cca ggt ggc ttc aaa ttc taa gg-3′. To detect deletion of Met in the collecting duct, primer 5′-cag ccg tca gac aat tgg cac-3′ and primer 5′-cca ggt ggc ttc aaa ttc taa gg-3′ were used. The expected sizes of wild-type allele, floxed allele and deleted allele were 380 bp, 300 bp and 650 bp, respectively. To determine Cre expression, CreF 5′-ccgggctgccacgaccaa-3′ and CreR 5′-ggcgcggcaacaacattttt-3′ primers were used, generating a 400 bp fragment. In all experiments, homozygous littermates from the same breeding pair were used as controls. Waved 2 (wa-2) mice on a C57BL/6JEiC3H/HeSnJ/CD-1 background were purchased from Jackson Laboratory. Genotyping of wa-2 mice was performed using wa-2F (5′-ata acc tga cac ttg tca gag tac-3′) and wa-2 R (5′-ttt gca atc tgc aca cac cag ttg-3′) primers followed by digestion with *Fok*I. The expected sizes for wild-type and wa-2 alleles are 326 bp and 160 bp, respectively. All mouse experiments were performed under approval of the Yale IACUC.

Protein isolation and western analysis

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg), kidneys extracted and renal papilla surgically removed. The papilla was homogenized in saline with EDTA-free protease inhibitor (Complete; Roche, Indianapolis, IN, USA) using a Dounce homogenizer and centrifuged at 100,000 ***g***. The pellet was resuspended in 100 μl of PBS and 40 μg of protein/sample separated by SDS-PAGE, electrophoretically transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA), immunoblotted with the appropriate antibody and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Pittsburgh, PA, USA).

Kidney immunofluorescence and histology

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine followed by perfusion fixation with 40 ml of 4% PFA. The kidneys were frozen, sectioned at 4 μm, and subjected to antigen retrieval (Retrievagen; BD Biosciences, San Jose, CA, USA) followed by blocking with 1% bovine serum albumin for 1 hour. Immunostaining was performed with the appropriate primary antibody overnight at 4°C, followed by Alexaflour donkey anti-rabbit 488 or donkey anti-goat 594 secondary antibodies for visualization. 4,6,-diamidino-2-phenylindole (DAPI) was included in the mounting medium as a counterstain (Vector Laboratories, Burlingame, CA, USA). For kidney histology, mice were perfusion fixed as above followed by Hematoxylin and Eosin or Trichrome staining performed by the Yale Pathology Department. E17 kidneys were harvested, fixed with 4% PFA and cryosections stained with anti-Egfr antibody and DBA lectin.

Calculation of glomerular size

Hematoxylin and Eosin stained sections from kidneys of 12-week-old and 1-year-old male mice were used to calculate the relative glomerular cross-sectional area. Quantitation of 15 randomly chosen juxtamedullary glomeruli was performed in a blinded fashion by two independent investigators on sections from two separate mice for each age and genotype. Image J software was used to determine the relative area in square pixels. To determine total glomeruli/kidney cross-section, all glomeruli in 15 sagittal sections from *Met$^{+/+}$*, *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{fl/fl}$;HoxB7-Cre;wa-2/wa-2* mice were counted and averaged.

Glomeruli isolation for quantitation of nephron number

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine then perfused through the heart with 7.5×10^7 dynabeads diluted in 40 ml Hanks Buffered Saline Solution. The kidneys were removed and digested in collagenase (1 mg/ml) containing 100 U/ml deoxyribonuclease I in HBSS at 37°C for 30 minutes with gentle agitation. The collagenase-digested tissue was pressed through a 100 μm cell strainer using a pestle and washed with 5 ml HBSS. The cell suspension was centrifuged at 200 ***g*** for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml HBSS. The suspension containing the dynabeads was harvested with a magnet and washed three times with HBSS and aliquots counted under the microscope in a blinded fashion.

Kidney explant culture

E12.5 embryos were harvested from pregnant *Met$^{fl/+}$;HoxB7-Cre* females mated with *Met$^{fl/+}$;HoxB7-Cre* males. To ensure that the gestational age was identical, only embryos from the same female were directly compared. The embryos were microdissected and kidneys cultured on a Transwell clear polyester filter (0.4 mm; Costar) with L-15 Leibovitz Medium for 2 days at 37°C with or without Egf (20 ng/ml; Sigma Chemical Company, St Louis, MO, USA). The embryonic kidneys were then incubated for 30 minutes with 0.050% saponin followed by 0.050% saponin with 0.1% gelatin overnight. Kidneys were then incubated with FITC-conjugated Dolichos biflorus (DBA) (Vector, Burlingame, CA, USA) at a 1:40 dilution for 24 hours at 37°C in the saponin/gelatin mixture, washed with saponin three times and then visualized using Nikon Epiflourescence Microscopy.

Terminal ureteric bud branches were quantified for each explant in a blinded fashion. Kidney size in square pixels was determined using Image J software in a blinded fashion. Embryos were genotyped as above using tail tissue.

Quantitative PCR

Kidneys were obtained on E14.5 from *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{+/+}$;HoxB7-Cre* littermates and total RNA isolated using the RNeasy kit (QIAGEN, Valencia, CA). Total RNA was extracted using the RNeasy Kit (QIAGEN) and 1 μg of RNA was reverse transcribed using random hexamer primers according to the manufacturer's instructions (SuperScript II, Invitrogen). qPCR was conducted using power SYBR green mix (Applied Biosystems) with a 7300 AB Real-time PCR machine (Applied Biosystems). The primers used for PCR were selected for an efficiency of 90-100%, details can be provided on request. Results for each factor were normalized to Gapdh expression from the same PCR reaction (dCt) and then the expression in *Met$^{fl/fl}$;HoxB7-Cre* kidneys plotted relative to expression in control *Met$^{+/+}$;HoxB7-Cre* kidneys (2^{-ddCt}). A value of 1 would indicate equal expression in both genotypes.

Clinical chemistry and statistics

Plasma and urine electrolytes were analyzed using the Yale University School of Medicine Core Mouse Metabolic Phenotyping Center. All data are expressed as mean±s.e.m. Statistical significance was determined using the Student's *t*-test.

RESULTS

Collecting duct-specific deletion of the Met receptor

Met$^{fl/fl}$ mice, in which exon 16 of the *Met* gene is flanked by loxP sites (Huh et al., 2004), were mated with *HoxB7-Cre* mice that express the Cre recombinase in the Wolffian duct and ureteric bud-derived structures (Yu et al., 2002) (Fig. 1A). Offspring that were heterozygous for the floxed Met allele (*Met$^{fl/+}$;HoxB7-Cre*) were mated to generate *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{+/+}$;HoxB7-Cre* offspring, confirmed by DNA genotyping of the tail (Fig. 1B). *Met$^{fl/fl}$;HoxB7-Cre* mice were born in the expected Mendelian

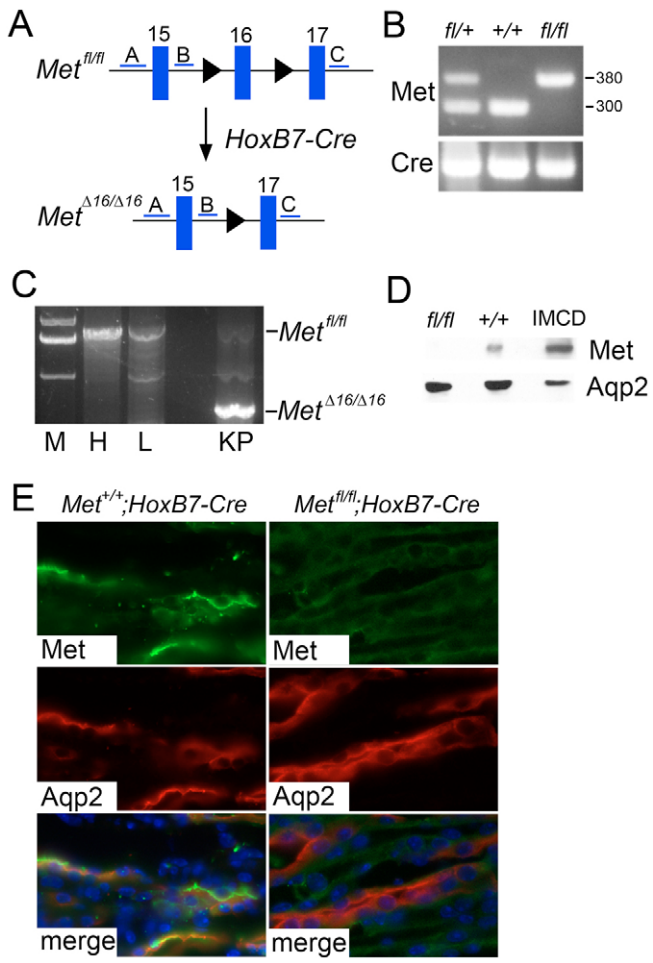

Fig. 1. Collecting duct knockout of Met expression. (**A**) Schematic of the *Met*$^{fl/fl}$ allele showing the location of the loxP sites flanking exon 16 and the primers (A,B,C) used for genotyping. (**B**) PCR of tail DNA using primers B and C reveals the expected band at 300 bp in wild-type mice, 380 bp in *Met*$^{fl/fl}$ mice, and both bands in heterozygotes. All three contain the Cre recombinase gene. (**C**) PCR of DNA from the heart (H), liver (L) and kidney papilla (KP) of *Met*$^{fl/fl}$*;HoxB7-Cre* mice using primers A and C reveals the selective excision of the floxed allele in the kidney to generate the smaller *Met*$^{Δ16/Δ16}$ fragment (M,DNA markers). A faint band is present at the expected size of the *Met*$^{fl/fl}$ allele in the papilla, presumably owing to the small number of thin limb cells present in this tissue. (**D**) Western analysis of renal papilla from *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice. Aqp2 was used as a loading control and immortalized IMCD cells were included as a positive control. (**E**) Immunostaining of the renal papilla from *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice with α-Met and α-Aqp2 reveals a strong Met signal in the collecting duct cells of wild-type mice that is markedly diminished in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice.

frequency, comprising 18% of the offspring (12/68). PCR analysis of DNA from the renal papilla (comprised primarily of collecting duct and thin limb segments), liver and heart revealed that exon 16 of *Met* had been deleted in cells from the renal papilla but not in other organs (Fig. 1C).

Western blot analysis of renal papilla confirmed that Met protein expression was markedly diminished in this region of the *Met*$^{fl/fl}$*;HoxB7-Cre* mice (Fig. 1D), as had been previously reported for Met expression in hepatocytes of the *Met*$^{fl/fl}$*;AlbCre* mouse (Huh et al., 2004). Immunofluorescent staining of kidney cryosections from *Met*$^{+/+}$*;HoxB7-Cre* mice revealed that Met is most highly expressed both apically and basolaterally in collecting duct cells of the papilla (Fig. 1E) and in cells of the proximal tubule (data not shown), consistent with previous reports (Konda et al., 2004; Liu et al., 1996). *Met*$^{fl/fl}$*;HoxB7-Cre* mice exhibited a marked decrease in Met protein expression in the collecting duct cells.

Adult *Met*$^{fl/fl}$*;HoxB7-Cre* mice exhibit decreased nephron number and glomerular hypertrophy

Met$^{fl/fl}$*;HoxB7-Cre* mice appeared to grow and behave normally. At 12 weeks of age there were no significant differences between *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* littermates with regards to body weight, plasma electrolytes or urinary electrolytes (Table 1). The kidneys of *Met*$^{fl/fl}$*;HoxB7-Cre* mice appeared grossly normal and renal histology revealed no obvious abnormalities in cortical architecture or collecting duct morphology (see Fig. S1 in the supplementary material), with no increase in interstitial fibrosis detected on trichrome stained sections (data not shown). Of note, the glomeruli demonstrated a slight increase in mesangial cellularity without evidence for sclerosis or mesangiolysis.

Hematoxylin and Eosin stained sections of kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre* mice at 1 year of age revealed that the glomeruli appeared larger than those from *Met*$^{+/+}$*;HoxB7-Cre* littermates (Fig. 2A). Quantification of average glomerular surface area confirmed that juxta-medullary glomeruli from 1-year-old *Met*$^{fl/fl}$*;HoxB7-Cre* mice were larger than those from either 1-year-old *Met*$^{+/+}$*;HoxB7-Cre* littermates or 12-week-old *Met*$^{fl/fl}$*;HoxB7-Cre* mice (Fig. 2B). In these older *Met*$^{fl/fl}$*;HoxB7-Cre* mice, there was no evidence of either an increase in glomerular or interstitial fibrosis or an increase in albuminuria (data not shown).

Glomerular hypertrophy can occur in the setting of a reduction in total nephron number owing to sustained hyperfiltration (Cullen-McEwen et al., 2003; Hostetter et al., 1981; Novick et al., 1991). Glomeruli from 12-week-old *Met*$^{fl/fl}$*;HoxB7-Cre* mice were quantified using magnetic bead isolation as described by Takemoto et al. (Takemoto et al., 2002) (Fig. 2C). Kidneys from *Met*$^{+/+}$*;HoxB7-Cre* mice contained 20,912±252 glomeruli/mouse [consistent with previous reports for wild-type mice (Takemoto et al., 2002)], whereas *Met*$^{fl/fl}$*;HoxB7-Cre* mice had an average of only 13,660±600 glomeruli/mouse (Fig. 2D).

UB branching is reduced in explants of *Met*$^{fl/fl}$*;HoxB7-Cre* mice

As final nephron number is determined by ureteric bud branching, we examined the possibility that UB branching is reduced in *Met*$^{fl/fl}$*;HoxB7-Cre*. *Met*$^{fl/+}$*;HoxB7-Cre* heterozygous mice were

Table 1. Physiology of *Met* mutant mice

	Met$^{+/+}$*; HoxB7-Cre*	*Met* $^{fl/fl}$*; HoxB7-Cre*
Blood urea nitrogen (mg/dl)	20.8±2.2	19.9±3.3
Plasma Na^+ (mM)	149±2.6	148.3±1.5
Plasma K^+ (mM)	3.2±0.4	3.2± 0.7
Plasma Cl^- (mM)	106.7±4.8	105.7±1.8
Urine Na^+ (mM)	34.5±10.2	39.7±16.2
Urine K^+ (mM)	141.7±47.2	123.7+55.8
Urine Cl^- (mM)	153.5±66.9	131.3±59.7
Body weight (female, g)	18.7±0.1	18.5±0.8
Body weight (male, g)	26.2±2.0	27.9±1.2

Values are mean ± s.e.m., *n*=3 for urine and plasma electrolytes for each genotype and *n*=6 for male and female body weight at 12 weeks of age for each genotype. None of the differences is significant.

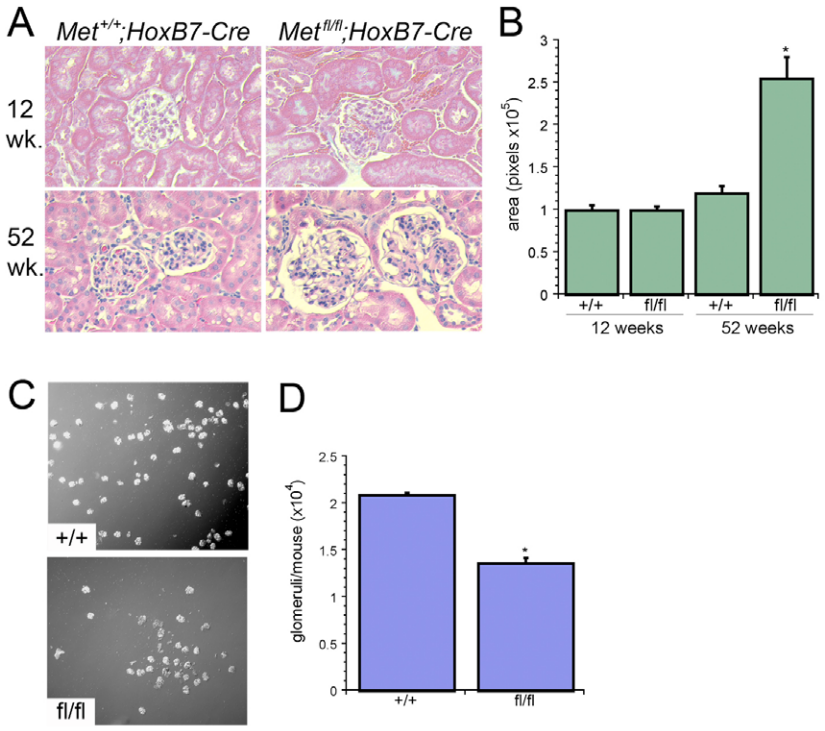

Fig. 2. *Met*$^{fl/fl}$*;HoxB7-Cre* mice have reduced nephron number. (**A**) Representative images of glomeruli at 12 and 52 weeks from *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice, as indicated. (**B**) Quantification of glomerular surface area (in pixels) from kidney sections as in A (n=15 glomeruli/genotype, *P<0.001 versus *Met*$^{+/+}$*;HoxB7-Cre* glomeruli). (**C**) Representative image of isolated glomeruli from *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice. (**D**) Quantification of total glomeruli/mouse in *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mouse (n=5 mice/group, *P<0.001).

mated and embryonic kidneys harvested at E12.5. Consistent with previous reports (Bladt et al., 1995), E12.5 kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre* embryos were indistinguishable from that seen in *Met*$^{+/+}$*;HoxB7-Cre* littermates (Fig. 3A, quantified in Fig. 3B,C), demonstrating that early UB branching is intact in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice. Examination of kidneys harvested at E14.5 also revealed no difference in size (Fig. 3B), although accurate quantification of UB branching could not be performed at this later time point. However, when E12.5 kidneys were explanted and cultured for 48 hours in the absence of exogenous growth factors, kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre* embryos grew less well and exhibited significantly less ureteric bud branches when compared with *Met*$^{+/+}$*;HoxB7-Cre* littermates (Fig. 3A bottom panels, quantified in Fig. 3D,E).

It has been found that knockout of a single gene may lead to the upregulated expression of related or redundant pathways, resulting in phenotypic compensation. To address this possibility, *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* embryonic kidneys were harvested at E14.5 and total RNA isolated followed by quantitative PCR to define expression levels of a number of factors known to potentially regulate UB branching. These experiments revealed that the expression of two factors that positively regulate branching were increased in the *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys: Egfr and Fgf2 (Fig. 3G). There were also modest reductions in the expression of negative regulators of branching such as Bmp4 and 7 that did not reach statistical significance. Expression of Fgf1, Fgf7, Fgf10, Fgfr2, Gdnf, heregulin (Hrg) and pleiotrophin (Ptn) were unaltered.

Egf can stimulate in vitro morphogenic responses similar to those described for Hgf (Sakurai and Nigam, 1998; Sakurai et al., 1997b). As we have previously found that immortalized cells derived from kidneys of E12.5 *Met*$^{-/-}$ embryos express the Egfr and exhibit branching morphogenesis in response to Egf stimulation (Kjelsberg et al., 1997), we examined the possibility that increased Egfr signaling in *Met*$^{fl/fl}$*;HoxB7-Cre* embryonic kidneys might be providing a compensatory UB growth and branching stimulus that accounts for the relatively modest loss of final nephron number in the adult *Met*$^{fl/fl}$*;HoxB7-Cre* mouse. To determine whether the addition of Egf could rescue the defect in UB branching seen in the explanted *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys, some explants were treated with Egf (20 ng/ml) in addition to the defined medium. Both *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* explants responded to exogenous Egf with increased UB branching and increased kidney size (Fig. 3F, quantified in Fig. 3D,E), resulting in *Met*$^{fl/fl}$*;HoxB7-Cre* explants in the presence of Egf being indistinguishable from *Met*$^{+/+}$*;HoxB7-Cre* control kidneys.

Increased Egf receptor expression and activation in kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre* mice.

The observation that EGF could rescue the decreased branching morphogenesis seen in explanted *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys led us to determine whether or not this pathway was upregulated in vivo in the absence of Hgf-Met signaling. Immunostaining of E17 kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre* mice revealed increased Egfr expression in multiple cells including the UB (arrows) and renal vesicles (arrowhead) as compared with wild-type mice (Fig. 4A). This staining was seen on basolateral as well as apical surfaces of the cells. Western analysis of papilla isolated from 6-week-old mice confirmed an increase in Egf receptor expression in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice (Fig. 4B).

Immunoblotting with antibodies that detect phosphorylation of the Egfr at the autophosphorylation sites Y992 and Y1068 revealed increased Egf receptor phosphorylation in *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys (Fig. 4C,D). Of note, there was no detectable difference in phosphorylation at tyrosine 845 (data not shown). The Y1068 site has been shown to be heavily phosphorylated following receptor activation with Egf or heparin-binding Egf (HB-EGF) and to mediate Grb2 binding and Erk1/2 activation (Ward et al., 1996; Wu et al., 2004; Yamauchi et al., 1998). A second MAPK family member, Erk5 (also known as Bmk1), is activated downstream of Egfr activation and has been shown to positively regulate both

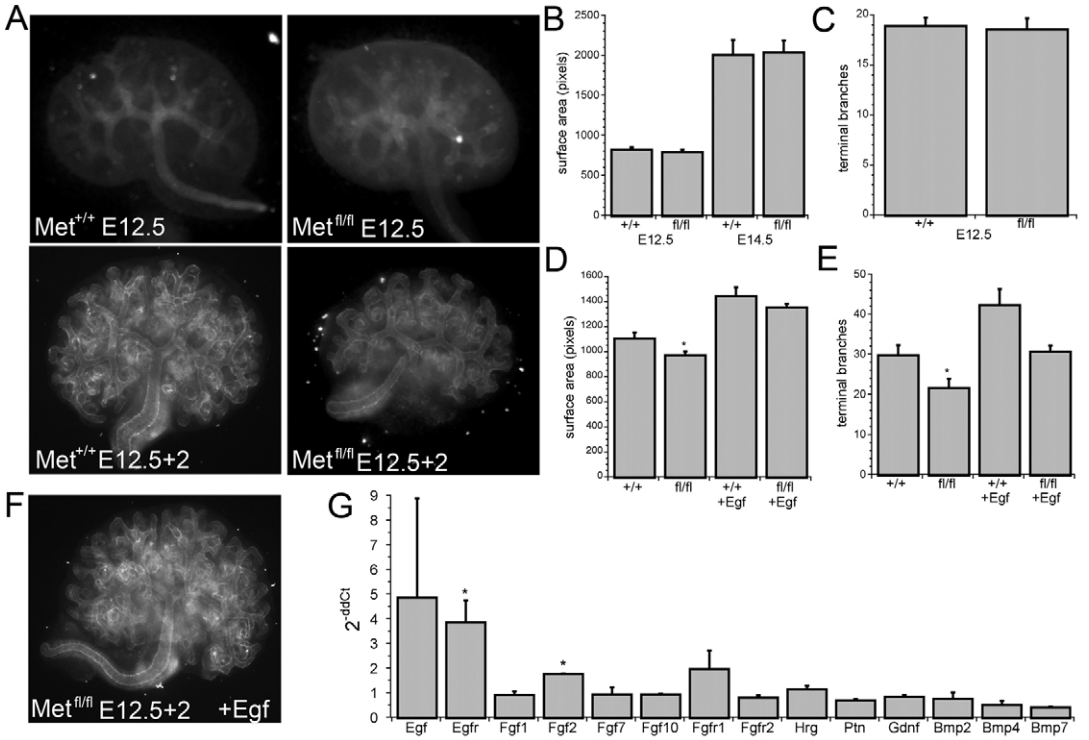

Fig. 3. *Met*$^{fl/fl}$*;HoxB7-Cre* kidney explants have decreased ureteric bud branching. (**A**) Representative images of E12.5 kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice (top panels) and E12.5 kidneys grown for 2 days in explant culture (bottom panels), as indicated, and stained with Dolichos biflorus (DBA). (**B**) Quantification of kidney surface area (in pixels) from E12.5 (n=7) and E14.5 (n=5) kidneys. (**C**) Quantification of terminal UB branches from E12.5 kidneys stained as in A (n=7). (**D**) Quantification of surface area of E12.5 kidneys grown as in A for 2 days in explant culture with or without Egf (n=10, *P<0.03 versus +/+ and P<0.001 versus fl/fl+Egf). (**E**) Quantification of terminal UB branches from E12.5 kidney explants grown as in A for 2 days in culture with or without Egf (n=12, *P<0.02 versus +/+ and P<0.01 versus fl/fl+Egf). (**F**) Representative image of E12.5 *Met*$^{fl/fl}$*;HoxB7-Cre* explant as in A cultured in the presence of Egf. (**G**) Real-time PCR for mRNA levels of the indicated factors in E14.5 *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys expressed relative to *Met*$^{+/+}$*;HoxB7-Cre* E14.5 kidneys. n=3 for each factor. *P<0.05 versus *Met*$^{+/+}$*;HoxB7-Cre* kidneys.

proliferative and morphogenic actions of Egf (Karihaloo et al., 2001; Kato et al., 1998). Immunoblotting with an antibody that detects the phosphorylated form of Erk5 confirmed that this downstream effector is activated in papillary cells from *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys (Fig. 4E). These results demonstrate that the Egfr is upregulated and activated in the absence of normal Met signaling by UB-derived structures, and that this activation is maintained even after development is complete.

Loss of Egfr signaling in *Met*$^{fl/fl}$*;HoxB7-Cre* mice results in decreased UB branching and renal failure

Cumulatively, these observations suggest that upregulation of Egfr signaling might act to partially compensate for the lack of Hgf-Met signaling in *Met*$^{fl/fl}$*;HoxB7-Cre* mice, resulting in the relative preservation of UB branching and final nephron number observed in these animals. To test this possibility, *Met*$^{fl/fl}$*;HoxB7-Cre* mice were mated with waved-2 (wa-2) mice that have a spontaneous point mutation in Egfr that decreases receptor activation (Fowler et al., 1995; Luetteke et al., 1994). These mice exhibit decreased lactation, curly (waved) hair and small size, but are otherwise phenotypically normal with normal kidney morphology and function (Wang et al., 2003). Consistent with data from liver homogenates of *wa-2* mice (Luetteke et al., 1994), stimulation with Egf failed to induce significant Egfr phosphorylation in kidneys from these mice (data not shown).

Male and female *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/+* mice were viable and fertile. These mice were mated and pregnant females sacrificed for embryo harvest at E14.5. Kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* embryos were found to be substantially smaller with markedly reduced UB branches when compared with *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ littermates (Fig. 5A,B). To determine whether these kidneys could respond to exogenous Egf, embryos were harvested on E12.5 and kidneys maintained in explant culture for 2 days with or without Egf. Kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* embryos grown in control media exhibited less surface area and less ureteric bud branching when compared with *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ kidneys (Fig. 5C, quantified in Fig. 5D,F), and treatment with Egf failed to rescue kidney growth or ureteric bud branching in the explanted *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* kidneys (Fig. 5E, quantified in Fig. 5D,F).

Although compound heterozygotes were born at the expected Mendelian frequency, only seven viable pups were obtained with the *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* genotype rather than the predicted 19 (Fig. 6A). *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice were small in size compared with heterozygous littermates at 3 weeks (7.9±0.7 g versus 12.8±0.5 g, P<0.001). Kidneys from these mice were small compared with either wild-type or *wa-2* mice (Fig. 6B, *Met*$^{+/+}$*;Egfr*$^{+/+}$=0.036 g; *Met*$^{+/+}$*:wa-2/wa-2*=0.032 g; *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2*=0.011 g) and contained substantially fewer glomeruli/cross-section than did wild-type, *wa-2* or *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys (Fig. 6C). Kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/+*

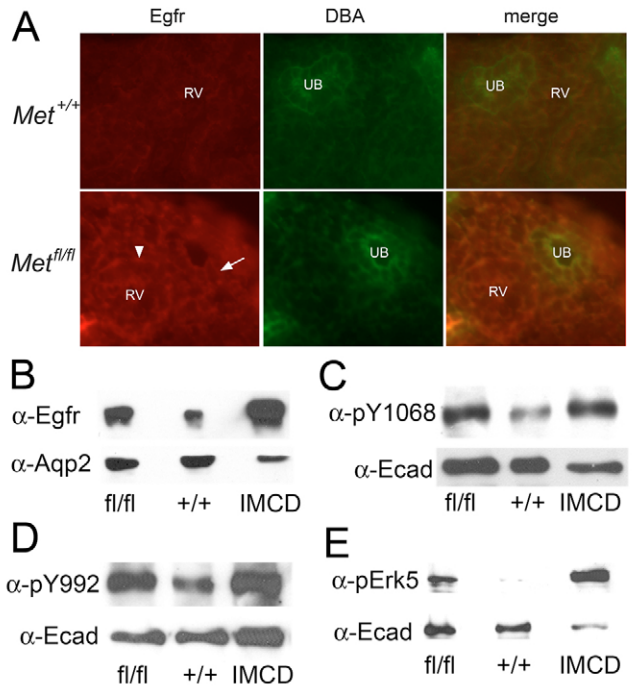

Fig. 4. Increased expression and activation of Egf receptor in UB-derived cells of *Met*$^{fl/fl}$*;HoxB7-Cre* mice. (**A**) Immunostaining of E17 explant cryosections from *Met*$^{+/+}$*;HoxB7-Cre* and *Met*$^{fl/fl}$*;HoxB7-Cre* mice with α-Egfr and DBA. Cells from the DBA-positive UB tips (arrow) as well as the adjacent renal vesicle (arrowhead, RV) and metanephric mesenchyme show increased Egfr fluorescence. (**B-E**) Western blot analyses of renal papilla obtained from 6-week-old *Met*$^{+/+}$*;HoxB7-Cre* and *Met*$^{fl/fl}$*;HoxB7-Cre* mice. (B) Immunoblotted with α-Egfr and α-aquaporin-2, (C) α-pEgfr (Y1068) and α-E-cadherin, (D) α-pEgfr (Y992) and α-E-cadherin, (E) α-pErk5 and α-E-cadherin.

heterozygotes exhibited a loss of the upregulated Egfr activation seen in the *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ kidneys and an intermediate phenotype in regards to both kidney size and glomerular number (Fig. 6B,C).

Examination of kidney histology showed abnormal collecting duct morphology with significant interstitial fibrosis in the papilla and outer medulla of *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice, but not in the cortex (Fig. 6D). Despite the reduction in nephron numbers, glomerulosclerosis was minimal at this early age (data not shown). Serum BUN levels were significantly elevated compared with either *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ or *Met*$^{+/+}$*;HoxB7-Cre;wa-2/wa-2* littermates (Fig. 6E), and five out of seven mice died prior to 1 month of age. These data demonstrate that loss of Egfr signaling in the *Met*$^{fl/fl}$*;HoxB7-Cre* mouse leads to a severe decline in UB branching and that these factors act cooperatively to maintain normal tubule architecture and prevent fibrosis in the mature collecting duct.

DISCUSSION

Using a conditional knockout approach to selectively eliminate Met expression in the ureteric bud, we obtained viable mice in which kidney development appeared grossly normal but resulted in a final nephron number that was only 70% of predicted. Based on prior studies showing that Hgf-Met signaling can stimulate epithelial branching morphogenesis, we pursued the possibility that this reduction in nephron number was due to a defect in ureteric bud branching. Consistent with the published studies of Met and Hgf knockout mice, UB branching was not significantly diminished in kidneys of *Met*$^{fl/fl}$*;HoxB7-Cre* embryos at E12.5. The three-dimensional complexity of the ureteric bud made it technically difficult to accurately count terminal branches in vivo beyond this point, so an explant approach was used to assess later branching quantitatively. Explanted kidneys continue to undergo ureteric bud branching, nephrogenesis and growth, albeit at a reduced rate as compared with in vivo development [compare surface area of wild-type E14.5 kidneys (Fig. 3B) with that of wild-type explants at E12.5 + 2 days culture (Fig. 3D)]. In these experiments, we found that in the absence of exogenous growth factors the rate of branching and growth of E12.5 *Met*$^{fl/fl}$*;HoxB7-Cre* kidney explants was significantly less than that of wild-type littermates.

Although the decreased UB branching seen in our explant experiments provides a plausible explanation for the reduction in nephron number seen in adult *Met*$^{fl/fl}$*;HoxB7-Cre* mice, the number of UB branches in vivo at E12.5 was normal in these mice. The discrepancy between the in vitro and in vivo data has several possible explanations. As UB branching proceeds through E21 (Costantini, 2006), the decrease in final nephron number seen in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice may result from a selective loss of UB branching after E12.5. Alternatively, it is possible that a subtle defect in branching is present throughout development and that the cumulative effect of this defect is not quantitatively detectable at E12.5 without the examination of a significantly larger number of embryos than was performed in this study.

Our finding that a clear branching defect was detectable in explanted E12.5 *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys suggested to us that alternative signaling pathway(s) might be activated in vivo, which partially compensate for the loss of Hgf-Met signaling. Our examination of several candidate factors in the *Met*$^{fl/fl}$*;HoxB7-Cre* embryonic kidneys revealed significant increases in two factors known to promote ureteric bud branching: the Egf receptor and Fgf2 (Qiao et al., 2001; Zhao et al., 2004). There was also a non-significant decrease in the expression of several factors known to inhibit branching [Bmp4 and Bmp7 (Bush et al., 2004; Luo et al., 1995)]. By contrast, there was no difference in mRNA expression of other well known UB branching regulators, including Fgf7, Fgf10, Gdnf and pleiotrophin (Ohuchi et al., 2000; Pichel et al., 1996; Qiao et al., 1999; Sakurai et al., 2001). Although only the levels of Egfr and Fgf-2 reached statistical significance, these trends suggest that several factors may be involved in compensating for the loss of Hgf signaling in these developing kidneys.

Several lines of evidence have supported the idea that Hgf and Egf can signal cooperatively to induce ureteric bud branching and tubulogenesis, at least in vitro. Early studies by Barros and co-workers using an embryonic kidney explant-renal tubular cell co-culture system revealed that both Hgf and Egf receptor ligands are made by the explanted kidney and induced tubulogenic responses in the co-cultured cells (Barros et al., 1995). Furthermore, Sakurai et al. found that cells derived from the metanephric mesenchyme secrete both Hgf and Egf receptor ligands, and that both factors were able to induce branching morphogenesis in ureteric bud-derived cells (Sakurai et al., 1997a). Consistent with this, *Met*$^{-/-}$ epithelial cells isolated from E12 kidneys exhibited cell migration and branching tubulogenesis in response to Egf and Tgfα (Kjelsberg et al., 1997; Sakurai et al., 1997b). It has also been suggested that Egf receptor ligands play a significant role in remodeling the collecting system during the later stages of kidney development. Examination of rat kidneys has demonstrated that apoptosis occurs as the first

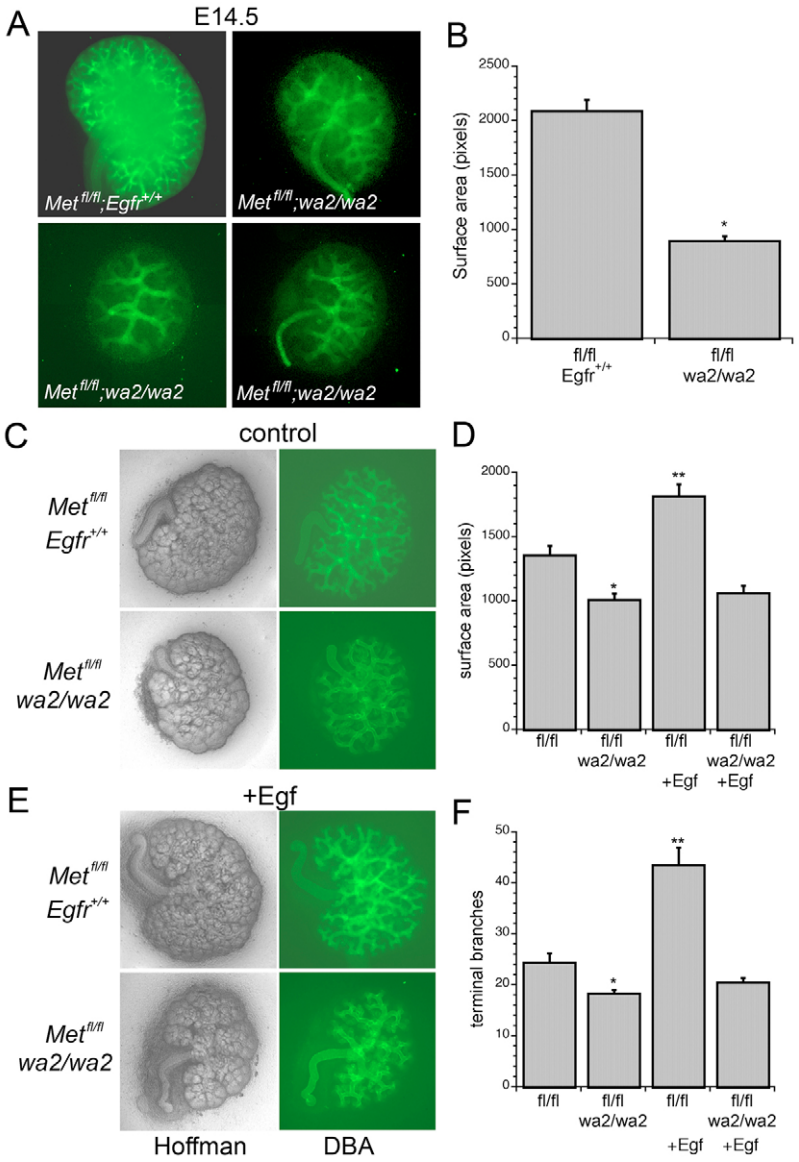

Fig. 5. Embryonic kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre:wa-2/wa-2* mice have decreased UB branching and fail to respond to exogenous Egf. (**A**) Representative images of E14.5 kidneys from a *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ embryo and three separate *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* littermates stained with DBA to show UB branches. (**B**) Quantitation of surface area from images of E14.5 kidneys (n=4, *P<0.005). (**C**) Representative images of E12.5 kidney explants from *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ and *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice. Hoffman images on left and corresponding DBA stain on right. (**D**) Quantification of surface area from explanted kidneys as in C (n=6, *P<0.03 vs. fl/fl; **P<0.003 vs. fl/fl;wa2/wa2+Egf). (**E**) Kidneys as in C treated with or without Egf (20ng/μl). (**F**) Quantification of terminal branch number from explanted kidneys as in A (n=5, *P<0.004 versus fl/fl; **P<0.001 versus fl/fl;wa2/wa2+Egf).

branches of the ureteric bud dilate in order to form the collecting system, and that treatment with Egf can substantially diminish this (Coles et al., 1993).

Our finding that Egfr expression is increased at both the mRNA and protein levels in *Met*$^{fl/fl}$*;HoxB7-Cre* embryonic kidneys, coupled with the ability of exogenously added Egf to partially rescue the in vitro branching defect in these kidneys, suggests that Egf receptor signaling may be an important in vivo pathway to maintain UB branching during development of the *Met*$^{fl/fl}$*;HoxB7-Cre* mouse. To more rigorously test this hypothesis, we generated *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* embryos in which Egfr signaling is impaired in all cells and Met receptor signaling is selectively lost in the UB-derived cells. As opposed to the kidneys of parental *Met*$^{fl/fl}$*;HoxB7-Cre* or *wa-2/wa-2* mice, the E14.5 *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* embryos demonstrated markedly decreased kidney size and ureteric bud branching in vivo, and an exaggerated loss of branching and growth during explant culture. Because very few of these animals survived beyond 3 weeks, we were unable to perform magnetic bead quantitation of nephron number. However, the number of glomeruli/kidney cross-section was reduced by 55% in fully developed kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice when compared with wild-type or *wa-2* mice. The relative accuracy of this approach is suggested by our finding that *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ kidneys demonstrated a 26% reduction in nephron number relative to wild-type mice, similar to the 35% reduction observed using the magnetic bead approach. Thus, these data support the conclusion that Egfr signaling in vivo acts to partially rescue ureteric bud branching defects in *Met*$^{fl/fl}$*;HoxB7-Cre* mice, and that these two signaling pathways act cooperatively during normal development of the collecting system of the kidney. Based on our real-time PCR results, it is likely that Fgf signaling pathways are also altered as part of this compensatory response.

To our surprise, upregulated expression of the Egfr persisted in the collecting duct of adult *Met*$^{fl/fl}$*;HoxB7-Cre* mice. Sufficient protein for western analysis was obtained from the adult papilla and revealed that the overexpressed Egfr was activated and that increased downstream signaling was present. Interestingly, collecting duct morphology and kidney function were normal for over 12 months in *Met*$^{fl/fl}$*;HoxB7-Cre* mice, whereas *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice demonstrated severe fibrosis of the renal papilla with renal

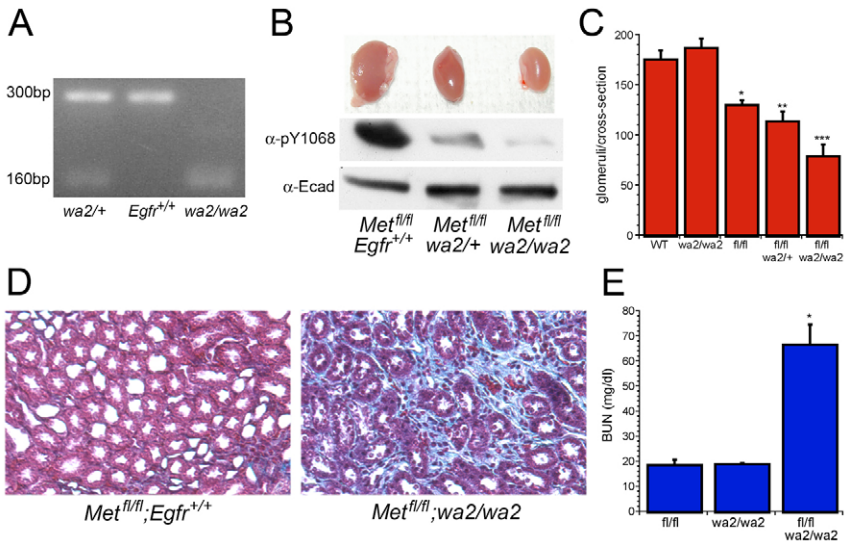

Fig. 6. *Met*$^{fl/fl}$*;HoxB7-Cre:wa-2/wa-2* mice have reduced glomerular number and papillary fibrosis. (**A**) PCR analysis of genomic DNA from *Egfr*$^{+/+}$, *wa-2/wa-2* and heterozygous mice. (**B**) Upper panel shows representative kidneys from *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$, *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/+* and *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice at 21 days. The lower panels show western analysis from renal papillas of these same genotypes immunoblotted with antibodies to detect the activated Egfr (α-pY1068) and a loading control (Ecad). (**C**) Quantification of glomeruli/section in *Met*$^{+/+}$*;HoxB7-Cre;Egfr*$^{+/+}$ (WT), wa-2/wa-2, *Met*$^{fl/fl}$*;HoxB7-Cre*, *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/+* and *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice (n=4 kidneys/genotype, **P*<0.04 versus WT; ***P*<0.04 versus *Met*$^{fl/fl}$*;wa2/wa2*; ****P*<0.03 versus *Met*$^{fl/fl}$). (**D**) Representative images of trichrome stained sections of the renal papilla from 3-week-old *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$ and *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice. (**E**) Serum BUN values from 3-week-old *Met*$^{fl/fl}$*;HoxB7-Cre;Egfr*$^{+/+}$, *Met*$^{+/+}$*;wa-2/wa-2* and *Met*$^{fl/fl}$*;HoxB7-Cre;wa-2/wa-2* mice (n=6, **P*<0.002).

failure and death by 3-4 weeks in the majority of animals. This observation is consistent with studies that have shown that Hgf-Met signaling can prevent fibrosis after unilateral ureteral obstruction (Yang et al., 2002), and that Egf signaling is required for maintenance of normal collecting duct architecture (Threadgill et al., 1995). Thus, similar to their role in UB development, Met signaling and Egfr signaling appear to play complementary roles in normal maintenance of the collecting duct.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/337/DC1

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