FGF/EGF signaling regulates the renewal of early nephron progenitors during embryonic development

Aaron C. Brown¹, Derek Adams¹, Mark de Caestecker², Xuehui Yang¹, Robert Friesel¹ and Leif Oxburgh¹,*

SUMMARY
Recent studies indicate that nephron progenitor cells of the embryonic kidney are arranged in a series of compartments of an increasing state of differentiation. The earliest progenitor compartment, distinguished by expression of CITED1, possesses greater capacity for renewal and differentiation than later compartments. Signaling events governing progression of nephron progenitor cells through stages of increasing differentiation are poorly understood, and their elucidation will provide key insights into normal and dysregulated nephrogenesis, as well as into regenerative processes that follow kidney injury. In this study, we found that the mouse CITED1⁺ progenitor compartment is maintained in response to receptor tyrosine kinase (RTK) ligands that activate both FGF and EGF receptors. This RTK signaling function is dependent on RAS and PI3K signaling but not ERK. In vivo, RAS inactivation by expression of sprouty 1 (Spry1) in CITED1⁺ nephron progenitors results in loss of characteristic molecular marker expression and in increased death of progenitor cells. Lineage tracing shows that surviving Spry1-expressing progenitor cells are impaired in their subsequent epithelial differentiation, infrequently contributing to epithelial structures. These findings demonstrate that the survival and developmental potential of cells in the earliest embryonic nephron progenitor cell compartment are dependent on FGF/EGF signaling through RAS.

KEY WORDS: Nephrogenic mesenchyme, Cited1, Sprouty, EGF, FGF, Tgfα, Mouse

INTRODUCTION
Nephrogenesis serves as an important model system for the study of mesenchymal-epithelial interactions in development. Both organ culture and gene inactivation studies have provided a detailed understanding of many interactions between the nephrogenic mesenchyme and ureteric bud that underlie the development of the fully formed organ (Grobstein, 1953; Grobstein and Dalton, 1957; Vainio and Lin, 2002). These investigations have recently been complemented with high-resolution mapping of gene expression and demonstrate that the population of nephron progenitor cells capping the ureteric bud (the ‘cap mesenchyme’) can be divided into a series of subcompartments based on the expression of distinct transcriptional regulators (Boyle et al., 2008; Brunskill et al., 2008; Kobayashi et al., 2008; Mugford et al., 2009). Expression of the transcriptional co-factor CITED1 characterizes early nephron progenitors, whereas loss of CITED1 and gain of the LEF1 transcription factor define the pretubular aggregate compartment that precedes epithelialization of the nascent nephron during differentiation. The functional significance of division of the cap mesenchyme into discrete progenitor cell compartments is at present not fully understood. One possibility is that early CITED1⁺ progenitors are resistant to the inductive activities of Wnt signaling because they do not express the Wnt transcription factor LEF1. Insulation from inductive signals would ensure the maintenance of a progenitor cell pool for continued rounds of nephron differentiation. Compartmentalization, which is a feature of numerous progenitor cell niches in the adult, prevents premature exhaustion of the highest order progenitors, and it is an intriguing possibility that progenitor cell organization in the developing kidney might fit this paradigm (Greco and Guo, 2010).

Previous investigations identified a number of genes required for nephron progenitor cell maintenance. Within nephrogenic mesenchyme, expression of the Wt1 transcription factor is essential for mesenchyme survival, whereas Six2 is crucial for self-renewal and is required to suppress the premature differentiation of nephron progenitor cells (Kobayashi et al., 2008; Kreidberg et al., 1993; Self et al., 2006). Eya1, which lies upstream of Six2, is necessary for induction of the mesenchyme (Kiefer et al., 2010; Nishinakamura et al., 2001; Xu et al., 1999). Genetic inactivation of Bmp7, which is expressed both in cap mesenchyme and ureteric bud tissue, results in reduced proliferation and premature depletion of nephron progenitor cells (Blank et al., 2009; Dudley et al., 1995; Luo et al., 1995). Inactivation of FGF receptors 1 and 2 in nephrogenic mesenchyme completely abrogates its growth, suggesting an essential role for FGF signaling (Poladia et al., 2006). FGF2 is secreted from the ureteric bud and recombinant FGF2 maintains nephrogenic mesenchyme in culture, making it an attractive candidate signal for progenitor cell maintenance in vivo (Plisov et al., 2001). However, the lack of a kidney development phenotype in the Fgfg2 null mouse argues that a group of redundant ligands might be responsible for progenitor maintenance (Dono et al., 1998; Ortega et al., 1998).

Although genetic and biochemical studies have revealed essential functions for specific genes and pathways in maintenance of the cap mesenchyme as a whole, we have yet to define the pathways required for the maintenance of specific progenitor subcompartments. In this study, we utilize a recently established system for the culture of primary cells derived from the mouse embryonic kidney to screen for growth factors that promote maintenance of the early CITED1⁺ nephron progenitor cell...
compartment. We find that a specific group of FGF and EGF ligands supports CITED1+ progenitor maintenance by means of the intracellular signaling mediator RAS (HRAS1 – Mouse Genome Informatics). We test this hypothesis in vivo by driving overexpression of sprouty 1 (Spry1), an inhibitor of the receptor tyrosine kinases (RTKs) that activate RAS, specifically in the CITED1+ progenitor compartment. We find that progenitor cell survival is impaired and that there is a reduced contribution of Spry1-expressing progenitors to the development of epithelial structures.

MATERIALS AND METHODS

Cell culture

Nephrogenic zone cells (NZCs) were extracted from E17.5 mouse kidneys and cultured as previously described (Blank et al., 2009). Growth factors used are listed in Table 1. The FGF inhibitor PD173074 (50 nM, Sigma), RAS inhibitor FPT III (25 μM, EMD Biosciences), PI3K inhibitor LY294002 (50 μM, Cell Signaling Technology) and ERK activation inhibitor peptide I (2.5 μM, Tocris Bioscience) were used at concentrations verified by the respective vendors to be specific for the kinases under study, with limited off-target effects.

Immunofluorescence, X-gal staining and microscopy

Monolayer cells were fixed and stained as previously described (Blank et al., 2009). Antibodies used were: CITED1, 1:100 (NeoMarkers); SIX2, 1:50 (Santa Cruz Biotechnology); PAX2, 1:100 (Invitrogen); pHH3, 1:100 (Cell Signaling Technology); GFP, 1:50 (Santa Cruz Biotechnology); LEF1, 1:50 (Cell Signaling Technology); PAX8, 1:100 (Abcam); and WT1, 1:100 (Santa Cruz Biotechnology). Paraffin sections were immunostained as previously described (Blank et al., 2009).

Table 1. Growth factors used in this study

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Validated range for biological activity</th>
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<tr>
<td>Biglycan (BGN)</td>
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<td>BMP7</td>
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Quantitative (q) PCR

RNA was extracted from NZCs plated in monolayer derived from pools of E17.5 mouse embryonic kidneys using the RNeasy Micro Kit (Qiagen). cDNA synthesis was performed using qScript cDNA SuperMix (Quanta Biosciences). qPCR was carried out using iQ SYBR Green SuperMix (Bio-Rad). Each assay was performed in triplicate and fold changes were calculated using the ΔΔCT method, normalized to endogenous Actb levels and displayed relative to the controls. Specificities of primer sets were determined by melt curve analysis on qPCR-generated amplicons. Average values (± s.d.) of three technical replicates from NZCs of 20-24 pooled embryonic kidneys are shown in the figures.

Statistics

P-values for protein quantitation of immunofluorescent images and TUNEL staining were derived using a two-tailed Student’s t-test. For qPCR, owing to the differing variance of data points as the cycle threshold increases, P-values shown were calculated using a two-tailed heteroscedastic Student’s t-test. P<0.05 was considered significant. The standard deviation was calculated for technical replicates derived from NZCs of 20-24 pooled kidneys or the standard error was calculated for true biological replicates derived from independent mice.

Adenoviral vectors and transduction

Transduction with adenovirus was carried out as previously described (Blank et al., 2009) for 24 hours with a multiplicity of infection of 500 in fibronectin-coated wells using serum-free medium.

Mouse strains and tamoxifen injection

Animal care was in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee of Maine Medical Center. NZCs were derived from kidneys of E17.5 ICR mice. Cited1-CreER2 mice, R26RloxP/loxP mice and Spry1-overexpressing transgenic mice are maintained on an FVB/NJ background (Soriano, 1999; Boyle et al., 2008; Yang et al., 2008). Pregnant mice were injected at the times indicated with 6 mg tamoxifen in corn oil per 40 g mouse.
RESULTS
FGF/EGF signaling maintains an early nephron progenitor phenotype

To identify signaling pathways that promote renewal of early nephron progenitor cells, a recently established ex vivo nephrogenic zone cell (NZC) culture system was used to screen a panel of factors with known expression and/or function in kidney development (Table 1) (Blank et al., 2009). Briefly, the capsules of embryonic day 17.5 (E17.5) mouse kidneys are removed and the underlying nephron progenitor cells and cortical interstitial cells are enzymatically separated from ureteric bud structures. The liberated nephron progenitor cells and cortical interstitial cells (collectively the NZCs) are then cultured in serum-free conditions on fibronectin-coated plates in the presence or absence of candidate growth factors.

A primary screen for effects on proliferation was conducted by measuring phospho-histone H3 (pHH3)-positive nuclei in cultured NZCs. Compared with untreated controls, FGF2 was the most potent proliferative factor after 24 hours, increasing proliferation by over 300%, followed by TGFα, BMP7 and EGF (Fig. 1A). Morphologically, cultures treated with FGF2 showed increased spreading, cellularity and attachment of NZCs to the well surface at 24 hours (Fig. 1A, inset).

Nephron progenitor cells are arranged into distinct compartments within cap mesenchyme, with expression of the transcriptional coactivator CITED1 identifying the earliest nephron progenitor cells and the β-catenin-interacting transcription factor LEF1 marking the more differentiated pretubular aggregate cells (Fig. 1B) (McMahon et al., 2008; Mugford et al., 2009). Analysis of CITED1 protein expression in growth factor-treated NZC cultures

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Fig. 1. FGF/EGF signaling maintains an early nephron progenitor phenotype in vitro. (A) Proliferation assay of nephrogenic zone cells (NZCs) treated with recombinant proteins or retinoic acid. Shown is the number of pHH3-positive mitotic events per 50,000 cells. Inset shows NZC morphology after 24 hours in culture medium with or without FGF2. (B) Subcompartments in the nephrogenic zone. Arrows indicate direction of increasing differentiation. (C) Summary of the ability of growth factors to promote CITED1 expression in NZCs (24 hours). (D) Immunofluorescence showing that the RTK ligands FGF2, TGFα and EGF promote the greatest increase in CITED1 (red, 10× image) of all growth factors tested. FGF2 also promotes increased expression of SIX2 (red, 20× image). (E) FGF2 treatment dramatically increases Cited1 transcription after 24 hours. Error bars indicate s.d. (F) Increased CITED1 expression by FGF2 occurs within the PAX2+ nephron progenitor population. PAX2+ progenitors (green) from Cited1-CreERT2 mice that drive a GFP reporter co-express GFP (red) when treated with FGF2, but not when cultured in medium alone. PAX2/GFP double-positive nuclei are yellow in the image overlays. Insets show representative positive and negative cells. (G) FGF2 treatment maintains Cited1 transcription up to 48 hours after harvest.
revealed that the proliferative effects of FGF2, TGFα and EGF, which are known ligands of the RTK signaling family, correlated with increased numbers of these early progenitors; this was in contrast to the proliferative effects associated with the RTK-independent ligand BMP7 (Fig. 1C,D; supplementary material Fig. S1). To ensure that CITED1+ cells promoted by FGF2 treatment retained the capacity to differentiate to the LEF1-expressing pre-tubular aggregate, we subjected FGF2-treated cultures to treatment with the canonical Wnt signaling activator BIO 18 hours after FGF2 addition. BIO-treated cultures showed a significant increase in LEF1 expression, indicating that they indeed retain the potential to differentiate (supplementary material Fig. S2).

The combination of TGFβ2, LIF, FGF2 and TGFα has a strong regulatory effect on nephron progenitor cells in the rat (Plisov et al., 2001), and we therefore tested whether these factors might regulate CITED1. We find no evidence of a synergistic effect (data not shown), possibly owing to species differences in response to these factors. Other ligands from our panel known to activate RTKs during kidney development, including FGF8, IL6, PLGF (PGF – Mouse Genome Informatics), PDGF, VEGFA and INS (insulin, data not shown), showed no significant increase in CITED1 protein expression (supplementary material Fig. S1). Thus, in the mouse, CITED1 expression might be controlled by a specific group of RTK ligands that activate FGF/EGF receptors.

Having ascertained that FGF2, TGFα and EGF promote proliferation of NZCs and the expansion of a CITED1+ early nephron progenitor cell population, we analyzed a more extensive panel of molecular markers characteristic of distinct compartments within the nephrogenic zone. Expression of compartment-specific signature genes in FGF2-treated cultures was assayed, as this growth factor showed the greatest effect of the three on CITED1 protein expression (Fig. 1C,D). As anticipated, Cited1 showed the greatest increase in expression versus the medium control at 24 hours, indicating expansion or maintenance of the earliest progenitor compartment (Fig. 1E). Meox1, which marks a newly identified subpopulation within the Cited1 compartment, was highly elevated, as were the more generalized cap mesenchyme markers Six2, Eya1, Sall1 and Pea3 (Eya4 – Mouse Genome Informatics), which are also known to be expressed within the CITED1+ progenitor compartment in vivo (Mugford et al., 2009). Based on analysis of this functionally essential gene set, we conclude that FGF signaling promotes the early nephron progenitor cell state in NZC cultures.

Immunofluorescent staining clearly showed that increased CITED1 and SIX2 protein expression correlated with the transcriptional activation caused by FGF2 (Fig. 1D,E). Previous results from our laboratory have revealed that although the NZC culture is composed of greater than 50% PAX2+ nephron progenitors derived from the cap mesenchyme, nearly 40% of the cells in these cultures represent cortical interstitium. To verify that CITED1 expression is increased in PAX2+ nephron progenitors, but not in cortical interstitial cells, following FGF2 treatment, NZCs derived from the Cited1-CreERT2 transgenic strain, which expresses GFP under the control of Cited1, were fixed and co-stained with anti-PAX2 (green) and anti-GFP (red) antibodies. FGF2 treatment resulted in robust GFP expression only in cells that express PAX2, whereas the majority of PAX2+ progenitors lack GFP expression when cultured in medium alone. CITED1 expression in response to FGF2 thus occurs in nephron progenitor cells derived from the cap mesenchyme rather than the cortical interstitium (Fig. 1F).

To understand whether FGF2 treatment increases the abundance of early nephron progenitor cells in NZC cultures, or prevents their decline from the time of harvest, we compared Cited1 expression in FGF2-treated and control NZCs over time and normalized the results to those from freshly isolated cells. In medium alone, NZCs progressively lose Cited1 expression over the course of 48 hours, whereas FGF2 treatment causes persistence of Cited1 expression throughout the time course at a level similar to that seen in freshly isolated NZCs (Fig. 1G). Taken together, these results suggest that FGF acts on nephron progenitors to promote a highly proliferative state and a transcriptional profile that is consistent with the earliest progenitor compartment.

Select FGFs that display cap mesenchyme-specific expression maintain early nephron progenitor cells

Results presented thus far suggest that FGF2 or an FGF2-like protein regulates the renewal program of the primitive CITED1+ progenitor compartment within the cap mesenchyme in vivo. To identify potential FGF candidate genes we reviewed the transcriptome data provided in the GenitoUrinary Molecular Anatomy Project (GUDMAP) database (http://www.gudmap.org). FGF genes expressed within specific subcompartments of the nephrogenic zone include Fgf1, 7, 8, 9, 10, 12 and 20 (Fig. 2). Multiple candidates are strongly expressed in the cap mesenchyme and several, including Fgf1, 7, 9, 10 and 20, show decreasing expression associated with the cessation of nephrogenesis at postnatal day 3, coincident with the loss of primitive cap markers such as Cited1, Six2, Meox1 and Dpf3 (Mugford et al., 2009).

To determine which of the FGFs expressed in the nephrogenic zone maintain the early nephron progenitor compartment, NZCs were stimulated with the corresponding recombinant FGFs and maintenance of CITED1 protein expression was measured at 24 hours (Fig. 3A). Ligand concentrations well exceeded the effective dose (ED50) range as determined in proliferation assays for each individual lot of recombinant FGF (Table 1). FGF1, 9 and 20 maintained CITED1 protein expression similarly to FGF2, and induced transcription of the primitive cap markers Cited1, Pea3, Meox1 and Six2, whereas FGF7, 8, 10 and 12 showed minimal or no effect (Fig. 3A,B). Interestingly, the three FGFs capable of substituting for FGF2 in promoting progenitor cell identity are strongly expressed in the cap mesenchyme (Fig. 2). FGF8, also expressed in the later cap mesenchyme and renal vesicle, showed only a modest effect on early cap marker transcription, even though the specific activity was comparable to, or greater than, that calculated for FGF1, 2, 9 and 20 (Table 1). The maintenance of early progenitors could however be achieved by treatment with extremely high concentrations of FGF8 (1600 and 3200 ng/ml; supplementary material Fig. S3A).

FGFs can be functionally grouped according to the receptors that they activate (Eswarakumar et al., 2005). Through alternate splicing, two isoforms of FGF receptors 1 and 2 (the b and c isoforms) are generated. These isoforms are predominantly expressed in either epithelial (b) or mesenchymal (c) tissues and display distinct ligand-binding specificities. FGF receptors 1c and 2c are known for their high binding affinity to FGF1, 2, 9 and 20, and we therefore examined whether these particular receptor isoforms are expressed in NZCs (Eswarakumar et al., 2005; Lavine et al., 2005; Ohmachi et al., 2003). qPCR analysis after reaction efficiency corrections for each FGF receptor isoform demonstrates that Fgfr1c and Fgfr2c are highly enriched in mesenchyme derived
from the nephrogenic zone compared with Fgf1b and Fgf2b (Fig. 3C; supplementary material Fig. S3B). This suggests that CITED1+ progenitors are restricted to responding to specific FGFs owing to their predominant expression of FGF receptors 1c and 2c.

Paradoxically, although Fgf1, 9 and 20 are expressed in the cap mesenchyme, the addition of exogenous FGF to cultures of these cells is required to maintain nephron progenitor cells. Predicting that expression of these FGFs might be lost during the culture period, we wished to determine whether reduced Cited1 expression correlated with reduced expression of Fgf1, 9 and 20. Analysis of NZCs cultured in medium alone for 24 hours demonstrates that transcription of Fgf1, 9 and 20 decreases significantly over the culture period, as compared with expression levels in freshly isolated NZCs (Fig. 3D). Attenuated expression of these FGFs might contribute to the reduction of higher-order cap markers that is seen following culture in medium alone. Since Fgf1, 9 and 20 are strongly expressed in the cap mesenchyme compared with other tissues, it is possible that nephron progenitor cells contribute to the maintenance of their own renewal through FGF signaling.

**FGF/EGF maintenance of early nephron progenitors requires RAS- and PI3K-mediated signaling**

The EGF receptor ligands EGF and TGFα maintain the nephron progenitor population in a similar manner to FGF (Fig. 4A; Fig. 1D). We therefore hypothesized that EGF receptor signaling might promote progenitor maintenance by increasing the expression of Fgf1, 9 and 20. Indeed, EGF receptor ligands do promote the expression of Fgf1 and Fgf9, as does FGF2 treatment (Fig. 4A, inset).

To functionally test the possibility that EGF receptor ligands promote the progenitor cell state indirectly by activating FGF signaling, we treated NZCs with either EGF or TGFα in the presence or absence of the small molecule PD173074, a specific ATP-binding inhibitor of FGFR1, 2 and 3 (Bansal et al., 2003; Pardo et al., 2009). As expected, treatment of NZCs with PD173074 abolished the CITED1 expression promoted by FGF2; however, EGF and TGFα were still able to maintain the CITED1 population in the presence of the FGF receptor inhibitor (Fig. 4B). Thus, endogenously produced FGFs do not appear to be responsible for the effects of EGF and TGFα on CITED1+ progenitor maintenance, at least in vitro.

FGF and EGF receptors frequently utilize overlapping intracellular signal transduction mediators, in particular RAS (Schlessinger, 2004). To test the possibility that both EGF and FGF receptors promote the progenitor cell state through RAS, we repeated the inhibition experiment using a small-molecule RAS inhibitor. In contrast to the results with the FGF inhibitor, CITED1 expression was extremely sensitive to the RAS inhibitor FPT III at one-quarter of the established IC50 dose, which significantly lowered CITED1 expression in response to FGF and EGF receptor ligands (Fig. 4B).

FGF and EGF signaling pathways can be further separated into ERK and PI3K branches. Immunofluorescent analysis in the presence of ERK and PI3K inhibitors demonstrates that CITED1 expression is controlled by the PI3K pathway (Fig. 4B). An interesting feature of this experiment is that ERK inhibition appears to have a slight stimulatory effect on CITED1 expression. Although this was not statistically significant, we wanted to determine whether ERK inhibition might promote Cited1 expression by inhibiting Spry1 transcription. qPCR reveals that this is not the case (supplementary material Fig. S4).

Overall, we conclude that FGFs, EGF and TGFα maintain CITED1+ progenitors through activation of a common RAS/PI3K-dependent RTK signaling program.

**RTK-mediated RAS signaling is required to maintain the expression of primitive nephron progenitor markers in vivo**

The NZC culture system propagates a mixture of cell types representative of the nephrogenic zone. To ascertain whether RTK signaling might be acting directly on nephron progenitors, rather than secondarily through other cells in the culture, we designed an in vivo experiment to specifically abrogate the response of CITED1+ nephron progenitor cells to RTK signaling. Sprouty (Spry) proteins inhibit RTK-mediated RAS activation, affecting both the FGF and
EGF signaling pathways (Mason et al., 2006). The Spryl gene is normally expressed in the collecting duct of the kidney and acts as an essential modulator of RTK signaling through the c-Ret receptor, negatively regulating ureteric bud branching (Basson et al., 2005). To develop an in vivo system with which to study the effects of FGFR/EGFR-RAS signaling in the CITED1+ progenitor compartment, we used a Spryl Cre-inducible mouse strain, in which expression of the mouse Spryl cDNA is driven by a ubiquitous CMV enhancer and β-actin promoter (Yang et al., 2008).

We first tested the ability of Spryl overexpression to block CITED1 expression in NZCs derived from embryos of Spryl transgenic (Spryl-Tg) mice. Transduction of NZCs with Cre-expressing adenovirus demonstrated that a ~3-fold increase in Spryl transcription results in a significant reduction in CITED1 protein expression, as compared with controls treated with adenovirus containing a GFP cassette (Fig. 5A). To obtain the necessary spatial and temporal specificity of Spryl expression in vivo, we bred the Spryl-Tg transgene onto the Cited1-CreERT2 transgenic strain that expresses the Cre:estrogen receptor fusion protein (CreER<T2>) under the control of Cited1 regulatory elements (Fig. 5B) (Boyle et al., 2008). Previous studies have demonstrated that recombination can be induced specifically in nephron progenitors of Cited1-CreER<T2> transgenic mice by tamoxifen treatment of the dam at a variety of stages throughout kidney development. To gain insight into the degree of recombination expected following a 24-hour tamoxifen induction, Cited1-CreERT2 transgenics were bred to R26RlacZ mice, which constitutively express β-galactosidase (β-gal) under the control of the Rosa26 promoter after Cre excision of loxP-flanked stop sequences (Soriano, 1999). Pregnant dams were injected with tamoxifen intraperitoneally at E16.5 and embryonic kidneys were harvested after 24 hours from R26RlacZ mice that were either positive or negative for the Cited1-CreERT2 driver. As shown by X-gal staining of frozen kidney sections from Cited1-CreER<T2>;R26RlacZ transgenics, some variability is seen within the same kidney, with recombination varying from 50% to 90% of cells in individual cap
mesenchymes (Fig. 5C; supplementary material Fig. S5). Because β-gal expression is heritable, cells contributing to nascent nephron structures, such as renal vesicles, can be detected. Within the 24-hour period following the tamoxifen pulse, many progenitors of the CITED1 compartment had progressed to the induced mesenchymal compartment, whereas a more limited number were found in renal vesicles.

From our primary cell analyses, we predicted that FGFR/EGFR signaling through RAS would be necessary to maintain the progenitor cell state within the cap mesenchyme, and we therefore compared the expression of WT1, PAX2, SIX2 and CITED1 by immunostaining in wild-type (Spry1-Tg) and mutant (Cited1-CreERT2;Spry1-Tg) kidneys at E17.5. The expression of the general cap mesenchyme markers WT1 and PAX2 is very similar in wild-type and mutant kidneys, and, together with Hematoxylin and Eosin staining, demonstrates that cap mesenchyme is still present in the mutant (Fig. 6A,B; supplementary material Figs S6, S7). However, as predicted from the in vitro overexpression of Spry1 in NZCs (Fig. 5A), the majority of CITED1 protein expression is lost from the nephrogenic zone of mutant kidneys as compared with the wild type (Fig. 6A-C; supplementary material Fig. S8). Also, a marked decrease in SIX2 protein expression was observed (Fig. 6B,C; supplementary material Fig. S8). Transcriptional analysis of freshly isolated NZCs from mutant and wild-type kidneys verified decreases in early nephron progenitor markers including Cited1, Meox1, Pea3 and Six2, whereas markers of the cortical interstitium (Foxd1, Sfrp1) remained largely unchanged (Fig. 6D). Considering that CITED1 progenitors represent only 50% of the mixed NZC isolate, a 1.5-fold increase in Spry1 expression may translate to a 3-fold increase specifically within the CITED1 compartment, as was observed in Spry1-Tg NZCs infected with Cre+ adenovirus (Fig. 6D; Fig. 5A). From this analysis we conclude that progenitors in the CITED1 compartment require RTK-mediated RAS signaling to maintain their cellular identity in vivo.

Fig. 4. FGF/EGF signaling maintains early nephron progenitors through RAS-mediated signaling. (A) EGF and TGFrα promote a transcriptional profile of higher-order nephron progenitor markers, similar to that of FGF2 (see Fig. 1E), including increased expression of Fgf1 and Fgf9 (inserts). Error bars indicate s.d. (B) The FGF receptor inhibitor PD173074 blocks CITED1 maintenance promoted by FGF2, but not that promoted by EGF or TGFrα. The RAS (FPT III) and PI3K (LY294002) inhibitors block CITED1 protein expression promoted by all three ligands, demonstrating a common RAS/PI3K dependency.
To ascertain whether RTK signaling through RAS is required for nephron progenitor maintenance at developmental stages earlier than E16.5, we repeated our transgenic experiment with tamoxifen injection at E12.5 and harvest at E13.5. Although a tendency can be seen toward reduction of CITED1 and SIX2 expression in the nephrogenic zone of \textit{Cited1-CreERT2;Spry1-Tg} kidneys, both markers remain expressed, indicating that the effect of SPRY1 expression on nephron progenitors is less pronounced in the early kidney (supplementary material Fig. S9).

\textbf{Spry1 perturbs the developmental fate of early nephron progenitors}

To determine the functional consequences of \textit{Spry1} overexpression in CITED1\textsuperscript{+} nephron progenitors, we intercrossed \textit{Cited1-CreERT2;R26RlacZ} double heterozygotes with \textit{Spry1-Tg} heterozygotes in order to lineage trace \textit{Spry1}-overexpressing CITED1\textsuperscript{+} progenitors by X-gal staining. We observed a clear reduction in the number of β-gal\textsuperscript{+} progenitors from \textit{Cited1-CreERT2;R26RlacZ;Spry1-Tg} mice after 24 hours compared with \textit{Cited1-CreERT2;R26RlacZ} controls lacking \textit{Spry1} overexpression (Fig. 7A). In addition, those cells that could be found in \textit{Spry1}-overexpressing mice were largely restricted to the cap mesenchyme and did not contribute significantly to the pretubular aggregate compartment in comparison with \textit{Cited1-CreERT2;R26RlacZ} controls (Fig. 7A). In agreement with this finding, transgenic \textit{Spry1} overexpression was also associated with a ~50% loss of the pretubular aggregate marker LEF1 (Fig. 7B,C; supplementary material Fig. S10). Comparison of glomerular counts in \textit{Cited1-CreERT2;Spry1-Tg} kidneys versus \textit{Cited1-CreERT2} or \textit{Spry1-Tg} wild-type controls injected with tamoxifen at E14.5 and harvested at E17.5 revealed a 5-10% decrease in glomerular numbers in mutant versus wild-type kidneys. This trend failed to reach $P<0.05$,
Fig. 6. RTK-mediated RAS signaling is required to maintain the expression of primitive nephron progenitor markers in vivo.

(A) Overexpression of mouse Spry1 in the Cited1 progenitor compartment reduces CITED1 protein expression, but not PAX2 or WT1 expression, in the E17.5 embryonic kidney 24 hours after tamoxifen injection. (B) Representative images of the nephrogenic zone demonstrate downregulation of CITED1 and SIX2, but not PAX2 or WT1, in Spry1-expressing cap mesenchyme. (C) Quantitation of CITED1 and SIX2 protein expression from kidney sections derived from Spry1-Tg mice with or without the Cited1-CreERT² driver. Error bars indicate s.e. (D) Transcription of early progenitor markers is decreased in NZCs derived from tamoxifen-injected Cited1-CreERT²;Spry1-Tg transgenic mice as compared with Cited1-CreERT² negative controls. Shown are average fold changes ± s.e.m. derived from two independent experiments of NZCs extracted from a total of five Cited1-CreERT²-positive and 11 Cited1-CreERT²-negative embryonic kidney pairs, respectively.
DISCUSSION

High-resolution gene expression studies have revealed that nephron progenitor cells are located in a series of compartments within the nephrogenic zone (Mugford et al., 2009). The signals that regulate the distribution of nephron progenitor cells in distinct compartments are poorly understood, and their elucidation will provide essential insights into normal nephrogenesis.

Fig. 7. The fate of nephron progenitors is disrupted in Spry1-overexpressing mice. (A) Representative X-gal staining from Cited1-CreERT2;R26RlacZ;Spry1-Tg E17.5 frozen kidney sections 24 hours after tamoxifen treatment demonstrates a loss of the induced mesenchymal and renal vesicle marker LEF1 as compared with control Spry1-Tg mice. (B) Quantitation of LEF1 expression from the full panel of immunofluorescence images shown in supplementary material Fig. S10. Error bars represent the s.e.m. from four mice analyzed in each group.

and the overall effect on nephrogenesis must be considered very mild, raising the possibility that a 50% reduction in LEF1 might have only a marginal impact on the number of nephrons formed.

As we have shown in both primary cell studies and the Spry1 transgenic model that early nephron progenitor cells lose their marker identity in the absence of FGF/EGF-mediated RAS signaling, we tested the possibility that CITED1 progenitors overexpressing Spry1 are prematurely removed from the progenitor pool. Indeed, we observed a ~5-fold increase in TUNEL labeling in mesenchyme cells surrounding ureteric bud tips, supporting the conclusion that cells overexpressing Spry1 are more susceptible to cell death (Fig. 7A,B). This finding was confirmed in kidneys harvested 12 hours after tamoxifen injection by immunostaining for cleaved caspase 3, an earlier marker of cell death (Fig. 8C). Because little or no morphological change other than an accumulation of pyknotic nuclei is apparent in the nephrogenic zone of mutant kidneys, we tested the hypothesis that increased proliferation of cap mesenchyme cells might compensate for the loss of cells in the mutant. Indeed, we find a ~2-fold increase in the number of cells positive for the mitosis marker pH3 within the cap mesenchyme of mutant kidneys (Fig. 8D,E). Furthermore, analysis of β-gal-positive and -negative progenitors from Cited1-CreERT2;R26RlacZ;Spry1-Tg mice reveals that those progenitors that have not undergone recombination display a very significant proliferative advantage over those that have recombined (Fig. 8F,G). Although we do find significant increases in both cell death and proliferation within the cap progenitor compartment of Cited1-CreERT2;Spry1-Tg kidneys, these effects might not entirely explain the profound dilution of transgenic cap mesenchyme cells shown in the lineage-tracing experiments. Rather, we hypothesize that transgenic cap cells are diluted out due to a combined effect of cell cycle slowing/exit, cell death and compensatory expansion of wild-type progenitors.

Fgf1 and Fgf9 are expressed by the cap mesenchyme and maintain primitive nephron progenitor marker expression in vitro (Fig. 2; Fig. 3A,B). Furthermore, we demonstrate in vitro that transcription of Fgf1 and Fgf9 was induced by FGF/EGF ligands that promote early progenitor markers in a RAS-dependent manner (Fig. 4A). A transcriptional survey of NZCs derived from Cited1-CreERT2;Spry1-Tg transgenic mice revealed that Fgf1 and Fgf9 expression was decreased by ~70% and ~40%, respectively, suggesting that these ligands are indeed regulated by RTK-mediated RAS signaling in vivo (Fig. 9A). A global reduction in the expression of FGFs that normally act in an autocrine manner to maintain early progenitor markers might help to explain why remaining β-gal-negative progenitors in the cap of Cited1-CreERT2;R26RlacZ;Spry1-Tg mice display decreased levels of early progenitor markers (Fig. 7A; Fig. 6).

Recently, canonical Wnt signaling has been identified as a requirement for maintenance of the Cited1-expressing progenitor population of the E11.5 kidney (Karner et al., 2011). To establish whether FGF could be maintaining the CITED1 population by modulating Wnt signaling, we treated our NZC cultures with the canonical Wnt activator BIO. Wnt activation does not affect the capacity of FGF to maintain CITED1 progenitors (Fig. 9B; supplementary material Fig. S2). However, a subset of the genes that have been identified as Wnt targets in the early kidney are activated by FGF in the presence or absence of the Wnt inhibitor IWR1, showing that FGF activates these targets in a Wnt-independent manner (Fig. 9C). Furthermore, the expression of these genes is reduced in NZCs from CreERT2;R26RlacZ;Spry1-Tg kidneys (Fig. 9D). These data suggest that the maintenance of nephron progenitors might have a differential requirement for Wnt and FGF signaling during early (E11.5) versus later (E17.5) nephrogenesis.
developmental kidney malformations. The earliest compartment, which expresses CITED1, is predicted to possess the greatest capacity for self-renewal and differentiation (Boyle et al., 2008). Although CITED1 is an important molecular marker for the earliest nephron progenitor cell compartment, the transcription factor itself is not essential for kidney development (Boyle et al., 2007). However, redundancy with CITED2 remains a possibility, and the early lethality of the Cited2 null mouse has precluded this analysis at the later developmental time points that we focused on in our study. We found that RTK activation by FGF and EGF receptor ligands maintains the early CITED1+ nephron progenitor phenotype in a RAS-dependent manner. A number of FGF and EGF receptor ligands are expressed within the nephrogenic zone, suggesting extensive redundancy in vivo. Blockade of RTK signaling by transgenic Spry1 overexpression resulted in the loss of early progenitor cell markers and in increased apoptosis in the cap mesenchyme. Moreover, lineage tracing demonstrated that remaining labeled progenitors overexpressing Spry1 were unable to contribute to the pretubular aggregate and renal vesicle compartments. We conclude that redundant FGF/EGF-mediated RAS signaling regulates the maintenance and developmental potential of the early nephron progenitor cell population.

Several reports suggest that EGF receptor ligands might regulate the survival of nephron progenitor cells. Neutralizing antibody treatments of kidney organ culture show that TGFα is required for kidney development in vitro (Rogers et al., 1992). Furthermore, in combination with FGF2 or conditioned medium from ureteric bud cells, TGFα promotes the survival of isolated E13 rat metanephric mesenchyme, indicating that it has a survival-promoting effect on early nephron progenitor cells (Barasch, 1999; Karavanova et al., 1996). However, unlike FGF2, TGFα treatment is not sufficient to rescue isolated metanephric mesenchyme from death in culture, indicating that its role is limited to augmenting survival (Karavanova et al., 1996). The EGFR ligand amphiregulin is under the transcriptional control of the transcription factor WT1, which is expressed in nephron progenitor cells. Amphiregulin protein is abundant in the nephrogenic zone, and it promotes the growth of cultured kidneys suggesting that it might act as a proliferative factor for nephron progenitor cells (Lee et al., 1999). One caveat to the interpretation of organ culture treatment experiments is the fact that many studies have been conducted using rat kidneys, which respond differently to growth factor treatment than mouse kidneys. Compound genetic inactivation of EGF, TGFα and amphiregulin in the mouse does not perturb kidney development.
Genetic and organ culture experiments indicate roles for FGF signaling in nephron progenitor cell maintenance. FGF2 supports proliferation and inhibits apoptosis of isolated metanephric mesenchymes (Barasch et al., 1997; Dudley et al., 1999; Perantoni et al., 1995), promoting expression of the anti-apoptosis gene Wt1 (Perantoni et al., 1995). Similar to the findings of our study, this effect cannot be reproduced with FGF7 but can be reproduced with FGF1 (Barasch et al., 1997). Compound conditional inactivation of FGF receptors 1 and 2 in the mesenchyme of the developing kidney results in arrest of nephrogenesis at ~E11.5, demonstrating an essential role for FGF signaling in the development of the metanephric mesenchyme (Poladia et al., 2006). Several FGF ligands are expressed within the developing kidney, and genetic inactivation studies have revealed phenotypes for Fgfl, Fgf8 and Fgfl0 mutants. Inactivation of Fgfl7 and Fgfl0, known ligands for FGF receptor b isoforms, result in defects in collecting duct branching (Michos et al., 2010; Ohuchi et al., 2000; Qiao et al., 1999). In this study, we found evidence that early nephron progenitors residing within the cap mesenchyme express a class of FGFs (Fgfl, 9 and 20) that normally bind FGF receptor c isoforms and are capable of promoting renewal of early CITED1+ progenitors. In accordance, our gene expression analyses indicate that NZCs display predominant expression of Fgfr1c and Fgfr2c. Recent genetic studies in mice further support this notion and demonstrate that, in the absence of Fgfr1, there is a specific requirement for the c isoform of Fgfr2 in the development of the metanephric mesenchyme, including the maintenance of the Six2 and Pax2 progenitor compartments. Taken together, these data suggest that early nephron progenitors might rely, in part, on FGF receptor/ligand signaling that acts in an autocrine manner for their survival (Sims-Lucas et al., 2011).

Genetic inactivation studies have shown a requirement for Fgf8 in the survival of CITED1+ nephron progenitors within the nephrogenic zone (Grieshammer et al., 2005; Perantoni et al., 2005). However, in contrast to Fgfl1, 2, 9 and 20, we find that Fgf8 has a very weak effect on the CITED1+ progenitor phenotype. This is surprising considering that all of these FGFs are predicted to signal through the c isoforms of the FGF receptors. Although a technical explanation for this effect cannot be entirely ruled out, we have titrated independent lots of Fgf8 with defined ED50 values over an extensive concentration range, finding a high concordance of results. Our finding that nephron progenitor cells are insensitive to Fgf8 might be explained by the fact that the decoy receptor Fgfrl1 is expressed specifically in the cells of the cap mesenchyme (GUDMAP). In direct binding studies, Fgfrl1 has high affinity for Fgf8, but shows little or no binding with Fgfl1, 9 and 20, and intermediate binding with Fgf2 (Steinberg et al., 2010). Thus, Fgfrl1 might modulate the sensitivity of CITED1+ cells to stimulation by FGFs, suppressing the response to FGF8. Although the Fgfrl null mouse displays a strong developmental phenotype in the kidney, inferences regarding interactions with FGF8 cannot be made from the mutant analysis because Fgf8 is not expressed due to the blockage of nephron differentiation (Gerber et al., 2009).

Wnt signaling is required for maintenance of the nephron progenitor cell compartment in the E11.5 kidney (Karner et al., 2010), and we therefore speculated that Wnt signaling might lie downstream of FGF/EGF in the cap mesenchyme. Our experiments using both an activator and an inhibitor of canonical Wnt signaling...
demonstrate that this is not the case. However, a subset of gene targets activated by canonical Wnt signaling are also activated by FGF. This partial overlap in gene activation might indicate that FGF/EGF lies downstream of Wnt, as has previously been described in development of the forebrain (Paek et al., 2011). Alternatively, it might indicate that Wnt and FGF/EGF are two independent circuits that activate a common program required for nephron progenitor maintenance. The finding that Sprouty antagonism of RTK signaling impacts CITED1 and SIX2 progenitor maintenance more severely at E17.5 than at E12.5 supports the notion that these two pathways are required at different times during nephrogenesis.

Our results might have important implications regarding the pathophysiology of neoplasms such as in Wilms’ tumor, which resemble renal progenitors in a state of arrested differentiation. Failure in FGF/EGF feedback control by genes such as Sprouty might contribute to the undifferentiated metanephric blastema found in Wilms’ tumor, where the advanced stage of the disease is known to be correlated with persistent expression of early blastemal markers such as Cited1 and Meox1 (Williams et al., 2004; Li et al., 2005; Lovvorn et al., 2007a; Lovvorn et al., 2007b). The FGF/EGF signaling pathway components that are disrupted by Spry1, or genes that alter Spry1 activity, might represent novel therapeutic targets to combat Wilms’ tumor and other cancers that arise from parent stem cells that normally display a requirement for FGF signaling during self-renewal.

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