GDNF-independent ureteric budding: role of PI3K-independent activation of AKT and FOSB/JUN/AP-1 signaling

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Summary
A significant fraction of mice deficient in either glial cell-derived neurotrophic factor (GDNF) or its co-receptors (Gfrα1, Ret), undergoes ureteric bud (UB) outgrowth leading to the formation of a rudimentary kidney. Previous studies using the isolated Wolffian duct (WD) culture indicate that activation of fibroblast growth factor (FGF) receptor signaling, together with suppression of BMP/Activin signaling, is critical for GDNF-independent WD budding (Maeshima et al., 2007). By expression analysis of embryonic kidney from Ret(−/−) mice, we found the upregulation of several FGFs, including FGF7. To examine the intracellular pathways, we then analyzed GDNF-dependent and GDNF-independent budding in the isolated WD culture. In both conditions, Akt activation was found to be important; however, whereas this occurred through PI3-kinase in GDNF-dependent budding, in the case of GDNF-independent budding, Akt activation was apparently via a PI3-kinase independent mechanism. Jnk signaling and the AP-1 transcription factor complex were also implicated in GDNF-independent budding. A model is proposed for signaling events that involve Akt and JNK working to regulate GDNF-independent WD budding.

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Key words: Ureteric bud, Kidney development, Wolffian duct, AKT, Jun-Fos, JNK signaling, ret, Fibroblast growth factor

Introduction
Development of the mammalian (metanephric) kidney begins when the Wolffian duct (WD), a paired mesonephric organ in mammalian embryos, is induced by signals arising from adjacent metanephric mesenchyme (MM) cells to form a localized epithelial outgrowth known as a ureteric bud (UB). Growth and branching of the UB will ultimately give rise to the tree-like collecting system of the kidney from the connecting segment to its insertion into the bladder. Timely induction and proper growth of the UB is critical for the appropriate formation of the kidney as subsequent elongation and branching of this epithelial bud dictates renal architecture (i.e. spatial arrangement of nephrons via its induction of mesenchymal-to-epithelial transformation of the MM), a fundamental determinant of kidney function (Shah et al., 2004; Costantini and Shykya, 2006; Shah et al., 2009).

Glial cell-derived neurotrophic factor (GDNF), a member of the transforming growth factor beta (TGF-β) superfamily of growth factors, is the main soluble factor that induces formation of the UB from the WD by way of signaling through the Ret receptor tyrosine kinase and its co-receptor GFRα1 (Sariola and Saarla, 2003). GDNF-null mice are characterized by renal agenesis, dysgenesis, or hypogenesis (Moore et al., 1996; Piche et al., 1996; Sánchez et al., 1996), while mice lacking either GFRα1 (Cacalano et al., 1998) or Ret (Schuchardt et al., 1994; Schuchardt et al., 1996) display similar phenotypes. Deletion of upstream mediators of GDNF expression, such as Eya1, Pax2,
and Gdf11, also results in renal agenesis (Xu et al., 1999; Bouchard et al., 2002; Esquela and Lee, 2003; Li et al., 2003; Brodbeck and Englert, 2004; Shah et al., 2004). Nevertheless, up to one-half of GDNF, Ret, and GFRα1 knockout mice continue to form a UB for reasons that remain unclear (Schuchardt et al., 1994; Moore et al., 1996). In addition, ex vivo data support the notion that budding of the UB can occur in the absence of GDNF-Ret mediated signaling (Maeshima et al., 2007). FGF signaling and suppression of BMP/Activin appear to play a key role in GDNF-independent budding; a notion supported by both in vitro and in vivo data (Maeshima et al., 2006; Maeshima et al., 2007; Michos et al., 2007; Michos et al., 2010). Thus, while it is clear that GDNF signaling is an important promoter of UB outgrowth, it is also clear that this is not the only growth factor signaling cascade capable of regulating this process.

Here we have utilized a combination of ex vivo/in vitro wet lab perturbation and transcriptomic analyses of Ret(−/−) early embryonic kidneys in an attempt to identify growth factor signaling cascades potentially important in GDNF-Ret independent WD budding. The data support a central role for both FosB/Jun/AP-1 signaling and PI3-kinase-independent activation of Akt in GDNF-independent budding. A model is proposed for growth factors and downstream signaling events regulating GDNF-independent WD budding.

Materials and Methods

Reagents

JNK inhibitor II, LY294002 (PI3K inhibitor), Akt inhibitor IV and recombinant rat FGF1 were from CalBiochem (EMD, San Diego, CA). Recombinant rat GDNF, FGF7, follistatin, and goat anti-GFRα1 were from R&D Systems (Minneapolis, MN). Fetal bovine serum (FBS) was from Biowhitaker (Walkerville, MD). DMEM/F12 was from Gibco (Grand Island, Carlsbad, CA). Mouse anti-ZO-1 and mouse anti-E-Cadherin were from Zymed (Invitrogen). Alexa Fluor 488 or 594 secondary antibodies were from Molecular Probes (Invitrogen). All other reagents were from Sigma (St. Louis, MO).

Isolation and culture of Wolffian ducts

Wolffian ducts (WDs) isolated from E13.5 Sprague-Dawley rat embryos (Harlan, Indianapolis, IN) were dissected free from surrounding mesonephric tissues such that a thin layer of intermediate mesoderm remained associated with the epithelial tube (Zhang et al., 2012). These so called “semi-clean” WDs were cultured on top of Transwell filters (0.4 μm pore size; Costar, Cambridge, MA) for up to 7 days in DMEM/F12 supplemented with 10% FBS in the absence or presence of various growth factors and/or inhibitors as indicated (Maeshima et al., 2007; Rosines et al., 2007; Choi et al., 2009; Tee et al., 2010).

Microarray

Mice heterozygous for Ret in the 129/Sv background were mated to generate Ret knockout animals and wild-type controls. Embryos were genotyped (Schuchardt et al., 1994) and kidneys were visually inspected for the presence of a ureteric bud before processing for microarray analysis. Wild-type and mutant kidneys were lysed, total RNA was extracted (RNeasy Micro kit; Qiagen, Germantown, MD), processed and hybridized to the GeneChip Mouse Genome 430 2.0 microarray (Affymetrix) by the UCSD genechip core by as previously described (Choi et al., 2009; Tee et al., 2010). GeneSpring GX 11.5 (Agilent, Santa Clara, CA) was used to analyze fold-change data. Data was preprocessed by converting any value less than 0.01 to 0.01. Data was normalized per chip to the 95th percentile. Data was normalized per gene to the median. Network/pathway analysis was performed using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) plugin for GeneSpring (Choi et al., 2009; Tee et al., 2010).

Immunohistochemistry

Isolated WD cultures fixed in 4% PFA for 1 hour at room temperature were processed for immunohistochemical fluorescent staining as previously described (Choi et al., 2009; Tee et al., 2010). Samples were visualized with a Nikon D-Eclipse 80i confocal microscope.

Real-time quantitative PCR

Total RNA was extracted from WDs (RNaqueous-Micro RNA Purification kit; Ambion, Foster City, CA) and amplified into cDNA with the SuperScript III system (Invitrogen, Carlsbad, CA) with ~100 ng of RNA per reaction. Primers for genes were generated using Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using Syber Green/ROX (Invitrogen) and Fast Real-Time PCR 7500 (Applied Biosystems). Cycle thresholds (Ct) values were normalized to GAPDH using the formula 2ΔΔCt. Triplicate samples were analyzed and significant fold changes were determined using Student’s T-Test.

Small interfering RNA (siRNA)

On-TargetPlus Rat FosB siRNA was purchased from Dharmacon (Chicago, IL) with a target sequence of CAACAGGCGCAUAAGGCAGU. On-TargetPlus non-targeting siRNA #1 (D-001810-01-05, Dharmacon) was utilized as a non-targeting mismatch control oligo. Isolated WDs were cultured on top of Transwells in the presence of DME/F12 supplemented with 10% FBS for four to six hours before transfection to allow for adhesion of the WDs to the membrane. DharmaFECT I (Dharmacon) was diluted to 3% in Opti-MEM (Gibco) and siRNA was diluted to 1 μM in Opti-MEM. Following separate 5 minute incubations at room temperature, the siRNA mixture was combined with the DharmaFECT I mixture to generate a final siRNA oligomer concentration of 500 nM. The mixture was gently mixed together at room temperature for 20 minutes and then applied on top of the Transwell filter, directly in contact with the isolated WDs. 125 ng/ml GDNF and FGF1 were added to the media in the well below the Transwells and the culture was allowed to proceed for 48 hours.

Results

The GDNF-ret signaling pathway, which induces the outgrowth of the UB from the WD, is perhaps the best studied pathway for kidney development and is sometimes considered essential for the first step in nephrogenesis. Nevertheless, a significant number (i.e. 20–50%) of knockouts of either GDNF or one its coreceptors (ret and GFRα1) undergo budding and form rudimentary kidneys (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1996). Despite the fact that these kidneys are generally hypoplastic with a reduced capacity to undergo branching morphogenesis, the presence of even a rudimentary kidney indicates that UB outgrowth (the initiating event in metanephric kidney development) must have occurred even in the absence of...
canonical GDNF-ret-mediated signaling demonstrating the existence of an in vivo “bypass” pathway.

Evaluation of gene expression reveals increases in the expression of a number of FGFs in the Ret

This “bypass” pathway has been reconstituted in an in vitro isolated WD culture system and reliable GDNF/Ret-independent budding has been achieved with the exogenous addition of an FGF (i.e. FGF1 or FGF7) together with simultaneous inhibition of activin signaling with follistatin (Fig. 1) (Maeshima et al., 2007; Rosines et al., 2007; Choi et al., 2009; Tee et al., 2010). Although GDNF-independent budding will occur in cultures of the whole mesonephros, in order to limit potential extraneous signaling events, the epithelial WD is mechanically microdissected away from the majority of the surrounding mesonephric mesenchyme leaving all but a thin layer of mesodermal cells associated with the WD epithelial tissue (Maeshima et al., 2007; Rosines et al., 2007; Choi et al., 2009; Tee et al., 2010). Although the exact FGF remains unknown, a roughly analogous condition has been used to demonstrate GDNF-independent budding in vivo, where FGFs 7, 10 or a combination have been suggested as possible mediators of an in vivo GDNF-independent budding “bypass” pathway (Chi et al., 2004; Maeshima et al., 2007; Choi et al., 2009; Michos et al., 2010; Pitera et al., 2012).

As described above, the appearance of a rudimentary kidney (albeit hypoplastic) in some Ret knockouts indicates that a stimulus for UB outgrowth which “bypasses” canonical GDNF-Ret signaling is active in these mice (Fig. 2). To investigate this, global gene expression patterns were compared between wildtype and Ret

Table 1. Expression of select genes in Ret

Although downstream signaling events have only recently been examined in GDNF-independent budding. For example, in cultures of whole mesonephros, we found that, in addition to activation of PI3K/Akt signaling, GDNF-independent WD budding also leads to the activation of MEK/ERK signaling (Table 2) (Maeshima et al., 2007). In this study, we utilized the in vitro isolated WD culture system to probe intracellular signaling pathways potentially involved in GDNF-independent WD budding. As expected, inhibition of PI3K signaling (but not p38 MAPK or MEK/ERK signaling) in isolated WDs cultured in the presence of GDNF blocked UB emergence from the WD (Fig. 3; Table 3). However, the same effect was not seen in GDNF-independent budding conditions with the same PI3K inhibitor. In this case, perturbation of PI3K had no effect on budding (Fig. 3; Table 3), however inhibition of Akt activity blocked WD budding in GDNF-independent budding (Fig. 3; Table 3). In fact, perturbation of Akt activity blocked budding in both GDNF-dependent and GDNF-independent budding. As the PI3K pathway is generally considered to be common to the activation of AKT (Brugge et al., 2007; Mahajan and Mahajan, 2012), the data suggest that GDNF-independent budding involves signaling pathways which mediate activation of AKT without activation of PI3K-kinase – i.e. GDNF-independent budding involves PI3K-independent AKT activation.
GDNF-independent budding is mediated by JNK signaling

We have previously shown that in addition to AKT and ERK activation, GDNF-independent outgrowth of the UB also activates the JNK pathway (Maeshima et al., 2007) (Table 2), suggesting that this signaling pathway plays a role in WD budding in the absence of GDNF. Supporting this notion, pathway analysis of the 180 developmentally annotated genes with increased expression in the Ret(-/-) kidney versus the wildtype (Fig. 4) resulted in several networks one of which demonstrated the existence of a signaling hub for the Jun oncogene (Fig. 5). Taken together with the fact that c-Jun N-terminal kinases (JNKs) have been reported to be capable of activating Akt signaling independent of PI3K (Shao et al., 2006; Chaanine and Hajjar, 2011), the role of the JNK signaling pathway in GDNF-independent budding was investigated.

FosB regulates GDNF-independent WD budding

Inhibition of JNK-mediated signaling selectively blocked WD budding in the absence of GDNF, but not in its presence (Fig. 6). JUN family members can dimerize with other proteins to form the AP-1 transcription factor complex (Eferl and Wagner, 2003). Inhibition of AP-1 transcription factor activity (with SR11032) similarly inhibited GDNF-independent WD budding but not GDNF-dependent budding (Fig. 6). Thus along with PI3K-independent Akt activation, both JNK signaling and AP-1 activation appear to play key roles in GDNF-independent WD budding.

In addition to the JUN protein family, the AP-1 complex is also composed of members of the Fos, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families (Eferl and Wagner, 2003). Importantly, the gene displaying the highest expression in the knockout relative to the wild-type was FosB, a finding validated by qRT-PCR (Table 1). Immunohistochemical analysis using an anti-Fosb antibody revealed the presence of activated Akt even in the absence of JNK inhibitor (Fig. 8). Thus, in the presence of 20 μM JNK inhibitor (Fig. 8), the presence of phosphorylated Akt (pAkt) was examined in isolated WDs cultured under GDNF-independent budding conditions in the presence and absence of JNK inhibitor (Fig. 8). Immunohistochemical analysis using an anti-pAkt antibody revealed the presence of activated Akt even in the presence of 20 μM JNK inhibitor (Fig. 8). Thus, in the developing kidney activation of Akt in GDNF-independent budding was independent of JNK activity.

**Discussion**

We sought to provide mechanistic insight into how animals without Ret, Gdnf, or Gfra1 form a ureteric bud and rudimentary kidneys 20–50% of the time (Schuchardt et al., 1994; Moore et al., 1996). Employing a combination of global gene expression analysis of embryonic kidneys from Ret(-/-) animals and ex vivo wet-lab analyses using a well-established ex vivo model of WD budding (Maeshima et al., 2007; Rosines et al., 2007; Choi et al., 2009; Tee et al., 2010), we found that: 1) perturbation of PI3K activity by PI3K/AKT inhibition with 5 μM Akt inhibitor IV inhibited both GDNF-dependent and GDNF-independent budding. No evidence of budding was seen with the addition of the inhibitors in 3 or more independent cultures. Scale bar: 200 μm.

In contrast to the JNK activity, GDNF-independent budding was not inhibited by PI3K/AKT inhibition. However, GDNF-independent budding was blocked by PI3K/AKT inhibition with 5 μM Akt inhibitor IV indicating that GDNF-independent budding is mediated by JNK signaling in the absence of GDNF.

**Table 2. Signaling pathways activated in WD budding.**

| Signaling pathway | Activated in budding | Reference |
|-------------------|----------------------|-----------|
|                   | GDNF dependent       | GDNF independent |
| p38 MAPK          | Yes                  | ND        |
| MEK/ERK           | Yes                  | Yes       |
| PI3K/AKT          | Yes                  | Yes       |
| JNK               | ND                   | Yes       |
|                   | (Maeshima et al., 2006) | (Maeshima et al., 2006; Maeshima et al., 2007) |
|                   | (Maeshima et al., 2006; Maeshima et al., 2007) | (Maeshima et al., 2007) |

*ND, not determined.

**Table 3. Inhibitors of signaling pathways.**

| Signaling pathway | Effect on budding | Reference |
|-------------------|-------------------|-----------|
|                   | GDNF dependent    | GDNF independent |
| p38 MAPK          | No inhibition     | No inhibition  |
| MEK/ERK           | No inhibition     | No inhibition  |
| AKT               | Inhibition*       | Inhibition*    |
| PI3-kinase        | Inhibition*       | No inhibition  |
| JNK               | No inhibition     | Inhibition*    |
|                   |                   |               |
*No evidence of budding was seen with the addition of inhibitors in 3 or more independent cultures.
both conditions; 3) a signaling hub for the Jun oncogene exists in GDNF-Ret independent budding and that perturbation of this pathway (by blocking either c-Jun N-terminal kinases (JNKs) or the AP-1 complex) selectively inhibited GDNF-independent budding; 4) the most highly differentially expressed gene in the Ret\((\sim^{+/+})\) hypomorphic kidney was the c-Jun binding partner, FosB; 5) siRNA-mediated suppression of FosB selectively inhibited GDNF-independent WD budding; and 6) activation/phosphorylation of AKT in GDNF-independent budding is independent of c-Jun mediated signaling. Taken together, the data suggest that GDNF-Ret independent UB outgrowth is likely to be due to signaling cascades requiring activation of AKT independent of both PI3K and the JNK-FosB-AP-1 signaling complex.

Here, a well-established ex vivo model of WD budding was employed to analyze GDNF-independent budding in comparison to wild-type WD budding.
to GDNF-dependent budding. A number of FGFs were upregulated in the kidneys of mutant animals compared to the wildtype (Table 1). Although a recent study demonstrated the expression of FGF8 and FGF10 in human WD epithelial and mesenchymal cells (Carey et al., 2008), there is little information on the expression of FGFs in kidney development during these very early stages of kidney development. Nevertheless, expression analysis has been performed on later stages of kidney development subsequent to UB outgrowth which supports the observations presented here. For example, a recent examination of the GUDMAP database revealed the expression of several FGFs in the early wildtype kidney, including 1, 7, 8, 9, 10, 12, and 20 (Brown et al., 2011). In addition, FGF receptors (Fgfr) appear to be appropriately expressed at this developmental time point and recent data indicates that deletion of Fgfr2 (the receptor for FGF7 and FGF10) from the stromal cells surrounding the WD results in perturbed induction of the ureteric bud (Walker et al., 2013). Thus, data support the notion that the expression of various FGFs may serve as compensatory factors mediating signaling mechanism(s) necessary for the formation of the UB in the absence of canonical GDNF-Ret signaling (Chi et al., 2004; Michos et al., 2010; Pitera et al., 2012). For example, FGF7, which is upregulated in the ret knockout when budding manages to occur and a rudimentary kidney forms (Maeshima et al., 2007), as well as FGF2 and FGF10, is capable of inducing ectopic bud formation in WDs expressing human Sprouty2 (Spry2, a negative regulator of receptor tyrosine kinase signaling) (Chi et al., 2004). In addition, kidney agenesis can be rescued in either Ret(−/−) or Gdnf(−/−) mice by crossing these mutant strains with mice deficient in Spry1, which is believed to allow normal kidney organogenesis through a mechanism dependent on FGF10 (Michos et al., 2010). Thus, as with the in vitro/ex vivo data, in vivo data support the notion that the expression of FGFs may be serving as a compensatory mechanism for activating signaling pathways to form the UB in the absence of Gdnf-Ret signaling.

A reduction in BMP/Activin signaling activity also appears to be important, and this is supported by in vivo and ex vivo data (Maeshima et al., 2006; Maeshima et al., 2007; Choi et al., 2009; Tee et al., 2010). Such modulation of the BMP/Activin pathway has been shown to play a role in in vivo UB emergence in mice (Michos et al., 2007). For instance, Six1 knockout mice display renal agenesis despite apparently normal levels of GDNF mRNA (Kreidberg et al., 1993; Xu et al., 2003). In addition, recent evidence indicates that Six1 also regulates the expression of Grem1, an antagonist of Bmp4 (Nie et al., 2011), a factor which suppresses GDNF activity (Miyazaki et al., 2000; Brophy et al., 2001). Treatment of renal tissues isolated from Grem1 knockout animals with recombinant grem1 protein induced UB outgrowth (Michos et al., 2007). Thus, while GDNF appears to be the predominant soluble growth factor involved, it is becoming increasingly clear that this critical morphogenetic process is modulated by an interplay of stimulatory and inhibitory growth factors (Bush et al., 2004; Maeshima et al., 2006).

Inhibitors of various signaling pathways demonstrated that Akt activation was key to the emergence of the epithelial bud in both GDNF-dependent and GDNF-independent budding. However, in the case of GDNF-independent budding, activation of Akt was apparently via a PI3K-independent mechanism since inhibition of PI3K did not hinder budding in the absence of GDNF (Fig. 3). Examination of a number of other potential signaling pathways implicated the JNK/AP-1 signaling pathway as playing a potential role in GDNF-independent WD budding. Microarray expression analysis also found that FosB (which can dimerize

**Fig. 8.** Inhibition of JNK does not block activation of Akt in GDNF-independent WD budding. (A,B) Darkfield photomicrographs of isolated WDs induced to bud in the presence of 125 ng/ml FGF7 and 500 ng/ml follistatin with (A) or without (B) 20 μM JNK inhibitor. Scale bar: 200 μm. (C,D) Confocal fluorescent photomicrographs showing localization of phospho-Akt (pAkt) in WDs cultured under GDNF-independent budding conditions with or without JNK inhibitor. No evidence of budding was seen with the addition of the inhibitor in 3 or more independent cultures. Red-pAkt; green-Dolichos biflorus lectin. Scale bar: 25 μm.

**Fig. 9.** Proposed signaling process for GDNF-independent UB outgrowth. A possible schema for the signaling processes involved in GDNF/Ret-independent budding of the Wolffian duct, incorporating the study’s *in vitro* pathway findings and existing knowledge. Arrowheads indicate stimulatory signal. T-capped lines indicate inhibitory signal. Observations from the results of this study are highlighted in blue. A role for BMP4, PKA and activin in budding regulation has been previously established (Miyazaki et al., 2000; Maeshima et al., 2007; Tee et al., 2010). PI3K = phosphoinositide 3-kinase; PKA = protein kinase A; BMP4 = bone morphogenetic protein 4; JNK = Jun N-terminal kinases; FosB = FB3 murine osteosarcoma viral oncogene homolog B; AP-1 = activator protein-1 transcription factor.
with c-Jun to form the AP-1 transcription factor complex) was the most highly differentially expressed gene in the Ret^+/− metanephros (Table 1), but its potential role in the developing kidney has remained largely unexplored. FosB has been implicated in the regulation of cell proliferation and differentiation in other organ systems (Haasper et al., 2008). Moreover, in the brain, increased FosB expression has been demonstrated in Gdnf^+/− mutant mice and has been associated with increased dendritic branching (Airavaara et al., 2004; Kim et al., 2009). Treatment of isolated WDs with either siRNA against FosB (Fig. 7) or an inhibitor of the AP-1 transcription factor complex (Fig. 6) supported the notion that GDNF-independent WD budding was dependent upon FosB/Jun/AP-1 signaling. Although the direct stimulant for the JNK pathway remains unclear, GFGs have been implicated in JNK signaling in other systems. For example, in alveoli, the effects of FGF7 on genes can be arrested by JNK inhibition (Chang et al., 2005; Qiao et al., 2008). Moreover, exogenous in vivo administration of FGFI5 has been shown to activate JNK in the livers of mice genetically modified for the study of bile-acid synthesis (Kong et al., 2012).

In summary, although both GDNF-dependent and GDNF-independent budding from the WD ex vivo require RTK and Akt activation, GDNF-dependent budding requires PI3K activation while GDNF-independent budding appears to require PI3K-independent activation of Akt, as well as JNK/FosB signaling. The data indicate that both of these signaling pathways are necessary, but neither is sufficient on its own for GDNF-independent budding. The accumulated data on signaling pathways is summarized in Table 3. By adding these new results to previously obtained data on BMP4 (Miyazaki et al., 2008), the signaling pathways is summarized in Table 3. By adding these new results to previously obtained data on BMP4 (Miyazaki et al., 2008), the signaling pathways is summarized in Table 3. By adding these new results to previously obtained data on BMP4 (Miyazaki et al., 2008), the signaling pathways is summarized in Table 3. By adding these new results to previously obtained data on BMP4 (Miyazaki et al., 2008), the signaling pathways is summarized in Table 3.

Competing Interests

The authors have no competing interests to declare.

References

Airavaara, M., Planken, A., Gäärdnäs, H., Piepponen, T. P., Saarma, M. and Ahtee, M. (2011). Six1 regulates Grem1 expression in the metanephric mesenchyme to initiate branching morphogenesis. Dev. Biol. 351, 145-151.

Bouchard, M., Souabni, A., Mandler, M., Neubüser, A. and Busslinger, M. (2011). FGF/EGF signaling regulates the renewal of early nephron progenitors during embryonic development. Development 138, 5099-5112.

Brugge, J. F., Pang, M. and Mills, G. B. (2007). A new mutational AKTivation in the PI3K pathway. Cancer Cell 12, 104-107.

Bush, K. T., Sakurai, H., Steer, D. L., Leonard, M. O., Sammogna, P. V., Mayer, T. N., Schwesinger, C., Qiao, J. and Nigam, S. K. (2004). TGF-beta superfamily members modulate growth, branching, shaping, and patterning of the ureteric bud. Dev. Biol. 266, 285-298.

Cacalano, G., Fariñas, I., Wang, L. C., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A. M., Rechardt, L. F. et al. (1998). GFRalpha 1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron 21, 53-62.

Carev, D., Saraga, M. and Saraga-Babic, M. (2008). Involvement of FGF and BMP family proteins and VEGF in early human kidney development. Histol. Histopathol. 23, 853-862.

Channane, A. H. and Hajjar, R. J. (2011). AKT signalling in the failing heart. Eur. J. Heart Fail. 13, 825-829.

Chang, Y., Wang, J., Lu, X., Thewke, D. P. and Mason, R. J. (2005). GKF induces lipogenic genes through a PI3K and JNK/SREBP-1 pathway in H9C2 cells. J. Lipid Res. 46, 2624-2635.

Chi, L., Zhang, S., Liu, Y., Prunskaitė-Hysyläinen, R., Voelteehaan, R., Itäranta, P. and Vainio, S. (2008). Sprouty proteins regulate ureteric branching by coordinating reciprocal epithelial Wnt11, mesenchymal Gdnf and stromal Fgft signaling during kidney development. Development 131, 3345-3356.

Choi, Y., Tee, J. B., Gallegos, T. F., Shah, M. M., Oishi, H., Sakurai, H., Kitamura, S., Wu, W., Bush, K. T. and Nigam, S. K. (2008). Nedd4 like as a member of the PI3K-independent c-Jun N-terminal kinase (JNK) signaling pathway in the developing kidney. J. Clin. Invest. 119, 117-127.

Efend, R. and Wagner, E. F. (2003). PI3K: a double-edged sword in tumorigenesis. Nat. Rev. Cancer 3, 859-868.

Esquela, A. F. and Lee, S. J. (2003). Regulation of metastatic kidney development by growth/differentiation factor 11. Dev. Biol. 257, 356-370.

Haasper, C., Jagodziński, M., Drescher, M., Moller, R., Wehmeier, M., Krettek, C. and Hesse, E. (2008). Cyclic strain induces FosB and initiates osteogenic differentiation of mesenchymal cells. Exp. Toxicol. Pathol. 59, 355-363.

Kim, Y., Teylan, M. A., Baron, M., Sands, A., Nairn, A. C. and Greengard, P. (2009). Methylphosphatidate-induced dendritic spine formation and DeltaFosB expression in nucleus accumbens. Proc. Natl. Acad. Sci. USA 106, 2915-2920.

Kong, B., Wang, L., Chang, J. Y., Zhang, Y., Klaassen, C. D. and Guo, G. L. (2012). Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. Hepatology 56, 1034-1043.

Kreidberg, J. A., Sarolina, H., Loring, J. M., Armanini, M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). WT-1 is required for early kidney development. Cell 74, 679-691.

Li, X., Ogih, K. A., Zhang, J., Krones, A., Bush, K. T., Glass, C. K., Nigam, S. K., Agarwal, A. K., Maas, R., Rose, D. W. et al. (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature 426, 247-254.

Maeshima, A., Vaughan, D. A., Choi, Y. and Nigam, S. K. (2006). Activin A is an endogenous inhibitor of ureteric bud outgrowth from the Wolffian duct. Dev. Biol. 295, 473-485.

Maeshima, A., Sakurai, H., Choi, Y., Kitamura, S., Vaughan, D. A., Tee, J. B. and Nigam, S. K. (2007). GDNF expression in vivo during nephrogenesis: the Pax/ Eya/Six1 transcriptional network regulates the emergence of the ureteric bud. Development 134, 355-363.

Majahak, K. and Majahak, N. P. (2012). PI3K-dependent AKT activation in cancers: a treasure trove for novel therapeutics. J. Cell. Physiol. 227, 3178-3184.

Michos, O., Goncalves, M., Lopez-Rios, J., Klaassen, C. D. and Guo, G. L. (2012). Initiation of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. Hepatology 56, 1034-1043.

Moore, M. W., Klein, R. D., Farías, I., Sauer, H., Armanini, M., Phillips, H., Rechardt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. Nature 382, 76-79.

Nie, X., Xu, J., El-Hashash, A. and Xu, P. X. (2011). Six1 regulates Grem1 expression in the metanephric mesenchyme to initiate branching morphogenesis. Dev. Biol. 352, 145-151.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996). Deficits in enteric innervation and kidney development in mice lacking GDNF. Nature 382, 73-76.

Pitera, J. J., Woolf, A. S., Basson, M. A. and Scammell, P. J. (2012). Sprouty1 haploinsufficiency prevents renal agenesis in a model of Fraser syndrome. J. Am. Soc. Nephrol. 23, 1790-1796.

Qiao, R., Yan, W., Clavijn, C., Mehriz-Shai, R., Zhong, Q., Kim, K. J., Ann, D. C., Comfort, E. D. and Borek, Z. (2008). Effects of GKF on adventitial cellular epithelial transdifferentiation are mediated by JNK signaling. Am. J. Respir. Cell Mol. Biol. 38, 239-246.

Rosinés, E., Sammogna, R. V., Jochkura, K., Vaughan, D. A., Choi, Y., Sakurai, H., Shah, M. M. and Nigam, S. K. (2007). Staged in vivo reconstitution and...
implantation of engineered rat kidney tissue. *Proc. Natl. Acad. Sci. USA* **104**, 20938-20943.

Sánchez, M. P., Silos-Santiago, I., Frisén, J., He, B., Lira, S. A. and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-73.

Sariola, H. and Saarma, M. (2003). Novel functions and signalling pathways for GDNF. *J. Cell Sci.* **116**, 3855-3862.

Schuchardt, A., D’Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380-383.

Schuchardt, A., D’Agati, V., Pachnis, V. and Costantini, F. (1996). Renal agenesis and hypoplasia in ret-k- mutant mice result from defects in ureteric bud development. *Development* **122**, 1919-1929.

Shah, M. M., Sampogna, R. V., Sakurai, H., Bush, K. T. and Nigam, S. K. (2004). Branching morphogenesis and kidney disease. *Development* **131**, 1449-1462.

Shah, M. M., Tee, J. B., Meyer, T., Meyer-Schwesinger, C., Choi, Y., Sweeney, D. E., Gallegos, T. F., Johkura, K., Rosines, E., Kouznetsova, V. et al. (2009). The instructive role of metanephric mesenchyme in ureteric bud patterning, sculpting, and maturation and its potential ability to buffer ureteric bud branching defects. *Am. J. Physiol.* **297**, F1330-F1341.

Shao, Z., Bhattacharya, K., Hsich, E., Park, I., Walters, B., Germann, U., Wang, Y. M., Kyriakis, J., Mohanlal, R., Kuida, K. et al. (2006). c-Jun N-terminal kinases mediate reactivation of Akt and cardiomyocyte survival after hypoxic injury in *vitro* and in *vivo*. *Circ. Res.* **98**, 111-118.

Takahashi, M. (2001). The GDNF/RET signaling pathway and human diseases. *Cytokine Growth Factor Rev.* **12**, 361-373.

Tang, M. J., Cai, Y., Tsai, S. J., Wang, Y. K. and Dressler, G. R. (2002). Ureteric bud outgrowth in response to RET activation is mediated by phosphatidylinositol 3-kinase. *Dev. Biol.* **243**, 128-136.

Tee, J. B., Choi, Y., Shah, M. M., Dnyanmote, A., Sweeney, D. E., Gallegos, T. F., Johkura, K., Ito, C., Bush, K. T. and Nigam, S. K. (2010). Protein kinase A regulates GDNF/RET-dependent but not GDNF/Ret-independent ureteric bud outgrowth from the Wolffian duct. *Dev. Biol.* **347**, 337-347.

Walker, K. A., Sims-Lucas, S., Di Giovanni, V. E., Schaefer, C., Sunseri, W. M., Novitskaya, T., de Caestecker, M. P., Chen, F. and Bates, C. M. (2013). Deletion of fibroblast growth factor receptor 2 from the peri-Wolffian duct stroma leads to ureteric induction abnormalities and vesicoureteral reflux. *PLoS ONE* **8**, e56062.

Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.

Xu, P. X., Zheng, W., Huang, L., Maire, P., Laclef, C. and Silvius, D. (2003). Six1 is required for the early organogenesis of mammalian kidney. *Development* **130**, 3085-3094.

Zhang, X., Bush, K. T. and Nigam, S. K. (2012). *In vitro* culture of embryonic kidney rudiments and isolated ureteric buds. *Methods Mol. Biol.* **886**, 13-21.