FAK–Src signalling is important to renal collecting duct morphogenesis: discovery using a hierarchical screening technique

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Summary

This report describes a hierarchical screening technique for identification of pathways that control the morphogenesis of the renal collecting duct system. The multi-step screen involves a first round using a 2-dimensional, cell-line-based scrape-healing assay, then a second round using a 3-dimensional tubulogenesis assay; both of these rounds use new cell lines described in this report. The final stage is *ex vivo* organ culture. We demonstrate the utility of the screen by using it to identify the FAK–Src-pathway signalling as being important for collecting duct development, specifically for the cell proliferation on which this development depends.

Key words: Kidney, Ureteric bud, Collecting duct, Tubulogenesis, Screening, Src, FAK, Branching morphogenesis, Cell proliferation

Introduction

This report has two main purposes: first, to describe an embryonic cell-line-based system for screening pathways potentially involved in renal collecting duct development and second, to present a novel discovery about the importance of Src-family signalling to collecting duct branching made using this system.

Driven by the need to understand congenital disease and to invent methods for renal regeneration, there has been a strong and steady growth in research into the basic mechanisms of metanephric development. If the exponential increase in discovery is to continue, especially in a period of fiscal retrenchment, research methods will have to become significantly more efficient than the transgenic approaches that have been common so far. In hierarchical screening, common in the pharmaceutical industry, simple cell-line-based assays are used as a preliminary screen for interesting effects of pathway modulators. Successively more sophisticated (but more labour-/time-intensive) assays are then performed to test the short-listed candidates. The selectivity of each stage greatly reduces the resources wasted on candidate pathways that turn out not to be involved in an event of interest; the approach also means that, by the time funding and ethical permission have to be raised to support *in vivo* experiments, a wealth of data from culture-based assays will already exist.

Assays to be used in the early steps of a hierarchical screening programme demand well-characterised, immortal cell lines. The traditional method for raising immortal cell lines – passaging a primary culture to the Hayflick Limit (Hayflick and Moorhead, 1961) and cloning surviving colonies – has two disadvantages: it is slow, and immortalisation depends on a stochastic, low-probability event such as a mutation, the exact nature of which will not be known and may interfere with subsequent experiments. More deterministic alternatives exist, using the conditional expression of an immortalising protein. Examples include the 'Immortomouse', a transgenic animal whose cells can be induced by gamma interferon to express a thermo-labile mutant of the SV40 large T antigen (Jat et al., 1991; Salmon et al., 2000; O'Hare et al., 2001), and ectopic expression of hTERT on its own or with SV40 (Meyerson, 1998). In order to make cell lines for hierarchical screening, we have raised immortal cell lines from the ureteric buds of embryonic kidneys of 'Immortomouse' embryos. We have then characterised them in terms of marker expression (expression of 'anchor genes' in the language of Thiagarajan (Thiagarajan et al., 2011)) and morphogenetic ability.

To demonstrate the application of a hierarchical screening approach to renal development, we have designed a screening strategy for candidate pathways that might control the morphogenesis of the collecting duct system. The mature urinary collecting duct system has the form of a branched tubular tree, and it develops by multiple rounds of branching from an initially unbranched ureteric bud (for reviews, see Davies, 2001; Costantini, 2010). Its development is already known to respond to a number of external signalling molecules, including Gdnf, Fgfs, Bmps, angiotensin and components of the basement membrane, and to a number of intracellular signalling paths including MAP-kinase, PI-3-kinase Wnt/PCP and Rock (for reviews, see Sakurai, 2003; Yosypiv, 2004; Michos, 2009). Because maldevelopment of the collecting ducts is implicated in

a number of renal diseases, including the relatively common polycystic disease (for a review of the mechanisms of which, see Al-Bhalal and Akhtar, 2008), there is strong interest in obtaining a more complete view of all of the pathways that control its development, and of their interactions. There are two complementary approaches to this: the first is to gather high-throughput expression data that can suggest candidate paths, an approach exemplified by the studies of Brunskill et al. (Brunskill et al., 2008) and the GUDMAP database (Harding et al., 2011); the second is to test these candidates, ideally by an efficient and relatively high-throughput technique.

The screening strategy described here begins with a simple, two-dimensional screen for morphogenetic activity, based on the 'healing' of a scrape in an epithelial monolayer. This is then followed, for short-listed candidates, by a three-dimensional culture system using the same cell lines, and only then by testing in *ex vivo* organ rudiments. Using this system, we show that signalling by Src-family proteins and by focal adhesion kinase (FAK) is critical for the normal development of the ureteric bud/collecting duct system.

Results

Production and characterisation of ureteric bud cell lines

Kidney rudiments were isolated from 'Immortomouse' (H-2k-tsA58) embryos, 11.5 days post coitum, and ureteric buds (Fig. 1b) were isolated with the aid of dispase. These were disaggregated enzymatically into cell suspensions that were plated at low density and maintained in 5% CO_2 at 33°C in the presence of IFNγ. About 40 (s.d.=4) clones per 5000 cells formed over 5∼10 days; 3 clones (6TA1,2,3) were expanded and characterised further.

Each clone showed epithelial morphology and expressed the ureteric bud marker protein, Pax2 (Dressler et al., 1990) (Fig. 2a,b,c). Analysis by RT-PCR showed that they expressed *Ret* and *Hoxb7* (expressed throughout the early ureteric bud (Pachnis et al., 1993; Watanabe and Costantini, 2004)), *Sox9* and *Wnt11* (characteristic of ureteric bud tips (Kent et al., 1996; Lako et al., 1998)) and *Wnt9b* and *Col18* (characteristic of ureteric bud stalk (Lin et al., 2001; Qian et al., 2003)) (Fig. 2d–f). All three lines expressed all of these markers at different levels; as populations, none had clearly 'stalk' or 'tip' identity in 2-dimensional culture but showed a mix of both characteristics. Given that tip and stalk can inter-convert freely depending on their environment (Sweeney et al., 2008), expression of both may reflect limitations of monolayer culture. All three cell lines also expressed *emx2* (empty spiracles homeobox 2), *Axin2* (Axis inhibition protein 2) and *Zo1* (zona occludens 1) (Fig. 2d), all of which are expressed in the normal ureteric bud (Miyamoto et al., 1997; Marlier et al., 2009; Kiefer et al., 2010).

In culture, the cell lines formed confluent monolayers typical of epithelial cells, making them suitable for use in scrape assays. In this type of assay, a pipette tip is used to scrape a cell-free zone across the monolayer; the subsequent 'healing' of the scrape involves many aspects of epithelial morphogenesis, such as cells detecting the loss of their neighbours, planar polarisation, formation of motile leading edges, rearrangement of adhesive contacts, coordination of 'purse-string' contraction and, when the edges meet, formation of new adhesions, disassembly of motile structures and more changes in polarity (Kirfel and Herzog, 2004; Lee et al., 2010). This type of assay therefore has great potential as the first round in a hierarchical screen (Yarrow et al., 2005). Our cell lines 'healed' scrapes steadily over the course of 24–27 hours (micrographs of the process are shown in the controls for the experiment in Fig. 4).

In vivo, the ureteric bud undergoes branching morphogenesis to form the collecting duct system. Collecting duct-derived cells such as mIMCD3 can make branching tubules in 3-dimensional matrices when provided with mesenchyme-derived ramogenic factors (Sakurai et al., 1997; Sakurai and Nigam, 1997). To test whether our cell lines do this, we placed them in 3-dimensional Matrigel gels and cultured them either in medium conditioned by a metanephric mesenchyme line, Six5N6 (Tai et al., 2012) (Fig. 3a–c) or in medium supplemented with the known ramogens pleiotrophin, FGF1 and GDNF (Fig. 3d–f) (Sakurai and Nigam, 1997). Cell lines 6TA1 and 6TA2 readily formed branching systems of tubules. Cell line 6TA3 formed some short tubes but it more typically made rounded cysts.

Use of the cell lines in a screen for pathways regulating morphogenesis

Our hierarchical screening scheme (Fig. 1a) begins with scrape assays, which are cost and labour-efficient, then promising candidates are taken on to the more resource-intensive 3-dimensional cell line tubule culture and only later move on to *ex vivo* assays.

Using cell line 6TA2, which closes 95–100% of a scrape in 24–27 hours (Fig. 4a,b), we used a range of small-molecule

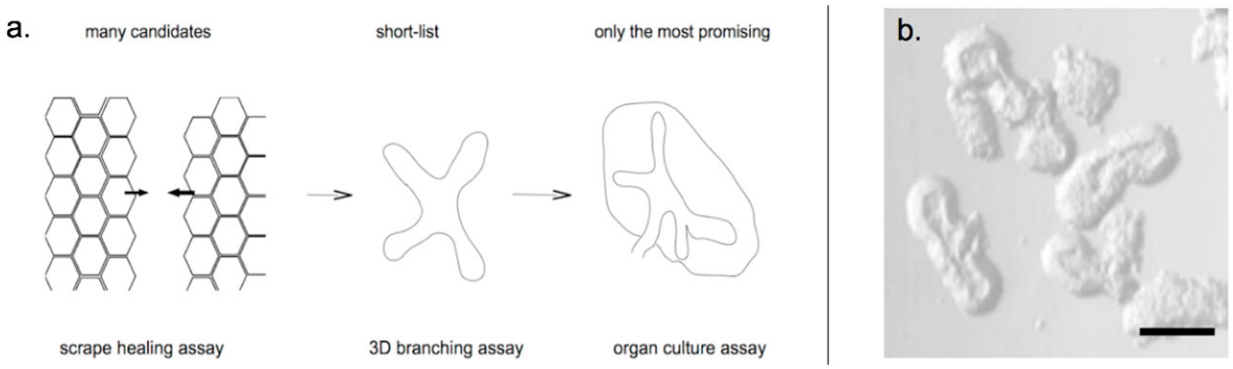

Fig. 1. Scheme and starting material. (**a**) The successive stages of the hierarchical screening strategy. (**b**) Isolated ureteric buds, examples of those used for the creation of cell lines 6TA1, 2 and 3. Scale bar: 100 µm.

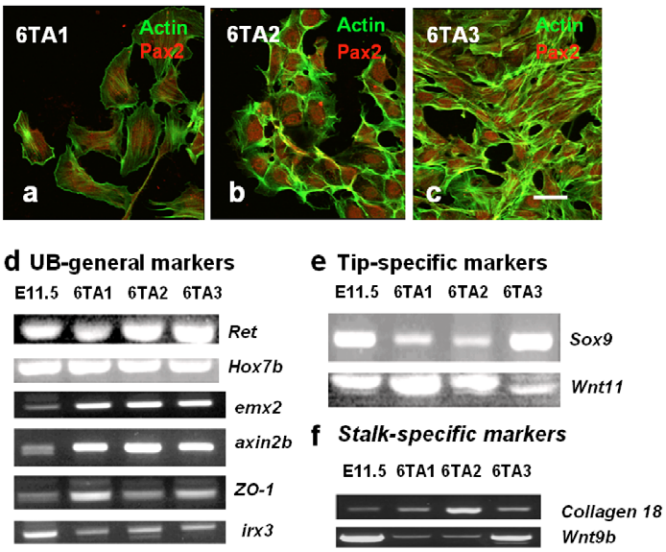

Fig. 2. Characterisation of cell lines 6TA1, 6TA2 and 6TA3. (**a–c**) All three cell lines express nuclear Pax2 protein (red). Scale bar: 10 µm. (**d**) All three cell lines, and embryonic kidney, express the general ureteric bud marker mRNAs encoding *Ret*, *Hox7b*, *emx2*, *axin2b*, *ZO-1* and *irx3*. (**e**,**f**) All cell lines express the tip-specific mRNAs encoding *Sox9* and *Wnt11*, and stalk-specific markers *Collagen 18* and *Wnt9b*; each cell type expresses all of these markers. These are not at the same ratios, but in each case both stalk and tip markers are present in two-dimensional culture. For reasons of space, these images have been trimmed to show only the data bands; an example of a full experiment with all primers and positive and negative RT-PCR controls can be viewed in supplementary material Fig. S1.

inhibitors to test their effects on the rate of scrape closure. Some acted on pathways known to be important in ureteric bud/collecting duct morphogenesis, and were a positive control for the assay, while others addressed pathways that have not been explored in this tissue. The set of small molecules included inhibitors of Akt kinase (124005), PI-3-kinase (LY294001), Erk1/2 (U0126), JNK 1/2/3 (SP600125), p38 (SB203580), CXCR4 receptor (AMD3100), G proteins (Pertussis toxin), canonical Wnt signalling (IWR1), FAK (PF573228) and the Src family (PP2 – with PP3 as an inactive relative – and SU6656). Of these, IWR1, Pertussis Toxin and SB203580 had no discernible effect at the concentrations used, AMD3100 accelerated scrape closure, and all of the others delayed or inhibited scrape closure (Fig. 4c,d; Table 1).

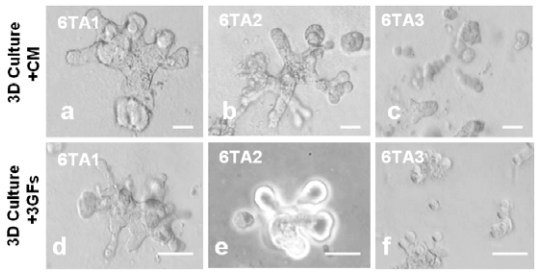

Fig. 3. Tubulogenesis by 6TA1,2,3 cells in 3-dimensional Matrigel, driven by conditioned medium (a–c) or a mix of the ramogenic growth factors pleiotrophin, FGF1 and GDNF (d–f). In both conditions, cell lines 6TA1 and 6TA2 form branching systems of tubules efficiently but line 6TA3 forms structures that are more commonly cyst-like than tubular. Scale bars: 50 µm (a–c); 100 µm (d–f).

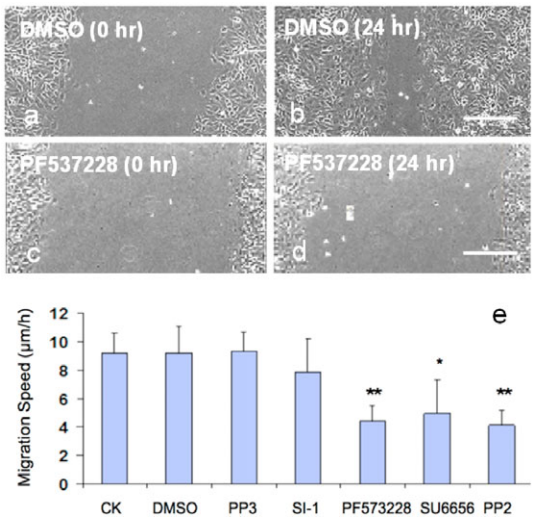

Fig. 4. A primary screen based on scrape closure. In controls, a scrape in a monolayer of 6TA2 cells, visible as the cell-free zone in **a**, is almost closed by 24 hours (**b**). In the presence of some inhibitors, scrape closure is greatly inhibited (**c**,**d**). The effect of a range of inhibitors is summarised in Table 1; the effects of inhibitors that focus on the Src–FAK system are shown quantitatively in (**e**). CK is Control Kidney medium, DMSO is the vehicle control, while the other labels are the names of drugs used at the concentrations shown in Table 1. Error bars show s.e.m; *indicates significance at $P<0.05$, **indicates $P<0.01$, by a *t*-test comparison with the control. Scale bars: 100 µm.

The significant inhibition of closure produced by inhibition of Akt and Jnk, by 124005 and SP600125 respectively, was expected as these pathways had already been shown to be important in collecting duct development (Tang et al., 2002; Karner et al., 2009). The responses did, however, act as a positive control for the assay. The responses to PP2 and PF573228 (Fig. 4e), which inhibit the Src family and FAK respectively, were interesting because they suggested that Src-family signalling might be important in collecting duct development, something that has not been shown before.

Having established that Src-family signalling is required for scrape closure in the cell-line-based primary screen, we went on to test the effects of inhibiting this pathway in 3-dimensional Matrigel culture of 6TA2 cells. The cultures were incubated for 7 days, and the number of distinct branches formed was used as a quantitative measure of branching morphogenesis. The results (Fig. 5) show that control cultures branched well, forming about six branches, as did cultures in the inactive PP2 relative, PP3. Cultures in the presence of the Src-family inhibitors PP2, SI-1 and SU6656, however, showed significantly reduced branching. As was seen with the scrape assays, the FAK inhibitor PF573228 inhibited morphogenesis to the same extent as Src-family inhibitors.

Verification of the importance of Src-family signalling to collecting duct development, using organ culture

With FAK–Src-family signalling identified as being potentially interesting in the first round screen, and shown to be required for the branching morphogenesis of tubules in the second round screen, there was sufficient evidence to justify final verification in intact tissues. E11.5 mouse kidneys were cultured in control medium or in the presence of the Src-family inhibitor PP2 or SU6656, or in the FAK inhibitor PF573228, as used for the 6TA2

Table 1. Effect of small-molecule pathway inhibitors on scrape closure by 6TA2 cells.

Drug	Concentration	Target	Effect
124005	20 µM	Akt kinase	Inhibited closure
LY294002	20 µM	PI-3-kinase	Inhibited closure
U0126	20 µM	MKK activation of Erk 1/2	Inhibited closure
PD98059	10 µM	Mek1	Inhibited closure
SP600125	0.5 µM	JNK 1/2/3 kinase and others	Inhibited closure
SB203580	10 µM	P38 MAP kinase, Src family	No effect detected
AMD3100	20 µM	CXCR4 receptor	Accelerated closure
Pertussis toxin	1 µg/ml	G-protein	No effect detected
IWR1	10 µM	Canonical Wnt	No effect detected
PP2	20 µM	Src family, RIP2	Inhibited closure
PP3	20 µM	Inactive analogue	No effect observed
Src Inhibitor 1	20 µM	Src	Inhibited closure
SU6656	5 µM	Src family, CAMK	Inhibited closure
PF573228	10 µM	FAK	Inhibited closure

cell line. PP2 and SU6656 inhibited ureteric bud branching in a dose-dependent manner (Fig. 6). Nephron progenitors formed in the surrounding mesenchyme even in the presence of the Src-family inhibitors. This indicates that the drugs were not non-specifically toxic and also that the induction and early development of nephron progenitors is much less dependent on Src-family signalling than is ureteric bud development. Treatment with the FAK inhibitor PF573228 had similar effects up to 100 nM (Fig. 6i–l), although at 1 mM it inhibited nephron formation as well and loss of tissue integrity suggested that, at this concentration, it was simply toxic.

Cell proliferation in the tips of the ureteric bud is an important driver of ureteric bud growth and branching (Michael and Davies, 2004). This, along with the facts that proliferation of a wide variety of epithelial cells depends on signalling by the FAK–Src pathway (Chaturvedi et al., 2007; Owen et al., 2011) and that oncogenic mutations within this pathway can result in uncontrolled proliferation (Fu et al., 2011; Serrels et al., 2012), leads naturally to the hypothesis that the ureteric bud requires Src–FAK signalling to maintain its normal rate of proliferation. We tested this idea by examining the effect of FAK inhibition on the frequency and distribution of phosphohistone H3-positive

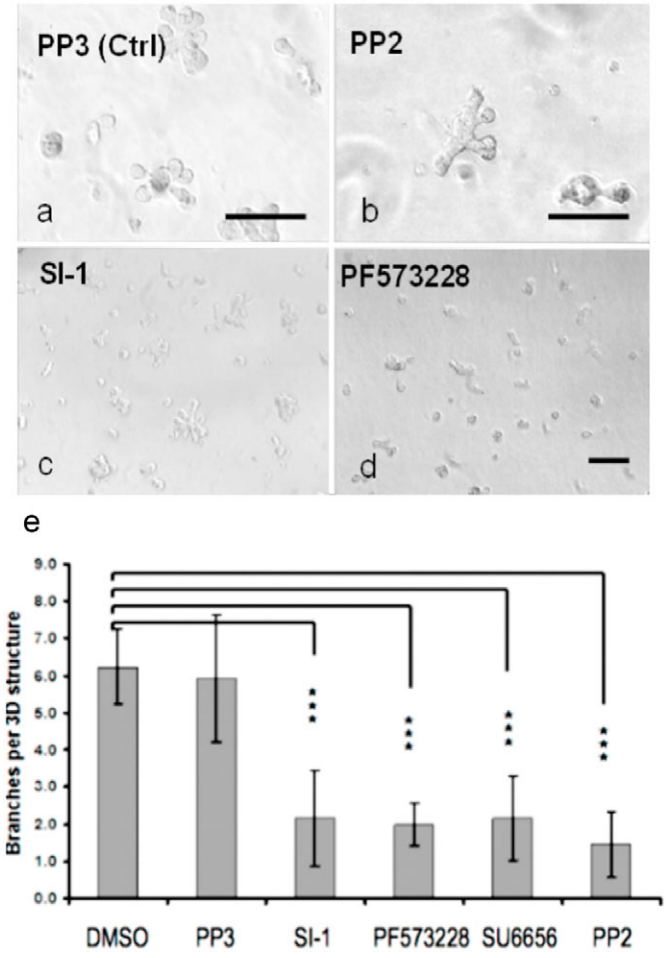

Fig. 5. Effect of inhibiting Src or FAK signalling on branching morphogenesis in 3-dimensional Matrigel culture. There is vigorous branching in cells treated with the control (inactive) drug PP3 (**a**), but there is less branching on average in PP2, SI-1 and PF573228 (**b–d**), although occasional cysts do still branch, as can be seen in b. Scale bars: 100 µm. The graph (**e**) shows the data quantitatively: DMSO is a solvent control and PP3 is the inactive control for PP2. ***$P<0.05$.

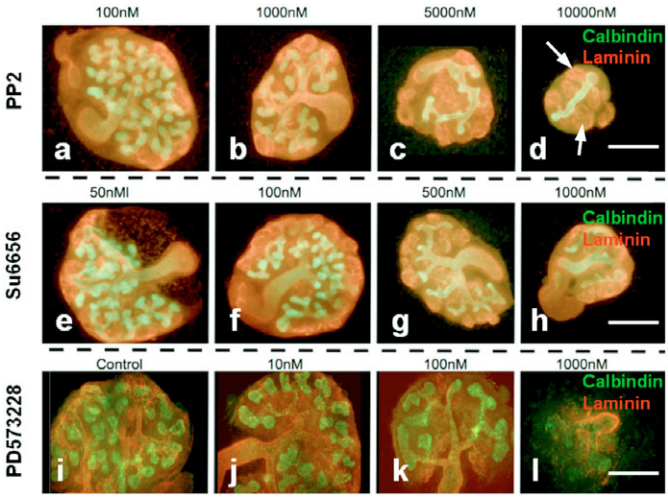

Fig. 6. Inhibition of Src or FAK also inhibits collecting duct morphogenesis (green: Calbindin-D-28K (Davies, 1994)) in cultured kidneys, in a dose-dependent manner (a–l). Nephron progenitors, visible by their having laminin-rich basement membranes (red) but no green stain, continue to form; examples are marked with arrows in d and can be seen in all images except l, in which the tissue is breaking down and the high concentration of the drug appears to be toxic. Scale bars: 100 µm.

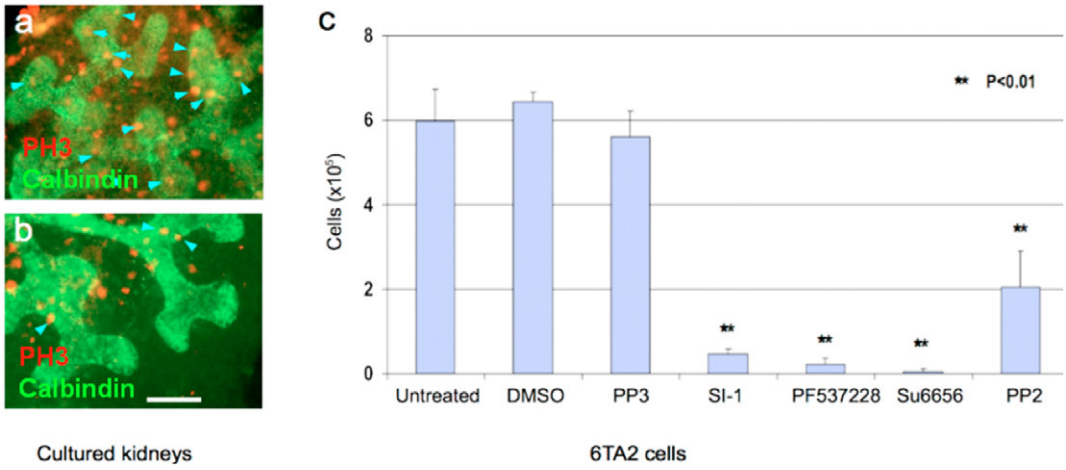

Fig. 7. Inhibition of Src or FAK reduces cell proliferation in the developing collecting duct. E11.5 mouse kidneys were cultured in control medium or in the presence of Src- or FAK-inhibiting drugs and stained for Calbindin-D-28K (green, to reveal the developing collecting ducts) and phosphohistone H3 (PH 3: red, to reveal proliferating cells). Examples of phosphohistone 3-positive cells in the collecting duct are marked with blue arrowheads in **a** (control) and **b** (PF573228); there are clearly fewer in the presence of the inhibitor. A quantitative analysis for this and other inhibitors on cell proliferation in 6TA2 cells is also shown (**c**); $^{**}P<0.01$. Scale bar: 100 μm.

(i.e. mitotic cells) cells in cultured embryonic kidneys. Controls (Fig. 7a) showed abundant mitoses in the ureteric bud/collecting duct system as well as in the surrounding mesenchyme, whereas FAK-inhibited kidneys (Fig. 7b) showed far fewer in the collecting ducts, and a modest reduction in the mesenchyme. To separate the effects of inhibition on the bud from indirect effects via the mesenchyme, and also to make the assay quantitative, we compared the increase in cell number during 2 days of culture. The results (Fig. 7c) showed that inhibition of either FAK or Src reduced proliferation significantly.

Expression of Src-family proteins in the cell lines and the kidney

Given that our results suggest that signalling by Src and FAK proteins is important in renal morphogenesis, we established which members of the Src family are expressed in this tissue. The Src family of proteins is divided into two sub-families, SrcA (Src, Fgr, Fyn and Yes) and SrcB (Blk, Hck, Lck, Lyn). To gain insight into which are most likely to be involved in collecting duct morphogenesis, we assessed the expression of the family members in 6TA1 and 6TA2 cells and in embryonic kidneys. 6TA1 and 6TA2 cells express Src, Fyn, Yes, Lyn and Hck, but no detectable Blk or Lck; the absence of this latter pair is not surprising as they are restricted mainly to the immune system and brain (Thomas and Brugge, 1997). Embryonic kidneys from E11.5, E13.5 and adult show a similar pattern, except that Blk is expressed transiently during development, being absent at E11.5, present at E13.5 and absent again in the adult (Fig. 8). FAK is expressed by the cells and by the kidney at all stages examined.

Discussion

In this report, we have described the development of conditionally immortal cell lines from embryonic mouse kidney, and have described the design of a hierarchical screening strategy that uses the cell lines for identification of signalling pathways important in ureteric bud development. We have illustrated the utility of this system by using it to show that signalling by Src-family proteins and by FAK is required for collecting duct branching, something that was not known before.

That both the Src family and FAK are important is not surprising. In typical cells, the non-receptor tyrosine kinase FAK is activated by clustering of integrins as they bind to the extracellular matrix (Mitra and Schlaepfer, 2006). Once activated, FAK can go on to activate other pathways; amongst the pathways that can be activated are those mediated by the Src family, which lead on to paths such as MAP kinase and Jnk (Kim et al., 2009; Cox et al., 2006). It is already known that interactions between specific integrins (e.g. α6β1 and α8β1 (Falk et al., 1996; Linton et al., 2007)) and specific components of the extracellular matrix (e.g. laminin and nephronectin (Yang et al., 2011; Zhao and Guan, 2011)) are required for ureteric bud development. Our data presented here suggest that FAK–Src-family signalling may be one essential consequence of this

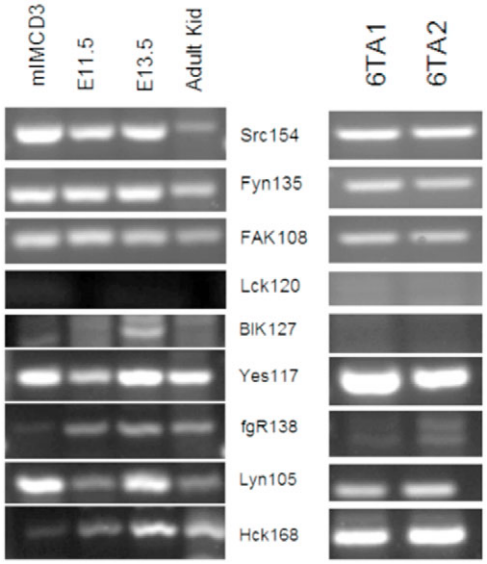

Fig. 8. Expression of Src-family proteins and FAK, in embryonic kidneys and 6TA1/2 and mIMCD-3 cell lines, by RT-PCR. See supplementary material Fig. S1 for an example of gels with control as well as data lanes.

Table 2. Polymerase chain reaction (PCR) primers used in this study.

PCR primers of differentiation markers

Accession number	Name	Sequence	Size of PCR product
NM_001080780.1	Ret	GCGTCAGGGAGATGGTAAAG CATCAGGGAAACAGTTGCAG	210
NM_010460	Hoxb7	TTCCCCGAACAAACTTCTTG CGGAGAGGTTCTGCTCAAAG	216
NM_011448	Sox9	GTGGCAAGTATTGGTCAA GAACAGACTCACATCTCT	318
NM_011719.4	Wnt9b	AGGAGACGGCCTTCCTGTAT GACAGCCGTGTCATAGCGTA	398
NM_009929	Col18	TATCCGTGGAGCAGATTTCC CCTGACCTGTAGCCCCAGTA	181
NM_010132.2	Emx2	ACCTTCTACCCCTGGCTCAT TCTCCACCGGTTAATGTGGT	354
NM_008393.3	Irx3	GCAAAACTAGCGACTCGGAC GGCTGGAAAGCTGTCTTGAG	586
NM_015732.4	Axin2	GAGGTGGTACCTTGCCAAAA TGCCAGTTTCTTTGGCTCTT	539
NM_009386.2	Zo1	CAGAAAACCATCACTCCGGT TTGCCAGGTTTTAGGGTCAC	624
NM_011587.2	Tie1	CTCACTGCCCTCCTGACTGG CGATGTACTTGGATATAGGC	226
NM_010612.2	Flk1	AGCTCTCCGTGGATCTGAAA CCAAGAACTCCATGCCCTTA	197
NM_010228.3	VEGFR1	CGGAAGCTCTGATGATGTGA TATCTTCATGGAGGCCTTGG	198
NM_011380	Six-2	AGCACCTCCACAAGAATGAAAG GTTCTTGAACCAGTTGCTGACTT	401
NM_011037	Pax2	AGTGTCTCATCCATCAACAGGAT GGTAGAGTGGTGCTCGTCATATC	626
NM_144783	WT1	CCACTCCTTCATCAAACAGGA AGCTCCTAGGTTCATCTGATTCC	532
NM_001081088.1	Megalin	CAGGGACTCCTCTGACGAAG CCTCTCCTTCTGGACAGTCG	376
NM_130456.3	nphs2	GCATCAAGCCCTCTGGATTAG AGACGGAGATCAACCTTGTGATA	232
NM_009788	CaBp-D28k	AGTGGTTGTGGTCACCAACTCT CCAGGTTACTACCAGTGCAGGA	305
XM_001479851	GAPDH	TGATGACATCAAGAAGGTGGTGAAG TCCTTGGAGGCCATGTAGGCCAT	217
EF579662.1	SV40Ts	GAGTTTCATCCTGATAAAGGAGG GTGGTGTAAATAGCAAAGCAAGC	720

PCR primers of Src family members

Accession number	Name	Sequence	Size of PCR product
NM_009271	Src	GAACCCGAGAGGGACCTTC GCGGGAGGTGATGTAGAAACC	154
NM_008054	Fyn	ACCTCCATCCCGAACTACAAC CGCCACAAACAGTGTCACTC	135
NM_007982	FAK	GAGTACGTCCCTATGGTGAAGG CTCGATCTCTCGATGAGTGCT	108
NM_010693	Lck	TGGAGAACATTGACGTGTGTG ATCCCTCATAGGTGACCAGTG	120
NM_007549	Blk	GAGGCAGGTCAGTGAGAAGG GTCCTGGTTAGGAGATGGTGG	127
NM_009535	Yes	AGTCCAGCCATAAAATACACACC TGATGCTCCCTTTGTGGAAGA	117
NM_010208	fgR	CGGCTGAAGAACGCTATTACC GGGCGACGAATATGGTCACTC	138
NM_010747	Lyn	GTGACATTGTGGTGGCCTTAT ACCATTCCCCATGCTCTTCTA	105
NM_010407	Hck	TCCTCCGAGATGGAAGCAAG ACAGTGCGACCACAATGGTAT	168

interaction. Indeed, the pathway might provide a critical link between cell–matrix interactions and the intracellular pathways already known to be essential for ureteric bud branching (e.g. MAPkinase and PI-3-kinase). It is also interesting to note that FAK–Src signalling is required for invasion of neoplastic cells (Zhao and Guan, 2011), highlighting the parallel between invasive behaviour of normal epithelia during developmental events such as branching, and metastatic invasion by tumours.

For any experiment based on pharmacological inhibitors, the question of specificity must be addressed. Fortunately, the

activities of the inhibitors used here have been studied carefully against a large range of enzymes (Bain et al., 2007). Of the drugs used here, PP2 has somewhat low specificity, inhibiting other kinases such as Ck1delta (IC_{50}=170 nM), Csk (IC_{50}=640 nM) and p38-MAP kinase (IC_{50}≈650 nM) as well as Src-family kinases (IC_{50}=53 nM for Src, 40 nM for Lck) (Bain et al., 2007). Src Inhibitor 1 (SI-1) inhibits Src and Lck (IC_{50}=44 nM and 88 nM respectively according to manufacturer's data sheet, IC_{50}=180 nM for Src according to Bain et al. (Bain et al., 2007)); it also inhibits Csk (Bain et al., 2007). SU6656 shows close specificity for Src and its closely related kinases, Fyn, Yes and Lyn, (IC_{50}=280, 170, 20 and 130 nM respectively), but not Lck (IC_{50}>6000 nM) or other kinases (Blake et al., 2000). PP2 and SI-1 are known also to inhibit the kinase activity Receptor Interacting Protein 2 (RIP2), which shares a very similar active site (Bain et al., 2007). The other drugs may also show this activity, although it has not been reported. This would be a concern in cells that express Rip2, e.g. keratinocytes (Adams et al., 2010) and T cells (Ruefli-Brasse et al., 2004), but published microarray analysis of laser captured ureteric bud shows the protein to be absent in this tissue, and indeed absent in all other tissues of the early kidney (later, it becomes detectable only in mature podocytes and glomerular epithelia; see the GUDMAP website (http://www.gudmap.org)). This potential off-target reaction is therefore not a concern here. Also, the FAK inhibitor, which has the same effect on ureteric bud branching as the Src-family inhibitors, has no known activity against Rip2. Taken together, the effects of all of the drugs examined in this report suggest strongly that the FAK–Src pathway is necessary for ureteric bud development.

Beyond describing the importance of a specific pathway, this report sets out to illustrate how a 3Rs-compliant, hierarchical screening strategy can be used as an approach to identifying pathways involved in renal development. The principle can be used for a wide variety of investigations of renal development. We have made the cell lines themselves freely available to the community ('free' except for the cost of shipping) via the GUDMAP website (http://www.gudmap.org; go to the mouse lines and cell lines section).

Materials and Methods

Production of cell lines from 'Immortomouse' embryos

Kidney rudiments were obtained from embryos of H-2k-tsA58 ('Immortomouse') mice at 11.5 days of gestation (the morning of vaginal plug discovery being considered 0.5 days). The kidney rudiments were isolated by manual dissection in Dulbecco's Minimum Essential Medium (DMEM: D5546, Sigma Chemical Corporation, Poole, UK). Rudiments were incubated for 5–10 minutes in 2 U/ml dispase in PBS at room temperature (14–16℃) and fine needles were used to pull the ureteric bud out of the mesenchyme, in an action similar to removing a hand from a glove. Tissues were rinsed in DMEM, crudely chopped up using needles and incubated for 2 minutes in Trypsin–EDTA in DMEM at 37℃. They were then transferred to 'IM' (Immortalisation Medium): 1% ITS supplement (I2521, Sigma), 100 U/ml IFNγ (cyt-358, ProSpecBio), 1× glutamine–penicillin–streptomycin from a joint stock solution (10378016, Invitrogen), 1× antioxidants (A1345, Sigma) and 10% heat-inactivated foetal calf serum (Invitrogen) in 1:1 DMEM:F12 (D8437, Sigma). The tissues were reduced to a cell suspension by trituration in a yellow tip, and plated on Matrigel-coated dishes in IM supplemented with 10 µM Y27632 (Y0503, Sigma), an inhibitor of Rock, and incubated at 33℃, 5% CO_2 for 48 hours. After this, the medium was replaced with plain IM and samples were incubated for a further 72–240 hours. In the clonogenicity study, 10% FCS serum in DMEM medium was initially used to optimise cell culture condition.

After this incubation, clones were isolated using cloning rings, and used to seed new cultures. These were maintained at 33℃ in PM, which has the same constitution as IM except that it contains only 20 U/ml IFNγ. They were passaged every 72 hours, using Trypsin–EDTA, being diluted 1:3 (and samples were frozen in 90% FCS, 10% DMSO).

Reverse transcription polymerase chain reaction (RT-PCR)

Cells were collected from sub-confluent cultures and their total RNA was isolated using QIAshredder and an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesised using ImProm-II™ Reverse Transcriptase (Promega). PCR was then performed using gene-specific DNA primer pairs that were designed using the Primer3 programme and synthesised by Eurofins MWG. The primers are listed in Table 2. The PCR programme was: initial denaturation at 94℃ for 2.5 minutes, followed by 35 cycles of 94℃ for 30 seconds, 58℃ for 45 seconds 72℃ for 45 seconds, then final extension at 72℃ for 10 minutes. Controls include 'no mRNA', and 'no RT'. Electrophoresis was performed on a 0.8% agarose gel.

Scrape assays

Ureteric bud cell lines were passaged by brief digestion in pre-warmed 1× Trypsin–EDTA for 2 minutes, washed, and a suspension of separated cells (verified by microscopic examination) was plated and cultured for 24~36 hours to form a confluent monolayer. Scratch wound lines were made with a fine sterile needle. The scraped monolayer was gently washed and incubated for 2 hours (37℃, 5% CO_2), before drugs were applied. Images were captured immediately after drug was added (deemed 0 hours) and at times thereafter as indicated in the results section.

Three-dimensional branch assays

Matrigel (BD Bioscience), stored at −20℃, was thawed on ice and 5 µl aliquots were used to coat 13 mm glass coverslips, the Matrigel being spread on the coverslip in a 24-well plate and the plate then being transferred to a 37℃ incubator for ~5 minutes. A suspension of 5×10^4 6TA1 or 6TA2 cells was mixed with 500 µl Matrigel solution (final concentration 9% in 5% FCS MEM medium) and plated on to the coated coverslips, 50 µl (5000 cells) per coverslip. 50 µl conditioned medium from a culture of the metanephric mesenchymal cell line Six5N6 was added per well to induce branching morphogenesis. Complex trees (6~8 tips) appeared between 5 and 10 days.

Ex vivo kidney culture

Kidney rudiments were isolated from E11.5 embryos of wild-type CD1 mice by micro-dissection. They were cultured for 4 days on Millipore™ polycarbonate filters supported at the air-liquid interface in MEM medium (Invitrogen) with 10% serum (FCS), with or without drugs (as specified in the Results section). Fixation was in cold methanol for 10 minutes or (for anti-phosphohistone staining) in 4% formaldehyde in PBS for 1 hour. After washing in PBS (with 0.1% Triton X-100 for formaldehyde-fixed specimens), specimens were incubated overnight at 4℃ in 1/100 anti-laminin (Sigma L9595) and 1/100 anti-calbindin (Abcam ab82812) and 1/200 anti-phosho-histone H3 (Cell Signalling Technologies 9701), in PBS. Controls were maintained in PBS alone. After another PBS wash, samples were incubated in a 1/100 dilution of the relevant secondary antibody (FITC anti-mouse, Sigma F2012; FITC anti-rat, Sigma F6258; TRITC anti-rabbit, Sigma T6778) in PBS for 4 hours at 4℃, washed in PBS and viewed on a Zeiss AxioScope epifluorescence microscope.

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Competing Interests

The authors have no competing interests to declare.

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