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.

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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% CO₂ 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
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).

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
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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Target</th>
<th>Effect</th>
</tr>
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<tbody>
<tr>
<td>124005</td>
<td>20 μM</td>
<td>Akt kinase</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>LY294002</td>
<td>20 μM</td>
<td>PI-3-kinase</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>U0126</td>
<td>20 μM</td>
<td>MKK activation of Erk 1/2</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>PD98059</td>
<td>10 μM</td>
<td>Mek1</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>SP600125</td>
<td>0.5 μM</td>
<td>JNK 1/2/3 kinase and others</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>SB203580</td>
<td>10 μM</td>
<td>P38 MAP kinase, Src family</td>
<td>No effect detected</td>
</tr>
<tr>
<td>AMD3100</td>
<td>20 μM</td>
<td>CXCR4 receptor</td>
<td>Accelerated closure</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>1 μg/ml</td>
<td>G-protein</td>
<td>No effect detected</td>
</tr>
<tr>
<td>IWR1</td>
<td>10 μM</td>
<td>Canonical Wnt</td>
<td>No effect detected</td>
</tr>
<tr>
<td>PP2</td>
<td>20 μM</td>
<td>Src family, RIP2</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>PP3</td>
<td>20 μM</td>
<td>Inactive analogue</td>
<td>No effect observed</td>
</tr>
<tr>
<td>Src Inhibitor 1</td>
<td>20 μM</td>
<td>Src</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>SU6656</td>
<td>5 μM</td>
<td>Src family, CAMK</td>
<td>Inhibited closure</td>
</tr>
<tr>
<td>PF573228</td>
<td>10 μM</td>
<td>FAK</td>
<td>Inhibited closure</td>
</tr>
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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<0.001.

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.
(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...
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. MAP kinase 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.
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\textsubscript{50}=170 nM), Csk (IC\textsubscript{50}=640 nM) and p38-MAP kinase (IC\textsubscript{50}=650 nM) as well as Src-family kinases (IC\textsubscript{50}=53 nM for Src, 40 nM for Lck) (Bain et al., 2007). Src Inhibitor 1 (SI-1) inhibits Src and Lck (IC\textsubscript{50}=44 nM and 88 nM respectively according to manufacturer’s data sheet, 2007); it also inhibits Csk (Bain et al., 2007). SU6656 shows Src Inhibitor 1 (SI-1) inhibits Src and Lck (IC\textsubscript{50}=44 nM and 88 nM respectively according to manufacturer’s data sheet, 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\textsubscript{50}=280, 170, 20 and 130 nM respectively), but not Lck (IC\textsubscript{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 community (‘free’ except for the cost of shipping) via 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.

### 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°C) and fine needles were used to pull renal vesicles from the amnion. The kidney rudiments were then incubated in 90% FCS, 10% DMSO. 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 RNase 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°C for 2.5 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 45 seconds 72°C for 45 seconds, then final extension at 72°C 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°C, 5% CO₂), 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°C, 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°C incubator for ~5 minutes. A suspension of 5×10⁶ GTa1 or 6Tα2 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 branch 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°C in 1/100 anti-laminin (Sigma L9595) and 1/100 anti-calbindin (Abcam ab828212) 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°C, 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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