Published in final edited form as:

Genesis. 2009 February ; 47(2): 61–66. doi:10.1002/dvg.20452.

A transgenic mouse that reveals cell shape and arrangement during ureteric bud branching

Xuan Chi¹, Anna-Katerina Hadjantonakis², Deborah Hyink³, and Frank Costantini¹

¹ Department of Genetics and Development, Columbia University Medical Center, 701 W. 168th St. W. 168th Street, New York, NY 10032

² Developmental Biology Program, Sloan-Kettering Institute, New York, New York, 10021

³ Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA

Abstract

Understanding the cellular events that underlie epithelial morphogenesis is a key problem in developmental biology. Here we describe a new transgenic mouse line that makes it possible to visualize individual cells specifically in the Wolffian duct and ureteric bud, the epithelial structures that give rise to the collecting system of the kidney. myr-Venus, a membrane-associated form of the fluorescent protein Venus, was expressed in the ureteric bud lineage under the control of the *Hoxb7* promoter. In *Hoxb7*/myr-Venus mice, the outlines of all Wolffian duct and ureteric bud epithelial cells are strongly labeled at all stages of urogenital development, allowing the shapes and arrangements of individual cells to be readily observed by confocal microscopy of freshly excised or cultured kidneys. This strain should be extremely useful for studies of cell behavior during ureteric bud branching morphogenesis in wild type and mutant mouse lines.

Introduction

During the development of the excretory system, the Wolffian duct gives rise to an evagination, the ureteric bud (UB), which grows and branches extensively within the developing kidney to form the collecting duct system. Defects in either the initial outgrowth, or the subsequent branching and elongation, of the UB can result in severe renal developmental defects including agenesis or hypodysplasia, which are relatively common birth defects in humans (Pohl *et al.*, 2002) (Airik and Kispert, 2007) (Dressler, 2006). The mouse has been used extensively as a model system to study the genetic control of renal branching morphogenesis, and many genes that participate in this process have been identified using knockout mice (Yu *et al.*, 2004) (Dressler, 2006). However, relatively little is known about the cellular behaviors that are controlled by these genes, and that ultimately carry out the “work” of branching morphogenesis (Davies, 2002) (Costantini, 2006). Among the various processes that may participate in epithelial growth and branching are cell proliferation, cell movements and changes in cell shape (Ettensohn, 1985) (Meyer *et al.*, 2004). As an example of the latter process, the conversion of cells from cuboidal to wedge-shaped can cause the epithelium to fold (Ettensohn, 1985). In this paper, we describe a new transgenic mouse line that was designed to aid in the analysis of cell shape and arrangement during UB morphogenesis.

We previously described a transgenic mouse strain, *Hoxb7*/eGFP, which expressed enhanced GFP under the control of the *Hoxb7* promoter (Kress *et al.*, 1990), and thus

specifically in the Wolffian duct, the ureteric bud, and their derivatives during excretory system development, as well as in the collecting ducts of adult kidneys (Srinivas *et al.*, 1999). This strain has been very useful for studies of renal development, both *in vivo* and in organ cultures, as it allows ureteric bud morphogenesis to be visualized by fluorescence microscopy in live tissues (Watanabe and Costantini, 2004) (Shakya *et al.*, 2005) (Davies, 2005). However, one limitation of this line is that eGFP is a cytoplasmic protein which labels the ureteric bud cells rather uniformly (Srinivas *et al.*, 1999). Thus, while *Hoxb7*/GFP mice are useful for studying morphogenesis at the tissue level, they are less useful for studies of individual cell behaviors or cell shapes. To overcome this limitation, we have generated a new transgenic strain that uses the same *Hoxb7* promoter to drive expression of myr-Venus (Rhee *et al.*, 2006), a membrane-targeted form of Venus, which is a variant of EYFP (enhanced Yellow Fluorescent Protein). While mice expressing this protein in a widespread pattern have been previously described (Rhee *et al.*, 2006), the use of tissue-specific regulatory elements, such as the *Hoxb7* promoter, makes it possible to focus on a specific structure of interest within a complex organ such as the kidney.

Materials and Methods

Construction of the *Hoxb7*/myr-Venus transgene

The myr-Venus DNA fragment, originally in pCX vector (Rhee *et al.*, 2006) was recloned into the EcoRI site of pBluescript SK vector. The 756bp myr-Venus fragment was then excised with BamHI and EcoRV, and ligated upstream of a human β -globin 3' sequence (in pKS-h β globin3' vector) at the BamHI and XbaI sites (the XbaI site was filled in with Klenow DNA polymerase, to be compatible with the EcoRV blunt end). The ligated myr-Venus and β -globin DNA was cut with NotI, Klenow-filled, then digested with EcoRV to release a 2.5kb fragment containing myr-Venus- β -globin 3', which was ligated downstream of the *Hoxb7* promoter at the SmaI site of a *Hoxb7* promoter construct (in pBluescript KS vector). The final plasmid is called p*Hoxb7*/myr-Venus.

Generation of and identification of transgenic mice

The insert of plasmid p*Hoxb7*/myr-Venus was excised with NotI and XhoI, gel-purified, and microinjected into B6CBAF1/J x B6CBAF1/J zygotes by standard procedures (Hogan *et al.*, 1994). Transgenic lines were identified by PCR analysis of tail tip DNA, using a pair of Venus primers (5'-GCC CGC TAC CCC GAC CAC ATG A-3' and 5'-CGG CGG CGG TCA CGA ACT CC-3') that yielded a 469-bp product. The PCR conditions were: 2 min at 94 °C, then 32 cycles of 30 s at 94°C, 30 s at 55°C and 45s at 72°C. The founder transgenic mice were confirmed by Southern blot analysis using restriction enzyme SpeI and HindIII and a 469bp probe for Venus. Two independent transgenic mouse lines (#10 and #17) were evaluated, and both showed indistinguishable patterns of expression of myr-Venus, which were strong and heritable, in the Wolffian duct/ureteric bud lineage. The studies in this paper were carried out using line #17, which was propagated in the hemizygous state.

Kidney organ culture

Embryos were dissected at various stages of development and transgenic embryos were identified by examination on a stereomicroscope equipped with epifluorescence (Leica MZ16FA). Renal organ cultures were carried out as previously described (Srinivas *et al.*, 1999) (Watanabe and Costantini, 2004).

Immunofluorescence

Kidney explant cultures were fixed in 4% PFA for 3 hours at 4 °C. Anti-phosphohistone H3 (Ser10) (6G3) (Cell Signaling, 9706S) staining of organ cultures was performed on

Transwell filters, using a protocol described previously (Basson *et al.*, 2006). Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch.

Confocal microscopy and image processing

Both live kidneys and fixed kidneys were imaged by confocal laser scanning microscopy. For live imaging experiments, E14.5 Hoxb7/myr-Venus kidneys were placed in cover slip-bottomed dishes (MatTek, Ashland, MA) in PBS. Confocal images were acquired as z-stacks of x,y images taken at 2 μ m z-intervals, using a Zeiss LSM510 META on a Zeiss Axiovert 200M, with a Plan-Neofluar 40 $\times$ /NA1.3 and a Plan-Apochromat 20 $\times$ /NA0.75. Fluorophores were excited with a 488-nm argon laser (EGFP/Venus). E15.5 kidneys fixed overnight in 4% paraformaldehyde were imaged with a Bio-Rad laser scanning confocal microscope equipped with an OlympusUM PlanFl water immersion lens 10 $\times$ /NA 0.3 and Olympus U PlanApo/IR water immersion lens 60 $\times$ /NA1.2, at 0.5-5.3 μ m z-intervals. Volume-rendering of image stacks and rotations were performed using Volocity (Improvision, www.improvision.com) and Voxx (<http://www.nephrology.iupui.edu/imaging/voxx/index.htm>). Image segmentation and isosurface models were generated by Amira 3.1 by TGS Template Graphics Software, Inc. (www.amiravis.com).

Results and Discussion

Kidneys from Hoxb7/myr-Venus transgenic mice were examined at various stages of development to analyze the subcellular expression of the myr-Venus fluorescent protein, and to examine the organization and shapes of ureteric bud epithelial cells during kidney development. Figure 1a shows a low magnification image of the urogenital system of a Hoxb7/myr-Venus mouse at stage P0 (newborn), revealing the expression of myr-Venus in the ureteric bud tips throughout the periphery of the kidney, as well as in the ureters, but not in the bladder, reproductive organs, or adrenals. Figure 1 b and c show 3-D reconstructions of the ureteric tree of a Hoxb7/myr-Venus E15.5 kidney, generated from stacks of low-magnification (10 $\times$) confocal images, and Figure 1 d-g show z-projections at different levels of the kidney, revealing that myr-Venus expression is limited to the ureteric bud and its derivatives, the collecting ducts. Thus, the tissue-specificity of expression is identical to that previously observed for other Hoxb7-driven transgenes, and it can be used similarly to the Hoxb7/GFP mice to examine the development and overall organization of the collecting duct system. The faint honeycomb-like staining surrounding the UB tips visible in some images (e.g., Figure 1d) was the results of brief staining with Alexa488-conjugated collagen (type I) antibody (Sigma cat # C2456, Molecular Probes Zenon Conjugation kit, cat # Z-25002), which stains some mesenchymal/stromal cells.

Figure 2 shows somewhat higher-magnification optical sections of Hoxb7/myr-Venus kidneys explanted at E12.5 (a, b) or E14.5 (c, d) and cultured for 72 hours (a, b) or overnight (c, d). The kidney in c, d was fixed and stained with phosphohistone H3 (pH3) antibody to detect mitotic cells. Fluorescence of myr-Venus is preserved well after fixation in 4% PFA, and the overlap with anti-pH3 staining indicates the location of mitotic cells within the UB epithelium. The myr-Venus protein clearly demarcates the ureteric bud epithelium from the surrounding structures (e.g., metanephric mesenchyme, nephron epithelia and renal stroma), which derive from different cell lineages and do not express Hoxb7-transgenes (Kress *et al.*, 1990) (Srinivas *et al.*, 1999). Within the UB cells, myr-Venus fluorescence is present only at the cell membrane, as expected. It shows the organization of cells into a simple cuboidal epithelium in the tips (arrows) as well as the stalks (arrowheads), and also reveals the cross-sectional shapes of the individual cells.

Figure 3 a-o shows two series of image stacks through the outer nephrogenic zone of E14.5 kidneys that had developed *in vivo*. At these magnifications, the shapes of individual cells are clearly visible, as is the generally cuboidal nature of the UB epithelium, with a distinct lumen (Meyer *et al.*, 2004). For comparison, Figure 3p shows an optical section thorough a Hoxb7/eGFP transgenic kidney, which shows two collecting ducts in cross section, but fails to reveal the shapes of individual cells. Thus, the Hoxb7/myr-Venus transgenic line represents a considerable advance for studies in which individual cell morphology and cell arrangement is important.

Figure 4 a-f shows several 3-dimensional reconstructions of the surface of an E15.5 kidney, which were rendered from multiple confocal images such as those shown in Figure 3. These reconstructions reveal several interesting features of the organization of ureteric bud tips, such as the highly regular arrangements and similar sizes of most individual tip cells (Figure 4b), the single-layered nature of the tip epithelium (Figure 4f), and the apparent “indentations” where the ureteric buds join the “connecting tubules” (asterisks in Figure 4d, e), a segment of the nephron that does not express Hoxb7 transgenes. For comparison, Figure 4g shows a volume rendered image of a Hoxb7/eGFP kidney, where the overall shape of the UB tips is visible, but the individual cells are not.

While the images in Figure 4 show the 2-D cell shapes at the surface of the UB epithelium, confocal image stacks of Hoxb7/myr-Venus kidneys can be further processed by segmentation to reveal the 3-dimensional shapes and the spatial arrangements of individual cells, as shown in Figure 5. This reveals that the cells closer to the UB tip (6 and 8) are somewhat larger than those in the cleft (1-4).

These images demonstrate that the Hoxb7/myr-Venus mice are a useful tool to examine epithelial cell organization and cell shape in the developing ureteric bud, in live tissue, and without the need for staining with antibodies or lectins. The ability to image live tissue, together with the established methods for renal organ cultures (Saxen, 1987) (Srinivas *et al.*, 1999) (Watanabe and Costantini, 2004), should make it possible to examine, in real time, the changes in cell organization and shape that occur during branching morphogenesis. While methods are currently available for time-lapse epifluorescence imaging of kidneys growing in organ culture, and we have used them to examine the patterns of branching morphogenesis in Hoxb7/eGFP kidneys (Srinivas *et al.*, 1999) (Watanabe and Costantini, 2004), the resolution in this culture/microscopy system is not sufficient to follow individual cells in developing Hoxb7/myr-Venus kidneys (data not shown). Therefore, it will be necessary to adapt the renal organ culture system to the confocal microscope to take full advantage of potential of this new transgenic strain.

Acknowledgments

We thank Tingting Tam and Jacob Kreugel for experimental assistance, and Zaiqi Wu for DNA microinjections. This work was supported by grants DK055388 and DK075578 from the NIDDK (to F.C.), HD052115 from the NICHD (to A-K. H.) and a fellowship from the American Heart Association (to X.C). Confocal laser scanning microscopy and volume rendering with Volocity and Amira was performed at the MSSM-Microscopy Shared Resource Facility, and was supported by NIH-NCI shared resources grant 5R24 CA095823-04, NSF Major Research Instrumentation grant DBI-9724504 and NIH shared instrumentation grant 1 S10 RR0 9145-01.

References

- Airik R, Kispert A. Down the tube of obstructive nephropathies: the importance of tissue interactions during ureter development. *Kidney Int.* 2007; 72:1459–1467. [PubMed: 17928823]
- Basson MA, Watson-Johnson J, Shakya R, Akbulut S, Hyink D, Costantini FD, Wilson PD, Mason IJ, Licht JD. Branching morphogenesis of the ureteric epithelium during kidney development is

- coordinated by the opposing functions of GDNF and Sprouty1. *Dev Biol.* 2006; 299:466–477. [PubMed: 17022962]
- Costantini F. Renal branching morphogenesis: concepts, questions, and recent advances. *Differentiation.* 2006; 74:402–421. [PubMed: 16916378]
- Davies JA. Do different branching epithelia use a conserved developmental mechanism? *Bioessays.* 2002; 24:937–948. [PubMed: 12325126]
- Davies JA. Watching tubules glow and branch. *Curr Opin Genet Dev.* 2005; 15:364–370. [PubMed: 15964757]
- Dressler GR. The cellular basis of kidney development. *Annu Rev Cell Dev Biol.* 2006; 22:509–529. [PubMed: 16822174]
- Ettensohn CA. Mechanisms of epithelial invagination. *Q Rev Biol.* 1985; 60:289–307. [PubMed: 3901078]
- Hogan, B.; Bedington, R.; Costantini, F.; Lacy, E. *Manipulating the Mouse Embryo: a laboratory manual.* 2nd. Cold Spring Harbor: Cold Spring Harbor Laboratory; 1994. p. 332
- Kress C, Vogels R, De GW, Bonnerot C, Meijlink F, Nicolas JF, Deschamps J. Hox-2.3 upstream sequences mediate lacZ expression in intermediate mesoderm derivatives of transgenic mice. *Development.* 1990; 109:775–786. [PubMed: 1977573]
- Meyer TN, Schwesinger C, Bush KT, Stuart RO, Rose DW, Shah MM, Vaughn DA, Steer DL, Nigam SK. Spatiotemporal regulation of morphogenetic molecules during in vitro branching of the isolated ureteric bud: toward a model of branching through budding in the developing kidney. *Dev Biol.* 2004; 275:44–67. [PubMed: 15464572]
- Pohl M, Bhatnagar V, Mendoza SA, Nigam SK. Toward an etiological classification of developmental disorders of the kidney and upper urinary tract. *Kidney Int.* 2002; 61:10–19. [PubMed: 11786080]
- Rhee JM, Purity MK, Lackan CS, Long JZ, Kondoh G, Takeda J, Hadjantonakis AK. In vivo imaging and differential localization of lipid-modified GFP-variant fusions in embryonic stem cells and mice. *Genesis.* 2006; 44:202–218. [PubMed: 16604528]
- Saxen, L. *Organogenesis of the Kidney.* Cambridge: Cambridge University Press; 1987.
- Shakya R, Watanabe T, Costantini F. The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev Cell.* 2005; 8:65–74. [PubMed: 15621530]
- Srinivas S, Goldberg MR, Watanabe T, D'Agati V, al-Awqati Q, Costantini F. Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. *Dev Genet.* 1999; 24:241–251. [PubMed: 10322632]
- Watanabe T, Costantini F. Real-time analysis of ureteric bud branching morphogenesis in vitro. *Dev Biol.* 2004; 271:98–108. [PubMed: 15196953]
- Yu J, McMahon AP, Valerius MT. Recent genetic studies of mouse kidney development. *Curr Opin Genet Dev.* 2004; 14:550–557. [PubMed: 15380247]

Figure 1. Hoxb7/myr-Venus expression is specific to the ureter and ureteric bud

a, The entire urogenital system of a newborn transgenic mouse, showing expression of Hoxb7/myr-Venus in the UB tips and ureters but not the bladder, reproductive organs, or adrenals. **b** and **c**, 3D rendered images of a z-stack taken at 5.3 μ m intervals of the surface (**b**) and a deeper layer (**c**) of a fixed E15.5 kidney, showing that myr-Venus expression is limited to the UB branches, and absent from surrounding mesenchyme, stroma and nephron epithelia. **d – g**, z-projections at four different levels of the same kidney shown in **b** and **c**, revealing expression in the deeper collecting ducts as well as the UB tips. At these low magnifications (10 $\times$), individual cells are not readily visible. The image in panel **a** was taken using a fluorescence stereomicroscope (Leica MZ16FA). All other images were taken using

a Bio-Rad laser scanning confocal microscope with an OlympusUM PlanFl water immersion lens 10× /NA 0.3. The RGB-colored vector on the bottom left of b and c depicts the orientation of x-axis in green, y-axis in red and z-axis in blue. The grid size is 129 μM in b, c and 125 μM in d-g.

Figure 2. Hoxb7/myr-Venus reveals ureteric bud epithelial organization in cultured kidney explants

a-b, two optical sections (a at lower, b at higher magnification) through an E12.5 Hoxb7/myr-Venus kidney that had been cultured for 72 hours. The fluorescence image is superimposed with bright field to show the relationship between the mVenus-expressing ureteric bud and the surrounding structures, which derive from different lineages and do not express Hoxb7/mVenus. **c – d**, two optical sections at different levels of an E14.5 Hoxb7/myr-Venus kidney that had been cultured overnight and stained with anti-phospho histone H3 antibody (red) to reveal mitotic cells. Note the cuboidal nature of the epithelium in the tips (arrows) as well as the stalks (arrowheads) of the UB branches. Scale bars, 100 μ M.

Figure 3. Epithelial cell shape and arrangement in the UB tips of a Hoxb7/myr-Venus kidney
a – h and **i – o** are two series of optical sections through the surface of a kidney at E14.5. The fluorescence images in a-h are superimposed with bright field images to show the surrounding tissue. At these magnifications, Hoxb7/myr-Venus clearly delineates the outline of each cell in the UB epithelium. All images were taken using a Zeiss 510 META, with a Plan-Neofluar 40×/NA1.3 Oil DIC (a to h) and a Plan-apochromat 20×/NA0.75. **p** shows a fluorescence image of a Hoxb7/eGFP E14.5 kidney, in which the UB epithelial cells are uniformly labeled, showing the outline of the two tubules but not the individual cells. Scale bars, 50μM.

Figure 4. 3-D rendering of the ureteric bud tips at the surface of an E15.5 kidney
a-b and d-f show 3D rendered images of the UB tips at the surface of a transgenic kidney, tilted at different angles to reveal various features. **b** is an enlargement of **a**. Note the highly regular arrangement of individual cells, and the generally similar cell size at the epithelial surface. Asterisks in **d** and **e** indicate apparent concavities in the UB tip, representing the sites where the UB connects to the connecting tubule, which does not express Hoxb7/myr-Venus. Panel **c** shows a 3D volume-rendered image in a plane perpendicular to the surface of the kidney, in which the cuboidal epithelial organization is visible. Images a-f were taken using a Bio-Rad laser scanning confocal microscope, with Olympus U PlanApo/IR water lens 60×/NA1.2. The RGB-colored vectors depict the orientation of x-axis in green, y-axis in red and z-axis in blue. Grid size is 21 μm. Panel **g** shows a 3D volume rendered image of the UB tips of a Hoxb7/eGFP kidney, which does not label the cell outlines. This image was rendered using Voxx software (<http://www.nephrology.iupui.edu/imaging/voxx/index.htm>), with the help of Dr. Jeff Clendenon at the Indiana Center for Biological Microscopy, Indiana University Medical Center.

Figure 5. Isosurface models of the ureteric bud tips and individual cells

Confocal images of the same kidney shown in Figure 4 were segmented with Amira. The UB tip model (green) was segmented using active contours, and the individual cells (1-8) were segmented with a region-growing algorithm. Isosurface models were generated from the segmented images using Amira 3.1. Four cells at the branch point (1-4), and four near the tip of the ureteric bud (5-8) were analyzed. Panels **a** and **c** show two different views of the segmented cells in relation to the UB branch, and **b** and **d** show the eight cells in isolation. Panels **e** and **f** show a cut through cells 1-4 (yellow line). An optical section was superimposed over the model to show outlines of individual cells (**f**). The bright green areas in the transparent UB (**a**, **c**, **e**) are shadows. The apparent holes in the undersides of the UB

tips in e and f (asterisks) show the sites where the UB joins the connecting tubule of the nephron.