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Podocin-Green Fluorescence Protein Allows Visualization and Functional Analysis of Podocytes

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ABSTRACT

Podocytes do not remain fully differentiated when cultured, and they are difficult to image *in vivo*, making the study of podocyte biology challenging. Zebrafish embryos are transparent and develop a single, midline, pronephric glomerulus accessible for imaging and systematic functional analysis. Here, we describe a transgenic zebrafish line that expresses green fluorescence protein (GFP) from the zebrafish *podocin* promoter. The line recapitulates the endogenous pronephric *podocin* expression pattern, showing GFP expression exclusively in podocytes starting 2 days postfertilization. Using the podocyte GFP signal as a guide for dissection, we examined the pronephric glomerulus by scanning electron microscopy; the surface ultrastructure exhibited fine, interdigitating podocyte foot processes surrounding glomerular capillaries. To determine whether the GFP signal could serve as a direct readout of developmental abnormalities or injury to the glomerulus, we knocked down the podocyte-associated protein *crb2b*; this led to a loss of GFP signal. Thus, podocin-GFP zebrafish provide a model for ultrastructural studies and *in vivo* visualization and functional analysis of glomerular podocytes. This model should also be useful for high-throughput genetic or chemical analysis of glomerular development and function.

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Podocytes are specialized epithelial cells of the kidney glomerulus, which extend long branched foot processes that enclose the kidney glomerular capillary loops. The foot processes of neighboring podocytes interdigitate to form filtration slits, through which water, solutes, and proteins smaller than 60 kD pass from the blood into the primary urine, whereas larger plasma proteins are retained in the blood plasma. The podocytes, their foot processes, and the diaphragms that connect the processes and span the filtration slits (the slit diaphragms) form together the outer and size-selective layer of the glomerular filtration barrier. Proteinuria in mammals is commonly associated with disruption of the normal architecture of the podocyte foot processes and their slit diaphragms. This is observed in monogenic hereditary human nephrotic syndromes, in which podocyte-specific proteins such as nephrin and podocin are mutated,[1,2] but also in more common glomerular disorders of sometimes unclear etiology and pathogenesis, such as autoimmune glomerulonephritis,[3] and in toxic or inflammatory animal models of proteinuria, such as LPS-induced nephropathy.[4] Podocytes are therefore recognized as critical components of the kidney filter and culprits in the development of many kidney disorders.[5,6]

To gain detailed insight into the functions of podocytes, cell culture systems and rodent models have been commonly applied. However, *in vivo* podocytes are terminally differentiated nonproliferative cells with elaborate morphologic distinctions, which are not fully recapitulated *in vitro*.[7,8] Moreover, whereas genetically engineered mouse models have been used to demonstrate the critical importance of several podocyte proteins,[9–13] a major disadvantage of mice, like almost all other mammalian systems, is that the location of the glomeruli is deep within the kidney cortex and the animals' bodies, which makes real-time studies, such as live imaging of glomeruli and podocytes, problematic. Access to Munich-Wistar rats that have glomeruli located at the renal surface circumvents some of these problems and has allowed real-time imaging of blood flow and glomerular filtration using two-photon microscopy.[14] Moreover, advances in multiphoton imaging technologies now also allows imaging of glomerular function in mice, which have their glomeruli located deeper in the kidney cortex.[15] Nevertheless, these types of studies remain complicated, they have limited spatial and

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temporal resolution, and they have low throughput.

In comparison, the small size, transparency, and external development of the zebrafish embryo offers an interesting alternative, because these embryos possess a pronephric kidney that resembles the mammalian metanephric kidney structurally and functionally.[16] The pronephros is the first and simplest kidney to form during embryonic development, and unlike mammals where it constitutes a nonfunctional evolutionary residue, the pronephros is playing an important role in fish and amphibians where it constitutes the functional kidney of early larval life, regulating water homeostasis.[17,18] Despite the difference in complexity between the zebrafish pronephros and the mammalian metanephric kidney, the basic glomerular structure and its cellular make-up are similar.[16] Ultrafiltration in the zebrafish pronephros begins at 2 days postfertilization (dpf),[19] *i.e.* when the embryo is fully transparent and, importantly, amenable to high throughput analyses, such as gene knockdown using morpholino antisense oligonucleotides, and drug screens using chemical compound libraries.

The molecular make-up of the podocytes and their slit diaphragms is also similar between the zebrafish pronephros and the mammalian metanephric kidney. The proteins nephrin and podocin, both key components of the slit diaphragm in mammals and implicated in hereditary nephrotic syndromes when mutated in humans, are present in the zebrafish pronephric glomerulus, and inactivation of either of them leads to renal dysfunction, with pericardial edema and early death as a result.[20] Podocin is present exclusively in the glomerular podocytes[1] and is therefore a specific marker for these cells.

Thus, the zebrafish pronephric glomerulus appears ideally suited for live imaging, and toward this end, transgenic fish with fluorescence reporter expression in podocytes would be helpful. A 2.5-kb DNA fragment corresponding to the 5′ end of the human podocin gene was previously shown to drive specific expression of a reporter gene specifically in podocytes in transgenic mice.[21] We therefore used a similar size DNA fragment corresponding to the 5′ end of the zebrafish *podocin* gene to generate transgenic zebrafish expressing green fluorescence protein (GFP). The 2.5-kb fragment was cloned into a *Tol2* transposon-based plasmid to generate transgenic GFP lines (Figure 1A). In total, 15 injected G_0 embryos that were mosaic with regard to GFP-positive cells in the pronephric glomerulus survived into adulthood. By outcrossing, five independent founders

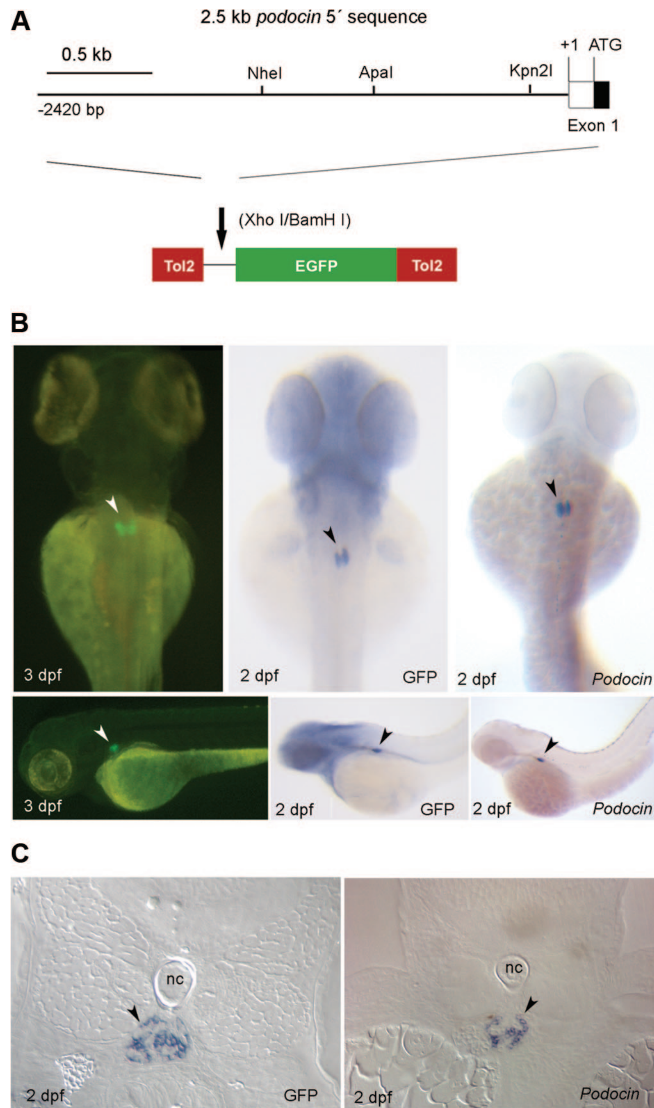

Figure 1. Generation and characterization of the podocin-GFP transgenic zebrafish line showing that the GFP expression overlaps with the endogenous podocin expression. (A) The *Tol2* transposon-based construct used to generate the line. A 2.5-kb zebrafish *podocin* 5′ sequence was cloned into the pT2KXIG plasmid. The flanking *Tol2* transposon sites are indicated (red boxes). (B) GFP expression driven by the *podocin* promoter recapitulates endogenous *podocin* expression pattern. GFP is exclusively expressed in the pronephric glomerulus (arrowheads; note that fluorescence signals in yolk and eyes represent autofluorescence), illustrated in dorsal and lateral views. The location of GFP is further confirmed by *in situ* hybridization using GFP and *podocin* probes (note that the GFP *in situ* hybridization signal in the head represents unspecific cross-reaction). (C) Cross-sections show the presence of GFP mRNA (left panel) at the location typical for podocytes, as confirmed by the stained obtained using a *podocin* probe (right panel). nc, notochord.

(30%) were identified. Embryos from each of the five founders displayed identical expression patterns in which GFP was expressed specifically in the pronephric glomerulus without signs of ectopic expression (Figure 1B). The glomerular GFP signal emerged at 2 dpf and was robust thereafter. The founder with the strongest GFP expression, *Tg(podocin:GFP)*, was used to collect homozygous fish for line maintenance and subsequent studies. To determine whether the GFP localization recapitulated the endogenous *podocin* expression pattern, we performed whole-mount *in situ* hybridization to compare the expression pattern of GFP with that of the endogenous *podocin* gene. These results showed a complete overlap between *podocin* and GFP expression (Figure 1, B and C). Consistent with the GFP expression pattern, *podocin* mRNA was undetectable at 1 dpf (data not shown). Thus, the 2.5-kb zebrafish *podocin* 5′ sequence used in this study appears to contain the transcriptional regulation elements necessary to recapitulate endogenous *podocin* expression in pronephric podocytes. A transgenic zebrafish expressing the fluorescence reporter mCherry from a 3.5-kb podocin promoter fragment was recently reported.[22] Although that study focused mainly on the mesonephros, the expression of the 3.5- and 2.5-kb podocin promoters appears similar in the pronephros as well as in the mesonephros (reference [22] and data not shown). Our study therefore refines somewhat the region containing podocyte-specific regulatory elements. Our ongoing analyses aim at defining this region more precisely.

Transmission electron microscopy (TEM) analysis has shown that, similar to podocytes of mammalian metanephric kidneys, the pronephric podocytes in zebrafish elaborate foot processes that are connected by slit diaphragms.[16,20] TEM analysis of the pronephric glomerulus is relatively straightforward because it relies on sectioning of whole embryos. It is more difficult to access the three-dimensional architecture of the embryonic pronephric glomerulus by scanning electron microscopy (SEM) because this requires microdissection of a tiny transparent single glomerulus located deep inside the embryo. The brightly fluorescence glomeruli of *Tg(podocin:GFP)* embryos greatly facilitated glomerular dissection and cutting of the embryo to expose the glomerulus for SEM analysis *in situ*. The SEM image shown in Figure 2A illustrates the surface architecture of a 6-dpf pronephric zebrafish glomerulus clearly displaying interdigitating podocyte foot processes. These observations are consistent with previous data obtained by TEM.[16,20] The SEM analysis thus provides further morphologic evidence for the presence of a functional filtration barrier in the embryonic zebrafish pronephric glomerulus.

Pericardial edema often occurs after knockdown of glomerulus-associated genes using morpholinos.[23,24] This phenotype is usually associated with loss of pronephric kidney function (osmoregulation),[20,25] but it is not specific for glomerular and/or podocyte dysfunction because it generally occurs also as a result of cardiac or vascular dysfunction.[26–28] Thus, the presence of pericardial edema

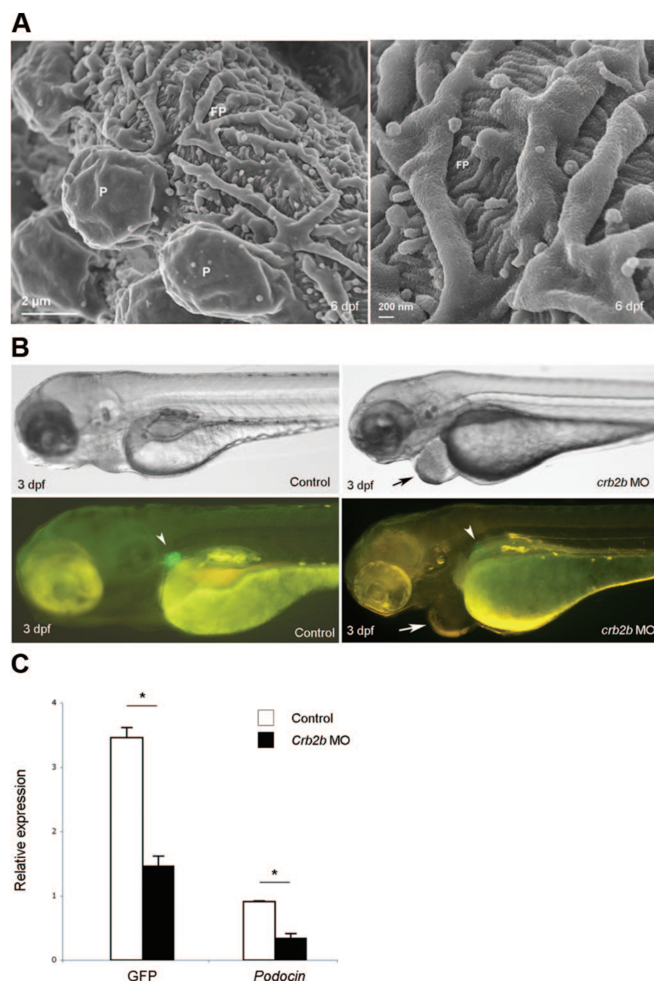

Figure 2. The *Tg(podocin:GFP)* line allows for ultrastructural studies and provides a direct readout for glomerular-specific phenotype in genetic studies. (A) Scanning electron micrograph of a 6-dpf glomerulus. Podocytes extend foot processes that interdigitate around capillary loops (left panel). The right panel shows branching foot processes at higher magnification. P, podocyte cell body; FP, primary (left panel) and interdigitating (right panel) foot processes. (B) C*rb2b* knockdown embryos. A *crb2b* morphant (right panel) shows a typical pericardial edema (arrow), coupled with loss of glomerular GFP signal (arrowhead), compared with the control (left panel). (C) Relative GFP and *podocin* mRNA levels in *crb2b* morphants ($n = 5$) and controls ($n = 3$) by determined by quantitative PCR. The error bars indicate the SD. $^{*}P < 0.01$ (*t* test).

in a genetic or chemical screen cannot be taken as evidence for kidney dysfunction without additional morphologic and/or functional analysis of the pronephros. A significant change in the GFP signal in the *Tg(podocin:GFP)* line could, however, be used as a direct readout of developmental aberrations or injury to the glomerulus. We tested this idea by injecting a translation-blocking morpholino against *crb2b* into *Tg(podocin:GFP)* embryos. Previous studies have demonstrated that *crb2b* knockdown leads to pericardial edema and pronephric cyst phenotype with compromised glomerular filtration barrier function and integrity in the early embryo.[23] As shown in Figure 2B, pericardial edema in *crb2b* morphants correlated with loss of glomerular GFP signal in *Tg(podocin:GFP)* embryos. The reduced GFP signal paralleled the drop in mRNA levels for GFP and *podocin* (Figure 2C).

In summary, we report the generation of transgenic *Tg(podocin:GFP)* zebrafish lines with strong and specific GFP expression in podocytes of the pronephric glomerulus. These lines provide powerful tools for podocyte research, because they can be used for real-time imaging, FACS sorting, and molecular profiling of zebrafish pronephric podocytes, mutant and chemical screens, and for high-throughput functional analysis of genes by morpholino injections.

CONCISE METHODS

Transgenic zebrafish were generated using the *Tol2* transposon system.[29] A 2.5-kb DNA fragment corresponding to the zebrafish *podocin* promoter was amplified from AB strain genomic DNA, cloned into the pCRII-TOPO vector (Invitrogen), and confirmed by sequencing. The fragment was then transferred to the pT2KXIG vector (kindly provided by K. Kawakami, National Institute of Genetics, Shizuoka, Japan) (Figure 1A) and injected into G_0 embryos, which were subsequently raised and outcrossed with TL zebrafish for the identification of germline founders. GFP expression was documented at ages 1 to 6 dpf under a stereomicroscope. For SEM, 6-dpf *Tg(podocin:GFP)* embryos were briefly fixed in glutaraldehyde followed by dissection under a stereomicroscope to expose the brightly fluorescence glomerulus. After further fixation, ethanol washes, infiltration with tetramethylsilane, and air-drying, specimens were mounted and analyzed in an Ultra 55 field emission SEM at 3 kV. Knockdown of Crb2b using translation-blocking morpholinos was done as recently described.[23]

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DISCLOSURES

None.

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SUPPLEMENTARY INFORMATION

Podocin-GFP zebrafish allows *in vivo* visualization and functional analysis of glomerular podocytes

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FULL METHODS

Zebrafish husbandry

The zebrafish wild-type AB and TL strains were maintained, mated and raised as described (8) at the Karolinska institutet zebrafish core facility. Embryos were kept in E3 water with or without 0.003% of PTU (1-Phenyl-2-thiourea) to inhibit pigmentation.

Generation transgenic zebrafish

Transgenic zebrafish were generated using the *Tol2* transposon system (4). The zebrafish *podocin* promoter sequence was amplified from AB strain genomic DNA. The 2.5-kb amplicon was first cloned into the pCRII-TOPO vector (Invitrogen) followed by sequencing. The cloned fragment was then transferred into the pT2KXIG plasmid (A gift from K. Kawakami, National Institute of Genetics, Shizuoka, Japan) (**Figure 1A**). The injected G_0 embryos were raised and outcrossed with TL zebrafish to identify germline founders. All experiments were approved by the Stockholm North Ethical Committee for animal research.

Embryo microinjection

The pT2KXIG-based construct, together with the *Tol2* transposase mRNA, were co-injected into the yolk of zebrafish embryos at the late one-cell or early two-cell stage as

described (3). We analyzed GFP expression in embryos at ages between 1-6 dpf using a stereomicroscope equipped for epifluorescence.

Scanning electron microscopy

Tg(podocin:GFP) embryos at 6 dpf were briefly fixed by immersion in 2,5 % glutaraldehyde in 0.1M PBS (pH 7.4). The glomerulus, localized by GFP fluorescence under fluorescence microscopy, was exposed by dissection followed by further fixation at 4°C. The specimens were briefly rinsed in distilled water and placed in 70% ethanol for 10 min, 95% ethanol for 10 min, absolute ethanol for 15 min at room temperature and in pure acetone for 10 min and then were transferred to Tetramethylsilane (Merck, Germany) for 10 min and air-dried (1). After drying, the specimens were mounted on an aluminum stub and coated with Carbon (Balzer, SCD 005, Liechtenstein). The specimens were analyzed in an Ultra 55 (Carl Zeiss, Oberkochen, Germany) field emission SEM at 3 kV.

In situ hybridization, histological analysis and crb2b knockdown

Whole-mount in situ hybridization was performed as described (7). Histological analysis was carried out as described (5). Knockdown of Crb2b using translation-blocking morpholinos was done as previously described (2).

qPCR

The qPCR was performed on cDNA using SYBR Green PCR Master Mix (Applied Biosystems) on the ABI PRISM 7300 System. The cDNA was reverse-transcribed from total RNA extracted from single 4-dpf whole embryos. The relative expression quantification was analyzed using the comparative threshold (Ct) method (6).

Primer sequences

All primers used in the present study are listed in Table S1.

Table S1 . Zebrafish primer sequences used

Gene	Primer sequence
Podocin 5′	5′-GGTGATTCTATGCTCTTTGCGCTTTGT
	5′-TTTCTCTATCTCCGCAGGAAGCATCGT
GFP probe	5′-GACGTAAACGGCCACAAGTT
	5′-TTCTCGTTGGGGTCTTTGCT
Podocin probe	5′-GTCTGGAATGCTAGCGAAGG
	5′-GTCTGGAATGCTAGCGAAGG
β-actin qPCR	5′-CGAGCAGGAGATGGGAACC
	5′-CAACGGAAACGCTCATTGC
GFP qPCR	5′-ACCACTACCTGAGCACCCAGTC
	5′-GTCCATGCCGAGAGTGATCC
Podocin qPCR	5′-CGAGAGATACTGGCCCATCA
	5′-CCACTTTAATACCCCACCTG

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