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A multicolor podocyte reporter highlights heterogeneous podocyte changes in focal segmental glomerulosclerosis

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In contrast to most glomerular diseases, the injury pattern in focal segmental glomerulosclerosis (FSGS) is highly heterogeneous, even though podocytes are genetically identical and exposed to the same environmental factors. To understand changes in individual podocytes, we generated and analyzed a stochastic multicolor Cre-reporter, encoding four fluorescent proteins. In these animals, podocytes were randomly labeled allowing individual cells and their foot processes to be distinguished. In healthy animals, podocyte size and structure showed little cell to cell variability. In the doxorubicin-induced FSGS model, fluorescent-labeled glomerular podocyte numbers decreased and fluorescent cells could be recovered from the urine. The size of the remaining podocytes showed a high degree of heterogeneity, some cells remained small, while others enlarged. Both enlarged and non-enlarged podocytes showed alterations in their foot process morphology. Thus, by the virtue of a multicolor cre-reporter, individual podocytes could be viewed in real time at a cellular resolution indicating a heterogeneous podocyte injury response during the pathogenesis of FSGS.

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Our kidneys filter approximately 180 liters of plasma every day. The glomerular filtration barrier has three layers: fenestrated endothelial cells, basement membrane, and the foot processes of glomerular epithelial cells.[1] The highly organized podocyte foot process architecture is critical for maintaining the filtration barrier.[2] Foot process effacement (FPE) or widening of the foot processes are almost universally observed in patients with nephrotic syndrome,[2] therefore it is considered to be the 'diagnostic lesion' of nephrotic syndrome. The highly dynamic podocyte slit diaphragm and associated actin cytoskeleton are responsible for maintaining the foot process architecture.[3] Genetic mutations of different actin cytoskeletal and slit-associated proteins cause nephrotic syndrome and FPE in humans. Most of these mutations show histopathological lesions consistent with focal segmental glomerulosclerotic (FSGS).[4,5] FSGS, however, most commonly occurs secondary to glomerular hyperfiltration in the setting of low nephron number, obesity, viral infection (human immunodeficiency virus), or medication use.[6] Podocyte injury, apoptosis, and loss of functional podocytes are thought to be the root cause of FSGS. In addition to podocyte apoptosis, FPE and hypertrophy are also shown to have an important role in the pathogenesis of FSGS. At present, it is not clear whether or not podocyte death, FPE, and hypertrophy are coupled mechanisms.

As the name indicates, glomerular lesions are heterogeneous in FSGS; only certain glomerular segments of some glomeruli are affected. The focal and segmental lesions are in striking contrast with other disease entities including diabetes, IgA and membranous nephropathy where cells and glomeruli are similarly affected. The cause and the mechanism of disease heterogeneity are not well understood. The cell-to-cell and glomerulus-to-glomerulus heterogeneity are even more puzzling as coding mutations, viral infections, obesity, or drugs should influence all cells similarly.[7] With the exception of electron microscopy (EM), present methods of analysis do not really allow us to distinguish individual podocytes. EM is time consuming, tedious, only a few cells

and glomeruli can be studied and it requires the use of harsh fixatives. To understand events occurring in individual podocytes, here we developed a podocyte-specific stochastic multicolor reporter, which allowed us to monitor individual cells. We found that in healthy mice podocyte architecture is highly organized, showing little cell-to-cell variation. Podocytes exhibited very heterogeneous structural changes following injury. This heterogeneous injury and adaptation might explain the development of heterogeneous histological pattern of FSGS. Defining pathways that control this heterogeneity will likely be important for the understanding of FSGS. This new mouse model and imaging modality will help us to achieve this.

RESULTS

Generation and characterization of podocyte-specific stochastic multicolor reporter

Understanding local changes and cell–cell interaction in podocytes requires visualization of individual cells and individual foot processes. As cultured podocytes show significant differences when compared with their native state, it is desirable to characterize them *in vivo*.[8] Here we generated a stochastic multicolor reporter mouse line by intercrossing the NPHS2Cre[9] (podocincre) line with a reporter strain that contained four different fluorescent reporters:[10] cytoplasmic targeted yellow fluorescent protein (YFP), cytoplasmic targeted red fluorescent protein (RFP), membrane targeted cyan fluorescent protein and nuclear targeted green fluorescent protein (GFP) (Figure 1a). Fluorescent proteins are expressed from the Rosa26 locus. The construct consists of the strong CAGG promoter, the LoxP-flanked neomycin resistance cassette, which serves as a transcriptional roadblock, and the original Brainbow-2.1 cassette. After Cre-mediated recombination, the roadblock is removed and the fluorescent marker proteins are stochastically placed under the control of the CAGG promoter. As the fluorescent proteins were cloned in reverse orientation, only one sequence per cell will be in the correct orientation to express. Therefore, in heterozygous Confetti animals ($NPHSP2^{Cre}/Confetti^{TG/WT}$) each cell where recombination took place will express only one fluorescent protein. As recombination is random, neighboring cells can be identified with high precision.[11] First, we analyzed images from 6-week-old control male $NPHSP2^{Cre}/Confetti^{TG/WT}$ mice (Figure 1b). The Cre-mediated recombination efficiency was relatively high and the model allowed for distinction of individual podocytes. As the spectra of GFP and YFP overlap, we focused on distinguishing these cells based on their nuclear versus cytoplasmic location of the fluorescent signal.

The percentage of fluorescent-labeled podocytes was similar in the $NPHSP2^{Cre}/Confetti^{TG/TG}$ homozygous mice and in the $NPHSP2^{Cre}/Confetti^{TG/WT}$ mice, however, in the homozygous mice we also observed double flourophore-labeled cells (Figure 1c). Using z-stack images obtained with the Leica SP5 confocal microscope, and freshly removed kidneys, we generated stacked reconstruction images of the glomeruli (Figure 1d). Volocity Software-based volume reconstruction gave the best overview of the glomerulus (Figure 1e). Furthermore, the complete three-dimensional reconstruction also helped to fully distinguish individual cells and foot processes. The YFP and RFP gave the strongest fluorescence and also allowed the most comparable visualization of cell architecture including foot processes and their interactions. Mice with a single Confetti allele, allowed for much better spectral discrimination of adjacent cells. In double transgenic animals, adjacent podocytes sometimes labeled with the same from each other fluorescent proteins making them difficult to distinguish from each other (Figure 1c). In summary, we found that the volume reconstruction method of YFP and RFP from a single allele allowed the best discrimination of the podocyte architecture (Figure 1e).

Healthy podocytes display little architectural variability

We compared established imaging methods with our new fluorescent method using control $NPHSP2^{Cre}/Confetti^{TG/WT}$ mouse. Periodic acid–Schiff stained light microscope (Figure 2a), confocal fluorescent microscope (Figure 2b and d–g), and scanning EM (Figure 2c) are shown side by side. Pictures obtained with the new confetti fluorescent imaging method showed structural details that were similar to scanning EM images (Figure 2c). The fluorescent detection method, however, was far superior in its ability to image entire glomeruli, thereby allowing the detection of podocyte heterogeneity and cell–cell interactions. As expected, individual podocyte foot processes interacted only with adjacent podocyte foot processes, and we never observed interdigitation between processes originating from the same cell body (Figure 2d–f). The images also seem to indicate that podocyte processes follow a monopodial branching pattern in the primary, secondary, and tertiary branches. The branching is likely to be guided as they carefully align and cover glomerular capillary loops. Primary processes were fairly long and we were able to follow them up to 20–25 μm. Secondary processes were of similar length to primary processes and we were able to identify tertiary processes to about 5 μm. The slit density was about 3–4 slits per μm, similar to previous publications.[12] The images appear to indicate that primary and secondary branches can differ in length; however, tertiary branch width and branch-to-branch distance seemed to be tightly controlled to create a highly organized pattern. In summary, podocytes in the healthy $NPHSP2^{Cre}/Confetti^{TG/WT}$ mice showed little architectural variability.

Next, we analyzed glomeruli of younger (5 and 10 days old) animals. We observed significant heterogeneity of glomerular maturation in kidneys taken from 5-day-old animals (Figure 3a and b). In immature glomeruli, the fluorescent podocyte number and the overall fluorescence level were much lower when compared with the 6-week-old animals. This is consistent with the observation that the podocin gene is only expressed in mature podocytes. Sometimes we also observed clusters of podocytes with the same color potentially indicating recently dividing cells (yellow cells at 6 o'clock in Figure 3b). Fairly simple podocyte architecture