Exp. Anim. 66(3), 183–189, 2017

—Original—

Nephron, Wilms' tumor-1 (WT1), and synaptopodin expression in developing podocytes of mice

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Abstract: Newborn mouse glomeruli are still immature with a morphological feature of an early capillary loop stage, but infant mice do not manifest proteinuria. Little is known about the molecular mechanism whereby infant mice are resistant to proteinuria. Nephrin and synaptopodin are crucial for slit diaphragm and foot process (FP) formation for avoiding proteinuria. Nephrin tyrosine phosphorylation means a transient biological signaling required for FP repair or extension during nephrotic disease. Using an immunohistochemical technique, we examined the natural course of nephrin, Wilms' tumor-1 (WT1) and synaptopodin at 16.5 days of embryonic age (E16.5d) and E19.5d, 7 days of post-neonatal age (P7d) and P42d during renal development of mice. As a result, nephrin and synaptopodin were detected at E19.5d in S-shaped bodies. WT1, a transcriptional factor for nephrin, was detected in nucleus in podocyte-like cells in all stages. Nephrin tyrosine phosphorylation was evident in glomeruli at P7d, and this was associated with an early-stage of FP extension. Inversely, nephrin phosphorylation became faint at P42d, along with maturated FP. Based on the present results, we suggest the sequential molecular mechanism to protect growing mice from proteinuria: (i) WT1-induced nephrin production by podocytes in S-shaped bodies at E19.5d; (ii) Synchronized induction of synaptopodin at the same period; and (iii) FP extension is initiated at a milk-suckling stage under a nephrin tyrosine-phosphorylated condition, while it is arrested at an adult stage, associated with a loss of nephrin-based signaling.

Key words: glomerulogenesis, nephrin, podocyte, synaptopodin, Wilms' tumor-1 (WT1)

Introduction

Renal glomeruli are mainly composed of four components, such as podocytes, endothelial cells, glomerular basement membrane (GBM) and mesangial cells [6]. Podocytes are located along outside of GBM and play a key role for blood-to-urine filtration via slit diaphragm (SD) formation and foot process (FP) extension [1, 21]. Importantly, specific depletion of podocytes by toxin treatment leads to proteinuria in mice [18, 34], thus establishing a "protective"function of podocytes to avoid proteinuria. In this homeostatic process, glomerular SD, a slit between interdigitating FP of podocytes, serves as a size-selective barrier and it is linked to the actin-based cytoskeleton by adaptor proteins [1, 21]. There is now ample evidence to show that podocyte damage or FP dysfunction is contributable for the onset or progression of proteinuria during acute or chronic kidney diseases

(Received 10 November 2016 / Accepted 10 January 2017 / Published online in J-STAGE 7 February 2017)
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[25, 29].

Kidney development is a very complex process that requires interactions between epithelial and mesenchymal cells, eventually leading to the coordinated growth and differentiation of highly specialized cell types [28, 31]. During earliest stages of glomerular development, podocytes arise from mesenchymal precursors in the induced metanephric mesenchyme, which in the S-shaped body stage acquires characteristics of epithelial cells [30]. At capillary loop stage of glomeruli, primitive podocyte appears and is characterized by transformation of columnar epithelial cells into mesenchymal-like cell type. This transition is associated with loss of lateral cell attachments and acquirement of cellular extensions and interdigitating cell processes [22].

Until now, we and other group have accumulated evidence that infant mice are resistant to proteinuria, although glomerular structure is still immature, with a feature of an early capillary loop stage [19, 23, 36]. These findings suggest the possible maturation of SD or FP extension in an embryonic or post-neonatal stage, but little is known about an initial event contributing to podocyte maturation. It is well known that nephrin is indispensable for SD formation [24, 25], and synaptopodin for FP extension [2, 3]. Furthermore, Wilms' tumor-1 (WT1) is shown to initiate nephrin transcription as a nuclear transcriptional factor through binding to the promotor region of nephrin cDNA [37]. These previous data prompted us to hypothesize that SD-associated molecules may be sequentially involved in the possible podocyte maturation.

To address this hypothesis, we used immunohistochemistry, because this method is suitable for determining distribution (and in part, staining intensity) of SD-associated molecules, including nephrin. Based on immunohistochemistry, we identified the sequential expression patterns of WT1, nephrin, and synaptopodin during glomerulogenesis of mice. This is the first report to comprehensively delineate the natural course of podocyte development in embryonic, infant and adult mice, with a focus on SD-associated proteins.

Materials and Methods

Animal experiment

We obtained three pregnant C57BL/6J strain mice for analyzing renal morphology at an embryonic day of 16.5 (E16.5d), E19.5d and postnatal day 7 (P7d) (Charles River, Osaka, Japan). The morning of copulatory plug detection was defined as E0.5d and the day of birth was defined as a postnatal day (P0d), as described [10]. Furthermore, 42-day-old mice were purchased from Charles River as an adult control (defined as P42d). All animal experiments were carried out according to the Guideline for Experimental Animal Care issued by the Prime Minister's Office of Japan and approved by the Committee on Animal Experimentation of Osaka University School of Medicine.

Histological analysis

Right kidney removed at each autopsy was fixed in cold 10%-buffered formalin for 24 h. Transversally trimmed kidney tissues were submitted to a routine process for paraffin embedding [14, 15]. The renal sections were prepared, deparaffined, and stained with Hematoxylin & Eosin (HE). Anti-nephrin IgG {sc-19000, Santa Cruz Biotechnology (SCB), Dallas, TX, USA}, anti-WT1 IgG (sc-7385, SCB), anti-synaptopodin (65194, Progene, Heidelberg, Germany) were used for immunohistochemistry. Anti-phospho-Y1208-nephrin IgG was produced in our laboratory, as reported [33]. Immunostaining assays were performed as described previously [14–16]. The sections were dewaxed and then were treated with 0.1 M citrate buffer at pH 6.0 in an autoclave for five minutes for antigen retrieval. The sections were incubated at 4°C for overnight with primary antibodies, washed with PBS and incubated with biotin-labeled anti-mouse and anti-goat antibody (Vector, Burlingame, CA) for 1 hour at room temperature. Avidin-biotin coupling reaction was performed on the sections using a kit (Elite®, Vector). All antigens were visualized as brown with 3-3" diamino benzidine (Nacalai, Kyoto, Japan).

Transmission electron microscopy

The extracted kidneys were fixed in cold 1% glutaraldehyde/1% paraformaldehyde in phosphate buffered saline at 4°C overnight, as reported [16]. Tissue specimens were post-fixed in osmium tetroxide for 2 h at 4°C, dehydrated through an ethanol gradient, and embedded in Epon. Ultrathin sections (90 nm) were cut with an ultra-microtome, stained with 4% uranyl acetate and 1% lead citrate, and then examined with an electron microscopy (HT7700; Hitachi, Tokyo, Japan) [16].

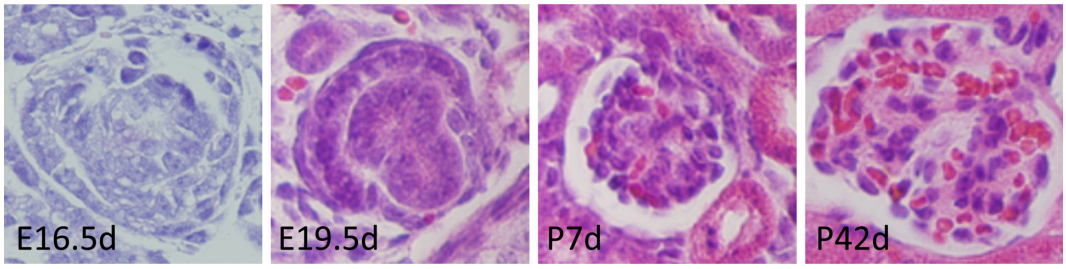

Fig. 1. Representative findings of renal tissue during glomerulogenesis in mice. The renal sections were harvested from embryonic (E16.5d and E19.5d), neonatal (P7d) and adult (P42d) mice and stained with HE (original magnification: ×400).

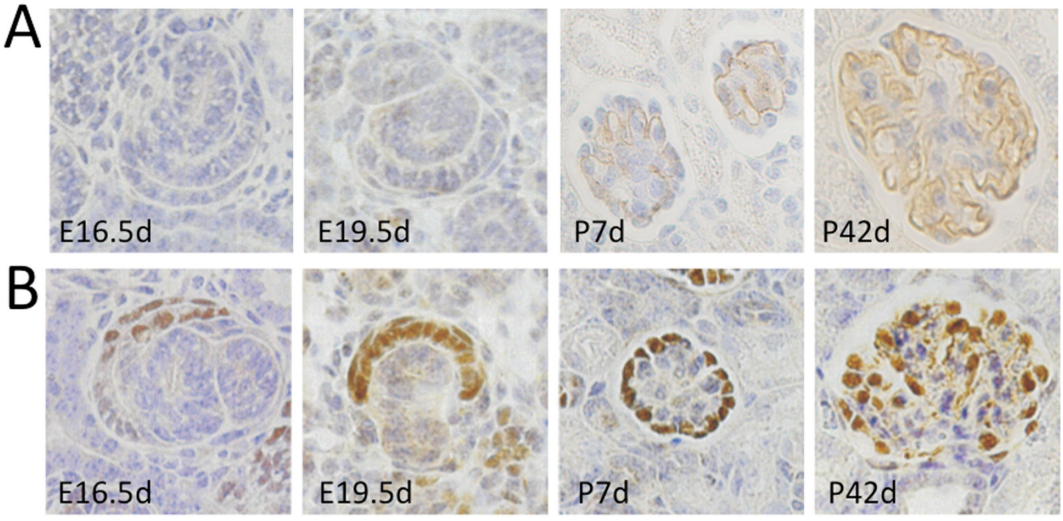

Fig. 2. Immunohistochemical findings of developing kidneys (×400). Nephrin (A) and WT1 (B) expression patterns were visualized by immunohistochemistry during glomerulogenesis in each age of mice.

Results

Morphological changes in glomeruli during renal development

At first, we confirmed the podocyte development on HE-stained renal sections. Renal histology revealed that vesicle stage was predominant at E16.5d, followed by S-shaped body stage around E19.5d. Afterward, capillary loop stage was observed at P7d. Moreover, all of most glomeruli became mature at latest by P42d (Fig. 1). These changes were similar to a previous report [28], hence indicating the typical model of mouse glomerulogenesis.

Alteration of nephrin and WT1 expression during glomerulogenesis

Using the typical model, we next investigated the functional protein expression in podocytes, with a focus on nephrin and WT1. Nephrin was not yet detectable in podocytes of E16.5d, but some extent of nephrin expression became detectable in podocytes of E19.5d, especially in S-shaped body. The nephrin expression was linearly detected at P7d, and eventually, it became extensive with a lobular distribution, at latest P42d (Fig. 2A).

WT1, known as a key master switch for renal development [20], was already detected at E16.5d (Fig. 2B). Furthermore, WT1-positive signals were strongly detected, especially in S-shaped body at E19.5d (Fig. 2B). After birth, WT1 was clearly detected in glomerular cells at P7d and P42d, especially in the outer areas of glomerular tufts (Fig. 2B).

Changes in synaptopodin and nephrin phosphorylation during FP extension

Synaptopodin is an actin binding protein for induction

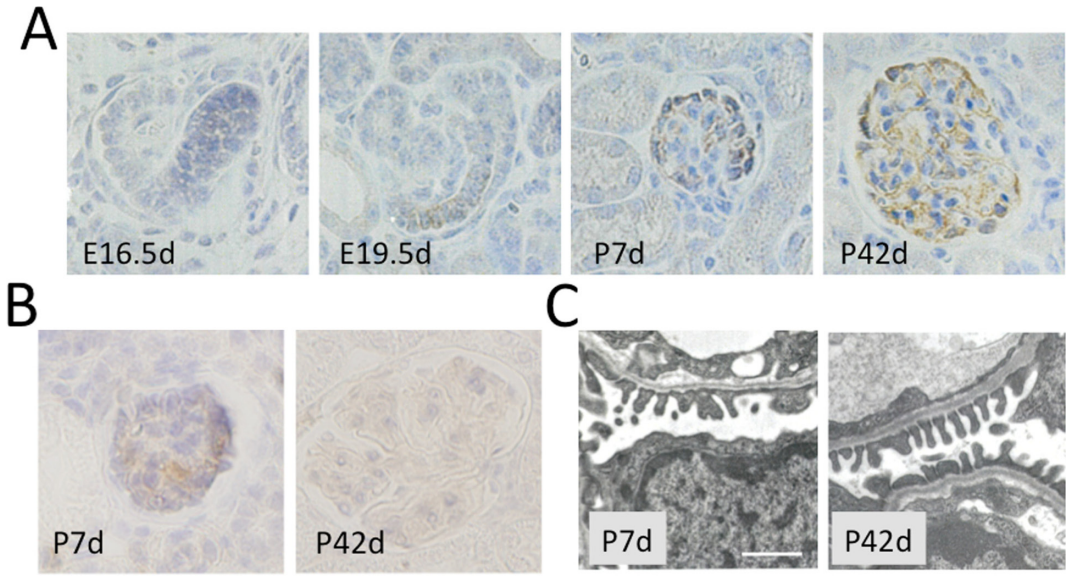

Fig. 3. Changes in synaptopodin and nephrin tyrosine phosphorylation during FP development in mice. (A & B) Immunohistochemical images of synaptopodin (A) and nephrin phosphorylation at a position of Y1208 (B) in each age of mice. (C) Electron microscopic findings of glomerular FP in each age. Scale bars = 2 μm.

of FP extension [2, 3]. Synaptopodin was not detected in podocytes at E16.5d (Fig. 3A). However, podocytes exhibited synaptopodin expression at E19.5d. The synaptopodin expression became evident in post-neonatal stage, especially between P7d and P42d (Fig. 3A).

With a focus on this time-frame (i.e., synaptopodin-increasing stage), we focused on nephrin phosphorylation, a key signal transducer for FP extension [33]. Immunohistochemical analysis revealed that nephrin was tyrosine-phosphorylated in podocytes at P7d (Fig. 3B). However, this tyrosine phosphorylation became faint in mature podocytes of P42d kidneys, suggesting developmental arrest of FP, as discussed later.

In contrast to the nephrin phosphorylation pattern, glomerular FP formation was mild to moderate at P7d, as evidenced by an electron microscopy (Fig. 3C). The glomerular FP formation was extensively seen in the adult mice (i.e., P42d) (Fig. 3C), associated with a loss in nephrin phosphorylation.

Discussion

Nephrin is originally identified as a loss-of-function gene that causes Finnish type congenital nephrotic syndorome [24, 25]. Clinical studies indicate that a decrease in glomerular nephrin is negatively linked with proteinuria in a wide range of renal diseases [29]. In this study, we detected nephrin in the embryonic kidney of E19.5d, where GBM or FP formation is immature [28]. Thus, it is important to discuss a relationship between nephrin and FP during renal development. With regard to this, Holzman's group demonstrated that nephrin localizes in pores between FP and FP in a newborn stage [11]. Nephrin is critical for FP-FP contact via hemophilic interaction of extracellular domain [17]. Anti-nephrin antibody induces severe proteinuria via destruction of SD in normal rats [32]. Importantly, nephrin-deficient mice manifest proteinuria, due to abnormality of SD and FP [26]. Collectively, it is likely that nephrin is a prerequisite for normal formation of FP during renal development.

In the present study, WT1 was detected in all stages of glomerulogenesis. Since WT1 initiates nephrin transcription via binding to promoter region of nephrin cDNA [37], it is reasonable that changes in WT1 expression are similar to those of nephrin. Nephrin undergoes proteolytic degradation via extracellular shedding [5]. Thus, podocytes constitutively stimulate *de novo* synthesis of nephrin, providing a rationale why WT1 is always located in nucleus of podocytes. It is important to discuss a role of WT1 at E16.5d, since nephrin is not yet detected in this stage. Vascular endothelial growth factor (VEGF)-secreted from podocytes is necessary for early-stage glomerulogenesis [7], while WT1 targets VEGF gene to initiate its transcription [20]. Taken together, it

is likely that WT1 also contributes to the nephrin-independent mechanism, such as VEGF-mediated vascular cleft formation.

It is important to discuss molecular events in S-shaped body. Wnt4-β-catenin signaling is necessary for mesenchymal-to-epithelialization [9] and subsequent Notch2 for podocyte specification during a late stage of S-shaped body [4]. Our study revealed that nephrin is detected along outside of S-shaped body at E19.5d. In contrast, other group indicated that nephrin was identified in capillary loop, but not in S-shaped body in newborn mice [11], raising a controversial issue. With regard to this, Takano *et al*. reported that nephrin was detected in S-shaped body in 24-week fetal kidneys of human [31]. Nephrin transgene mRNA is detectable in S-shaped body in a transgenic mouse [35]. Collectively, we predict the possible molecular hierarchy as followed: (a) Wnt4 signaling for epithelization in metanephros mesenchyme; (b) Notch2 pathway for podocyte specification; and (c) WT1 cascade for induction of nephrin in podocytes, all of which contributes to protection of newborn from proteinuria.

Synaptopodin is an actin-associated protein specifically expressed in podocytes and is also critical for stabilizing SD integrity [2, 3]. Synaptopodin plays a protective role in induction and mentenance of FP under a pathological condition [3]. We previously reported that not only nephrin but also synaptopodin is contributable for the attenuation of proteinuria in mouse models of acute or chronic kidney disease [14, 15]. These previous findings prompted us to check the time course of synaptopodin during renal development. As a result, synaptopodin was detectable at E19.5d, being similar to the nephrin expression. Synaptopodin is critical for cytoskeletal formation via a direct binding with α-actinin 4 [3]. Therefore, synaptopodin may be critical for FP extension even in renal development.

Finally, we would like to discuss the biological significance of nephrin tyrosine phosphorylation. Aside from being a structural molecule, nephrin is a signaling molecule that, after phosphorylation, mediates signal transduction in podocytes [8, 27, 33]. Once podocytes undergo damage, nephrin tyrosine phosphorylation is transiently initiated, and then a defect in FP integrity is restored through actin polymerization [24]. In this process, phosphorylated nephrin recruits down-stream adaptors, such as noncatalytic region of tyrosine kinase adaptor protein (Nck) [13], and synaptopodin is also involved in actin polymerization, possibly via an indirect pathway [3, 12]. Of note, nephrin phosphorylation is arrested when FP repair is completed under disease, hence implying phosphorylated nephrin as a transient biochemical marker for FP repair or development [24]. This may explain the decrease in nephrin phosphorylation, as seen at P42d, because FP development is completed in this adult age.

Overall, we delineated the natural course of molecular cascades in mouse podocytes during renal development. The possible molecular events are summarized as followed: (i) WT1 is induced in specified podocytes at E16.5d; (ii) nephrin is produced via WT1-induced transcriptional pathway from E19.5d; (iii) synaptopodin is also induced from E19.5d; (iv) eventually, FP formation becomes mature between 7 and 42 days after birth, in part, via regulating nephrin-mediated signaling pathways. These sequential events may be contributable for protecting neonatal mice from intractable proteinuria.

Conflicts of Interest

The authors have no conflicts of interest to this report.

Acknowledgments

This work was supported by a grant from the Osaka Kidney Foundation (OKF16-0006 to TK) and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 18590369 and No.15K08402 to SM).

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