



## Clinical Journal of Nephrology

Review Article

Open Access

### Downregulation of ephrin-B1 is a critical event of podocyte injury

Yoshiyasu Fukusumi, Veniamin Ivanov, Ying Zhang, Hidenori Yasuda and Hiroshi Kawachi\*

Department of Cell Biology, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, Japan

\***Corresponding Author:** Hiroshi Kawachi, MD, PhD, Department of Cell Biology, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, Niigata, 951-8510, Japan, Phone: +81-25-227-2159, Fax: +81-25-227-0770; Email: [kawachi@med.niigata-u.ac.jp](mailto:kawachi@med.niigata-u.ac.jp)

**Received Date:** Jun 17, 2021 / **Accepted Date:** Jun 29, 2021 / **Published Date:** Jun 30, 2021

#### Abstract

Proteinuria in several glomerular diseases results from dysfunction of the slit diaphragm, a cell-cell junction of glomerular epithelial cells (podocytes). Ephrin-B1 and its related molecule NHERF 2 are novel essential components of the slit diaphragm. Ephrin-B1 interacts with nephrin via the extra-cellular domain and interacts with NHERF2 via the cytoplasmic site. In the proteinuric state induced by the stimulation to nephrin, nephrin and ephrin-B1 are phosphorylated, and NHERF2 is de-phosphorylated and consequent disruption of the linkage and downregulation of nephrin, ephrin-B1 and NHERF2 are a critical pathogenic event of podocyte injury.

**Keywords:** Podocyte; Slit Diaphragm; Ephrin-B1; Nephrin; NHERF2

**Cite this article as:** Yoshiyasu Fukusumi, Veniamin Ivanov, Ying Zhang, et al. 2021. Downregulation of ephrin-B1 is a critical event of podocyte injury. *Clinic J Nephro.* 3: 01-06.

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#### Introduction

The elucidation of the pathogenic mechanism of proteinuria is one of the most important themes in nephrology field. Proteinuria in several glomerular diseases results from dysfunction of the slit diaphragm connecting the neighboring foot processes of glomerular visceral epithelial cell (podocyte) [1-3]. However, the mechanisms of the slit diaphragm injury were not fully clarified yet. In 2007, we reported that ephrin-B1 is expressed at the slit diaphragm and interacts with nephrin, a key molecule of the extra-cellular components of the slit diaphragm [4]. Then, we reported that if nephrin is stimulated, not only nephrin but also ephrin-B1 is evidently downregulated [5].

Recently, we reported that ephrin-B1 interacts with NHERF2, and NHERF2 is also downregulated by the stimulation to nephrin [6]. These reports demonstrated that ephrin-B1 is a crucial molecule for maintaining slit diaphragm function. In this article, first, we overview the molecular composition of the slit diaphragm and discuss the role of ephrin-B1 and its related molecule NHERF2 of the slit diaphragm in physiological and pathological states.

## **Overview: molecular structure of slit diaphragm**

The first molecule identified as a slit diaphragm component is zonula occludens 1 (ZO-1). ZO-1 was originally identified as a component of tight junction [7] and was observed to be concentrated along the cytoplasmic surface of the slit diaphragm [8]. A key molecule maintaining the barrier function of the slit diaphragm is nephrin. Nephrin was identified as a product of a gene mutated in familial steroid-resistant nephrotic syndrome (NPHS1) [9]. Another key molecule of the slit diaphragm is podocin. Podocin was identified as a gene product of NPHS2, the causative gene of autosomal recessive steroid-resistant nephrotic syndrome [10]. Podocin interacts with nephrin and CD2AP [11]. CD2AP is also a functional molecule of the slit diaphragm [12]. CD2AP is an adaptor molecule that was identified to bind to CD2, a membrane protein on T cells [13]. CD2AP was shown to interact with nephrin and could anchor nephrin to the cytoskeleton [12]. It is reported that some patients with focal segmental glomerular sclerosis had a mutation of CD2AP [14]. NEPH1 was identified as a nephrin-related protein with a technique of the gene trapping screen [15]. NEPH1 interacts with ZO-1 and nephrin [16].

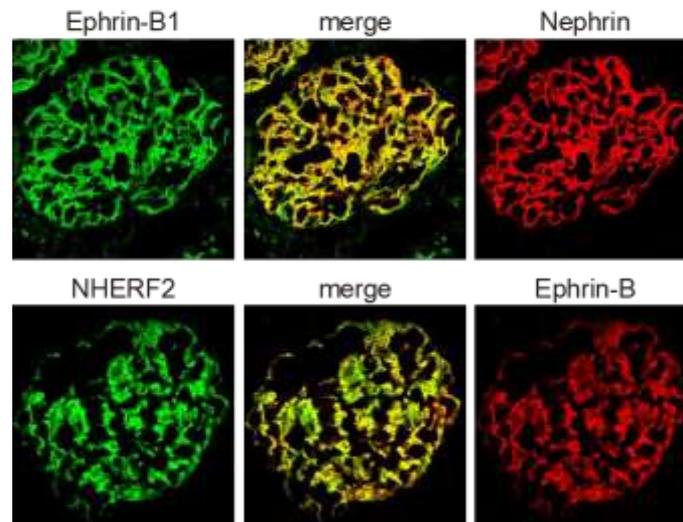
## **Ephrin-B1, a novel critical component of the slit diaphragm**

To explore a novel critical molecule that participates in the maintenance of the function of the slit diaphragm, we intended to purify the molecules whose expressions were downregulated in the proteinuric state resulted from slit diaphragm dysfunction. It is conceivable that the molecules downregulated in the proteinuric state might be a functional molecule of the slit diaphragm. Ephrin-B1 was identified as a molecule of which mRNA expression is decreased in the nephrotic state induced by the stimulation to nephrin [4]. Ephrin and its related protein Eph function as receptor-ligand pairs [17- 19]. The Eph-ephrin

family is reported to have many biological functions, such as formations of tissue-border, vascular development, and cell migration [20-22]. It is also reported that the Eph-ephrin-B family regulates the paracellular permeability of epithelial cells [20]. Dual labeling immunohistochemical analyses with a normal rat glomerular section showed that ephrin-B1 is colocalized with nephrin (Figure 1), which indicates that ephrin-B1 is a component of the slit diaphragm. Podocyte-specific ephrin-B1 conditional knockout (CKO) mice display clear alterations of the podocyte morphology, disarrangement of the critical molecules of the slit diaphragm, and the mice showed proteinuria. The in-vitro analyses with the HEK293 cell expression system revealed ephrin-B1 interacts with nephrin via the extracellular domain [5]. It was concluded that ephrin-B1 is one of the critical components of the slit diaphragm and is essential for the maintenance of the integrity of the slit diaphragm.

## **NHERF2, an ephrin-B1-associated molecule**

Then, we explored ephrin-B1-related molecules, then we found mRNA expression of NHERF2 was decreased in podocyte-specific ephrin-B1 CKO mice. The observation suggested that NHERF2 is a ephrin-B1-related molecule [6]. NHERF2, an isoform of NHERF proteins, was initially identified by two-hybrid screening using the cytoplasmic tail of Na<sup>+</sup>/H<sup>+</sup>-exchanger 3 (NHE3) [23]. Takeda et al. reported that NHERF2 interacts with podocalyxin, which is expressed at the apical surface of podocytes [24]. Dual-labeling immunohistochemical analyses with normal rat kidney section showed that some portions of NHERF2 are colocalized with ephrin-B1 (Figure 1). The interaction assay with HEK293 cells showed that NHERF2 interacts with ephrin-B1. These observations implied that NHERF2 is a component of the slit diaphragm complex.

**Figure 1**

**Figure 1:** Dual-labeling immunofluorescence of ephrin-B1 with nephrin and NHERF2. Ephrin-B1 is almost completely colocalized with nephrin (detected as yellow) (upper panel). Ephrin-B1 is colocalized with NHERF2 (seen as yellow), and some portions of NHERF2 are apart from ephrin-B1 (detected as green) (lower panel).

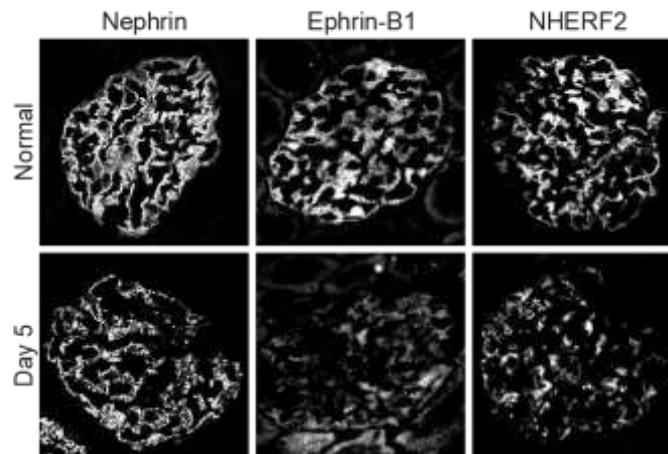
### **Ephrin-B1 and NHERF2 are downregulated in the proteinuric state caused by the stimulation to nephrin**

Immunostaining of ephrin-B1 and NHERF2, as well as nephrin, are evidently downregulated in rat nephrotic model induced by the injection with the antibody against nephrin [4-6] (Figure 2). The in-vitro analyses with the HEK293 cell expression system showed that if nephrin was stimulated by the antibody against the extracellular site of nephrin, nephrin and ephrin-B1 were tyrosine-phosphorylated, and serine/threonine of NHERF2 was de-phosphorylated, and the linkage of nephrin-ephrin-B1-NHERF2 was disrupted [5-6]. Thus, the observations implied that altered phosphorylated state of nephrin, ephrin-B1, and NHERF2 and downregulation of these molecules are involved in developing the slit diaphragm injury.

### **Conclusions**

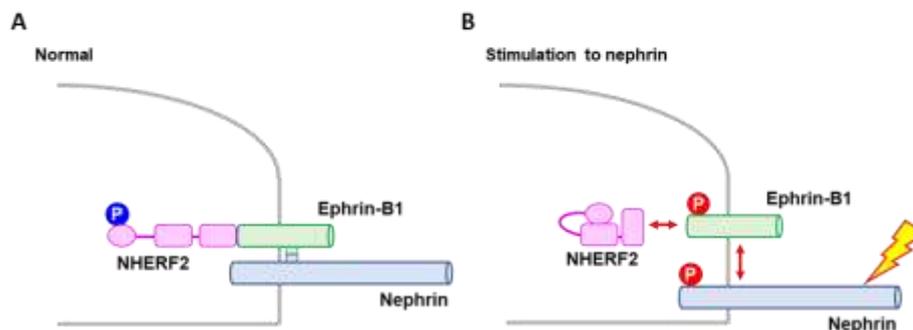
Ephrin-B1 and NHERF2 are essential components of the slit diaphragm of glomerular podocytes. The interactions of ephrin-B1 with nephrin and NHERF2 are essential for maintaining the proper molecular structure and the function of the slit diaphragm. Tyrosine phosphorylation of ephrin-B1 and consequent de-phosphorylation of serine/threonine of NHERF2 induced by the stimulation to nephrin leads to the disruption of the linkage of nephrin-ephrin-B1-NHERF2, and downregulation of these molecules is a critical pathogenic event of podocyte injury. A schematic diagram of the proposed pathogenic mechanism is shown in Figure 3.

**Figure 2**



**Figure 2:** Immunostaining of nephrin, ephrin-B1 and NHERF2 in proteinuric state of anti-nephrin antibody-induced nephropathy. Immunostaining intensity of nephrin, ephrin-B1, and NHERF2 is evidently decreased, and their staining pattern changed to be discontinuous on day 5 when proteinuria peaked.

**Figure 3**



**Figure 3:** Schematic diagram of the proposed pathogenic mechanism.

A: Ephrin-B1 interacts with nephrin via the extracellular domain and interacts with NHERF2 via the cytoplasmic site. B: If nephrin is stimulated, nephrin and ephrin-B1 are phosphorylated, and NHERF2 is de-phosphorylated, and consequently, the linkage of nephrin-ephrin-B1-NHERF2 is disrupted.

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