Planar cell polarity pathway regulates nephrin endocytosis in developing podocytes

Sima Babayeva1, Brittany Rocque1, Lamine Aoudjit1, Yulia Zilber1, Jane Li1, Cindy Baldwin1, Hiroshi Kawachi2, Tomoko Takano1 and Elena Torban1,3

1From the Department of Medicine, McGill University, Montreal, Quebec, Canada;
2Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

*Running title: PCP pathway and nephrin endocytosis

1To whom correspondence should be addressed: Elena Torban, Department of Medicine, McGill University, Montreal, Quebec, Canada, 3775 University street, Montreal, Quebec, Canada, H3A2B4. Tel.: (514) 398-8150; Fax: (514) 398-7446; Email: elena.torban@mcgill.ca

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Background: PCP pathway controls many cell processes during development.

Results: PCP pathway induces nephrin endocytosis when cultured podocytes are treated with Wnt5a. Loss of PCP protein Vangl2 decreases nephrin endocytosis.

Conclusions: During glomerular development, endocytosis of nephrin is regulated by the PCP pathway.

Significance: Implicating PCP pathway in nephrin endocytosis is important for understanding the complexity of the PCP signaling during mammalian development.

SUMMARY

The non-canonical Wnt/Planar cell polarity pathway controls a variety of cell behaviors such as polarized protrusive cell activity, directional cell movement and oriented cell division and is crucial for the normal development of many tissues. Mutations in the PCP genes cause malformation in multiple organs. Recently, the PCP pathway was shown to control endocytosis of PCP and non-PCP proteins necessary for cell shape remodeling and formation of specific junctional protein complexes. During formation of the renal glomerulus, the glomerular capillary becomes enveloped by highly specialized epithelial cells, podocytes, that display unique architecture and are connected via specialized cell-cell junctions (slit diaphragms) that restrict passage of protein into the urine; podocyte differentiation requires active remodeling of cytoskeleton and junctional protein complexes. We report here that in cultured human podocytes, activation of the PCP pathway significantly stimulates endocytosis of the core slit diaphragm protein, nephrin, via a clathrin/β-arrestin-dependent endocytic route. In contrast, depletion of the PCP gene Vangl2 leads to an increase of nephrin at the cell surface; loss of Vangl2 functions in Looptail mice results in disturbed glomerular maturation. We propose that the PCP pathway contributes to podocyte development by regulating nephrin turnover during junctional remodeling as the cells differentiate.

INTRODUCTION

The non-canonical Wnt/Planar cell polarity (PCP) pathway refers to a fundamental evolutionarily-conserved mechanism that establishes directional cell polarity essential for development of many tissues and organs (1). In vertebrates, PCP signaling is activated upon Wnt ligand binding to a Frizzled (Fz) receptor. In different cellular contexts, Wnt4 (2), Wnt5a (3), Wnt9b (4) and Wnt11 (5) have all been reported to activate the PCP pathway (6, 7); Wnt5a has emerged as the prototypical PCP Wnt ligand (8, 9). Wnt5a-Fz binding leads to the formation of asymmetrically positioned multiprotein complexes composed of the core PCP proteins Van Gogh-like (Vangl), Dishevelled, Prickle, Flamingo and Diego; function of additional PCP proteins Fat and Dachsous are also needed to achieve planar tissue polarity (1). PCP protein complexes interact with
the cell-cell junctions that act as the signaling hubs to propagate information from cell-to-cell (10). The asymmetric re-distribution of PCP proteins is crucial for initiating a chain of signaling events that regulate the polarized protrusions that remodel the extracellular matrix and underlie collective directional cell movements (1). Importantly, these cellular processes are essential for kidney morphogenesis (7, 11, 12).

Loss of PCP function during development adversely affects morphogenesis of many organs including the kidneys (7). Homozygous mutations in Fat 4 (13), Dachsous 1(14) or double Fat1/Fat4 mutants (15) disturb renal tubular elongation and tubular dilation and cause embryonic renal cyst formation. Knockout of Fat1 leads to the congenital nephrotic syndrome (16). In a mouse with a spontaneous homoygous mutation in the core PCP gene, Vangl2 (Looptail mouse) (17, 18), defects in kidney branching morphogenesis and glomerular morphology and maturation were recently reported (19). In our earlier work, we identified a complete complement of PCP transcripts (including Vangl2) in cultured human podocytes and showed that knockdown of Vangl2 or stimulation with the PCP ligand Wnt5a of cultured podocytes induced actin cytoskeletal reorganization, affected cell migration, and changed the distribution of the podocyte protein, nephrin (20).

Nephrin is an immunoglobulin-like transmembrane protein (21). In adult kidneys, nephrin expression is restricted to the visceral glomerular podocytes. Nephrin is uniquely localized to the slit diaphragm (SD) junctional contacts between adjacent podocytes which form the filtration barrier which restricts passage of protein into ultrafiltrate. At the SD, the extracellular domains of nephrin from adjacent podocytes interact with each other in a counter-parallel manner and serve as the SD structural backbone (21, 22). The cytoplasmic portion of nephrin is linked to the podocyte cytoskeleton via a number of adaptor proteins (23); nephrin gene mutations leads to profound changes of the podocyte cytoskeleton, loss of SD junctions and proteinuria (24). It is believed that SDs undergo continuous remodeling in response to physiologic changes in filtration pressure (25). Quack et al demonstrated that threonine phosphorylation of nephrin triggers recruitment of β-arrestin-2, an adaptor protein known to mediate endocytosis of G-protein coupled receptors (26), that induces nephrin endocytosis (27). Nephrin internalization was also shown to occur via CIN85-mediated ubiquitination (28) and raft-mediated endocytosis (29). So far, disturbances of nephrin endocytosis have been implicated in the context of disease states, for example in high glucose-mediated podocyte injury (27). However, nephrin turnover during glomerular development has not been studied and the role of the non-canonical Wnt/PCP pathway in nephrin endocytosis has not been addressed.

The purpose of the current work was to ascertain whether the PCP pathway regulates subcellular localization of nephrin during podocyte differentiation and to study its cellular mechanisms. We show that Wnt5a stimulates nephrin endocytosis via clathrin/β-arrestin-dependent route. We also reveal an important role for Vangl2 in nephrin internalization and subcellular distribution.

**EXPERIMENTAL PROCEDURES**

*Animal husbandry and embryo harvest. Looptail (Vangl2lp/+*) mice (17) were generously provided by Dr Gros (McGill). All animal manipulations were according to the Canadian Animal Act and have been approved by the McGill University Animal Care Committee (ACC Protocol # 5423). Heterozygous Lp mice were sister-brother mated to obtain homozygous embryos; a morning plug after overnight mating was counted as 0.5 days post coitum (embryonic day E0.5). At E17.5, pregnant dams were euthanized, embryos dissected, washed in phosphate buffered saline and fixed in 4% paraformaldehyde (PFA). Additional C57/Bl6 control animals were bred to obtain E17.5 embryonic tissues for protein localization studies. All embryos were either cryopreserved in OCT compound for Vangl2 staining or dehydrated in 25%, 50% and 75% ethanol and paraffin embedded at the Goodman Cancer Center Histology Service, McGill University.*

*Cell cultures. Human podocytes A8/13 (30) (gift from Dr. M. Saleem), were propagated and differentiated on cover slips in 12-well-plates prior to treatment and staining as described previously (20). Human embryonic kidney HEK293 cells stably transfected with full-length rat nephrin*
expression construct (HEK293/N) have been characterized previously (31). HEK293/N cells were grown on cover slips in 12-well plates or 24-well plates in DMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin solutions (all from Wisent Inc) and Hygromycin (Bishop) for 72 hours followed by incubation with the conditioned medium collected from L-Wnt5a expressing cells (ATCC CRL-2814) or paternal control L cells (ATCC CRL-2648) for various time as indicated in Results. L and L-Wnt5a cells were grown as recommended by the ATCC. In some experiments, HEK293/N cells (5x10^5 per well) were grown in 12-well plates for 24 hours and transfected with 100ng/well human Rab5-GFP, Rab7-GFP (both kindly provided by Dr Gruenheid) or human Rab11-GFP (Addgene). After 48 hours, cells were incubated with L-CM or Wnt5a-CM for various periods of time. The cells were fixed and processed for immunofluorescent microscopic studies.

siRNA and shRNA interference assays. 1x10^5 HEK293/N cells per well were grown in 24-well plate under conditions described above. Next day, cells were transfected with 20pmol of either control (Santa Cruz, sc-37007) or a pool of three anti-human Vangl2 siRNAs (Santa Cruz, sc-45595) by using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. After 6-hour incubation with siRNAs, medium was replaced with fresh DMEM medium, and cells were incubated for additional 48 hours. The shRNAs targeting β-arrestin-1 and β-arrestin-2 (provided by Dr Stefane Laporte, McGill) were described previously (32). The siRNAs targeting human clathrin heavy chain (provided by Dr Peter McPherson, McGill) were described previously (33). The HEK293/N cells with depleted expression of genes of interest were processed for ELISA assay of nephrin internalization as described below.

For quantitative RT-PCR or immunoblotting analysis, 2x10^5 HEK293/N cells were grown in 35mm plates, transfected with respective siRNAs or shRNAs as above and collected 48-72 hours post transfection. RNA was extracted with Trizol (Sigma) as described by manufacturer. qRT-PCR analysis to detect depletion of Vangl2 was carried with iTAG® SYBR® Green Super Mix kit (BioRad) as described by manufacturer. The following Vangl2 primers were used: Vangl2Forward: ccctctgaaggtgcctcttg; Vangl2Reverse: gtgaggtcatcatgggaga

**Immunofluorescent studies.** To detect endogenous nephrin expression in cultured human podocytes, A8/13 cells differentiated for 2 weeks were treated with L(control)- or Wnt5a-CM for 6 hours. The cells were fixed in 4% PFA at 4ºC, permeabilized with 0.5% Triton in PBS and blocked with 10% normal goat serum (Jackson Immuno Research Laboratories, West Grove, PA) and 0.1% Triton in PBS at room temperature. The cells were labeled with polyclonal rabbit anti-nephrin antibody that recognizes nephrin cytoplasmic domain (31) followed by secondary anti-rabbit IgG Alexa488 antibody (Molecular Probes). Actin was detected by AlexaFluor-568-conjugated phalloidin staining (Molecular Probes). Quantification of nephrin immunofluorescence intensity in the podocyte cortical zones was carried out with ImageJ software (Arch Version, ImageJ-win32) as described in Supplementary Figure 1.

For detection of rat nephrin, live HEK293/N cells were incubated for 1 hour at 37ºC with a monoclonal antibody (mAb5-1-6) (2.5 μg/ml) that recognizes the nephrin extracellular domain (31), followed by incubation with either Wnt5a-CM or L-CM for 20 or 60 min at 37ºC. Attempts to label cells with mAb5-1-6 at 4ºC or room temperature were ineffective. Cells were then fixed and blocked in 0.5% Triton-containing solutions as above. For images shown in Fig 3A, the 0.5% Triton permeabilization step was omitted. Nephrin was visualized with the anti-mouse IgG AlexaFluor488 or Cy3 (both Molecular probes). Z-projections of immunostained podocytes or HEK293/N cells were captured with the AxioObserver-100 microscope (Zeiss) using AxioVision 4.8 software.

5μm cryosections of the E17.5 wildtype C57/B16 embryos were post-fixed for 5 min in 4% PFA, boiled in citrate buffer for 2 min and blocked in 10% normal goat serum for 1 hour. Sections were incubated with polyclonal rabbit anti-Vangl2 (1:20) (34), at 4ºC overnight followed by incubation with guinea pig anti-nephrin antibody (1:150, Acris) for 1 hour at RT. Primary antibodies were visualized with goat anti-rabbit Cy3 and anti-
guinea pig AlexaFluor 488 (Molecular probes), respectively.

Paraffin-embedded 4μm sections were deparaffinized by standard histological methods using Xylene and various ethanol concentrations. Sections were boiled in citrate buffer, blocked as above and incubated with rabbit anti-WT1 antibody (1:400, Santa Cruz) and guinea pig anti-nephrin antibody (1:50, Acris) followed by incubation with the secondary goat anti-rabbit Cy3 and anti-guinea pig AlexaFluor488 antibodies, respectively. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). All sections were mounted in Prolong Gold Antifade (Molecular Probes). All images of developing glomeruli were taken on a LSM 780 Laser Scanning Confocal Microscope (Zeiss, Germany) at the Imaging Core Facility of the Research Institute of the McGill University Health Centre (RI-MUHC) using the Plan-Apochromat 63x/1.40 N.A. oil DIC objective. Lasers used: Argon multiline: 488nm (Green), DPSS 561nm (Red), Dipole 405nm (DAPI) using Zen 2010 software for image acquisition.

Protein interaction assays and immunoblotting. The HEK293T cells grown in 100mm plates were co-transfected with 5μg of yellow-fluorescent protein-tagged YFP-β-arrestin-2 and 5μg of pCDM8-human IgG or 5μg of pCDM8-human IgG-NephrinC using Calcium Phosphate Transfection Kit (Invitrogen) according to the manufacturer’s instructions. Twenty four hours post-transfection, cells were stimulated with L- or Wnt5a-CM for 3 h. Cells were lysed in the buffer containing 1% Triton X-100, 125 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 10 mM sodium pyrophosphate, 25 mM NaF, and protease inhibitor cocktail (Roche Diagnostics). Pull-down assays were performed with 1mg of cell lysate per condition mixed with 30μl of protein G Sepharose (Invitrogen). Precipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane, blocked with 5% dry milk and incubated with rabbit anti-GFP (Santa Cruz) or anti-nephrin (31) primary antibodies for 16 h at 4°C followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Medicorp Inc) and detection by enhanced SuperSignal West Pico chemiluminescence (Thermo Scientific).

HEK293/N cells were incubated with either L-CM or Wnt5a-CM medium for 1, 2 and 6 hours. Cells were lysed in the lysis buffer used for pull-down experiments. 25μg of protein per lane were separated on 8% SDS-PAGE and subjected to immunoblot analysis with rabbit anti-nephrin antibody (31) or rabbit anti-β-catenin antibody (Santa Cruz Biotechnology) and secondary HRP-conjugated antibody as in the pull-down experiments above. As a control for loading variation, immunoblotting with anti-GAPDH antibody (Santa Cruz Biotechnology) was used.

For si/shRNA–mediated depletion experiments, HEK293/N cells were lysed in the buffer as above, 50μg of lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose. The depletion of β-arrestin1/2 was detected with rabbit pan-anti-β-arrestin antibody (32); the clathrin heavy chain was detected with rabbit anti-CHC antibody (33); protein loading was monitored by the detection with mouse anti-α-tubulin antibody (Sigma-Aldrich). Band intensities corresponding to protein expression were quantified with AlphalImager EP (AlphalInnotech).

Analysis of nephrin internalization by ELISA. To measure nephrin internalization, we used an ELISA method described in (35) with some modifications. Briefly, HEK293/N cells were grown to 100% confluence in quadruplicate for each condition in 24-well plates. In each experiment, 4 wells were dedicated to the negative control (no antibodies). To detect nephrin at the cell surface, live HEK293/N cells were quickly washed in HBSS buffer (125 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 25 mM HEPES) followed by a 20 min wash in Ca2+-free medium at 37°C. The cells were incubated with mAb5-1-6 (2.5 μg/ml) in either L-CM or Wnt5a-CM for 1 hour at 37°C. Cells were washed 4 times in HBSS buffer, fixed for 10 min in 4% PFA followed by 10 min blocking in 5% skim milk/PBS at room temperature. The fixed cells were incubated with HRP-conjugated anti-mouse antibody (1:3000, Medicorp Inc) for 1 hour at room temperature followed by a detection step with freshly prepared o-phenylenediamine (OPD) substrate (Thermo Scientific Pierce) as recommended by the manufacturer. Final yellow-orange OPD soluble products formed in each well were detected at 492nm by ELISA Microplate.
reader ELx808 (Bio-Tec Instruments Inc). In some parallel experiments, cells were incubated with phorbol myristate acetate (PMA 250ng/ml, Sigma-Aldrich) for 1 hour at 37°C (instead of L- or Wnt5a-CM). In some experiments, HEK293/N cells were pre-incubated with 0.4nM PKCα inhibitor, bisindolylmaleimide I (EMD Millipore), for 30 min at 37°C prior to incubation with Wnt5a-CM or L-CM or PMA and mAb5-1-6.

To measure total amount of nephrin, HEK293/N cells grown in parallel under the same conditions, were incubated with either Wnt5a- or L-CM for 1 hour at 37°C and then fixed for 10 min in 4% PFA and permeabilized for 30 min in 0.1% Triton/PBS at room temperature. The cells were blocked as above and incubated with the mAb 5-1-6 for 1.5 hour followed by HRP-secondary antibody and OPD detection as above. Nephrin at the cell surface was normalized for both negative control (that was subtracted from the raw OPD data) and the total nephrin (that was used to calculate percentage of nephrin-positive signal detected in live un-permeabilized cells). The amount of internalized nephrin was calculated as a difference between 100% and % of the normalized nephrin measured at the cell surface. The effect of Wnt5a vs control (L)-induced nephrin internalization in majority of experiments was presented as a Wnt5a/L ratio of the percentages of internalized normalized nephrin. For each set of conditions, the experiments were repeated minimum 3 times in quadruplicate.

Statistical analysis. The Student t-test (two-tail unequal variance) was used to calculate statistical significance in all graphs except Figure 7. Mean and standard errors are shown in all graphs. In Figure 7, chi-test was used.

RESULTS
Expression of PCP protein Vangl2 in developing podocytes. We previously reported that the central PCP pathway protein, Vangl2, is expressed in developing podocytes in the mouse embryonic kidney (20) suggesting a role for PCP signaling during podocyte development. However, the dynamics of Vangl2 expression in podocytes as they undergo differentiation has not been studied, yet it might give a clue as to the function of the PCP pathway in glomerulogenesis. In mice, development of the glomerulus starts at ~E12.5 as renal mesenchyme converts into epithelium sequentially forming vesicles, comma- and S-shaped bodies. The proximal part of the S-shaped body gives rise to the future glomerulus that, at the early stage, is seen as a single loop-like structure enveloped by a row of cuboidal epithelial cells (early capillary loop-stage). As glomerulus matures, the capillary loop arrangement becomes progressively more complex with podocytes distributed in a less organized fashion (late capillary loop-stage) (36).

In sections of E17.5 mouse kidney, glomeruli at various developmental stages can be observed. Podocyte marker nephrin becomes detectable at the late S-shaped body to early capillary loop-stage along the lateral and basal aspects of cuboidal epithelial cells. As the podocytes mature, nephrin at the lateral membrane descends toward the basal aspect of podocyte where the tight junctions are being reorganized into SDs (Fig 1, capillary-loop-stage panels, magnified images, arrows), as reported previously (37). We observed that Vangl2 expression in podocytes precedes that of nephrin: strong Vangl2 expression was observed along the basal-lateral plasma membrane of cuboidal epithelial cells in comma- and S-shaped bodies (Fig 1, two top rows). At the capillary loop-stage, Vangl2 expression descends basally and both nephrin and Vangl2 localize to the basal aspect of the podocyte where foot processes and SDs are formed (Fig 1, loop-stage panels, arrowhead). At later developmental stages (> 3 capillary loops) as podocytes mature and express higher levels of nephrin, Vangl2 expression is downregulated and can be visualized only under high acquisition exposure (Fig 1, mature glomerulus panels). Vangl2 mRNA, however, can be detected by RT-PCR in adult kidneys, glomeruli and mouse and human podocyte cultures (20). By immunoblotting, Vangl2 protein can be detected in the adult mouse kidney (38).

Wnt5a treatment of cultured human podocytes leads to nephrin internalization. Since in developing podocytes Vangl2 expression co-localizes with nephrin where the future slit diaphragm is being formed, we hypothesized that activation of the PCP pathway might be involved in discrete nephrin localization during slit diaphragm assembly. Since the PCP pathway is
activated by Wnt5a in vitro (39), we incubated differentiated human podocytes with conditioned media (CM) from L-cells expressing Wnt5a or control L-cells (L). While the podocytes exposed to L-CM displayed nephrin in cell surface protrusions (Fig 2A-B, upper panels), podocytes incubated with Wnt5a-CM showed striking redistribution of nephrin from the cell surface to the cytoplasm (Fig 2A-B, lower panels). Intensity of nephrin immunofluorescence in the cortical area of Wnt5a-CM-treated cells (9.76±0.31 fluorescence intensity units per μm²) was significantly lower than that in the cells treated with L-CM (22.49±1.06) (Fig 2C and supplementary Figure1).

We also examined this phenomenon in a second cell system. In HEK293 cells stably transfected with full-length rat nephrin (HEK293/N), nephrin localization was assessed by live cell labeling with mouse anti-nephrin antibody 5-1-6 (mAb5-1-6) that recognizes the extracellular domain of rat nephrin (31). In the cells exposed to L-CM for 30 or 60 min, a strong nephrin signal (detected without detergent) was seen at the plasma membrane (Fig 3A, left panel, 60 min incubation is shown). However, surface nephrin staining was markedly reduced in cells exposed to Wnt5a-CM (Fig 3A, right panel). In the Wnt5a-CM-treated cells permeabilized with 0.1% Triton, nephrin staining appeared in cytoplasmic vesicles (Fig 3B, right panel, arrowheads). We then quantified plasma membrane nephrin (labeled in live cells) by ELISA, normalized for total nephrin (labeled in fixed cells). We found that only 8±4.7% of total nephrin was present in the cytoplasm of L-CM-treated cells whereas 51±5.1% of nephrin was internalized after 1 hour incubation with Wnt5a-CM (Fig 2C).

To better understand the dynamics of Wnt5a-induced nephrin internalization, we used a panel of endosomal markers: Rab5 (early sorting endosomes), Rab7 (late endosomes - early lysosomes) and Rab11 (recycling endosomes). HEK293/N cells were transfected with GFP-tagged Rab cDNAs, 48 hours later cells were live-labeled with mAb5-1-6 and then exposed to L-CM or Wnt5a-CM for 20 or 60 min. In the L-CM-treated cells, nephrin resided predominantly at the cell surface at all time points (Fig 4A-C, all L-CM images). In Wnt5a-treated cells, we detected nephrin in the Rab5(+) vesicles at both 20 and 60 min time points (Fig 4A, right panels). We did not see nephrin localization in recycling Rab11(+) endosomes at either 20 or 60 min (Fig 4B, right panels). Occasionally, we saw nephrin in Rab7(+) vesicles (Fig 4C, right panels). The presence of nephrin in Rab7(+) vesicles suggested that nephrin might be targeted for degradation. However, we detected no change in overall nephrin levels of HEK293/N cells incubated with Wnt5a-CM for 1-6 hours (Fig 4D).

**Wnt5a stimulates clathrin-dependent nephrin endocytosis.** Wnt5a was reported to induce endocytosis of the Fz4 receptor via a clathrin-dependent pathway (40). To examine whether Wnt5a stimulates nephrin endocytosis by a similar mechanism, HEK293/N cells were pre-treated (30 min) with 0.45 mM sucrose (41) to inhibit clathrin-dependent endocytosis prior to incubation with Wnt5a- or L-CM. Sucrose treatment led to retention of nephrin at the cell surface (Fig 5A). ELISA quantification revealed a significant (p<0.01) decrease in Wnt5a-stimulated nephrin endocytosis, from 3.53±0.18 to 2.02±0.43 Wnt5a/L-fold stimulation in cells exposed to sucrose (Fig 5B). To confirm this effect, we analyzed Wnt5a-stimulated nephrin endocytosis after depletion of clathrin heavy chain (CHC) with specific anti-CHC siRNAs (33); we achieved ~70% depletion of CHC protein expression with specific siCHC RNAs compared to the baseline CHC protein levels in mock- or control siRNA-transfected HEK293/N cells (Fig 5D). Wnt5a-induced nephrin internalization was reduced from 3.60±0.13-fold stimulation in cells transfected with control siRNAs to 1.68±0.26-fold stimulation after CHC knockdown (Fig 5C). These results indicate that Wnt5a-induced nephrin endocytosis is mediated by a clathrin-dependent mechanism.

**Wnt5a-induced nephrin internalization is mediated by β-arrestin.** Endocytosis via the clathrin-coated pit mechanism requires the multifunctional adaptor proteins, β-arrestins (42, 43). Both β-arrestin-1 and -2 are expressed in podocytes (44); β-arrestin-2 has been shown to interact with nephrin and to mediate clathrin-dependent nephrin endocytosis (27, 44). β-arrestin-2 was also implicated in Wnt5a-induced endocytosis of Fz4 (40). We, therefore, tested whether Wnt5a-dependent endocytosis of nephrin requires β-
arrestin. HEK293/N cells were transfected with either control, β-arrestin-2 or with a combination of both β-arrestin-1/2 shRNAs. β-arrestin-1 and 2 shRNAs showed some degree of cross-reactivity and the combination of the two resulted in a more effective knockdown of β-arrestins than β-arrestin-2 alone (Fig 6C,D). Seventy two hours post-transfection, the cells were live-labelled with mAb5-1-6 in the presence of either Wnt5a- or L-CM as above. In shControl-transfected cells exposed to Wnt5a, we detected nephrin mostly in the cytoplasm; little immunoreactive nephrin was noted at the cell surface (Fig 6A, left panel). In contrast, knockdown of β-arrestins-1/2 led to retention of nephrin at the cell surface after exposure to Wnt5a (Fig 6A, right panel). When quantified by ELISA, we observed 3.27±0.32-fold stimulation of nephrin endocytosis in cells transfected with shControl and treated with Wnt5a. Although a similar level of Wnt5a-stimulated endocytosis (3.14±0.63-fold) was seen in cells transfected with β-arrestin-2 shRNA alone, cells transfected with shRNA against both β-arrestins1/2 displayed a significantly reduced Wnt5a-induced nephrin internalization to 1.84±0.11-fold (Fig 6B).

Under conditions that stimulate nephrin internalization (e.g. high glucose in diabetes mellitus), an increase in nephrin endocytosis was accompanied by an increase in β-arrestin-2/nephrin interaction (27). We, therefore, tested whether Wnt5a promotes complex formation between nephrin and β-arrestin-2. We took advantage of a chimeric construct (Ig-nephrin), in which an extracellular domain of human immunoglobulin is fused with the cytoplasmic domain of human nephrin (45). HEK293 cells were co-transfected with YFP-tagged β-arrestin-2 and either Ig-control (no nephrin) or Ig-nephrin. Transfected cells were treated with L- or Wnt5a-CM or PMA (that served as a positive control (27). Ig-nephrin was then absorbed by affinity chromatography. As shown in Fig 6E, there was a modest interaction between nephrin and β-arrestin-2 in control (Fig. 6E, lane 2), however, this interaction was markedly increased when cells were stimulated with either Wnt5a (Fig 6E, lane 3) or PMA (Fig 6E, lane 4).

It has been reported that nephrin/β-arrestin-2 complex formation and nephrin endocytosis are facilitated under certain conditions by protein kinase C alpha (PKCa) (27). During embryonic vascular development, Wnt5a was also reported to activate PKCa (46). Thus, we tested if Wnt5a augments nephrin endocytosis via PKCa. We pre-treated HEK293/N cells with the 0.4 nM PKCa inhibitor prior to incubation with Wnt5a- or L-CM and performed ELISA as above. The PKCa inhibitor did not significantly reduce nephrin endocytosis induced by Wnt5a (3.69±0.8 Wnt5a/L fold stimulation without PKCa inhibitor vs 2.84±0.75 fold endocytosis stimulation) (Fig 6F). Tested in parallel, the same concentration of PKCa inhibitor significantly (p<0.001) reduced nephrin internalization induced by PMA (from 4.26±0.13 down to 1.11±0.12), indicating that the inhibitor was effective (Fig 6F). Our results suggest that Wnt5a induces β-arrestin-dependent nephrin endocytosis independently of PKCa.

Vangl2 depletion inhibits nephrin endocytosis. To test whether Wnt5a-stimulated nephrin endocytosis depends on the core PCP protein Vangl2, we depleted Vangl2 in the HEK293/N cells by transfecting the cells with anti-Vangl2 siRNAs. In the cells incubated with control L-CM, nephrin could be easily detected at the cell surface in the cells transfected with both control and anti-Vangl2 siRNA and live-labelled with mAb5-1-6 (Fig 7A, upper panels, arrows). In contrast, upon Wnt5-CM stimulation, nephrin became internalized in the cells transfected with siControl (Fig 7A, lower left panel, arrowheads) but not with the siVangl2, where we detected a strong mAb5-1-6-positive signal mainly at the cell surface (Fig7, lower right panel, arrows). Quantified by ELISA, the cells transfected with control siRNA displayed 4.68±0.46 Wnt5a/L fold increase in nephrin endocytosis, whereas Vangl2 depletion substantially suppressed nephrin endocytosis (1.44±0.22 fold increase, Fig 7B). Reduction in the level of nephrin internalization in siVangl2-treated cells correlated with the level of Vangl2 depletion measured by quantitative RT-PCR (Fig 7C).

Loss of Vangl2 affects glomerular maturation in vivo. The Looptail (Lp) mouse harbours a spontaneous semi-dominant mutation in the Vangl2 gene; homozygous Lp mice exhibit defects in multiple organs and die in utero of a severe neural tube defect in late gestation (17). We first
analyzed the gross morphology of E17.5 embryonic kidneys from 4 homozygous Lp/Lp (Lp allele) embryos and matching wildtype littermates (Fig 8A,B). Compared to controls, Lp/Lp kidneys showed variable dysplasia, ranging from nearly normal shape to smaller flattened appearance (Fig 8A, right panel). All Lp/Lp kidneys lacked clear demarcation between cortical and medullary zones and showed some degree of medullary hypoplasia with decreased evidence of tubular bundles and marked tubular dilation. Occasional glomerular cysts were seen (Fig 8B).

Confocal microscopy of wildtype E17.5 kidneys showed that >60% of glomeruli were at the more mature 4-5 capillary-loop stage (Fig 8C,D). At this stage, podocytes (detected with antibody against Wilm’s tumour protein, WT1) express nephrin predominantly at the basal surface, corresponding to the position of slit diaphragm complexes (Fig 8C, right panels). In contrast, we detected a significantly higher percentage of immature glomeruli (1-2 capillary loop-stage) in Lp/Lp kidneys (Fig 8D). In immature glomeruli, nephrin expression is detected both at the basal and the lateral plasma membrane surfaces and occasionally in the apical membrane domains (Fig 8C, left panels, arrows). The distribution of nephrin in mature glomeruli was similar in wildtype and Lp/Lp kidneys (Fig 8E). The mean number of WT1-positive podocytes was similar in wildtype and Lp/Lp glomeruli at the 1-2 capillary loop-stage (21 vs 22 podocytes/glomerulus) and the 3-5 capillary loop-stage (19 vs 19 podocytes/glomerulus).

**DISCUSSION**

The non-canonical Wnt/PCP pathway is indispensable for mammalian organogenesis; defects in PCP genes cause abnormalities in multiple organs. In the kidney, our observations suggest that the PCP signalling is important for nephrin endocytosis and for glomerular maturation.

We identified a significant decrease in nephrin endocytosis in cultured podocytes with depleted Vangl2 expression and a significant developmental delay in glomerular maturation in Vangl2 mutant Lp mice. Can Vangl2-regulated nephrin endocytosis contribute to podocyte and glomerular development and if so, how? The answer may reside in the known functions of the PCP pathway in lower organisms. During Drosophila wing development, a core PCP protein Flamingo (Fmi, atypical cadherin that possesses large extracellular domain) accumulates in discrete membrane domains that correspond to adherens junctions (47). Like nephrin, junction-associated Fmi interacts with Fmi from the adjacent cell to facilitate cell-cell adhesion. The PCP proteins Van Gogh (Vang, a fly homologue of Vangl2) and Fz also concentrate in junctions and stabilize Fmi there; the unbound Fmi is actively endocytosed in a PCP-dependent manner via Rab5- and dynamin-dependent processes (48). In the absence of Vang and Fz, Fmi does not localize stably to the junctions and accumulates at the apical membrane at increased rate. This ultimately leads to the loss of asymmetric PCP protein complex assembly and loss of planar polarization (47). By analogy, Vangl2 may be critical for the proper localization of nephrin at the developing slit diaphragm via controlling its endocytosis.

There are several examples in which the PCP pathway regulates endocytosis of the “non-PCP” cell surface proteins. The PCP pathway was shown to control polarized endocytosis and recycling of E-cadherin during remodelling of wing cells (49) and tracheal elongation (50) in the fly. The repacking of wing epithelia involves growth and shrinkage of the plasma membrane plus assembly and disassembly of junctional complexes that allow cells to assume a hexagonal shape. This requires a dynamic PCP-dependent polarized turnover of E-cadherin at adherens junctions. In PCP mutants, level of E-cadherin at the junctions increases (49, 50). Similarly, tracheal elongation involves polarized cell intercalation that is accompanied by junctional remodelling and a decrease in E-Cadherin at the junctions. Loss of PCP activity in PCP mutants (including Vang) leads to an increase in the stability and amount of E-cadherin in cell-cell junctions and a delay in tracheal branch intercalation (50). Another example can be drawn from the process of gastrulation during early vertebrate development. Gastrulation relies on the directional rearrangement of cellular cytoskeleton and convergent extension movements – processes controlled by the PCP pathway (1). During convergent extension in zebrafish, the PCP pathway regulates endocytosis of the metalloproteinase, MMP14, that is important for
remodelling of extracellular matrix (ECM). Loss of Vangl2 in trilobite mutants leads to a greater availability of MMP14 at the cell surface, disturbing normal ECM remodelling, miscoordination of polarized cell movements and convergent extension defects (51). Vangl2 was also shown to localize to the tips of filopodia in the commissural neuron axon growth cones (52) where it antagonized Wnt5-induced Fz3 phosphorylation and promoted Fz3 internalization. This allows “sharpening of PCP signaling locally on the tips of the filopodia to sense directional Wnt cues” (52). Thus it is conceivable that Vangl2-mediated nephrin endocytosis has a critical role in the development of the highly specialized structure of podocytes.

To achieve their unique mature architecture, developing podocytes must re-model their membranes and junctional complexes extensively. We previously reported that Vangl2 interacts with nephrin via the SD scaffold protein MAGI2 and may be a part of the SD complex (20). Based on our observations, we propose that Vangl2 participates in the control of podocyte shape by internalizing nephrin prior to foot process formation and slit diaphragm junction assembly needed for terminal differentiation. Conversely, loss of Vangl2 may contribute to abnormal glomerular maturation in Lp mice by permitting premature nephrin externalization, disturbing podocyte alignment. The importance of endocytosis in glomerular development was recently demonstrated in transgenic mice lacking genes required for clathrin-dependent endocytic machinery (53): mutant mice with knockout of three endophilin genes or the synaptotagmin failed to form normal foot processes and exhibited severe proteinuria and nephrotic syndrome at birth (53). These observations are in line with our results which show that loss of Vangl2 function impairs glomerular maturation.

The robust Wnt5a-induced nephrin endocytosis we observed in the current study has several similarity to other systems; Wnt5a was previously reported to induce clathrin-mediated endocytosis of its receptors Fz4 (40), Fz2 and Ror2 (non-canonical Wnt/PCP co-receptor), where Fz internalization may be required for regulation of its receptor activity (8). Similarly, we established that depletion of clathrin heavy chain by siRNA interference or clathrin inhibition by sucrose substantially decreased Wnt5a-induced nephrin internalization. Wnt5a-induced endocytosis of Fz4 was reportedly modulated by β-arrestin-2 (40). Indeed, we also identified involvement of β-arrestins in the Wnt5a-induced nephrin endocytosis by demonstrating that siRNA knockdown of β-arrestin-1/2 led to a significant decrease in nephrin internalization. We also found that Wnt5a strengthened interactions between the nephrin cytoplasmic domain and β-arrestin-2. Thus, Wnt5a-induced endocytosis of nephrin appears to utilize a clathrin/β-arrestin-mediated endocytic pathway similar to Fz endocytosis. Quack et al have also shown previously that nephrin is internalized via the clathrin/β-arrestin-2-dependent pathway in the high glucose milieu (27, 44).

Wnt5a-induced endocytosis of nephrin, however, displays some unique features. Chen et al reported that, by itself, Wnt5a-CM was unable to trigger Fz4 endocytosis but that the combination of Wnt5a and the PKCa activator PMA led to Fz4 internalization (40). Quack also reported a requirement for PKCa activity to stimulate nephrin internalization in the presence of pathologic hyperglycemia (27). Our data do not support involvement of PKC for the following reasons: a) addition of a PKC inhibitor did not significantly decrease Wnt5a-stimulated nephrin endocytosis, while it efficiently inhibited PMA-stimulated nephrin internalization in the same experiment; b) cell incubation with only Wnt5a was sufficient to increase nephrin internalization 3-5 fold without a requirement for PMA. c) Chen et al did not see Wnt5a stimulation of complex formation between Fz4 and β-arrestin-2 (40). However, we detected an increase in nephrin/β-arrestin-2 interactions in the presence of Wnt5a. Taken together, our results demonstrate that Wnt5a stimulates PKC-independent nephrin internalization.

In HEK293/N cells stimulated with Wnt5a, we detected nephrin predominantly in Rab5 (early endosomes) and occasionally in Rab7 (late endosomes/early lysosomes)-positive vesicles but not in the Rab11 (recycling endosomal marker) vesicles. Rab5-mediated PCP-dependent endocytosis of Fmi and E-cadherin was also demonstrated in the fly wing cells (47) and zebrafish (54). The Rab7 endosomal compartment is usually associated with degradation of proteins.

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such as EGFR (55). However, we did not detect any loss of nephrin in the Wnt5a-treated cells after 6 hours. Although low undetectable amounts of nephrin may have escaped detection, our results are in line with previous studies of human podocytes in which we saw no loss of nephrin after 24 hours of Wnt5a exposure (20). We, therefore, propose that Wnt5a causes a robust prolonged internalization of nephrin into endocytic vesicles, depleting the cell surface nephrin pool without marked nephrin degradation.

In adult kidneys, activation of the canonical Wnt pathway with subsequent β-catenin accumulation contributes to the podocyte dysfunction leading to proteinuria (56, 57). Although it was not the main focus of the study, Kato et al. also reported significant upregulation of the non-canonical Wnts in the glomerulus affected by diabetic nephropathy in humans (Wnt4) and in rats (Wnt4 and Wnt5a) (57). Similarly, Wnt4 was upregulated in the mouse model of adriamycin nephropathy, although the study was semi-quantitative (56). These reports, combined with our current results suggest a possibility that inappropriate stimulation of Wnt5a in the adult kidney may lead to increased nephrin internalization, which would lead to SD destabilization and proteinuria. Whether the Wnt/PCP pathway is re-activated during podocyte injury is an important question that warrants further investigation.

In summary, we show that the non-canonical/PCP pathway stimulates PKC-independent, clathrin- and Rab5-mediated internalization of podocyte-specific adhesion protein nephrin. We propose that during glomerular development, PCP pathway signalling suppresses nephrin externalization prior to terminal podocyte differentiation. Conversely, a dysfunctional PCP pathway might affect glomerular maturation by permitting premature nephrin accumulation at adherens junctions to form slit diaphragms. In adult tissues, inappropriate Wnt5a expression may cause increased nephrin internalization leading to proteinuria.

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FIGURE LEGENDS

Figure 1. Vangl2 is co-expressed with nephrin in developing podocytes.
A-D. E17.5 C57/Bl6 mouse sections co-stained with rabbit anti-Vangl2 (red) and guinea pig anti-nephrin antibodies (green), nuclei are revealed by DAPI (blue). A. Vangl2 expression (red) in comma-shape body (intemittent line denotes the most proximal domain which will give rise to future podocytes). B. Vangl2 protein expression (red) in S-shaped body (intemittent line denotes the most proximal domain of S-shape body where the future podocytes originate). C. Glomerulus at the early capillary-loop stage: Vangl2 is expressed at the baso-lateral domain of podocytes (red), nephrin (green) co-localized with Vangl2 at the baso-lateral domain of podocyte plasma membrane. C. High-power view of the selected area in box C: Vangl2 (red, arrowhead) co-localize with nephrin (green, arrow). D. Maturing glomerulus: Vangl2 (red) expression is low, nephrin (green) is detected as a thin multi-looped line along the basal domain of podocyte plasma membrane; bar size is 5μm.
Figure 2. Wnt5a induces nephrin redistribution from cell surface in human podocytes.
A. Wnt5a induces redistribution of nephrin from cell projections (arrows) to the cytoplasm in cultured differentiated human podocytes; nephrin (green, visualized with rabbit anti-nephrin antibody), actin (red, visualized by AlexaFluor568-conjugated phalloidin); bar size is 5μm. B. Magnified imaged of cell area designated by white boxes in A. C. Quantification of the fluorescent intensity corresponding to nephrin (detected with rabbit anti-nephrin antibody, green) in the cell cortical areas; 40 podocyte images per condition were analyzed by Image J software and normalized for the surface area as IF intensity per μm²; mean and standard error of mean are shown, p=2.04E-15 (see also Supplementary Figure 1).

Figure 3. Wnt5a induces nephrin endocytosis in HEK293 cells.
A. HEK293/N cells were live-labelled with anti-nephrin mAb5-1-6 antibody (green) that recognizes nephrin ecto-domain, then incubated for 60 min with L-CM (left panel) or with Wnt5a (right panel) and visualized with secondary antibody applied without permiobilization; bar size 40μm. B. Cell surface regions of HEK293/N cells live-labelled with mAb5-1-6 (red) and visualized with secondary antibody applied in the presence of detergent; left image shows cells treated with control L-CM for 60 min; right image shows cells treated with Wnt5a-CM; fluorescent signals are found in the cytoplasm (arrowheads) C. ELISA assay of nephrin internalization in HEK293/N cells, (*** ) p<0.0001.

Figure 4. Wnt5a induces nephrin localization to endocytic vesicles.
A. HEK293/N cells transfected with Rab5-GFP, were live-labeled with mAb5-1-6 (red) and then exposed to L- or Wnt5a-CM for 20min or 60 min; each inlet shows a magnified image of the area indicated in the low-magnification image. B. HEK293/N cells transfected with Rab11-GFP and then processed as in A. C. HEK293/N cells transfected with Rab7-GFP and then processed as in A. All size bars are 5μm. D. Western immunoblotting of nephrin in the HEK293/N cells exposed to either L or Wnt5a for indicated periods of time; control is the untreated cells. GAPDH levels in the same lysates serve as loading controls.

Figure 5. Wnt5a induces nephrin endocytosis via a clathrin-dependent pathway.
A. HEK293/N cells were incubated with Wnt5a (left panel) or pre-treated with 0.45M sucrose and then exposed to Wnt5a (right panel). Ectodomain of nephrin (ecNephrin, red) is visualized by mAb5-1-6 labelling of live cells; bar size 5μm. B. ELISA quantification of nephrin endocytosis in HEK293/N cells incubated with Wnt5a or Wnt5a+0.45M Sucrose; (*) p<0.01. C. ELISA quantification of nephrin endocytosis in HEK293/N cells transfected with either control siRNA or siRNAs against clathrin heavy chain, CHC; (**) p<0.001. All ELISA assays were repeated 3 times in quadruplicate for each condition; the data are presented as the mean ratio of nephrin endocytosis in cells treated with Wnt5a-CM to cells treated with L-CM. D. Immunoblot analysis (anti-CHC antibody) of lysates from mock transfected HEK293/N cells or cells transfected with siControl or siCHC RNAs. Immunoblotting with anti-α-tubulin antibody is used to monitor for equal loading.

Figure 6. Wnt5a stimulation of nephrin endocytosis depends on β-arrestins.
A. Nephrin endocytosis is inhibited in HEK293/N cells following knockdown of β-arrestin-1 and 2: left panel – nephrin (mAb5-1-6, red) is detected in the cytoplasm of HEK293/N cells transfected with shControl and incubated with Wnt5a-CM; right panel – nephrin (red) is detected at the plasma membrane in cells transfected with shRNAs against β-arrestin-1/2 and incubated with Wnt5a; bar size 5μm. B. ELISA assays in HEK293/N cells transfected with shRNAs against either control or β-arrestin-2 or a combination of β-arrestin-1 and 2. The ELISA assays were repeated 3 times in quadruplicate for each condition; the data are presented as ratio of nephrin endocytosis in cells treated with Wnt5a-CM to cells treated with L-CM. C. Immunoblot analysis (anti-β-arrestin antibodies) of lysates from mock transfected HEK293/N cells or cells transfected with shControl or siCHC RNAs. Immunoblotting with anti-α-tubulin antibody is used to monitor for equal loading; Immunoblot analysis was repeated 3 times. D. Densitometry quantification of immunoblotting analysis of β-arrestin expression from
3 different experiments, (*) p<0.01. **E.** Pull-down assays between Ig-nephrin and Ig-control proteins and YFP-β-arrestin-2. All proteins were expressed in HEK293T cells that were treated for 3 hours with either L- or Wnt5a or PMA (positive control). YFP- β-arrestin-2 was detected with anti-GFP antibody, Ig-nephrin was detected with polyclonal anti-nephrin antibody; pull-down experiments were repeated 4 times. **F.** Densitometry quantification of pull-down experiments, (**)p<0.001.

**Figure 7. Vangl2 mediates Wnt5a-stimulated nephrin endocytosis.**

**A.** upper panels: HEK293/N cells transfected with either siControl or siVangl2 RNAs, live-labeled with mAb5-1-6 and exposed to L-CM for 60 min; nephrin (green) is mostly detected at the cell surface. **Lower panel:** HEK293/N cells transfected with either Control or Vangl2 siRNAs, live-labeled with mAb5-1-6 and incubated for 60 min with Wnt5a-CM; note nephrin (green) is mostly detected in the cytoplasm of the Control siRNA-transfected cells, but is localized primarily at the plasma membrane in the cells transfected with Vangl2 siRNA; bar size 5μm. **B.** ELISA assays of Wnt5a-induced nephrin endocytosis in HEK293/N cells transfected with either siControl or siVangl2 RNAs. The ELISA assays were repeated 3 times in quadruplicate for each condition; the data are presented as the ratio of nephrin endocytosis in cells treated with Wnt5a to cells treated with L-CM; (***) p<0.0001. **C.** Quantitative RT-PCR of Vangl2 transcript in cells transfected with Control or Vangl2 siRNAs. Experiment was repeated twice in triplicate, (***) p<0.0001.

**Figure 8. Vangl2 Looptail (Vangl2) embryos exhibit defective glomerular maturation.**

**A-B.** H&E staining of E17.5 wildtype (left panel) and Lp/Lp (right panel) kidneys. Cortical areas are designated by yellow intermittent line, tubular bundles by long arrows, dilated tubules by (*), cystic glomeruli by short arrows. Note that images in B are from the same kidneys but not the same sections as shown in A; bar sizes are 50μm and 20μm. **4 17.5 Lp/Lp** kidneys from different litters and 4 wildtype kidneys were analyzed. **C.** Staging of glomerular maturation in E17.5 wildtype kidneys by confocal microscopy. **Upper panel** – merged images of double staining with guinea pig anti-nephrin antibody (green) and rabbit anti-WT1 antibody (red). In immature glomeruli at the one loop-stage (left image), nephrin is detected along the entire lateral-basal and apical (arrows) surfaces. In mature glomeruli at the >5 loop-stage (right image), nephrin is detected as a thin line exclusively at the basal podocyte surface. **Lower panel** – the same images as above showing only the nephrin channel; bar size 5μm. **D.** Quantification of glomerular developmental stage identified by nephrin/WT1 staining by confocal microscopy; >35 glomeruli were scored on sections prepared from two Lp/Lp and two wildtype (WT) E17.5 littermates from 2 different litters. **E.** Nephrin (green), WT1 (red) and DAPI (blue) expression in maturing glomeli of wildtype (left panel) and Lp/Lp (right panel) E17.5 embryonic kidneys; bar size 5μm. Inlets show magnified images of nephrin (green) channel.
FIGURE 1

A. comma-shaped body

B. S-shaped body

C. capillary loop-stage

C'.

D. maturing glomerulus
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