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Overexpression of Robo2 causes defects in the recruitment of metanephric mesenchymal cells and ureteric bud branching morphogenesis

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ABSTRACT

Roundabout 2 (Robo2) is a member of the membrane protein receptor family. The chemorepulsive effect of Slit2–Robo2 signaling plays vital roles in nervous system development and neuron migration. Slit2–Robo2 signaling is also important for maintaining the normal morphogenesis of the kidney and urinary collecting system, especially for the branching of the ureteric bud (UB) at the proper site. Slit2 or Robo2 mouse mutants exhibit multilobular kidneys, multiple ureters, and dilatation of the ureter, renal pelvis, and collecting duct system, which lead to vesicoureteral reflux. To understand the effect of Robo2 on kidney development, we used microinjection and electroporation to overexpress GFP-Robo2 in an *in vitro* embryonic kidney model. Our results show reduced UB branching and decreased glomerular number after *in vitro* Robo2 overexpression in the embryonic kidneys. We found fewer metanephric mesenchymal (MM) cells surrounding the UB but no abnormal morphology in the branching epithelial UB. Meanwhile, no significant change in MM proliferation or apoptosis was observed. These findings indicate that Robo2 is involved in the development of embryonic kidneys and that the normal expression of Robo2 can help maintain proper UB branching and glomerular morphogenesis. Overexpression of Robo2 leads to reduced UB branching caused by fewer surrounding MM cells, but MM cell apoptosis is not involved in this effect. Our study demonstrates that overexpression of Robo2 by microinjection in embryonic kidneys is an effective approach to study the function of Robo2.

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1. Introduction

The metanephros is a vital organ in mammals. The morphogenesis of the kidney arises through the inductive interaction between the UB cells and the MM cells. The signals generated by the UB cells can induce and maintain the differentiation of MM cells into nephrons. The UB grows outward and continues branching, which not only leads to the formation of the collecting ducts, the renal calyces, and the renal pelvis but also controls the kidney structure and the nephron number. UB branching defects also reduce the number of glomeruli. Such a malformation might render the individual more susceptible to kidney disease after birth, which can lead to hypertension and end-stage renal failure [1]. The restricted transition from MM to epithelium might cause malformation of glomeruli and abnormal apoptosis of mesenchymal cells [2]. Many *in vivo* and *in vitro* studies have demonstrated important effects of glial cell-derived neurotrophic factor (GDNF) and its receptors Ret and GFRa1 on the process of UB budding and branching [3,4]. A number of transcription factors expressed by MM, such as the Wilms' tumor protein WT1, Pax2, Eya1, and Six2, influence UB branching by regulating the expression of GDNF [5–7]. Another intercellular signaling system, involving Robo2 and its ligand Slit2, plays a key role in controlling UB formation. In mammals, the secreted Slit2 protein induces the function of Robo2 receptor, a transmembrane protein. Robo2 functions as a chemorepellent that causes cell migration [8,9]. Grieshammer et al. [10] found supernumerary UB development and abnormal distribution of the GDNF-expressing regions in homozygous *Robo2*- or *Slit2*-knockout mice. Their study showed that Robo2/Slit2 controls UB budding by inducing the GDNF-expressing regions to further restrict the UB budding to a single site. However, it remains unclear how overexpression of Robo2 influences kidney development and what effect of Robo2/Slit2 has on the morphogenesis of the kidney after UB budding.

Our previous study has shown that Robo2 is first expressed in the MM cell membrane and then gradually expressed by the condensed cap mesenchyme surrounding the UB during the early morphogenesis of the embryonic kidney. The postnatal expression of Robo2 remains low (data not shown). To further elucidate the biological function of Robo2 in kidney development, we applied

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microinjection and electroporation methods to overexpress Robo2 in *in vitro*-cultured mouse embryonic kidney and observed the developmental defects of the kidney.

2. Materials and methods

2.1. Experimental animals

Healthy adult C57BL/6 mice aged 6–8 weeks were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) [license number: SCXK (Jing) 2006–0009]. Animals were caged and fed with a female-to-male ratio of 4:1. We checked the female mice for pregnancy at 8:00 am and 8:00 pm every day. The first time at which vaginal plugs were observed was considered embryonic day 0.5 (E0.5).

2.2. Vector

The EGFP-Robo2 vector constructed from the full-length *Robo2* cDNA was kindly provided by Xiao-bing Yuan [11]. The RFP empty vector was kindly provided by Raman Das and Stuart Wilson [12].

2.3. Microinjection, electroporation, and culture of embryonic kidneys

The procedures for microinjection and electroporation were as described previously [13,14]. Embryonic kidneys harvested by microdissection at E12.5 were placed into petri dishes containing Dulbecco's modified Eagle's medium (DMEM) (Gibco) and were divided randomly into a control group (transfected with empty vector) and an experimental group (transfected with EGFP-Robo2). To inject vectors, we applied an IM-300 microinjection system (Narishiga, Japan) that used 15–20 μm diameter glass needles (the syringes had an inner diameter of 0.5 mm, outer diameter of 1.0 mm, and length of 10 cm) prepared by a PB-7 pipette puller (Narishiga). A vector volume of 9.2 nL at a concentration of 2–3 μg/μL was injected at 10 different sites of the MM. Each site was injected once. Every kidney was therefore injected with 0.184–0.276 μg of vector. Electroporation was conducted immediately following microinjection, using a square wave electroporator (BTX ECM830, Genetronics Inc., San Diego, CA) and rectangular electrodes (gold-plated, Model 516, Genetronics Inc.) placed in parallel on either side of the embryonic kidney after the injection (Fig. 1B). The parameters used here were voltage: 36 V; number of pulses: 5; pulse length: 50 ms; internal time: 100 ms. After the electroporation, kidneys were immediately placed in 0.4 μm Transwells and cultured at the air–liquid interface in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin sulfate (Sigma). The vector expression was observed under a fluorescence microscope after 24 h incubation at 37 °C in 95% air/5% CO_2. Immunofluorescence staining was performed after 3 days of culture.

2.4. Tissue immunofluorescence staining

Embryonic mice microdissected at E12.5 were fixed in 4% paraformaldehyde (Sigma) at 4 °C for 2 h, further dehydrated in 10%, 20%, and 30% sucrose, and embedded in OCT medium. Sections of 4 μm were cut with a microtome and air-dried at RT. After treatment with 0.1% casein blocking reagent (Vector Laboratories) at RT for 20 min, sections were incubated with primary antibodies against Robo2 (Santa Cruz) and E-cadherin (Abcam) at 4 °C overnight. After incubation, the sections were washed three times with phosphate-buffered saline (PBS) at RT for 5 min each. Then, the secondary antibodies, Cy-3 conjugated anti-goat IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit (Jackson ImmunoResearch), were added and incubated for 30 min at RT. After incubation, the sections were again washed three times with PBS at RT for 5 min each. After mounting the sections with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI), we observed the results under laser-scanning confocal microscope.

2.5. Immunofluorescence staining of in vitro kidney culture

The cultured kidneys were fixed in 100% methanol at −20 °C for 15 min, rinsed with PBS for 15 min, and further blocked against antigens in PBS/0.1% casein for 20 min at RT. After incubating with antibodies against WT1 (1:100) (Abcam) and E-cadherin (1:100) (R&D Systems) at 4 °C overnight and the next day at 37 °C for another hour, the kidneys were washed with PBS three times at RT and then incubated with anti-rabbit IgG-Cy3 and anti-goat-FITC at 37 °C for 1 h. Finally, the kidneys were washed three times for a total of 1 h and then mounted for observation under the fluorescence microscope. We counted the number of glomeruli and branch tips of the UB. Statistical analysis was performed by *t*-test. Differences were considered statistically significant when $p < 0.05$.

2.6. Staining for proliferation and apoptosis

The embryonic kidneys transfected with the vectors were cultured *in vitro* for 3 days. After fixation in 4% paraformaldehyde at RT for 30 min, permeabilization with 1% Triton X-100 for 1 h, and blocking in FCS containing blocking reagent for 20 min, the kidneys were incubated with primary antibodies against phospho-histone H3 (PH3, Cell signaling) and E-cadherin at 37 °C for 1 h. After three PBS washes at RT, TdT-mediated dUTP nick end labeling (TUNEL) solution (Roche) was applied for 1 h at RT according to the manufacturer's instructions. The kidneys were then washed for 1 h at RT and mounted in mounting medium containing DAPI. The results were observed under laser-scanning confocal microscope. The numbers of proliferative and apoptotic cells were counted in five random fields from each embryonic kidney, six kidneys per group. Statistical evaluation was performed by *t*-test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Robo2 is expressed in mm during early embryonic kidney development

During early morphogenesis of the mouse embryonic kidney, Robo2 expression was detected in the cell membranes of the MM, surrounding the UB as condensed clusters (Fig. 1A). To explore the effect of Robo2 overexpression on the development of the mouse embryonic kidney, we performed *in vitro* transfection of a Robo2-GFP vector (kindly provided by Xiao-Bing Yuan). An empty RFP vector was used in parallel as control. As shown in Fig. 1B, we first microinjected GFP-Robo2 or empty vector into the cultured E12.5 kidney. After multiple injections into the MM, electroporation and cell culture were immediately performed. The vector expression was observed under fluorescence microscope after 24 h culture (Fig. 1C). The green fluorescence protein encoded by the Robo2-overexpressing vector was strongly expressed in MM. The red fluorescence protein encoded by the RFP vector was also expressed in MM.

3.2. Reduced UB branching and glomerular number after Robo2 overexpression in vitro

We transfected the vectors into the E12.5 embryonic kidneys *in vitro*. The kidneys were divided into the no-transfection control

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