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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.

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

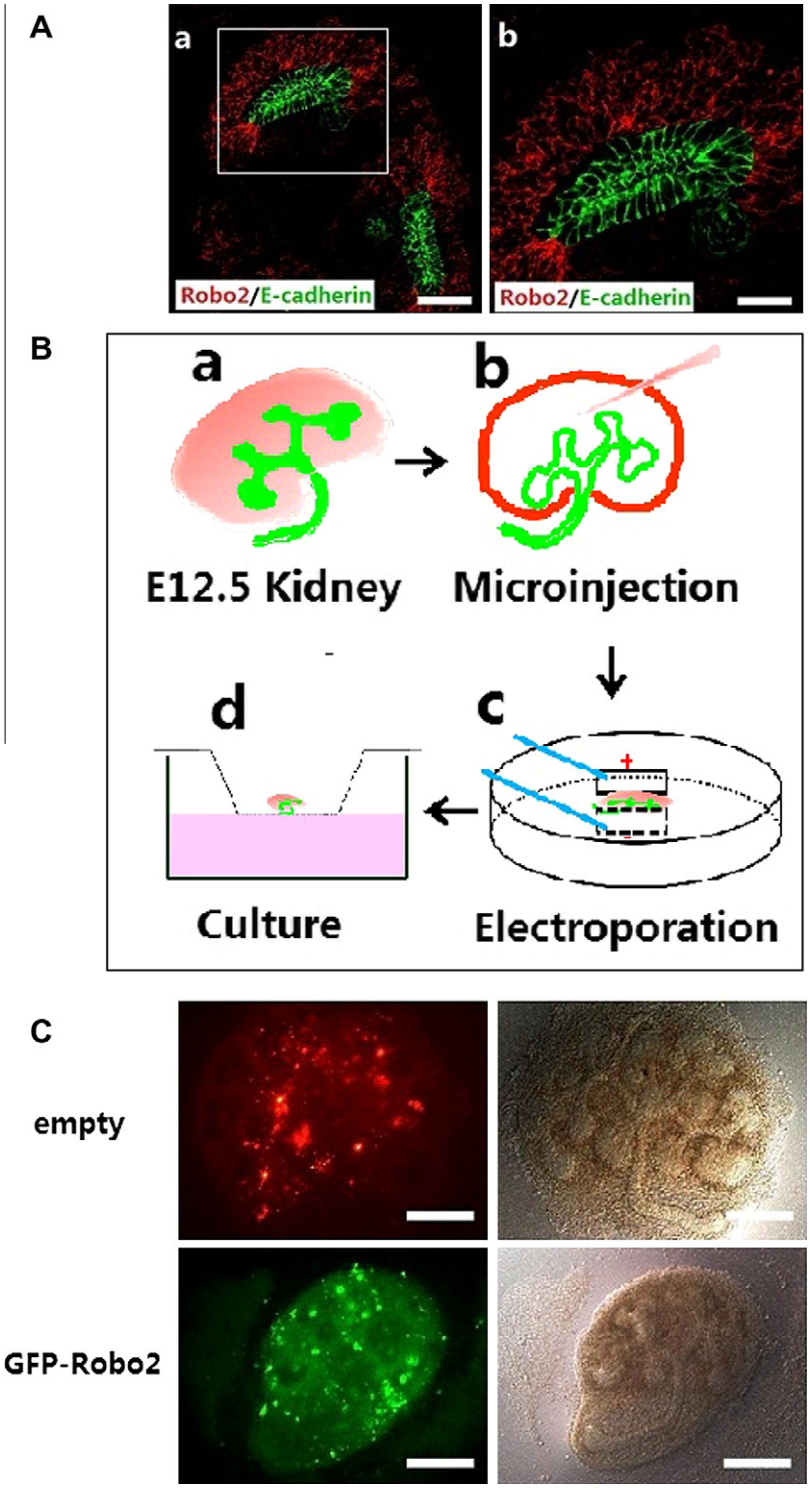

Fig. 1. (A) a – Immunofluorescence staining for Robo2 and UB marker E-cadherin in E12.5 *in vitro*-cultured mouse embryonic kidney. b – Higher magnification of the boxed region in a. Robo2 was expressed in the MM surrounding the ureter transplant. Magnification was 400× and 800×; the scale bar indicates 50 and 25 μm. (B) A model of microinjection and electroporation for the *in vitro* culture of embryonic kidney. a – An embryonic kidney from E12.5; b – microinjection into the condensed cap mesenchyme; c – two electrodes placed in parallel on either side of the embryonic kidney; d – *in vitro* culture at the air–liquid interface. (C) Fluorescence microscopy of E12.5 embryonic kidney 24 h after transfection with RFP empty vector or GFP-Robo2 vector. Magnification was 100×; the scale bar indicates 200 μm.

group, the RFP empty vector group, and the GFP-Robo2 vector group, with eight embryonic kidneys per group. The immunofluorescence staining of the UB marker E-cadherin after 3-day *in vitro* culture indicated normal embryonic kidney growth in the control and RFP groups, in which the UB bifurcated normally by lateral branching. In contrast, there was reduced UB branching in the GFP-Robo2 group (Fig. 2A). The cell count in the UB branch tip of the kidney from the GFP-Robo2 group decreased significantly compared to those from the control group and the RFP group (19.13 ± 6.71) ($p < 0.05$) (Fig. 2C). To observe the influence of Robo2 overexpression in the embryonic kidney on the development of the glomerulus, we stained for the glomerular marker WT1. The results show

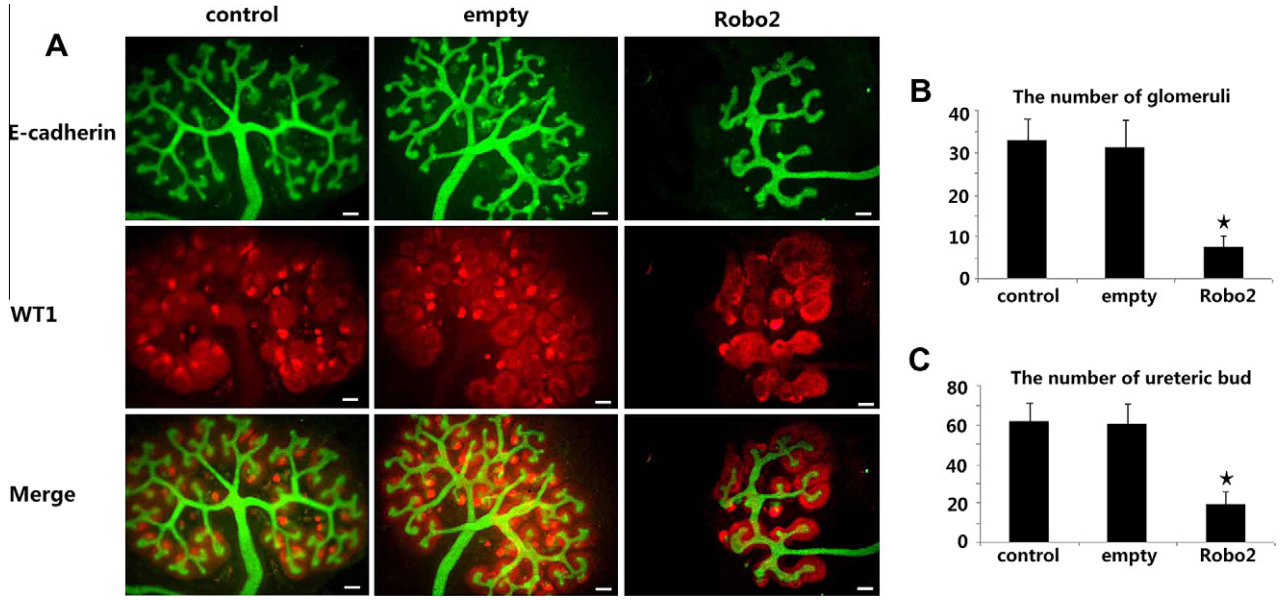

Fig. 2. (A) Immunofluorescence staining of the UB marker E-cadherin and the MM and glomerular marker WT1 in E12.5 embryonic kidney transfected with RFP empty vector or GFP-Robo2 vector after 3-day *in vitro* culture. Magnification was 100×; the scale bar indicates 200 μm. (B and C) Numbers of glomeruli and UB branch tips (*$p < 0.05$) ($n = 8$).

significantly fewer glomeruli (7.50 ± 2.73) ($p < 0.05$) (Fig. 2A, B) in the GFP-Robo2 group compared to the control group.

3.3. In vitro overexpression of Robo2 results in fewer MM cells

To further dissect the reasons for the abnormal UB branching and for the reduced number of glomeruli, we analyzed the whole embryonic kidney culture. The MM clustered around the UB and enclosed the UB during the kidney morphogenetic process (Fig. 3A). The MM inductively interacted with the UB and compelled the UB to continue branching with binary branching, triple branching, and bilateral branching patterns. Moreover, the MM underwent a series of morphological changes to from the glomeruli. Altogether, we found that overexpression of Robo2 in the mouse embryonic kidney *in vitro* led to reduced UB branching and fewer glomeruli. Further analysis showed that the cell count of the MM surrounding the UB branch tip decreased significantly in the embryonic kidney from the GFP-Robo2 vector group compared to the control group and the RFP empty vector group. This led to impaired clustering of the cap mesenchyme (Fig. 3C), which mostly happened in the lateral branch of the UB tip inside the kidney to form a blind end on the branch and prevent subsequent branching.

3.4. Normal epithelial morphology of UB and no abnormal proliferation or apoptosis of MM cells with in vitro overexpression of Robo2

Overexpression of Robo2 in the E12.5 mouse embryonic kidney *in vitro* reduced UB branching. To further understand the epithelial morphological changes in the UB branch, we performed immunofluorescence staining for the UB epithelial marker E-cadherin and observed the results under confocal microscope. Our data show that the expression of E-cadherin in the epithelium of the UB tip and the tightness and the order of the UB cell alignment were not significantly different from those in the control group and the RFP group (Fig. 4A–C). To study whether the diminished cap mesenchyme was related to excessive cell apoptosis, we performed TUNEL staining. Apoptotic MM cells increased significantly in the *in vitro* culture compared with *in vivo* development, but no significant difference was observed between the Robo2-overexpressing embryonic kidney and the control. Moreover, we found fewer MM cells around the UB (Fig. 4A–C). Through immunofluorescence staining for the proliferative marker PH3 no significant proliferation was found in the Robo2-overexpressing MM cells in the embryonic kidney (Fig. 4D–F). We picked five fields randomly from each embryonic kidney ($n = 6$) to count the apoptotic and proliferative cells and to perform the statistical analysis. No significant difference was found among the control, RFP, and GFP-Robo2 groups ($p > 0.05$) (Fig. 4G and H).

4. Discussion

During kidney development, the inductive interaction between the UB and MM is fundamental and necessary for kidney morphogenesis [2]. In the early stage of kidney development, the UB invades the MM, forming the characteristic "first generation." As the cells on the branch tip expand rapidly, the UB starts growing and forms the next two branches [15]. The UB expresses the genes that control its branching morphogenesis [16,17], and any abnormal alteration of these leads to malformation of UB branching morphogenesis. The overexpression or suppression of certain genes in MM might lead to kidney developmental defects [18], but few studies have examined the molecular signals in the UB during branching [19]. We report here that Robo2 overexpression in MM can cause kidney developmental defects, evidenced by little or no UB branching, abnormal morphology, and the failure of UB elongation. The initiation of UB branching morphogenesis relies heavily on the UB tip, and the elongation of the UB trunk partially depends on the UB branch site near the trunk [20], which is consistent with our results. Interestingly, however, the morphology of the outermost layer of UB was not strongly affected, which implies that different mechanisms govern the UB primary and subordinate branching.

UB branching is impaired when the MM cells are deficient in WT1 [21]. Likewise, overexpression of some genes in MM can result in kidney developmental defects [22,23]. However, overexpression of some genes has also shown no phenotype, even when the

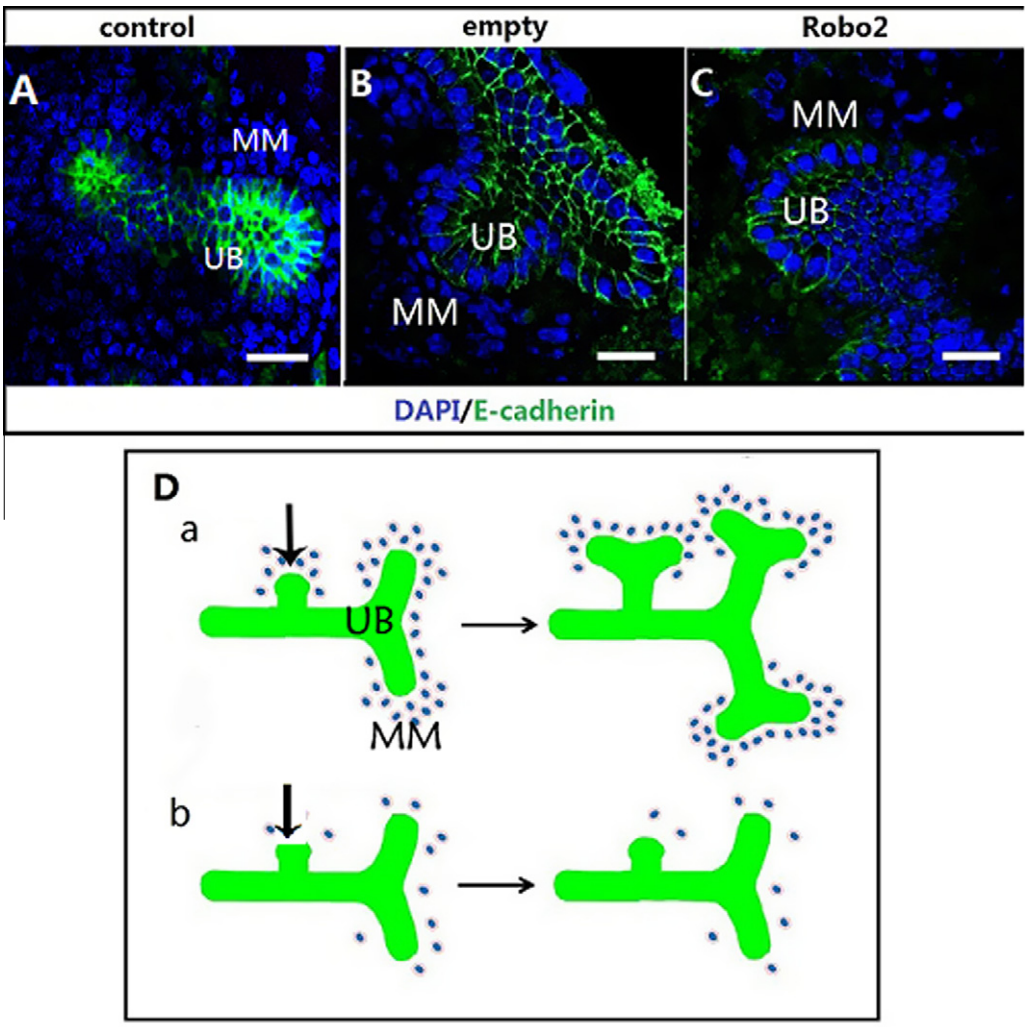

Fig. 3. Immunofluorescence staining for UB marker E-cadherin in E12.5 embryonic kidney transfected with RFP empty vector or GFP-Robo2 vector after 3-day *in vitro* culture. Magnification was 800×; the scale bar indicates 25 μm. (A) Control; (B) transfected with RFP empty vector; (C) transfected with GFP-Robo2 vector. (D) Schematic diagram of the interaction between the MM and UB during kidney development. Subpanel a shows normal UB branching through inductive interactions with the MM surrounding the branch tip of the UB. (↓) Lateral branch. Subpanel b shows defective UB branching after Robo2 overexpression, caused by a reduced number of MM cells surrounding the branch tip of the UB, leading to a lack of inductive effect.

knockout mutants of those genes have phenotypes. For example, overexpression of SALL1 does not lead to any defect in the kidney [24]. In this study, we found abnormal branching of the UB and fewer MM cells around the UB after Robo2 overexpression, which indicates that the earliest morphogenesis of UB subordinate or lateral branching does not rely completely on MM cells but that subsequent developmental processes, e.g., the elongation of UB, require the interaction between the UB branch tip and the MM cells.

Because the number of MM cells surrounding the UB was reduced by overexpression of Robo2, we hypothesized that Robo2 directly affects the migration and the accumulation of MM cells. Indeed, Robo2 plays important roles in nervous system development and neuron migration [25,26], and MM cells have the characteristics of migrating and clustering during kidney morphogenesis [27,28]. MM cells not only induce UB branching but also cluster to form the cap mesenchyme to proceed to epithelial-mesenchymal transition and to shape the glomerulus. Sims-Lucas et al. reported that MM-specific *Fgfr1*/*Fgfr2* double knockouts display a loss of condensed clustering of MM cells, the emergence of multiple UBs, and defective UB subordinate branching [18]. Our data concur with the above reports. We further postulate that after Robo2 overexpression, the reduced UB branching and the defective MM condensed clustering might lead to a decreased number of glomeruli.

Because the proliferation and/or transient apoptosis is correlated with abnormal UB branching [29], we investigated whether the abnormal UB branching and the reduced surrounding MM cells were correlated with the proliferation and apoptosis. We found that compared to the normal embryonic kidney in culture, the proliferation and apoptosis of the UB and MM cells from Robo2-overexpressing embryonic kidney were not different. Based on our results, it is likely that the reduction of MM cells in the UB tip after Robo2 overexpression is not caused by apoptotic induction of MM cells. Alternatively, Robo2 overexpression could lead to the defective MM clustering in the UB tip and influence UB branching morphogenesis. However, MM-specific *Fgfr1*/*Fgfr2* double knockouts display increased MM apoptosis and therefore impaired MM condensed clustering [30], which is not consistent with our Robo2 overexpression result. Similarly, inhibiting the apoptosis induced by caspase-3 leads to defective UB branching morphogenesis [31]. In contrast, we did not find any inhibition of caspase-3 in the embryonic kidney or any alteration of proliferation markers, such as PH3. These data indicate that Robo2 might directly affect MM clustering. Furthermore, we found increased apoptosis in the

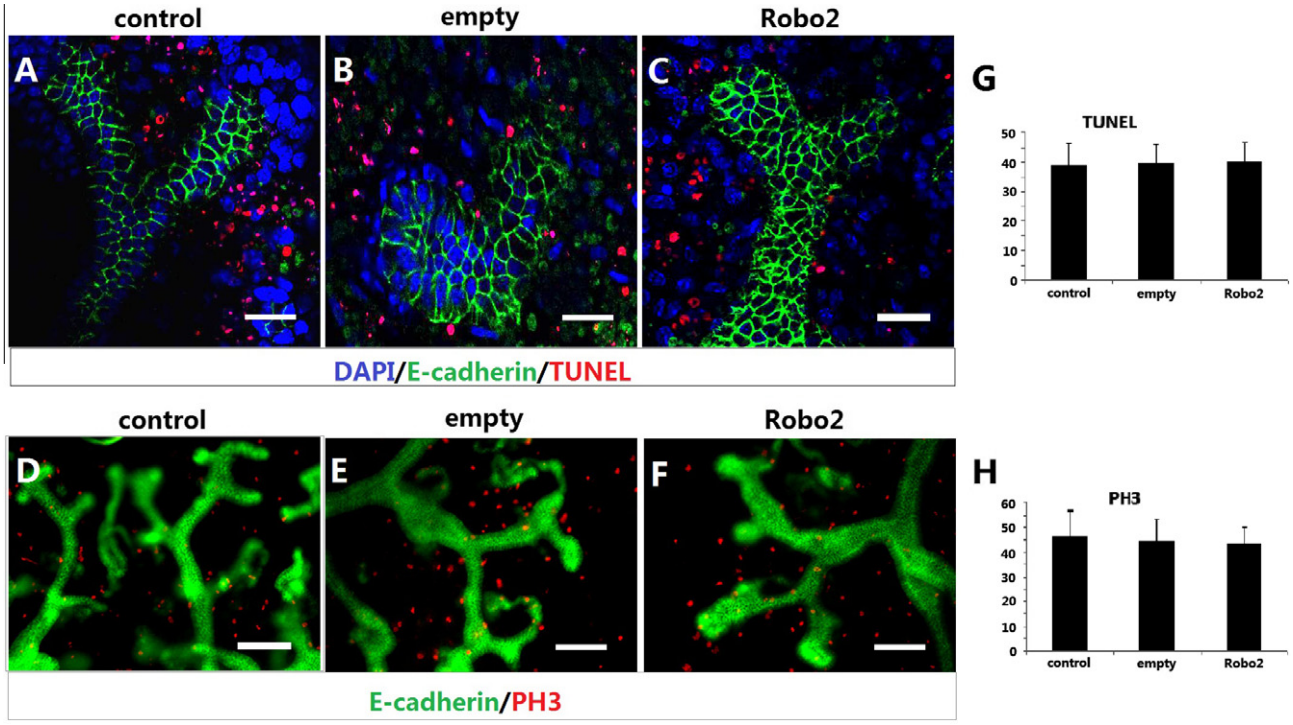

Fig. 4. Proliferation and apoptosis of MM cells in E12.5 embryonic kidney transfected with RFP empty vector or GFP-Robo2 vector after 3-day *in vitro* culture. (A–C) TUNEL staining, Magnification was 800×; the scale bar indicates 25 μm. (D–F) Immunofluorescence staining for proliferation marker phospho-histone H3. Magnification was 200×; the scale bar indicates 100 μm. (G and H) Cell counts of proliferative and apoptotic cells in five random fields from each embryonic kidney ($n = 6$ per group). There was no statistically significant difference among groups, $p > 0.05$.

cultured embryonic kidneys compared to the sections collected directly *in vivo*, which might have been due to the different surrounding environments *in vivo* and *in vitro*. However, when under the same *in vitro* environment, the embryonic kidney tissues overexpressing Robo2 showed no significant differences in apoptosis compared to the control group and to the empty-vector group.

Previous studies have also applied microinjection and electroporation to analyze the functions of genes during the kidney morphogenetic process [14,32,33]. Due to the fast development of the embryonic kidney, the UB starts budding from the Wolffian duct on E10.5 [27], but the embryonic kidney from E10.5 is too small to be isolated for microinjection. Therefore, it is difficult to study the gene regulatory functions in early kidney morphogenesis, whereas it is much easier to perform transfection on the E12.5 embryonic kidney by microinjection. For these reasons, we were not able to study the effect of Robo2 overexpression in earlier phase, such as E9.5, even though we found reduced UB branching in the embryonic kidney during Robo2 overexpression. So far, it is still unclear whether the phenomenon of multiple UB budding during Robo2 overexpression is similar to that in the *Robo2*-knockout mice. Therefore, the construction of transgenic mice is still needed to further investigate the effect of Robo2 overexpression on kidney morphogenesis.

In summary, we studied the function of Robo2 overexpression in *in vitro*-cultured embryonic kidneys by microinjecting a Robo2-expressing vector. Overexpression of Robo2 resulted in inhibition of MM cell migration and clustering and impaired the interaction between MM and partially inhibited UB budding. Therefore, UB branching was reduced, and meanwhile, the number of glomeruli decreased, leading to kidney malformation and smaller kidney size because of the lack of cap mesenchyme and subordinate branching. Our results indicate that microinjection is a simple and effective method to study gene functions in the embryonic kidney.

Conflict of interest statement

None declared.

Acknowledgments

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