BALANCING RENEWAL AND DIFFERENTIATION OF PROGENITOR CELLS IN THE DEVELOPING KIDNEY

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BALANCING RENEWAL AND DIFFERENTIATION OF PROGENITOR CELLS IN THE DEVELOPING KIDNEY

by

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DEDICATION

This thesis is dedicated to my late friend Arun Nedoungilli. Without him I would not have taken the necessary leap at the right time in my life to come this far.

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Tons and tons of appreciation to my husband for putting up with me and my craziness and in addition for supporting me in every possible way he could.

Lastly, these words don't do any justice to how much I really appreciate my husband and Tom.

ABSTRACT

Mammalian kidneys perform the important function of blood filtration. All the filtered wastes are concentrated into urine and excreted from the body. The kidney performs these functions through individual functional units called nephrons. On average, an adult human kidney contains ~1 million nephrons. There may be a decrease in the number of nephrons due to kidney injury. However, new nephrons cannot form in adult kidneys due to the absence of nephron precursor cells. Additionally, regeneration of kidneys upon injury is very limited. If nephron number falls below a certain threshold, then individuals are predisposed to a myriad of medical conditions including renal failure. Although dialysis is a treatment option for individuals with renal insufficiency, only 10% of those on dialysis survive beyond 10 years of treatment. Currently, kidney transplantation is the only available long term treatment, but sufficient numbers of transplantable kidneys are not available. Various stem cell therapies and kidney re-engineering are actively being pursued as viable treatment paths. For successful progress in those approaches, a thorough understanding of kidney development and the various signals that play important roles in nephron endowment is imperative. Despite many decades of work invested in this field, there are still many unknowns. Regulation of renewal and differentiation of the progenitors during the process of nephron formation is the main focus of my dissertation.

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1. **Polycystin-1 binds Par3/aPKC and controls convergent extension during renal tubular morphogenesis.** Nat Comm, 2013. Castelli M., Boca M., Chiaravalli M., Ramalingam H., Rowe I., Distefano G., Carroll T., Boletta A.

2. **Hemodynamic forces sculpt developing heart valves through a KLF2-Wnt9b paracrine signaling axis.** Dev. Cell, 2017**.** Lauren M. Goddard, Anne-Laure Duchemin, Harini Ramalingam, Bingruo Wu, Mei Chen, Sharika Bamezai, Jisheng Yang, Li Li, Michael Morley, Tao Wang, Marielle Scherrer-Crosbie, Dave B. Frank, Stephen C. Jameson, Edward E. Morrisey, Thomas J. Carroll, Bin Zhou, Julien Vermot, and Mark L. Kahn.

3. **Talin regulates integrin β1 dependent and independent cell functions in ureteric bud development.** Development, 2017. Sijo Mathew, Riya J Palamuttam, Glenda Mernaugh, Zhenwei Lu, Harini Ramalingam, Ming-Zhi Zhang, Shuta Ishibe, David R Critchley, Reinhard Fässler, Ambra Pozzi, Thomas Carroll, Charles R. Sanders, Roy Zent.

4. **Disparate levels of beta-catenin activity determine nephron progenitor cell fate.** Ramalingam H., Fessler A., Das A., Valerius M.T., Basta J., Robbins L., Brown A.C., Oxburgh L., McMahon A., Rauchman M., Carroll T.J. (PNAS, In submission process)

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ABBREVIATIONS

ACD - Asymmetric Cell Division

APC - Adenomatosis Polyposis Coli

c - floxed allele

Casp3 - Caspace 3

CK1α - Casein Kinase 1α

CAG-Wnt9b - Cagg-lox-stop-lox-Wnt9b-IRES-GFP

CK - Pan-Cytokeratin

DBA - Dolichos Biflorus Agglutinin

dKO - double knockout of Numb and Numblike (Six2-Cre;$N^{c/c}$;$NL^{c/c}$)

DMSO - Dimethyl Sulfoxide

E - Embryonic Day

ECM - Extracellular Matrix

Fzd - Frizzled receptor

GFP - Green Fluorescent Protein

Gsk3 - Glycogen Synthase Kinase-3

IWP - Inhibitor of Wnt Production

IWR1 - Inhibitor of Wnt Response -1

Lam - Laminin

Lef/Tcf - Lymphoid enhancer factor/T-cell factor

LiCl - Lithium Chloride

LTL - Lotus Tetragonolobus Lectin

MET - Mesenchymal-to-Epithelial Transition

MM - Metanephric Mesenchyme

Nb - Numb

Neph - Nephrin

NL - Numblike

NPC - Nephron Progenitor Cell

OCD - Oriented Cell Division

P - Postnatal day

Pla2g7 - Phospholipase A2 group 7

PP2A - protein phosphatase 2A

PTA - Pre-Tubular Aggregate

Rosa-Wnt1 - Rosa26-lox-stop-lox-Wnt1

Rspo3 - R-spondin 3

RV - Renal Vesicle

Tafa5 - expressed sequence AW049604

UB - Ureteric Bud

UGS - Urogenital System

WD - Wolffian Duct

WT1 – Wilm's Tumor 1

YFP - Yellow Fluorescent Protein

CHAPTER ONE

Introduction

Abstract

Mammalian kidneys perform the important function of blood filtration. All the filtered wastes are concentrated into urine and excreted from the body. The kidney performs these functions through individual functional units called nephrons. While nephron number may decrease during one's lifetime (due to various kidney injuries or hypoxic events), new nephrons do not form in adults. This is most likely due to the absence of nephron precursor cells. If nephron number falls below a certain threshold, the kidneys stop functioning properly pre-disposing individuals to a myriad of medical conditions including renal failure. While dialysis is a treatment option, the survival rate of a dialysis patients is poor. Currently, kidney transplantation is the only long-term treatment possible, but sufficient numbers of transplantable kidneys are not available. Various stem cell therapies and kidney re-engineering are actively being pursued as viable treatment paths. For successful progress in those approaches, a thorough understanding of kidney development and the various signals that play important roles in nephron endowment is imperative. Despite many decades of work invested in this field, there are still many unknowns. Regulation of renewal and differentiation of the progenitors during the process of nephron formation is the focus of my dissertation.

Introduction

Kidneys are the chief excretory organs of the body. They perform the function of blood filtration by removing bodily wastes in the form of urine. By carrying out these functions, they regulate water and salt levels, acid-base balance, detoxification and cellular metabolite levels. The kidney also act as endocrine organs. It secretes the following harmones – 1. erythropoietin to regulate RBC levels, 2. renin and angiotensin converting enzyme (ACE) to regulate blood pressure and 3. Calcitriol to regulate calcium absorption In sum, the organ called kidney (although considered disgusting, partly due to its end product, urine) is indeed an amazing organ that maintains the chemisty of blood and, thus, the chemistry of life itself.

The functional units of kidneys are nephrons. A nephron is a vascularized tubular structure made up of many different specialized segments that carry out specialized functions during urine formation. The main segments of the nephron are: 1. the glomerulus, 2. The proximal convoluted tubule, 3. proximal straight tubule, 4. thin descending limb of Henle, 5. thin ascending limb of Henle, 5. thick ascending limb of Henle, 6. distal straight tubule and 7. distal convoluted tubule. Many nephrons connect to one collecting duct. The kidney contains many collecting ducts, all of which open into the ureter. The ureter connects the kidney to the urinary bladder (Fig.1.1). Urine formed as a result of blood filtration, re-absorption and secretion in the nephrons is drained into the collecting ducts from which it is sent to the bladder through the ureter. The kidney tubules and vasculature reside on a substrate called the renal interstitium. Interstitial cells include vascular smooth muscle cells, mesangial cells of the glomerulus, pericytes and fibroblasts. These cells form the framework of the organ [1].

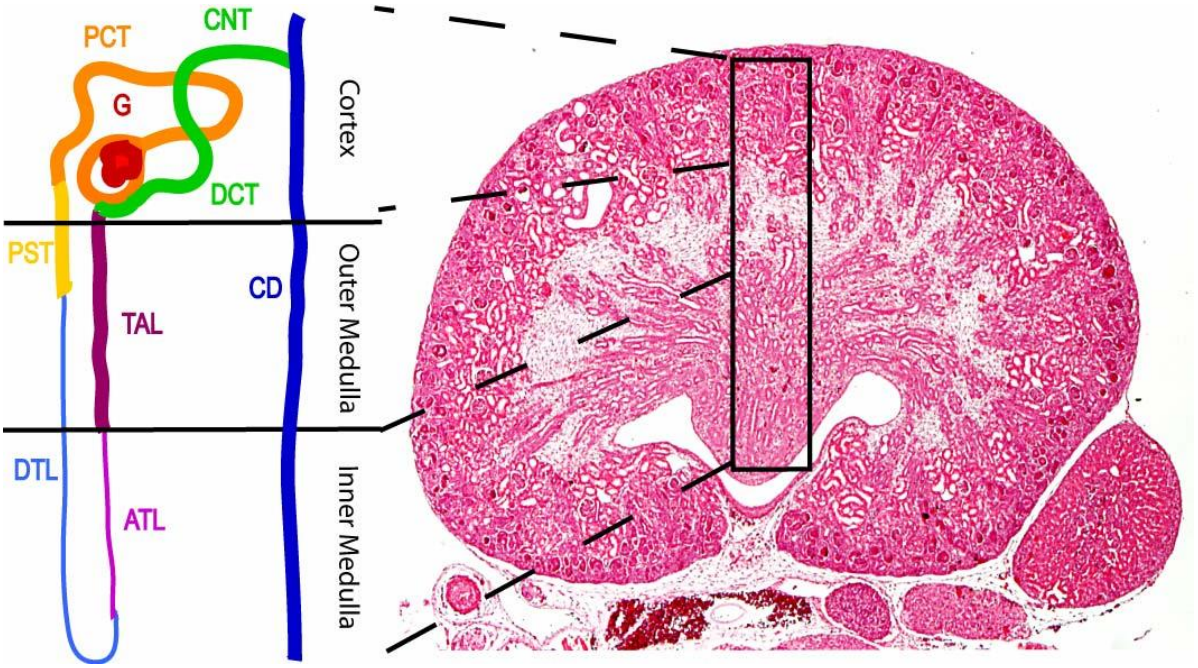


Fig. 1.1. Basic Kidney Anatomy: Hematoxylin and Eosin staining of a mouse metanephric kidney at E18.5 shows the numerous tubular structures of the kidney. At the very cortical region is the nephrogenic zone composing of ureteric bud tips, condensed renewing NPCs, differentiating nascent nephron structures and stroma progenitors. Depicted on the left is a schematic of a mature nephron showing the position of different nephron segments with respect to the cortico-medullary axis of the kidney. The glomerulus (G, in red), proximal convoluted tubule (PCT in orange), distal convoluted tubule (DCT in green) and connecting tubule (CT in green) are in the cortical region and the different parts of loop of Henle namely thick ascending limb (TAL, in magenta), ascending thin limb (ATL, in pink) and descending think limb (DTL, in light blue) are in the medullary region. The connecting tubule connects to the collecting duct (CD in dark blue). Image source: Carroll lab.

Overview of Kidney Development

The mammalian kidney develops from the intermediate mesoderm (IM). At embryonic day (E) 8.0 in mouse, a few precursors cells dissociate from the IM to give rise to a ductal structure called the Wolffian duct. The Wolffian duct (WD) starts at the cranial side of the IM, runs caudally, in immediate adjacency to the IM, and integrates with the cloaca at E11.0. During its formation and elongation, the WD induces the IM to form three different kidney types – the Pronephros, the Mesonephros and the Metanephros [2-4]. Pronephroi are the most primitive kidneys and exist between E8.5 - E9.5 after which they degenerate. They do not function as kidneys in mammals. However, their development is essential for the formation of the other two kidney types [3, 5]. The second type of kidneys is the mesonephros. These are the functional kidneys in lower organisms including frogs and fish. In vertebrates, they are functional for a brief period (E9.0 to E14.5 in mouse) in the embryo. Although the mesonephros degenerates during later stages of the embryo's development (E14.5 in mouse), its formation is required for the development of genital organs in all vertebrates [5].

Metanephric kidney development

The metanephros is the permanent kidney type in vertebrates. Development of the metanephric kidney begins when a subset of precursor cells of the intermediate mesoderm near the hind limb region specialize into a cell type called the metanephric mesenchyme (MM) [6-8]. Upon its specification, the MM induces the Wolffian duct to

develop an outgrowth into the MM called the ureteric bud (UB) [9, 10]. In mouse, specification of the MM begins ~E9.5 and UB outgrowth takes place at E11.0. The MM gives rise to nephron progenitor cells (NPCs) and stromal progenitors. The NPCs undergo self-renewal to maintain the progenitor population while a subset of NPCs undergo differentiation to form nephrons [11]. The stromal progenitors give rise to the renal interstitium [12].

The UB provides cues for the induction of MM [13, 14]. These cues include survival, renewal, and differentiation factors for the NPCs. Reciprocally, the MM provides factors for the maturation and dichotomous branching of the UB and, for the maintenance of stemness of the branching UB tips [15]. The UB, after several branching events, gives rise to the collecting duct system of the kidney [16, 17]. At each branching event, the MM condenses around the branch tip and a subset of NPCs undergo differentiation such that the renewing NPCs are present on the cortical side (dorsal) of the UB tip and the differentiating cells are present on the medullary side (ventral) of the UB tip at the cleft region. The reciprocal interactions between the UB and MM occur reiteratively throughout the development of the kidney. This leads to a centrifugal pattern of kidney development wherein the cortex or the outer region of the developing kidney is composed of UB tips, condensed MM and differentiating NPCs (this zone is called the nephrogenic zone) while the inner region or the medulla is composed of mature collecting ducts, nephrons and fibroblasts (Fig. 1.2) [18].

When the NPCs receive signals to differentiate, a subset of NPCs aggregate forming what is referred to as a pre-tubular aggregate (PTA), which always forms at the cleft of T-shaped UB tip. The PTAs then undergo mesenchymal-to-epithelial transition

(MET) to form an epithelial sphere called the renal vesicle (RV). This sphere-shaped RV is polarized along the apico-basal axis and along the proximo-distal axis. The half of the RV that is closest to the UB is the distal region and the other half is the proximal region. Distal and proximal regions are molecularly distinct. The RV further elongates along the proximo-distal axis to give rise to S-shaped epithelia called the S-body. The S-body expresses precursors of all the different segments of the nephrons namely the distal tubule, the loop of Henle, the proximal tubule and the glomerulus. These precursor segments eventually mature and differentiate into a fully functional nephron. Early in this process the distal most part of S-body fuses with the UB thereby forming a continuous lumen. Simultaneously, endothelial cells invade the cleft of prospective glomerulus at the proximal most part of S-body (Fig. 1.3) forming the renal vasculature. The origin of the vasculature of the kidney is unknown but there is some evidence suggesting that vascular precursors cells are part of the MM [6].

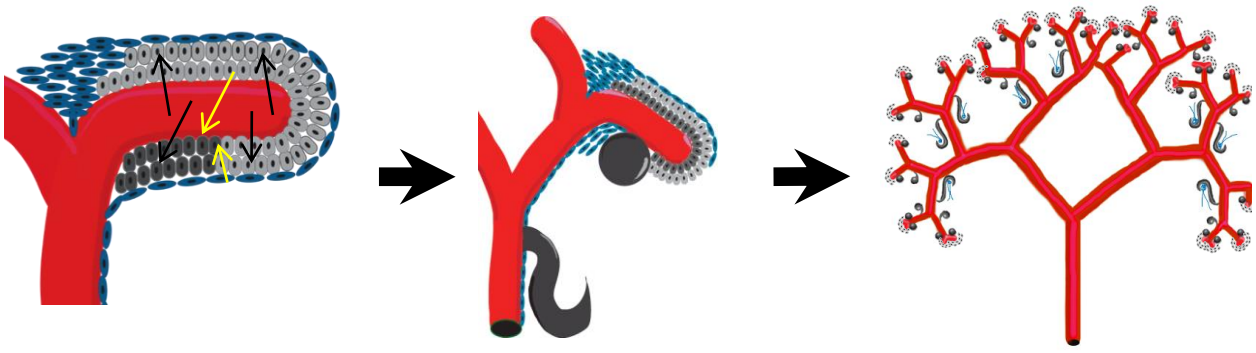


Fig. 1.2. Development of the embryonic kidney. Reciprocal signals (black and yellow arrows) between the ureteric bud (red) and the surrounding metanephric mesenchyme (MM) regulate kidney development. From E11.5, the kidney develops in a centrifugal pattern with mature nephrons in the center of the kidney and, nascent nephron structures and NPCs at the periphery. NPCs are shown as grey cells, stromal progenitors are shown

in blue and pre-tubular aggregates (PTAs) are shown in dark grey. Renal vesicle is shown as a black sphere. Source: Carroll Lab

MM specification and induction

Proper regulation of the NPCs is necessary for the formation of a healthy kidney. Functional genomics, gene expression profiling and genetic studies have shed light on some of the molecular mechanisms governing NPC specification, renewal and differentiation. Genes and pathways involved in this process are discussed below.

Odd-skipped related 1 (Osr1) is a zinc finger protein that is expressed throughout the intermediate mesoderm (IM) starting from E7.5 [19]. Osr1 cells can be lineage traced to most of the tissue types of metanephric kidney – UB, stroma, nephrons and vasculature [6]. Indeed, Osr1 transcriptional activity is necessary for the formation and maintenance of the Wolffian duct, outgrowth of the UB trunk and, specification and maintenance of the NPCs [19]. Although Osr1 cells give rise to stromal progenitors and NPCs, Osr1 activity is not necessary for stromal lineage specification or differentiation [6]. Osr1 mutants do not express several important MM specific genes including Gdnf, Pax2, Six2, Eya1, Sall1 and WT-1 [20]. These genes, with the exception of Gdnf, play a direct role in MM specification. NPC derived Gdnf (Glial cell line-derived neurotropic factor) signaling through the UB expressed receptor tyrosine kinase (Ret) is necessary for MM integrity albeit indirectly via its regulation of UB branching and growth [21]. Paired Box 2 (Pax2) is a transcription factor that is critical for the formation of Wolffian duct and is expressed in the IM, Wolffian duct and ureteric bud. Upon UB outgrowth, Pax2 shows up in the NPCs [6] where its activity is essential for NPC maintenance. Eya1 is another transcription factor that is necessary for Woffian duct specification, UB outgrowth and MM specification [22,

23]. Eya1 mutants precociously lose self-renewing NPCs. Eya1 functions genetically upstream of Pax2 and Six2 in MM specification [23]. Following MM specification, Eya1 expression gets restricted to the renewing NPCs [24] and is regulated by Six2 and B-catenin [25]. The homeobox protein, Six2 is an important NPC renewal factor [11]. Deletion of Six2 in kidneys results in ectopic epithelialization and thus precocious depletion of NPCs [26] unlike deletion of Pax2 or Eya1 which causes apoptosis. Mutants heterozygous for Six2 show reduced nephron endowment and hypoplasia [27]. How Six2 is regulated is not very clear. Some evidence shows that Hox11a, b, c transcription factors regulate Six2 expression [8, 28]. However, the Hox11 paralogs (Hoxa11, Hoxc11, Hoxd11) themselves are necessary for MM specification or for Six2 and Gdnf expression [29].

Recent work shows that a sub-fraction of Six2 cells are the true progenitors and that Six2 marks a broad group of progenitors. According to this work, Six2 is expressed in renewing progenitors as well committed NPCs en-route to epithelialization [30]. Cited1, Eya1 and Meox1 are the genes expressed specifically in the renewing NPCs [24, 25, 30].

Several secreted factors including Fgfs, Bmp7 and Wnts play important roles in MM survival, renewal and differentiation [31]. Fibroblast growth factors (Fgfs) are considered the chief survival factors of NPCs. Deletion of Fgf receptors results in abrogated growth of MM [32]. However, deletion of Fgf2 does not affect NPC growth or survival suggesting redundancy in Fgf ligands [33]. This led to the identification of other Fgfs. Fgf 9 produced by the UB, Fgf1 and Fgf20 produced by the NPCs regulate NPC survival and competency [33, 34]. Fgf8, the Fgf ligand secreted by PTAs is necessary for

the survival of NPCs [35]. Fgf8 is also necessary in the PTAs for nephrogenesis [36]. In Fgf8 mutants, lhx1 is not expressed and RVs do not progress to S-bodies [35, 36].

Another factor involved in NPC regulation is Bone morphogenetic protein 7 (Bmp7). Bmp7 is expressed by the NPCs. Bmp7 deletion affects NPC renewability post E14.5 suggesting that Bmp7 is not necessary for specification or initial renewal events [37]. Invitro studies show that Bmp7 induces proliferation of NPCs via the JNK signaling pathway [38]. Bmp7 also plays a role in differentiation of NPCs. Acting via the Smad receptors, Bmp7 signaling is necessary to induce Cited1-positive NPCs to differentiate into PTAs [30].

Wnt9b is expressed in the Wolffian duct and subsequently in the ureteric bud where it continues to be expressed throughout kidney development[39]. Wnt9b is secreted to the surrounding MM where it activates B-catenin to induce both renewal genes and differentiation genes [40]. Ablation of Wnt9b results in loss of Cited1 expression, and reduction in proliferation of NPCs as early as E11.5 [39, 40]. By E15.5, all NPCs are lost. Chromatin precipitation studies and functional assays show that Six2 and Wnt9b activated B-catenin co-operate to activate renewal genes [25, 40]. Wnt9b is also necessary for differentiation of NPCs. Wnt9b mutants do not show any signs of differentiation starting from E11.5. Some of the differentiation gene targets of Wnt9b include Wnt4, Fgf8, Pax8 and C1qdc2. Wnt4 activates genes including itself by signaling through the Calcium dependent non-canonical pathway [41]. Wnt4 target genes are necessary for MET of PTAs [42, 43]. Ablation of Wnt4 leads to kidney agenesis due to failure to develop renal vesicles [44]. Fgf8 and Wnt4 activity is required for the expression of Lhx1, a key gene inducing distal fate of RVs [35, 42, 45].

Yet another ligand that affect NPCs is the atypical cadherin, Fat4. It is expressed in cortical stroma and activates Dchs receptors in the NPCs [12, 46, 47]. This signaling is necessary for the induction of differentiation in NPCs. Fat4 mutants or stromaless mutants have a piled-up NPC compartment but do not have any differentiation [12, 48].

Cessation of nephrogenesis

Shortly before birth in humans, and around post-natal day 4 in mice, nephrogenesis ends. This is evidenced by the absence of NPCs at P4 and a concomitant absence of new PTAs post P4. In addition, the nephrogenic zone of the developing kidney is not present from here on.

During development, renewal of NPCs and differentiation into nephrons happen simultaneously suggesting that a balance between renewal and differentiation events must be struck in order to achieve correct nephron number. If the balance is skewed to renewal, then there will be low nephron endowment due to reduced differentiation. These kidneys may develop tumors (like Wilm's Tumor). On the other hand, if there is increased differentiation at the expense of renewal then there will be premature loss of NPCs and subsequent poor nephron endowment. In sum, the balance between renewal and differentiation of NPCs is critical for proper kidney function. How is the balance between renewal and differentiation of NPCs achieved? This is the focus of my dissertation.

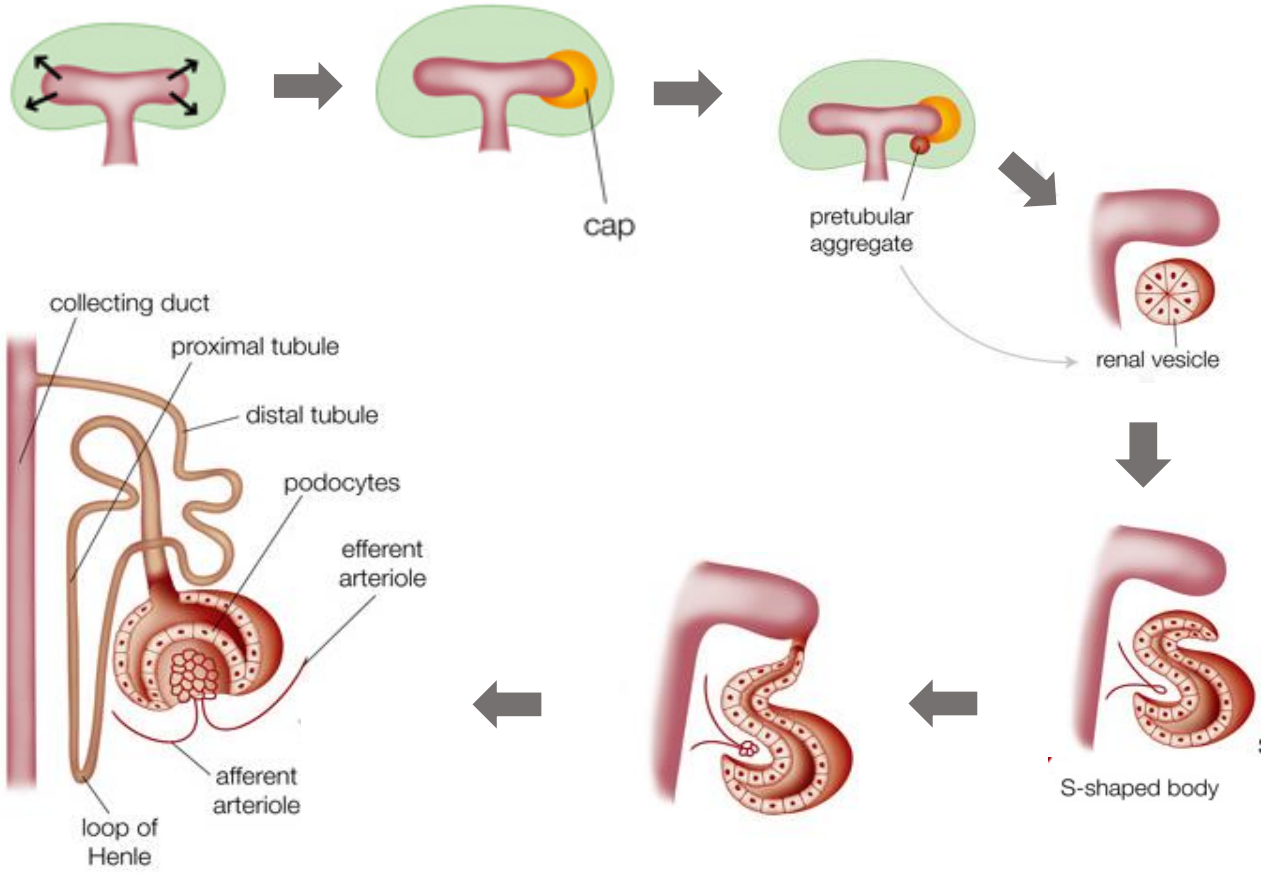


Fig. 1.3. Nephron Morphogenesis. The NPCs undergo a series of morphogenetic events to give rise to a fully patterned nephron. The UB provides induction cues (black arrows) to NPCs (labeled as cap). Upon signals to differentiate the NPCs form pre-tubular aggregates (PTAs), which are the earliest structures of differentiation. PTAs undergo mesenchymal to epithelial transition to form a renal vesicle which upon elongation give rise to the S-shaped body. The S body develops into a mature functional nephron. Source: *http://design.ninabosanac.com/?portfolio=kidney-development-illustrations*

Overview of Wnt signaling

Wnts are evolutionarily conserved secreted signaling proteins that play many diverse yet crucial roles in several biological and pathological processes. The name Wnt was derived from Wingless, the Wnt of *Drosophila* and Int1, the first Wnt identified in mammals [49]. Drosophila mutants lacking the Wnt gene had embryo patterning defects and had no wings (hence the name 'wingless') [50, 51]. Int1 was discovered as a proto-oncogene causing mammary tumor in mice [52]. Research related to Wnt signaling has exploded ever since the discovery of Wingless and Int-1 in the 1980's. At present, we have information regarding an entire family of Wnt proteins, their family of cognate receptor proteins, factors necessary for secretion and signal transduction. This has lead us to appreciate the realms of Wnt function in development and disease.

Certain key players are required for biogenesis of all Wnt ligands

There are 19 Wnt ligands in mammals (Wnt1, Wnt2a, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, Wnt16). All Wnt ligands are secreted glycoproteins carrying a palmitoyl (lipid) moiety. Addition of the palmitoyl group to the Wnt protein, through the process called lipid modification, is essential for its secretion [53]. If Wnts are not correctly lipid modified, then Wnt proteins are trapped inside the ER and do not get translocated to the Golgi network (for their subsequent secretion). Lipid modification is mediated by Porcupine, an O-acyltransferase present in the endoplasmic reticulum (ER) [54]. Porcupine was first

identified as a polarity gene required in the Wnt producing cell to regulate Wnt signaling [55]. Although it is accepted that Porcupine activity results in the palmitoylation of Wnt proteins, whether Porcupine is directly or indirectly involved in this process remains unknown. Synthetic inhibitors to Porcupine have been developed and are currently being used for controlled inhibition/modulation of Wnt signaling. Iwp, Iwp2 and Iwpl6 are well established Porcupine inhibitors and are used in cell and tissue culture experiments [56-58]. C59 and ETC-159 are orally administrable compounds currently tested on mice [59, 60]. Also, LGK974 is an oral drug under phase I clinical trial on cancer patients since December 1st, 2011 (https://clinicaltrials.gov/show/NCT01351103).

Intracellularly, upon successful addition of lipid moiety, a transmembrane protein called Wntless (Wls) tethers to the palmitoyl group of Wnt and, through its action as a cargo receptor, facilitates Wnt translocation across the golgi network and subsequent Wnt exocytosis [61]. Wls loss-of-function phenocopies Wnt loss-of-function [62] suggesting that Wls is an essential component of Wnt signaling. After Wnt is secreted, Wls is endocytosed and recycled for another round of Wnt secretion.

Recycling of Wls is mediated by the retromer complex that includes cargo selective subunits Vps35, Vps26 and Vps29 and, a sorting nexin protein SNX3 [63-66]. Vps35 is the subunit of the retromer complex that is specific to Wls retrograde transport [67, 68]. The retromer complex transports Wls from the endosomes to the golgi complex. In the absence of a functional retromer complex, Wls is stuck in the endosomes and is eventually degraded. Thus, there is no continuous secretion of Wnt ligands in these cells.[69, 70]. Indeed, a loss in Wnt target gene expression due to the loss-of-function of the retromer complex and/or failure to recycle Wls, can be compensated by providing

exogenous Wls [67]. This implies that Wls regulates the extent/strength of Wnt secretion and, that the retromer complex regulates Wnt signaling and gradient formation but not Wnt secretion. Until recently it was thought that Wls was not present in the ER. However, recent evidence has changed this understanding. Work in [71] shows that Wls is present in the ER, where it interacts with lipid modified Wnts and, shuttles between the ER and plasma membrane during the process of Wnt secretion. Just like Wnts, Porcupine, Wls and Vps35 are conserved across species and also, are specific to Wnt biogenesis.

Hydrophobic Wnts require mediators to reach target cells

Once secreted, Wnts require stabilization proteins or other specialized machinery to move across the extracellular space. Due to their hydrophobicity, they cannot freely diffuse between cells. Several models have been proposed to explain how Wnts travel through the extracellular space to reach their target receptors, Frizzled proteins. All evidence points to the idea that these mechanisms are specific to the cellular context and extracellular environment, more than to the Wnt itself [72]. In some contexts, Wnts endocytose from secreting cells as exosomal vesicles or associate with cell surface Heparan Suphate Proteoglycans (HSPGs) like Drosophila Dally and Dlp or with reggie proteins in lipid rafts or package into lipoproteins and, diffuse across cells [73, 74]. Through these simple diffusion methods, Wnts are presumed to form a ligand gradient such that cells closest to the Wnt producing cell receive high levels whereas cells at a distance receive lower levels of Wnts. This mechanism of simple diffusion is the oldest

and most primitive. Another relatively new mechanism for Wnt transport involves specialized filopodial cell projections called cytonemes or nanotubes [75]. Cytonemes are thin, tubular actin filaments that extend between the producing cell and the receiving cell. Ligands or receptors are transported in the cytonemes. When a contact is made between cytonemes of the sending and receiving cell, receptor activation occurs. Although cytonemes are seen in various lengths, rate of protein (ligand or receptor) movement appears to influence the concentration gradient across cells. The differential density of cytonemes between specific sender-receiver cell pairs, another explanation for gradient establishment, has not been explored yet. Cytonemes were first observed in Drosophila wing discs where these tubes extended between dpp producing cell and receiving cell[76]. Later, cytonemes were reported in all imaginal discs, gonads, lymph glands and embryos of Drosophila and in pigment cells of zebrafish[75]. Existence of cytonemes in mammalian development has not been shown yet. However, at least in Drosophila, there is compelling evidence that long range Wnt signaling occurs and that it occurs through cytonemes. In the mammalian system, there has not been any evidence of long range signaling. Hence, the current view of Wnt signaling (in vertebrates) is that Wnts are short range signals, activating receptors of only the neighboring cells [77]. Signal from these activated cells is then transduced either through cell division or through sequential activation. Evidence of the former was recently reported in the intestinal stem cell (ISC) niche[78]. In this context, Paneth cells are Wnt producers and ISCs are the receiving cells. Secreted Wnts interact with the Frizzled receptors expressed by the adjacent layer of ISCs only. As the Wnt bound ISCs divide, Wnt signal gets diluted, thus, generating a Wnt activity gradient.

Wnts elicit cellular response through a dedicated signaling cascade

Secreted Wnt ligands interact with their cognate receptor proteins, Frizzled. These receptors are a family of 10 transmembrane proteins (7TM) belonging to the GPCR group in mammals. The extracellular domain of Frizzled contains a cysteine rich CRD domain to which Wnt ligand binds. Lrp5 and Lrp6 are two known co-receptors of Wnt ligands. Frizzled and Lrp5 and -6 form a heterodimeric receptor complex to which Wnt binds. In some cases, interaction of Lrp5/6 with Wnt is essential for downstream signaling [79]. Ligand-receptor interaction is promiscuous and not specific. Specificity to Wnt signaling is, hence, achieved through differential expression of Wnts and Frizzled in tissues [77]. Wnts can activate Frizzled receptors that are expressed in their own cell (autrocrine mechanism) or in neighboring cells (paracrine mechanism). However, the components of downstream signaling do not differ between the autocrine and paracrine mode. Whether Wnt activates the same cell or the neighboring cell, the result is one of three major changes: 1. gene expression, 2. cytoplasmic changes including actin cytoskeletal rearrangement and mitotic spindle alterations or 3. calcium level alterations. The first mentioned effect of gene transcription is considered the canonical Wnt pathway and involves the downstream effector, b-catenin. The second and third pertain to the non-canonical signaling pathway. Based on the resultant cellular changes, the non-canonical pathway can be classified into Planar Cell Polarity (PCP) pathway and Ca+ dependent pathway. The B-catenin dependent, canonical Wnt signaling pathway will be further discussed below.

Canonical Wnt signaling

In the canonical Wnt signaling pathway, binding of a Wnt ligand to a Frizzled receptor activates a signal transduction cascade. The end result of this cascade is B-catenin dependent gene activation.

In the absence of a Wnt ligand, Axin, a cytoplasmic scaffolding protein, exists in a complex with adenomatous polyposis coli (APC), F-box protein β-Transducin repeat-containing protein (β-TrCP), and kinases Gsk-3 and CK1 in order to sequester B-catenin. When B-catenin is contained in this complex it is phosphorylated by Gsk3. Phosphorylated B-catenin is recognized by β-TrCP for ubiquitination and subsequent degradation. This is the 'off' state.

In the presence of a Wnt, the binding of Wnt to the Frizzled-Lrp5/6 receptor complex results in the increase of phosphatidylinositol (4,5)-bispho-sphate (PIP2) levels which induces oligomerization of Lrp5/6 and simultaneous Axin recruitment to Lrp5/6. This is the initiation step of Wnt/B-catenin signaling. Axin recruitment to Lrp5/6 brings the kinases in close proximity to the phosphorylation sites of Lrp5/6. Therefore, Gsk-3 and CK1 phosphorylate the PPPSP motif of Lrp5/6, thus, activating the protein. Activated Lrp5/6 recruits more Axin-kinase complex resulting in a positive-feedback loop. This is the amplification step of Wnt/B-catenin signaling. Once all the Axin bound complexes are recruited to Lrp5/6, and all the Lrp5/6 sites are phosphorylated, Lrp5/6 inhibits Gsk3 kinase activity. This results in the stabilization of B-catenin in the cytoplasm - the 'on' state. Stabilized B-catenin translocates to the nucleus to mediate gene transcription.

Axin is shown to be the limiting component of the destruction complex. This could be due to its scaffolding function and stoichiometric ratio. Exact role of APC is unclear however it seems to bind to Axin and B-catenin. Several inhibitors to Axin and Gsk3 have been synthesized that allows for manipulation of Wnt/B-catenin activity. SB-216763, CHIR99021, BIO, LY2090314 are the available Gsk3 inhibitors and, XAV939, IWR, G007-LK and G244-LM are the available Axin activators [58, 80-84]. Many of these compounds have been shown to be specific to B-catenin regulation and activity. Additionally, these compounds agonize or antagonize B-catenin activity in a dose dependent manner. These tools have been proven to be useful in exploring the Wnt pathway further.

B-catenin and Lef/Tcf mediate gene expression

The ultimate outcome of Wnt pathway activation (in the canonical Wnt pathway) is gene transcription. Depending on the cellular context, Wnts activate specific sets of genes that carry out specific functions. Gene expression is mediated by B-catenin and its co-activator, lymphoid enhancer factor/T cell factor (Lef/Tcf). In a Wnt 'off' state, the Lef/Tcf transcription factors repress target gene activation typically through its interaction with co-repressor Groucho/transducin-like enhancer (Gro/TLE1–3) [85, 86]. In a Wnt 'on' state, B-catenin translocates to the nucleus where it displaces the co-repressor and transactivates Lef/Tcfs to induce gene expression. There are 4 Lef/Tcf genes in vertebrates – Tcf7l (Tcf1), Tcf7l1 (Tcf3), Tcf7l2 (Tcf4) and Lef1. Through alternative promoter usage and mRNA splicing, multiple isoforms with distinct properties can be generated. All Lef/Tcfs contain the following domains - a conserved HMG DNA binding domain that binds to the consensus sequence referred to as the Wnt-Responsive

Element, CCTTTGWW (W = T or A), a conserved B-catenin interaction domain, conserved Gro/TLE interaction domain, a variable context dependent regulatory (CRD) domain and a variable DNA binding domain, called C-clamp [87]. The first three domains mentioned above are conserved across species and among Lef/Tcf members within a species. Alternative promoter usage generates dominant negative isoforms of Lef/Tcfs (dnLef1, dnTcf7l, Tcf7l2) that do not contain a B-catenin interaction domain and hence, are acting as constitutive repressors [88, 89]. The domains CRD and C-clamp have a high degree of size and sequence variability and are present in a limited number of splice variants only [87]. The exact role of CRD is not known yet, but studies done so far point to a role in gene repression. The C-clamp-DNA binding allows for variations in target gene expression pattern. These Lef/Tcfs can be post-translationally modified by the members and non-members of the Wnt pathway and upon Wnt signaling pathway activation [90]. Studies through biochemical methods have identified the following modifications: phosphorylation by kinases Gsk3, CK1, CK2, Hipk2, Nemo-like Kinase and TNIK [90], acetylation by CBP/p300 [91], Sumoylation by PIASy, a SUMO E3 enzyme [92, 93] and ubiquitination by NARF, a Nemo-like Kinase directed E3 ubiquitin ligase [94]. These modifications seem to further refine the interactions with B-catenin and repressor proteins [90]. Genetic studies involving gene alterations or deletions reveal that even though Lef/Tcfs can function as a repressor or activator, Tcf7l1 appears to predominantly function as transcriptional repressors and, Lef1 as transcriptional activators in all biological systems tested [95]. Tcf7l and Tcf7l2 are acting as transcriptional activators or repressors depending on the cellular context [95]. Since there is only one mediator, B-catenin, to relay the information from Wnt, an additional layer of mediation at the transcriptional level

is necessary to restrict transcription to specific gene sets. Multiplicity coupled with controlled regulation of Lef/Tcf protein may serve for the refinement of Wnt signaling. Indeed, a mechanism of Wnt target gene activation has been observed. In this mechanism, upon Wnt signaling pathway activation, a specific repressor isoform of Lef/Tcf gets removed from WRE sites resulting in the engaging of an activator isoform of Lef/Tcf and B-catein to inducing gene expression. [96].

Wnt targets genes have been found to play key roles in lineage specification, pattern formation and maintenance, and, when mis-regulated, in disease.

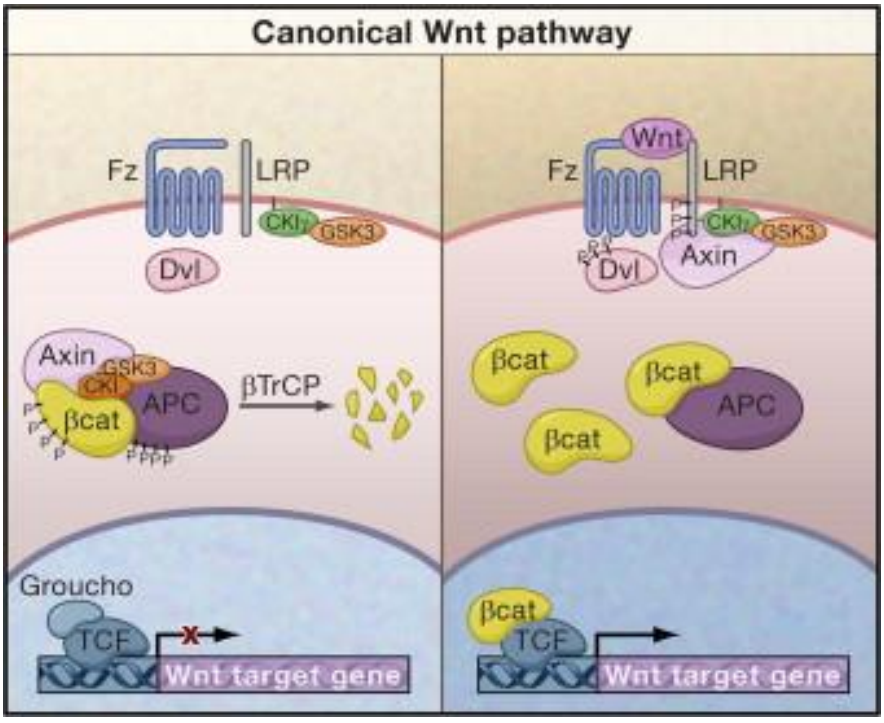


Fig. 1.4. Wnt signaling. (A) In the absence of a Wnt ligand, GSK3, a member of B-catenin destruction complex phosphorylates B-catenin thereby targeting it for proteolytic degradation. In this case, the pathway is inactive. (B) When Wnt binds to its receptor Frizzled, Wnt signaling pathway becomes active. Activated Frizzled receptor causes dissociation of the destruction complex which leads to stabilization and nuclear

translocation of B-catenin. When B-catenin is present in the nucleus it interacts with transcription factors Lef/Tcf to activate gene expression. Source : [97]

Question under pursuit

Wnt9b is expressed in the ureteric bud throughout kidney development. It is necessary and sufficient for differentiation of NPCs. Deletion of Wnt9b leads to kidney agenesis. These mutants completely lack expression of differentiation genes including Wnt4 [39]. In addition to inducing genes in the differentiating NPCs, ureteric bud produced Wnt9b also activates genes in the renewing NPCs. Renewal and differentiation targets genes are activated through the B-catenin dependent canonical Wnt signaling [40].

How does Wnt9b regulate two different processes through one transcription factor? Is the underlying mechanism in place to efficiently control the balance between renewal and differentiation of NPCs? These were the main questions driving my thesis work. There can be many ways how Wnt9b activates two different gene sets. I explored two mechanisms – 1. Wnt9b regulates asymmetric cell division of NPCs, 2. Wnt9b gradient across NPC population dictates cell fate.

We discovered that NPCs divide at perpendicular angles with respect to the UB. However, this orientation in NPC divisions was not disrupted in Wnt9b mutants. This suggests that the first proposed mechanism is ruled out. However, since NPCs undergo oriented cell divisions such that the resulting daughter cells have asymmetric cell-cell interactions, we looked for other factors that could regulate this asymmetric cell division (ACD) process. Numb and its paralog, Numblike acting as asymmetric cell fate determinants, have been shown to regulate renewal and differentiation in various model

organisms [98-100]. We generated NPC specific Numb and Numblike knock outs to test for a potential role of these genes in OCD. However, we found that oriented division of NPCs was not affected in Numb/Numblike mutants. In support of this result there was no obvious imbalance in renewal or differentiation of NPCs. However, these mutants show a complete absence of glomeruli suggesting that Numb/Numblike are essential for the specification and differentiation of a specific component of NPC lineage (chapter two).

With the aim of finding the mechanism of Wnt9b's activity, I tested whether Wnt9b signals via a concentration gradient. I have found that low levels of B-catenin activity induces renewal while high levels of activity induces differentiation and that, stromal signals agonize B-catenin activity in the differentiating NPCs. Our data suggest that a feedback mechanism involving Wnt signaling from the UB and signals from the stroma tightly controls the balance between renewal and differentiation. This is elicited by modulating B-catenin levels (chapter three).

CHAPTER TWO

Role of Numb/Numblike in Kidney Development

Abstract

Nephron progenitor cells (NPCs) of the mammalian kidney must maintain a balance between their self-renewal and differentiation events in order to achieve good nephron endowment. Asymmetric Cell Division (ACD) is a mechanism adopted by stem cells to maintain a tight control over the balance between renewal and differentiation. ACD is a process by which cell division results in cells with two different fates. We sought to understand if the balance between NPC renewal and differentiation is regulated through asymmetric cell division in the developing kidney. Data from our lab show that NPCs undergo Oriented Cell Division (OCD) such that one daughter remains in contact with the ureteric bud, its progenitor niche, while the other daughter moves away from the niche. To understand if the oriented division of NPCs contributes to asymmetric cell fate, we generated a nephron progenitor specific knock out of the asymmetric cell fate determinant Numb (Nb) and its paralog Numblike (NbL). Our data suggests that Nb/NbL deletion does not affect OCD. Additionally, these kidneys do not have an obvious imbalance in NPC renewal versus differentiation. However, mutant kidneys have a defect in the proximal most segment of nephron precursor resulting in complete absence of mature glomeruli. Molecular analysis suggests that this is due to regionalized apoptosis within the proximal-most portion of the S-body structures of developing nephrons. This suggests that Numb and Numblike do not regulate nephron progenitor cell fate but is necessary for formation of glomeruli.

Introduction

The elaborate tubular organ, mammalian kidney develops by the reciprocal interaction between an epithelium, the ureteric bud (UB) and an adjacent condensed progenitor population, the metanephric mesenchyme (MM). The MM is composed of nephron progenitor cells (NPCs) and stromal progenitors. Signals from the ureteric bud induce the NPCs to proliferate (self-renewal). Signals from the ureteric bud and adjacent stroma induce differentiation of NPCs into nephrons. When NPCs undergo differentiation, a subset of these cells aggregate into what we refer to as pre-tubular aggregates (PTAs), at the cleft of T-shaped UB branches. The PTAs undergo mesenchymal-to-epithelial transition (MET) to form the first epithelial nephron precursor called the renal vesicle, which is patterned with distinct distal and proximal regions. The renal vesicle undergoes morphogenesis to form a fully patterned functional nephron. Signals from the MM induce dichotomous branching of the ureteric bud at every differentiation event. At embryonic day (E) 11.5, the mouse kidney consists of: a T-shaped ureteric bud and a condensed MM. The ureteric bud tips continue to undergo branching until the cessation of kidney development. As the ureteric bud branches, the MMs condense around the newly formed UB tips while also giving rise to nephron precursors. This self-renewal and differentiation of NPC events reiterate throughout kidney development. These events cease few days post birth in mice (and few weeks before gestations in humans) resulting in cessation of new nephron formation. Individuals with poor nephron endowment are predisposed to renal diseases including renal failure, in addition to systemic conditions like hypertension or diabetes. Hence, attainment of correct nephron number is critical for proper functioning

of the kidney. Correct nephron number can be obtained only by establishing proper balance between NPC self-renewal and differentiation.

One way to maintain a tight control on the balance between renewal and differentiation is through asymmetric cell division (ACD). ACD is a process by which mitotic division of a cell results in two daughters with differing identities. This is the opposite of symmetric cell division where the resulting daughters of the cell division have identical identities. The differences in cell identity in ACD could be due to asymmetrical segregation of certain proteins called asymmetric cell fate determinants. Alternatively, specific orientation of mitotic spindles could drive differing cell identities. Regardless of the driving mechanism, the non-identical daughters take on different cell fates. The process of ACD is tightly regulated and evolutionarily conserved.

There are many known regulators of ACD, Wnt protein family and Numb being two very well studied molecules. In this context, Wnts act as cell fate determinants by regulating the orientation of cell division and/or by polarizing proteins for their asymmetric inheritance. Numb functions as an asymmetric cell fate determinant. During cell division, Numb proteins localize to one side on the cell membrane. Consequently, getting inherited by only one of the two daughters. This presence/absence of the Numb protein renders differential cell fates in daughters.

The NPCs undergo oriented cell divisions such that their mitotic spindles orient at perpendicular angles to the UB. Wnt9b, a Wnt molecule secreted by the ureteric bud induces both renewal and differentiation of NPCs in the developing kidney. Wnt9b signals via B-catenin to activate renewal and differentiation genes. How one Wnt molecule can activate one transcription factor to induce two different gene sets remains unknown. We

hypothesized that Wnt9b is regulating oriented cell division of NPCs, which, in turn dictates NPC fate. We also hypothesized that Numb regulates asymmetric cell division of NPCs.

In this study, we show that loss of Wnt9b does not disrupt oriented NPC division. To study the role of Nb and NL in asymmetric cell division, we generated NPC specific mutants. We found that loss of Numb (Nb) and Numblike (NL) in NPCs does not affect their cell division angles. Also, mutant kidneys do not show a loss or gain in NPCs. Instead, we find that ablation of Nb and NbL induces apoptosis of the proximal segment of nephron. Our data suggest that the defect occurs from the earliest stages of differentiation.

Results

Wnt9b does not regulate oriented cell division of nephron progenitor cells

The developing kidney has 2-3 layers of nephron progenitor cells (NPCs) encompassing the branches of ureteric bud. One layer of NPCs are in immediate adjacency to the UB. This layer of cells interact with the UB through adhesions such as Integrin B1-Fibronectin. The other two or more layers are not immediately adjacent to the UB. We reasoned that positional differences in NPCs with respect to their niche, the UB, could lead to oriented NPC division. To test our hypothesis we measured angles of NPC division in E11.5 wild type kidneys. We found that NPCs divided at perpendicular angles to the axis of ureteric bud. This suggested that when NPCs divide one of the daughters remain close to the UB while the other daughter moves away from the UB. Next, we tested whether this orientation of NPC division was regulated by Wnt9b. We measured cell division angles in $Wnt9b^{-/-}$ mutants. We found that angles of cell division were not perturbed (Fig. 2.1).

Although $Wnt9b^{-/-}$ mutants lack both renewing and differentiating NPCs, the orientation of NPC divisions do not change. This suggests that Wnt9b is not regulating nephron progenitor cell fate determination through OCD but perhaps, through a different mechanism (for mechanism of Wnt9b signaling refer chapter three).

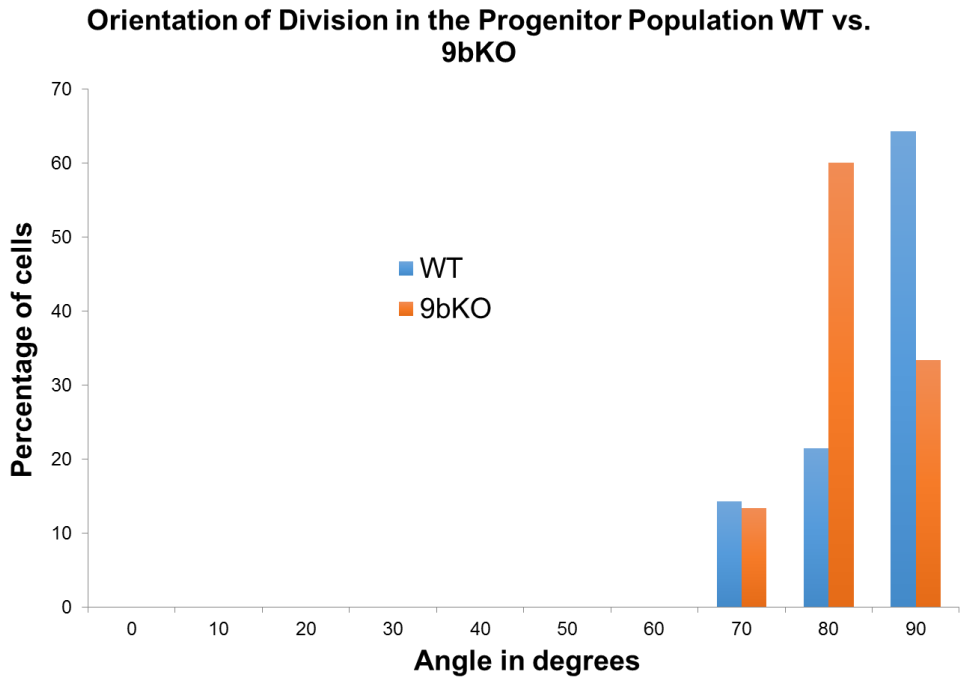


Fig. 2.1. Orientation of division in NPCs. Graph showing angle of division of NPCs in wild type (blue) and Wnt9b$^{-/-}$ (orange) E11.5 kidneys. Angles of division in all NPCs of both genotypes are greater than 70 degrees.

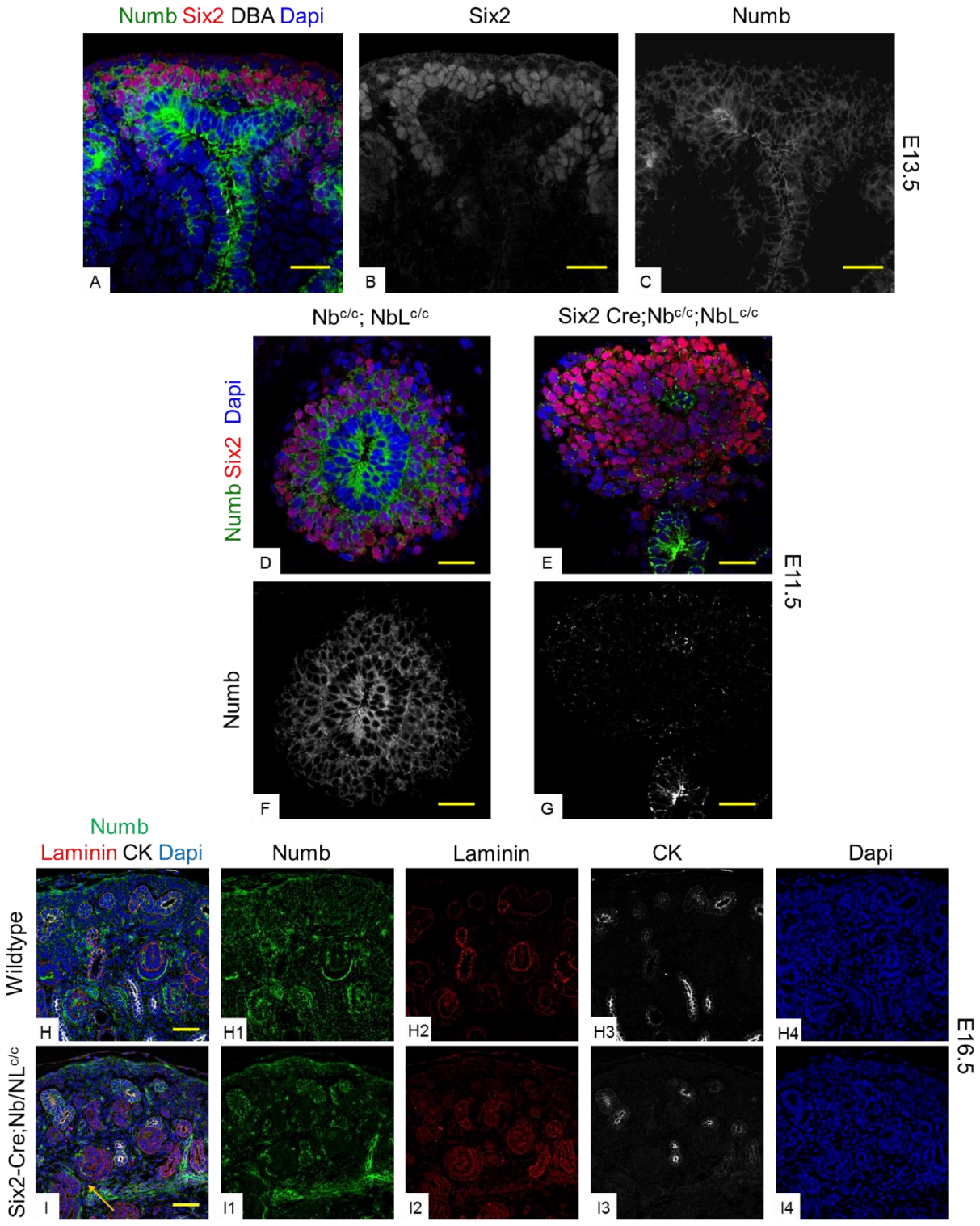


Fig. 2.2. Expression of mNumb. Immunostaining of wild type (A-F, H) and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$ mutants (E-G, I) kidney sections at E13.5 (A-C)

E11.5 (D-G) or E16.5 (H-I4) Tissues are stained with antibodies to Numb (green in A,D,E, H, H1, I, I1), NPC marker, Six2 (red in A,D,E), collecting duct marker, DBA (grey in A), ureteric bud marker, CK (H, H3, I, I3), basement membrane marker, Laminin (H, H2, I, I2) and counter stained with Dapi. Single channels are shown in B, C, F, G, H1-H4, I1-I4. Scale bars are 50uM. Arrow is pointing to endothelial cells entering the glomerular cleft.

mNumb and mNumblike deletion does not affect oriented cell division of NPCs

mNumb (Nb) and its homolog mNumblike (NL) are important cell fate determinants in mammals and invertebrate organisms [99, 100]. We wanted to test whether Nb regulates NPC fate determination. By immunostaining with an antibody raised against Nb, we found that Nb is expressed in the membranes of NPCs in addition to most of kidney epithelia (Fig. 2.2). In order to understand NPC specific role of Nb/NL, we deleted the genes using Six2-Cre, which is expressed exclusively in the nephron progenitors and its derivatives. We generated Six2-Cre;Numb$^{floxed/floxed}$; Numblike$^{floxed/floxed}$ double mutants (referred to as dKOs) and assessed the successful deletion of Numb by immunostaining. We found that most of Nb is deleted in the NPCs and in its derivatives as early as E11.5 (Fig. 2.2). There is no commercially available antibody raised against NL to assess its expression or its successful deletion in the mutant.

To test if Nb and NL regulate oriented NPC division, we measured the angle of cell division in dKOs and Cre negative controls. We found that NPCs divide perpendicularly to the UB in wild type kidneys, as expected. This orientation of NPC division was not disrupted in the dKOs. Although few cells divided parallel to the UB, this distribution of cell division was not significantly different from that of wild type (Fig. 2.3). This suggests that Nb and NL do not regulate oriented cell division of NPCs.

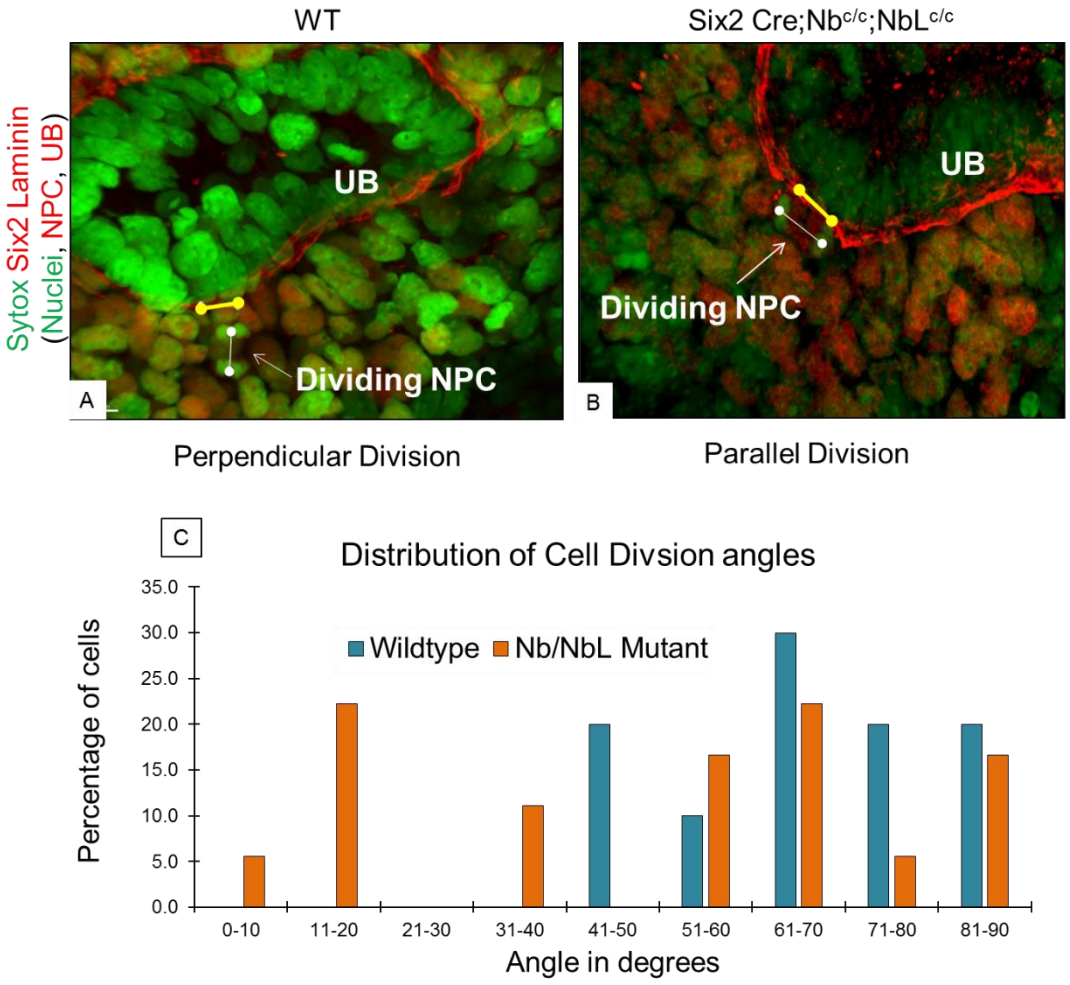


Fig. 2.3. Angle of division of NPCs in wildtype and Numb, Numblike knockout kidneys. A-B: Immunostaining of E11.5 wildtype (A) and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$ knockout (B) kidney sections with antibodies to NPC marker, Six2 and basement membrane marker, Laminin (in red) and counterstained with green Sytox dye shows axis of dividing NPCs at Anaphase stage of mitotic cell division (white dumbbell) and axis of ureteric bud (yellow dumbbell). C: Graph showing angle of division of NPCs in wild type (blue) and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$ (orange) E11.5 kidneys. N=2 wild type and 3 mutants. P value obtained through KS test = 0.5 (not significant).

Numb and Numblike deletion does not affect renewal

If Nb/NL are required for asymmetric cell fate determinants that affect NPC fate then deletion of Nb and NL will likely lead to either decrease or increase in NPC numbers. To test this, kidney sections were immunostained with antibodies to nephron progenitor marker, Six2, and ureteric bud marker, CK (Fig. 2.4). The dKOs had similar numbers of NPCs around each ureteric bud tip. Also, the size of dKO kidneys were not significantly smaller than the size of wild type littermates suggesting that there is no great reduction in renewal or differentiation (Fig. 2.5 C-D).This suggests that there is no obvious skewing of the balance between renewal and differentiation.

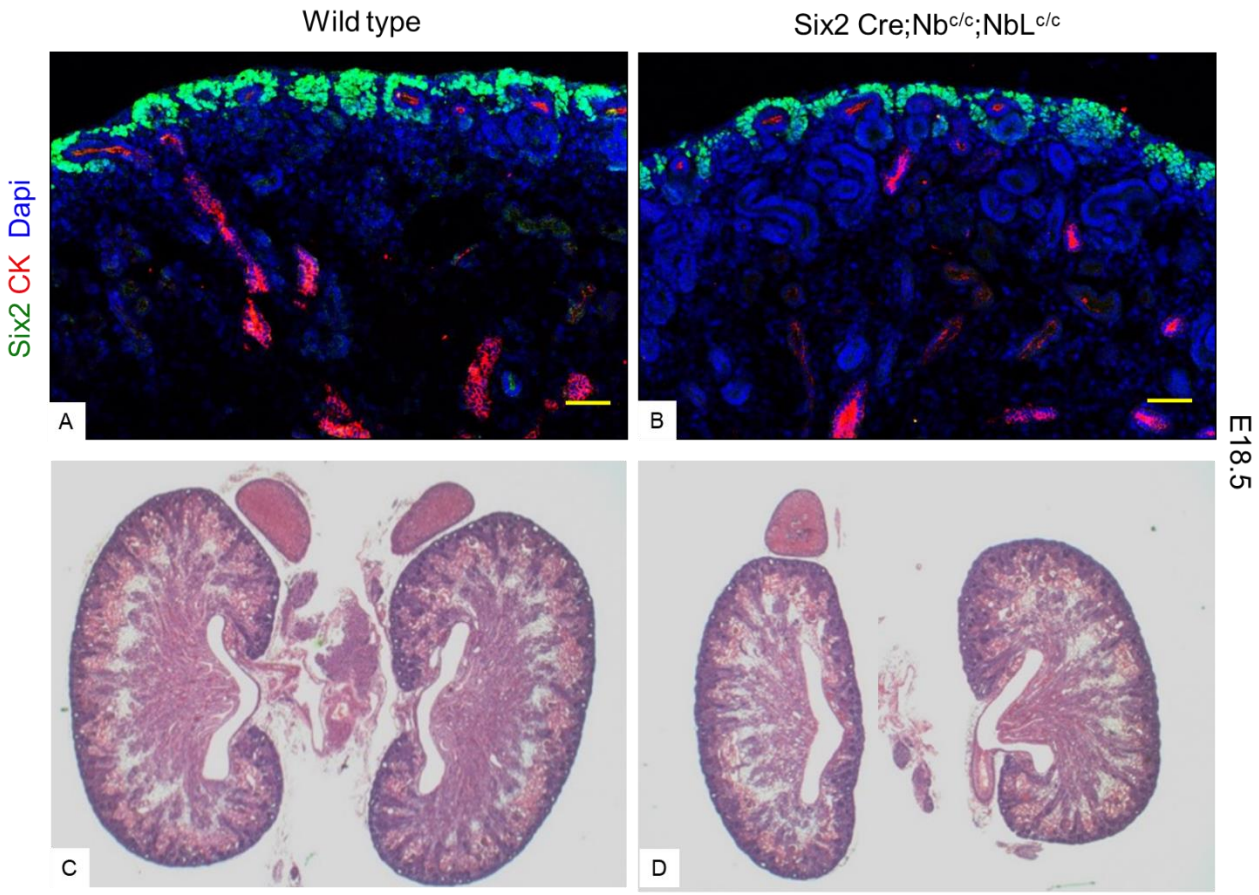


Fig. 2.4. NPCs are in similar numbers in wildtype and Numb, Numblike knockout kidneys. A-B: Immunostaining (A-B) or Hematoxylin and Eosin staining (C-D) of E18.5 wildtype (A, C) and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$ knockout (B. D) kidney sections with antibodies to NPC marker Six2 and ureteric bud marker, CK (in red). Scale bars = 50uM.

Nephron progenitor specific ablation of Numb and Numblike leads to severe decrease in glomeruli.

Although ACD was not affected in the Nb and NL mutants we noticed that the dKOs mutants showed a paucity in glomerular structures. Upon counting glomeruli on Hematoxylin and Eosin stained tissues sections we found that the dKOs have very significant reduction in glomeruli. In fact, most of the glomeruli contain almost empty Bowman's capsules (Fig. 2.6H and I). Confirming this finding, the mutants show very few Nephrin positive structures. We observed the birth rate and viability of mutants and found that the dKOs did not survive post-birth (Fig. 2.5) suggesting that the mutants do not have sufficient number of glomeruli to sustain life.

Stage	E11.5-E16.5	E17.5-E18.5	P1	>P14
No. of embryos /pups	224	129	168	57
No. of mutants alive	23	13	9	0
Ratio	0.102 (1 in 10)	0.100	0.053	0.000

Fig. 2.5. Deletion of Numb and Numblike in NPCs leads to death of pups shortly after their birth. Embryos of wild type and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$ knockouts were collected at different stages from E11.5 to P14. While number of knockout embryos obtained between E11.5 and E18.5 was according to Mendelian ratio, significantly fewer number of P1s were obtained. Also, there were no viable knock out mice post P14.

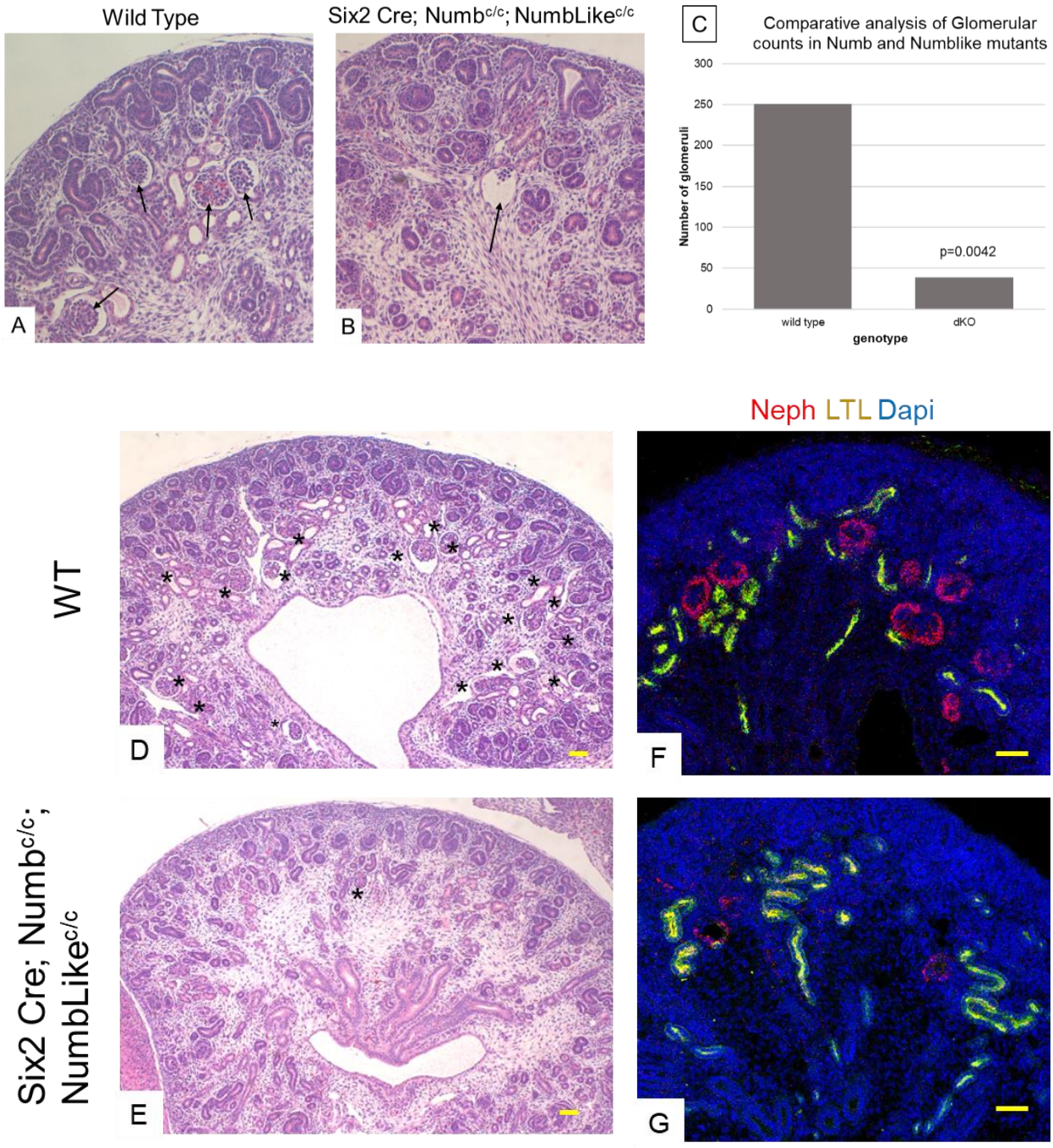


Fig. 2.6. Numb, Numblike double mutants do not have mature glomeruli. Hematoxylin and Eosin staining (A-B, D-E) or immunostaining with antibodies to mature glomeruli marker, Nephrin and proximal tubule marker, LTL (F-G) on wildtype (A,D,F) and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$ knockout (B,F,G) kidney tissues at E16.5 (E-H). * and arrow indicate glomeruli in tissue sections. Scale bars = 50uM. C: Graph showing an estimate on glomerular number between wildtype and dKO kidneys.

Nephron progenitor specific ablation of Numb leads to apoptosis.

The absence of glomeruli could be due to an arrest in morphogenesis and/or differentiation of RVs/S-bodies to glomerular segments or due to cell death. We did not see an increased number of S-bodies in the mutants but rather a decrease (not shown) suggesting that the reason for glomerular loss is apoptosis. Upon immunostaining with markers for apoptosis, we found that glomeruli of dKOs expressed Caspase-3. (Fig. 3.7). This suggests that deletion of Nb leads to apoptosis of glomerular derivatives of NPCs. We did not see apoptosis of other tubular segments including proximal tubules and distal tubules (not shown).

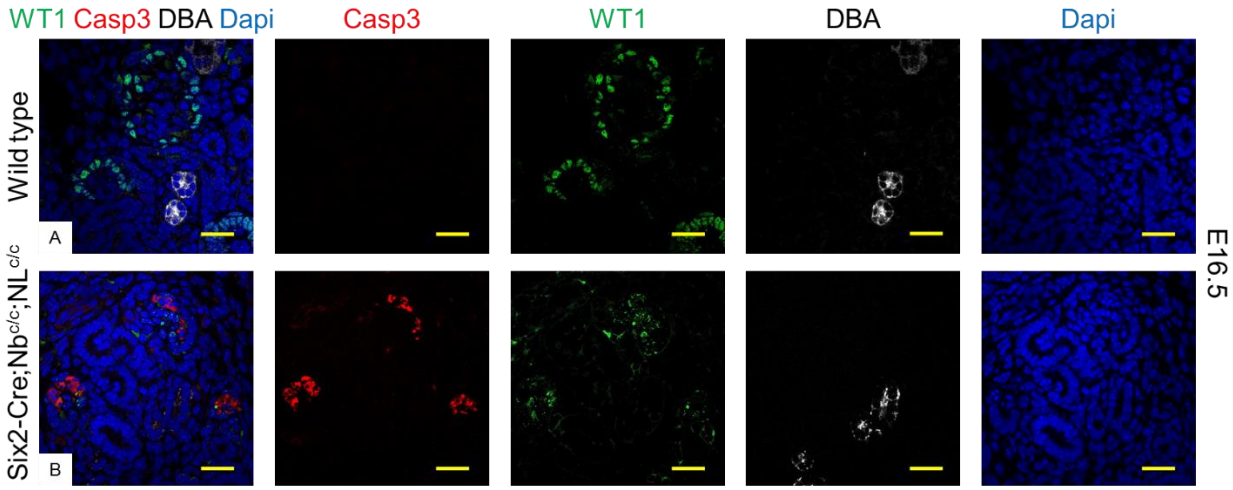


Fig. 2.7. Apoptosis in Numb and Numblike deleted mutants. Immunostaining with antibodies to podocyte marker, Wilm's tumor 1 (WT1, shown in green), apoptosis marker, Caspase 3 (Casp3, shown in red), collecting duct marker, Dolichos biflorus agglutinin (DBA, shown in grey) on wild type and Six2-Cre;Numb$^{floxed/floxed}$;Numblike$^{floxed/floxed}$

knockout kidney sections at E16.5. All tissues were counterstained with Dapi. Scale bars = 50uM.

Discussion

We have characterized Numb expression in the kidneys and have identified a role for Numb (mNb) and Numblike (mNL) in nephron progenitor cell (NPC) lineage. Deletion of mNb and mNL in the NPCs leads to apoptosis of the proximal most segment of developing nephrons resulting in glomerular absence. Although we see dying S-bodies and glomeruli, we do not know at what stage of nephrogenesis, apoptosis first occurs. Lack of unique stage specific markers of nephron formation makes this question difficult to answer. Further characterization with combinations of markers for nephrogenesis will be needed to get a better understanding of the exact time point at which mNb/NL deletion leads to apoptosis. Since mNb is expressed in NPCs and all NPC derivatives dissecting out developmental stage specific role of Nb during nephrogenesis is difficult.

Numb has very strong expression in the glomeruli. It is possible that Numb plays a role in the maintenance of glomerulus. It will be interesting to find out the role of Numb in glomerular formation and maintenance by using specific Cre lines.

Our data suggests that Nb is not required for proper orientation of NPC division. There does not seem to be an obvious skewing in NPC renewal or differentiation as seen by a lack of either loss or gain in NPCs. Hence, the contribution of orientated NPC division to the balance between renewal and differentiation remains under pursuit.

CHAPTER THREE

Disparate levels of B-catenin activity determine nephron progenitor cell fate

Abstract

Formation of a functional kidney depends on the balance between renewal and differentiation of nephron progenitors. Failure to sustain this balance can lead to kidney failure or stem cell tumors. For nearly 60 years, we have known that signals from an epithelial structure known as the ureteric bud were essential for maintaining this balance. More recently it was discovered that one molecule, Wnt9b, signals via beta-catenin to promote both renewal and differentiation of the nephron progenitor cells. How one ligand signaling through one transcription factor can promote two seemingly contradictory cellular processes has remained unclear. In this study, we show that Wnt9b/beta-catenin signaling alone is sufficient to promote both renewal and differentiation. Moreover, we show that discrete levels of beta-catenin can promote these two disparate fates, with low levels fostering progenitor renewal and high levels driving differentiation. However, these disparate responses take place in the absence of a clear gradient of ligand. These results provide insight into how Wnt9b regulates distinct target genes that balance nephron progenitor renewal and differentiation.

Introduction

Wnts are a family of evolutionarily conserved growth factors that play crucial roles in embryonic development, stem cell maintenance and tumorigenesis [101, 102]. Wnts can trigger distinct signal transduction cascades depending on the cellular context in which the signal is received. In the canonical signal transduction cascade, interaction between a Wnt ligand and its receptor leads to stabilization of beta-catenin, which, upon interaction with members of the Lef/Tcf family of DNA binding transcription factors, can mediate the expression of target genes [103, 104].

Formation of the adult or metanephric kidney of mammals depends on interactions between an epithelial structure known as the ureteric bud (UB) and a heterogeneous population of cells known as the metanephric mesenchyme (MM). During embryonic development, the UB undergoes branching morphogenesis within the MM, ultimately giving rise to the collecting duct network and the ureter. As the UB branches, the MM proliferates along with it so that each new tip is encompassed by mesenchyme. The MM consists of at least two cell types: a self-renewing, multipotent progenitor cell, known as the nephron progenitor cell (NPC) and a stromal fibroblast population. As the UB branches, a subset of the NPCs undergoes a mesenchymal to epithelial transition (MET) forming an epithelial vesicle that eventually becomes the nephron of the kidney. The stromal cells give rise to the smooth muscle, mural cells and peritubular fibroblasts of the mature kidney.

It has long been known that the UB is necessary for the survival, renewal and differentiation of the MM. The number of nephrons that form per kidney is determined by the relative rate of NPC renewal and differentiation/MET. As kidney function and

susceptibility to certain diseases is believed to be dependent on nephron number, it is essential to understand how NPC renewal and differentiation are balanced. For many years, it was believed that these processes were regulated by distinct signals produced by the UB. However, more recently, it was demonstrated that Wnt9b, produced by the UB, is necessary for the activation of two distinct transcriptional programs within the adjacent cap mesenchyme. One program correlates with differentiation and triggers the expression of several target genes (referred to as the differentiation program) within the pre-tubular aggregates including Pax8, C1qdc2 and Wnt4. The other program (referred to as the self-renewal program) is active within the renewing NPCs and includes targets Cited1, Fam19a5 (Tafa5), Pla2g7. Expression of at least a subset of each class of target genes is directly regulated by beta-catenin [39, 40, 105]. How beta-catenin promotes these two seemingly contradictory processes within the NPC population has remained unclear.

Pleiotropic roles for Wnt/beta-catenin signaling have been observed in multiple tissues including the mouse epiblast, hair follicles and intestinal crypts [106-110]. In each of these systems, similar to what is observed in the embryonic kidney, Wnt/beta-catenin signaling activates two distinct cellular/molecular programs. Although several hypotheses have been put forth as to how this can be accomplished, the predominant model posits that different levels of Wnt ligand differentially stabilize distinct levels of beta-catenin, which in turn triggers different transcriptional responses [111].

In this study, we show that within the NPCs, distinct levels of beta-catenin activity dictate cell fate. High levels of activity promote differentiation and low levels of activity promote renewal. Although we can alter response by adjusting Wnt ligand levels, we find

no evidence of a Wnt gradient of Wnt ligand in vivo. Our data are consistent with a model where signals provided by the stromal niche amplify beta-catenin activity in the adjacent NPCs, ultimately determining cell fate. Not only do these findings have significant impact on our understanding of kidney development, they will also contribute to our understanding of the regulation of beta-catenin levels and stem cell niches in other cellular systems.

Results

Homogenous production of Wnt ligand induces a singular cell fate in nephron progenitor cells.

We previously showed that Wnt9b signaling from the ureteric bud is necessary for activation of two distinct transcriptional programs within the adjacent cap mesenchyme. One program correlates with formation of the pre-tubular aggregates and is represented by the transcription of several target genes including C1qdc2, Pax8 and Wnt4. The other program is active within the renewing NPCs and is represented by the expression of a distinct set of targets including Cited1, Amphiphysin, Fam19a5 (Tafa5) and Pla2g7. How Wnt9b activates these two distinct programs is unclear, however a simple hypothesis is that another ureteric bud produced factor cooperates with Wnt9b to promote one or the other. Although it has previously been shown that Wnt ligands (Wnt1 and 9b) produced by fibroblast cells can induce survival, renewal and differentiation of isolated metanephric mesenchyme (MM) [39, 43, 112] it is possible that the fibroblasts produce additional factors that cooperate with the Wnt ligand to promote renewal or differentiation. In fact, it has never been determined whether a Wnt ligand alone is sufficient to activate either program.

To determine the sufficiency of Wnt ligand to induce renewal or differentiation, we first utilized a commercially available recombinant Wnt9b protein (rWnt9b). Isolated E11.5 MMs (devoid of UB) or Wnt9b mutant ($Wnt9b^{-/-}$) kidneys were cultured in the presence of varying concentrations of rWnt9b or BSA. In accordance with previous work showing an essential role for UB derived signaling in NPC survival [14, 113], isolated wildtype MM did not survive beyond 48 hours in all of the cultured MMs. Isolated MMs or $Wnt9b^{-/-}$ kidneys

treated with several different concentrations of rWnt9b also died within 48 hours (not shown) indicating that for this assay and under these conditions, rWnt9b was not a useful reagent. Therefore, we turned to explore a genetic strategy.

Previous studies showed that activation of a Wnt1 transgene (Rosa26-lox-stop-lox-Wnt1 - hereon referred to as Rosa-Wnt1) in the UB rescued kidneys lacking endogenous Wnt9b [39]. To test if this ligand alone was sufficient to induce renewal and differentiation (in the absence of additional factors from the UB or Wnt producing fibroblasts), Rosa-Wnt1 was activated specifically in isolated MMs (using Six2-Cre [11]). Six2-Cre;Rosa-Wnt1 and control MMs were isolated away from the UB at E11.5 and cultured for 2 days, then assessed for expression of the Wnt9b-independent NPC marker Six2, or Wnt9b-dependent differentiation targets (C1qdc2 or Wnt4) and renewal targets (Cited1 and Fam19a5). Although the vast majority of wildtype isolated MMs died within 48 hours (N=25/27, Fig. 2.1B and 2.1E), indicating that the UB was efficiently removed, all of the Six2-Cre;Rosa-Wnt1 MMs were still alive. In situ hybridization analysis revealed that 20/24 of the Six2-Cre;Rosa-Wnt1 MMs expressed renewal target genes (Fig. 2.1C, Fig. 2.2.F) while only 5/25 expressed differentiation targets (Fig. 2.1.F and 2.2.C). To determine whether the failure to express differentiation targets was simply a developmental delay, MMs were cultured for an additional 48 hours. After 4 days of culture, Six2-Cre;Rosa-Wnt1 MMs maintained expression of renewal markers Cited1 and Six2 but did not express differentiation markers nor did they form epithelial structures (as indicated by a lack of E-cadherin positive structures) suggesting that the mesenchymally produced Wnt1 was sufficient to induce renewal but not differentiation (N=3/3, Fig. 2.1.G-2.1.J). It is important to note that none of the Wnt targets assessed are affected by the

loss of other Wnt ligands including Wnt4 (a Wnt9b target), Wnt11 or Wnt7b. Thus, their expression is a specific indicator of Wnt9b activity ([40] and data not shown).

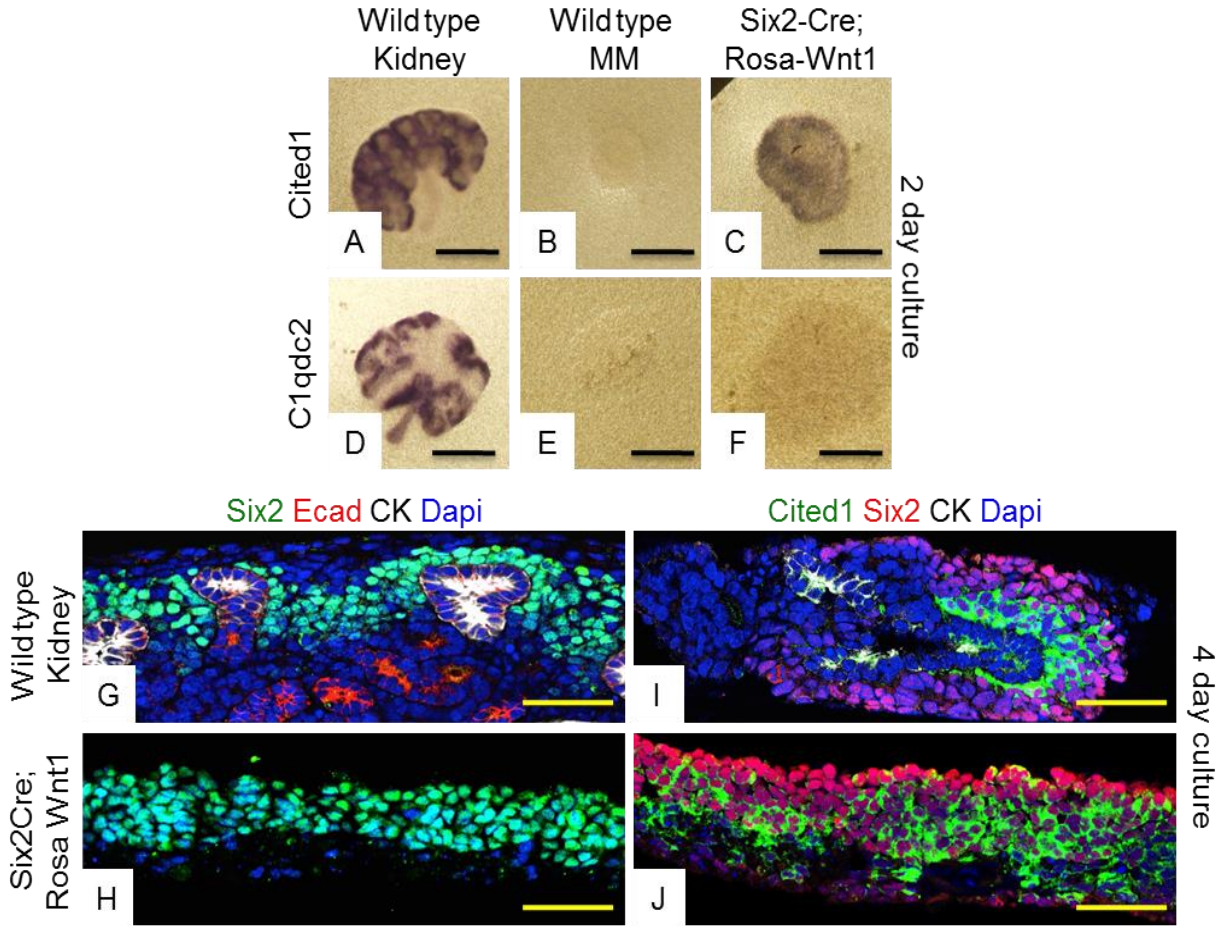


Fig. 3.1. Uniform activation of Rosa-Wnt1 ligand in NPCs of isolated metanephric mesenchymes promotes renewal. Wildtype (A, B, D, E, G, I) or Six2-Cre; Rosa-Wnt1 (C, F, H, J) kidneys (A, D) or isolated metanephric mesenchyme (B, C, E, F, H, J) were dissected at E11.5 and cultured for 48 (A-F) or 96 hours (G-J). Tissues were fixed and hybridized with antisense probes to the Wnt9b renewal target gene, Cited1 (A-C) or the Wnt9b differentiation target gene, C1qdc2 (D-F). Immunofluorescence staining with antibodies to the NPC marker Six2, epithelial tubule marker E-cadherin and the ureteric bud marker pan-cytokeratin (green, red, white respectively in G, H) or Cited1, Six2, Pan-Cytokeratin (green, red, white respectively in I, J) . All immunostained sections were counterstained with Dapi. Scale bars are 200uM for (A-F) and 100uM for (G-J).

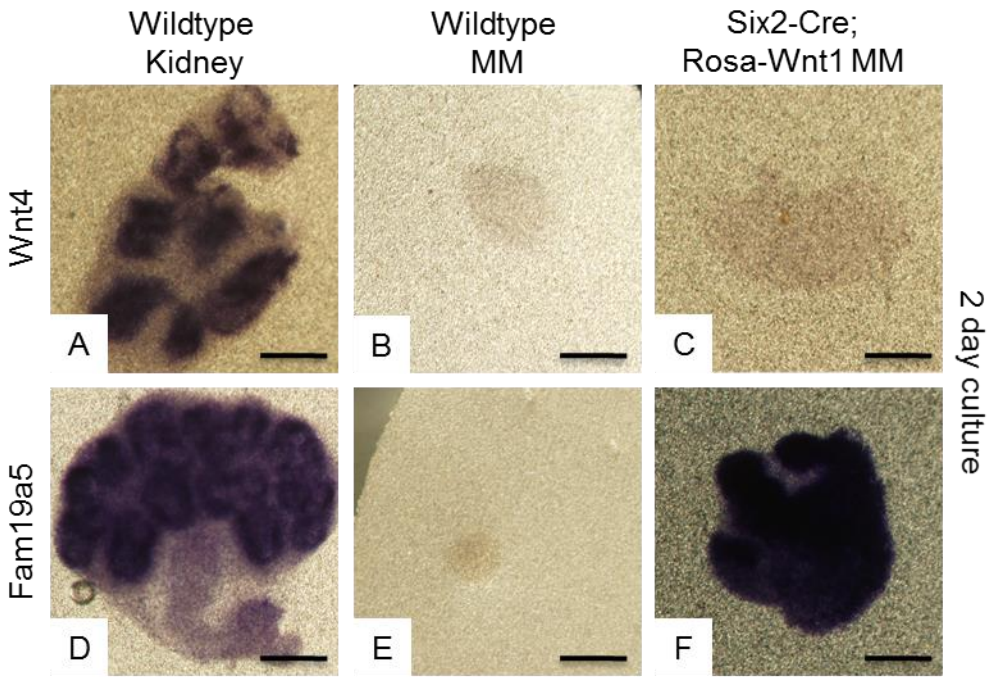


Fig. 3.2. Uniform activation of Rosa-Wnt1 ligand in NPCs promotes expression of renewal targets. In situ hybridization of E11.5 wildtype kidneys (A, D), isolated metanephric mesenchyme (B, E) or Six2-Cre; Rosa-Wnt1 isolated metanephric mesenchyme (C, F) after 2 days in culture. Tissues were hybridized with probes to the Wnt9b renewal target gene, Fam19a5 (D-F) or the Wnt9b differentiation target gene, Wnt4 (A-C). Scale bars are 200uM.

The ureteric bud does not produce additional factors that promote differentiation.

As previous work showed that Wnt1 produced by either the UB or fibroblast-derived cell lines induced both differentiation and renewal, the observation that expression of Rosa-Wnt1 in the NPCs only induces renewal was unexpected. There are several possible explanations for this result. We first considered whether this lack of differentiation was the result of a missing co-factor normally provided by the UB. To test

this possibility, we analyzed E11.5 intact (i.e. with the UB) Wnt9b mutant kidneys expressing Rosa-Wnt1 in the NPCs (Six2-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$). Like the isolated MMs, these mutants did not express differentiation genes C1qdc2, Wnt4 or Pax8 (Fig. 2.3.H, 2.4.H and not shown), but did express renewal targets Cited1, Fam19a5 and Six2 (Fig. 2.3.D, 2.4.H and not shown). Consistent with the lack of differentiation gene expression, these kidneys showed a complete absence of nephrons as seen by the lack of expression of E-cadherin (Fig. 2.3.L) but maintained a renewing progenitor population (as marked by the expression of Six2 and the renewal/class II target genes Cited1 and Fam19a5) throughout embryogenesis (Fig. 2.3.L and not shown). This suggests that the phenotype observed upon activation of Wnt1 within isolated MM is not simply due to the lack of additional UB derived signals.

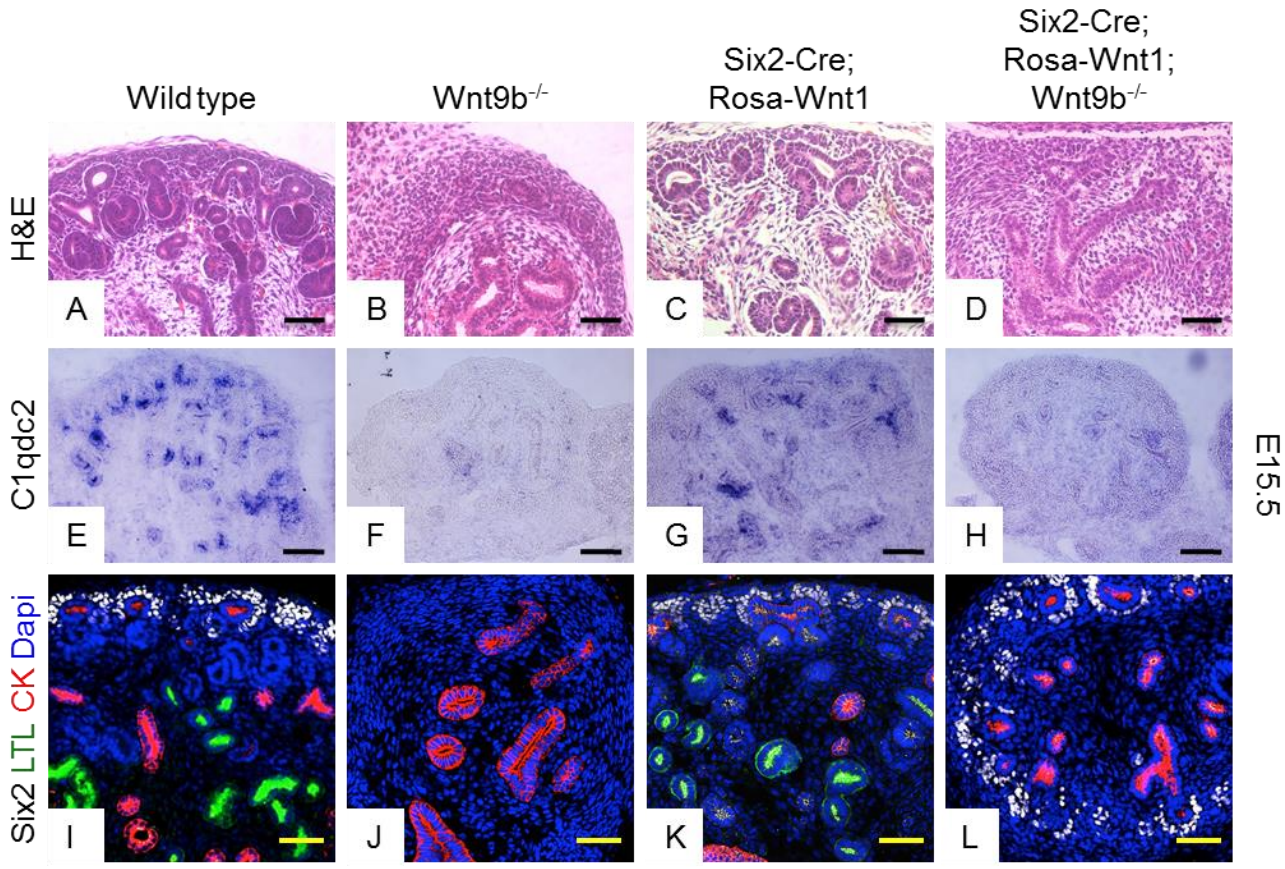


Fig. 3.3. Failure of Six2-Cre;Rosa-Wnt1 metanephric mesenchymes to differentiate is not due to lack of UB-derived signals or gain of function phenotype. Hematoxylin and Eosin staining (A-D), in-situ hybridization (E-H) or immunofluorescence staining (I-L) of sections of E15.5 wildtype (A, E, I), Wnt9b$^{-/-}$ (B, F, J), Six2-Cre;Rosa-Wnt1 (C, G, K), or Six2-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$(D,H,L) kidneys. Tissues were hybridized with anti-sense probes for the Wnt9b differentiation target gene, C1qdc2 (E-H). Antibodies are to the NPC marker Six2 (gray), the proximal tubule marker LTL (green) and the ureteric bud marker pan-cytokeratin (red) in I-L. All immunostained sections were counterstained with DAPI. Scale bars are 50uM for (A-H) and 100uM for (I-L).

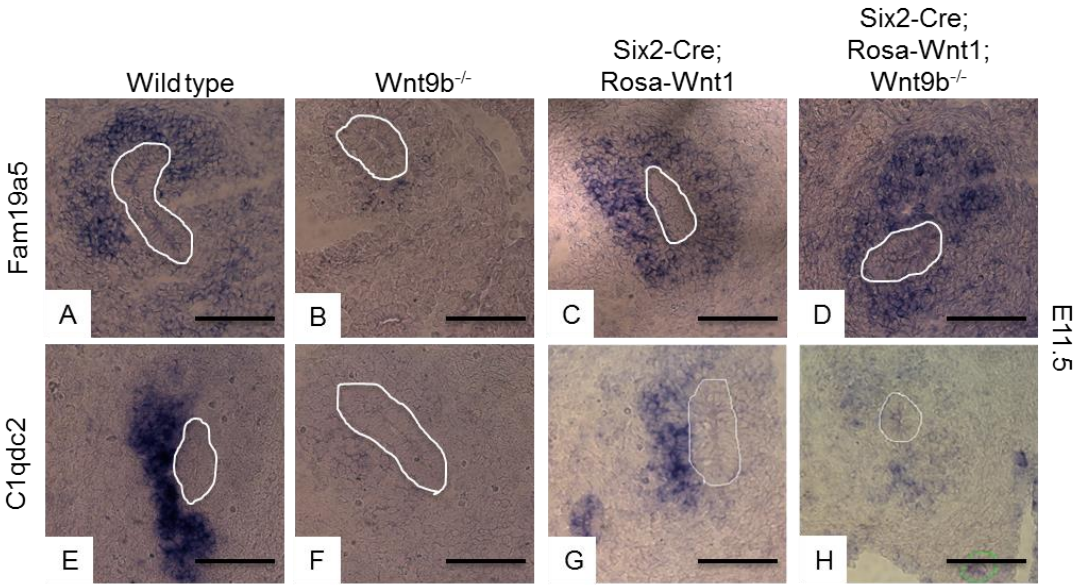


Fig. 3.4. Failure of Six2-Cre; RosaWnt1 MMs to differentiate is not due to lack of UB-derived signals. In situ hybridization on sections of E11.5 wildtype (A, E), Wnt9b$^{-/-}$ (B, F), Six2-Cre; Rosa-Wnt1 (C, G), Six2-Cre; Rosa-Wnt1; Wnt9b$^{-/-}$ (D, H) kidneys. Tissues were hybridized with probes to the Wnt9b renewal target gene, Fam19a5 (A-D) or the Wnt9b differentiation target gene, C1qdc2 (E-H). Scale bars are 100uM.

We next considered whether the observed phenotype was due to a dominant gain of function activity. This possibility seemed unlikely as previous work showed that

pharmacological or genetic activation of beta-catenin within isolated MMs resulted in differentiation, not renewal [105, 114, 115]. However, to test this hypothesis, we analyzed Six2-Cre;Rosa-Wnt1 kidneys with intact, wildtype UBs. In contrast to the Six2-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ kidneys, the Six2-Cre;Rosa-Wnt1;Wnt9b$^{+/+}$ kidneys were of normal size and histology and expressed both renewal targets (Six2, Cited1, Fam19a5, Pla2g7) as well as differentiation targets, C1qdc2, Wnt4, Pax8 (Fig. 2.3.C, 2.3.G, 2.3.K, 2.4.C, 2.4.G and not shown). These observations rule out a dominant gain of function activity for Wnt1 in cap mesenchyme.

The next possibility tested was that although Rosa-Wnt1 produced by the UB (using Hoxb7-Cre) was previously shown to be capable of rescuing the Wnt9b phenotype at the histological level, there may be functional differences between Wnt1 and Wnt9b ligand. To address this possibility, we performed a more detailed analysis of Hoxb7-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ kidneys. First, we assessed size and morphology of Wnt1 rescued kidneys and found that they were indistinguishable from wildtype kidneys (Fig. 2.5.D) suggesting that the renewal and differentiation programs stimulated by Rosa-Wnt1 were similar to that of Wnt9b. Next, we assessed the expression of Wnt9b specific targets in Hoxb7-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ kidneys and found that the abundance and spatial expression of all Wnt9b target genes examined were indistinguishable from wildtype kidneys (Fig. 2.5.J, 2.5.P). Thus, when produced by the UB, Rosa-Wnt1 is functionally indistinguishable from endogenous Wnt9b.

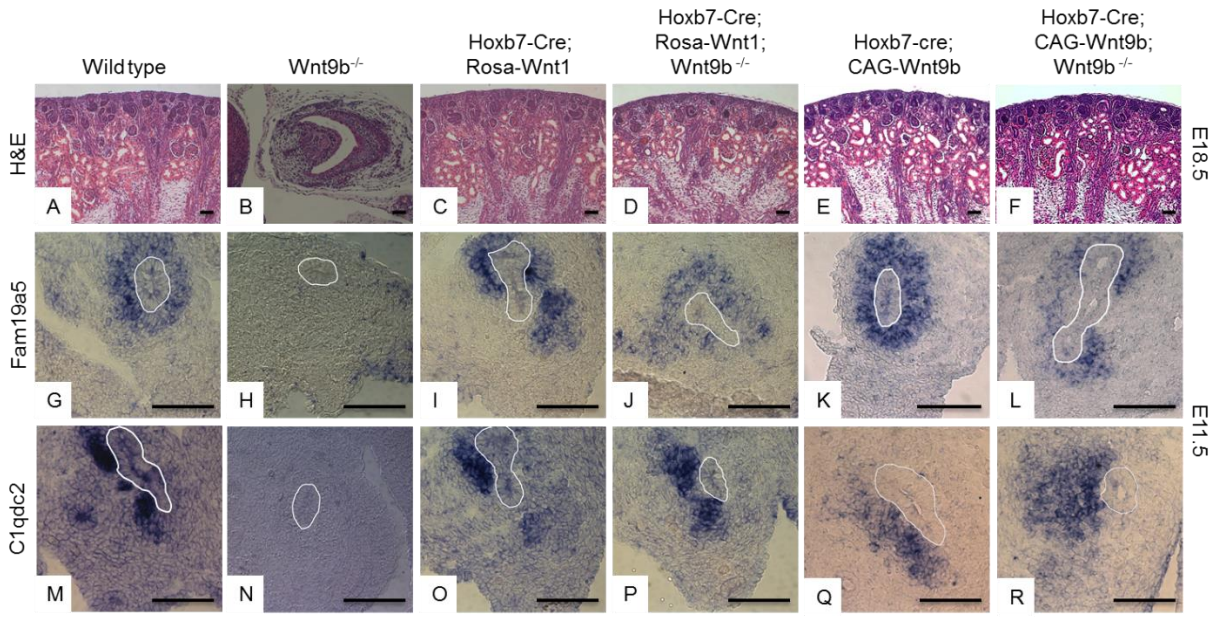


Fig. 3.5. Activation of either Wnt1 or Wnt9b transgene in the ureteric bud rescues the Wnt9b mutant phenotype. Hematoxylin and Eosin staining (A-F) or in situ hybridization (G-L) on sections of E18.5 (A-F) or E11.5 (G-R) wildtype (A,G,M), Wnt9b$^{-/-}$ (B,H,N), Hoxb7-Cre;Rosa-Wnt1 (C,I,O), Hoxb7-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ (D,J,P), Hoxb7-Cre;CAG-Wnt9b (E,K,Q), or Hoxb7-Cre;CAG-Wnt9b;Wnt9b$^{-/-}$ (F,L,R) kidneys. Tissues were hybridized with anti-sense probes to the Wnt9b renewal target gene, Fam19a5 (G-L) or the Wnt9b differentiation target gene, C1qdc2 (M-R). Scale bars are 50uM for A-F and 100uM for G-R.

To determine if the response to Rosa-Wnt1 requires beta-catenin, we utilized a pharmacological approach by culturing isolated Six2-Cre;Rosa-Wnt1 MM in media containing the beta-catenin antagonist IWR1 at 100uM, a concentration we previously showed blocks all beta-catenin activity [114]. The IWR1 treated Six2-Cre;Rosa-Wnt1 MMs behaved identically to wildtype MMs and died within 48 hours (N=8/9, Fig. 2.6.G). We next genetically assessed beta-catenin dependence by ablating beta-catenin at the same time that we activated Wnt1 (Six2-Cre;Rosa-Wnt1;beta-catenin$^{flox/-}$). Similar to wild

type MMs and IWR1 treated Six2-Cre;Rosa-Wnt1 MMs, Six2-Cre;Rosa-Wnt1;beta-catenin$^{flox/-}$ MMs died within 48 hours of culture (N=5/5, Fig. 2.6.D) suggesting that Wnt1 signals through beta-catenin to promote NPC survival/renewal.

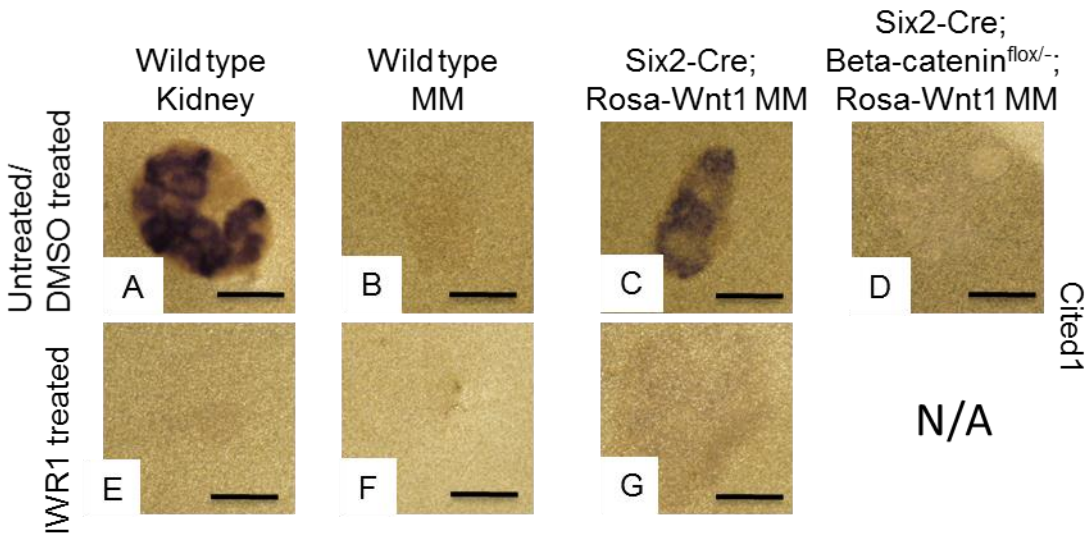


Fig. 3.6. Response of NPCs to agonists and antagonists of beta-catenin. Whole mount in-situ hybridization evaluating the expression of Wnt9b renewal target gene Cited1 in E11.5 wildtype kidney (A,E), wildtype isolated MM (B,F), Six2-Cre;Rosa-Wnt1 isolated MM (C,G), Six2-Cre;Rosa-Wnt1;beta-catenin$^{flox/-}$ isolated MM (D) cultured in normal media (D), or media containing DMSO (A-C) or 100uM of beta-catenin antagonist IWR1(E-G) for 48 hours. Scale bars are 200uM.

Cap mesenchyme is able to produce an active Wnt ligand.

Although the data presented so far suggest that Wnt1 produced by the UB is functionally equivalent to Wnt9b, it is possible that upon activation of Rosa-Wnt1 in the NPCs, the MM lacks the molecular machinery to produce and/or secrete a fully active Wnt ligand (e.g. enzymes necessary to post-translationally modify the protein or in some other way secrete an active ligand). To test whether mesenchymally produced Wnt ligand could be secreted and signal in a paracrine fashion, we generated clones of Wnt1 producing cells within NPCs using a Six2-CreERT2 transgene. To remove the influence of endogenous Wnt9b on NPCs, the analysis was performed on kidneys lacking both copies of Wnt9b. Rosa-Wnt1 was activated with a low dose of tamoxifen resulting in small clones of Wnt producing cells in $Wnt9b^{-/-}$ kidneys. E15.5 kidneys were subsequently assessed for the expression of Cited1 relative to the Wnt1 source, which was identified using Rosa26-lox-stop-lox-YFP.

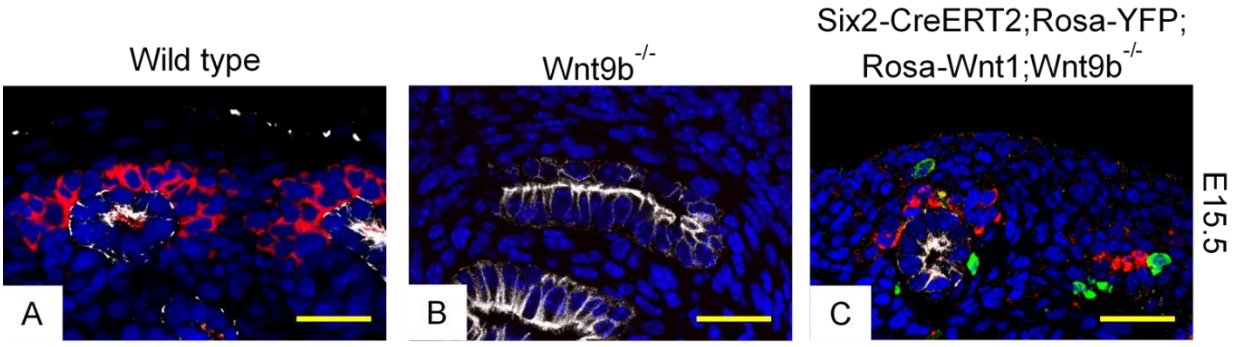


Fig. 3.7 NPCs can secrete functional Wnt proteins. Immunofluorescence staining (A-C) on sections of E15.5 wildtype (A), Wnt9b-/- (B) or Six2-CreERT2;Rosa-YFP;Rosa-Wnt1;Wnt9b-/- (C) kidneys. Antibodies are to GFP (green), renewal target Cited1 (red) and pan-cytokeratin (grey). All sections were counterstained with DAPI. Scale bars are 25uM.

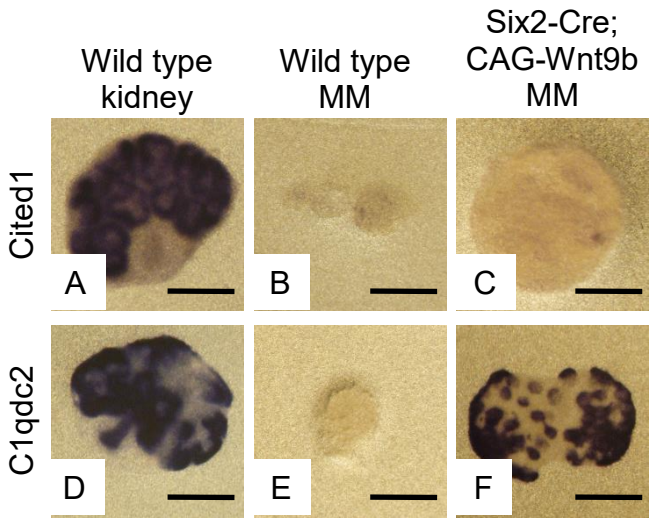
Six2-CreERT2;Rosa-Wnt1;Rosa-YFP;Wnt9b$^{-/-}$ kidneys were significantly larger than Wnt9b$^{-/-}$ mutants. They appeared to maintain a nephrogenic zone and their UBs underwent extensive branching, a process dependent on signals from the NPC progenitors. To determine if Wnt1 was produced and secreted properly when expressed in the NPCs, we examined expression of Wnt9b target genes with the hypothesis that if Wnt1 is able to be secreted, targets should be expressed adjacent to the Rosa-YFP positive, Wnt1-producing cells. However, if Wnt1 is only capable of signaling in an autocrine fashion, then only the Rosa-YFP positive cells will express Wnt9b target genes. In the Six2-CreERT2;Rosa-Wnt1;Rosa-YFP;Wnt9b$^{-/-}$ kidneys, the renewal target gene Cited1 was observed in YFP/Wnt1 negative cells that were adjacent to the YFP positive Wnt1 producing cells (Fig. 2.7.C). This demonstrated that the cap mesenchyme is capable of secreting a functional Wnt1 ligand.

Graded activity of Wnt9b induces nephron progenitor cell fate

Although Wnt1 can functionally substitute for Wnt9b when it is activated in the UB, we could not formally rule out the possibility that the phenotype observed upon activation of this factor in the nephron progenitors was ligand specific. As Wnt9b is the endogenous ligand, we repeated this analysis with a recently generated, Cre inducible Wnt9b allele (CAG-lox-stop-lox-Wnt9b-IRES-GFP, from here on referred to as CAG-Wnt9b) [116, 117]. We first confirmed that activation of this transgene within the UB could fully rescue Wnt9b mutant kidneys. Hoxb7-Cre;CAG-Wnt9b;Wnt9b$^{-/-}$ kidneys were indistinguishable

from Rosa-Wnt1 rescue (Hoxb7-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$) and wild type kidneys in both gross anatomy and expression of Wnt9b target genes (Fig. 2.5.F, 2.5.L, 2.5.R).

We next induced CAG-Wnt9b in the NPCs using Six2-Cre. In contrast to what was observed with Rosa-Wnt1, activation of CAG-Wnt9b in the NPCs resulted in expression of differentiation target genes (N=41/42) but not renewal targets (11/15,Compare Fig. 2.8.C and 2.8.F to Fig. 2.1). After 4 days of culture, the entirety of the Six2-Cre;CAG-Wnt9b NPC population had undergone MET as indicated by the expression of E-cadherin and the absence of the NPC marker Six2 (N=3/3, Compare Fig. 2.8.H to Fig. 2.1.I and 2.1.J). In vivo activation of CAG-Wnt9b in the NPCs of wild type or Wnt9b$^{-/-}$ intact kidneys, showed ectopic differentiation within the NPC domain at E12.5 and E15.5 (Fig. 2.9). Figure S6. Pax8-YFP expression in mouse UGS. Whole mount in-situ hybridization (A-B) or fluorescent images (C-E) evaluating the expression of Pax8 mRNA (A-B) and Pax8-YFP (C-E) in E13.5 (C) or E14.5 (A,B,D,E) isolated kidneys or intact urogenital systems. B and E are images of kidneys isolated at E11.5 and cultured for 72 hours ex-vivo. Note that YFP expression replicates the expression of Pax8 mRNA. The arrows indicate Mullerian duct and arrow heads point to pre-tubular aggregates/renal vesicles. Scale bars are 200uM (A,B,D) and 250uM (C,E).



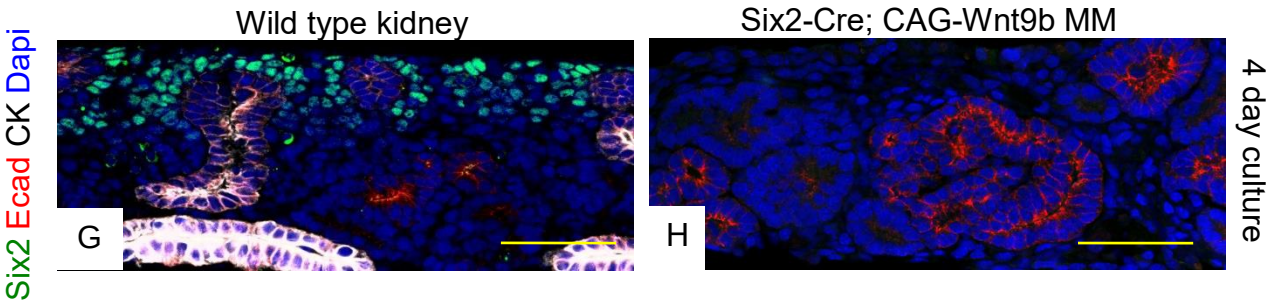


Fig. 3.8. Homogenous expression of Wnt9b in the NPCs induces differentiation but not renewal. A-F: Whole mount in-situ hybridization with probes to Wnt9b renewal target gene, Cited1 (A-C) or Wnt9b differentiation target gene, C1qdc2 (D-F) was performed on E11.5 wildtype kidneys (A,D), wildtype MMs (B,E) or Six2-Cre;CAG-Wnt9b MM (C, F) after 48 hours of culture. G-H Immunofluorescence staining on sections of wildtype kidneys (G), or Six2-Cre;CAG-Wnt9b isolated MM (H) cultured for 4 days. Antibodies are to Six2 (green), pan-cytokeratin (white) E-cadherin (red). All sections are counterstained with DAPI. Scale bars are 200uM for A-F and 100uM for G-N.

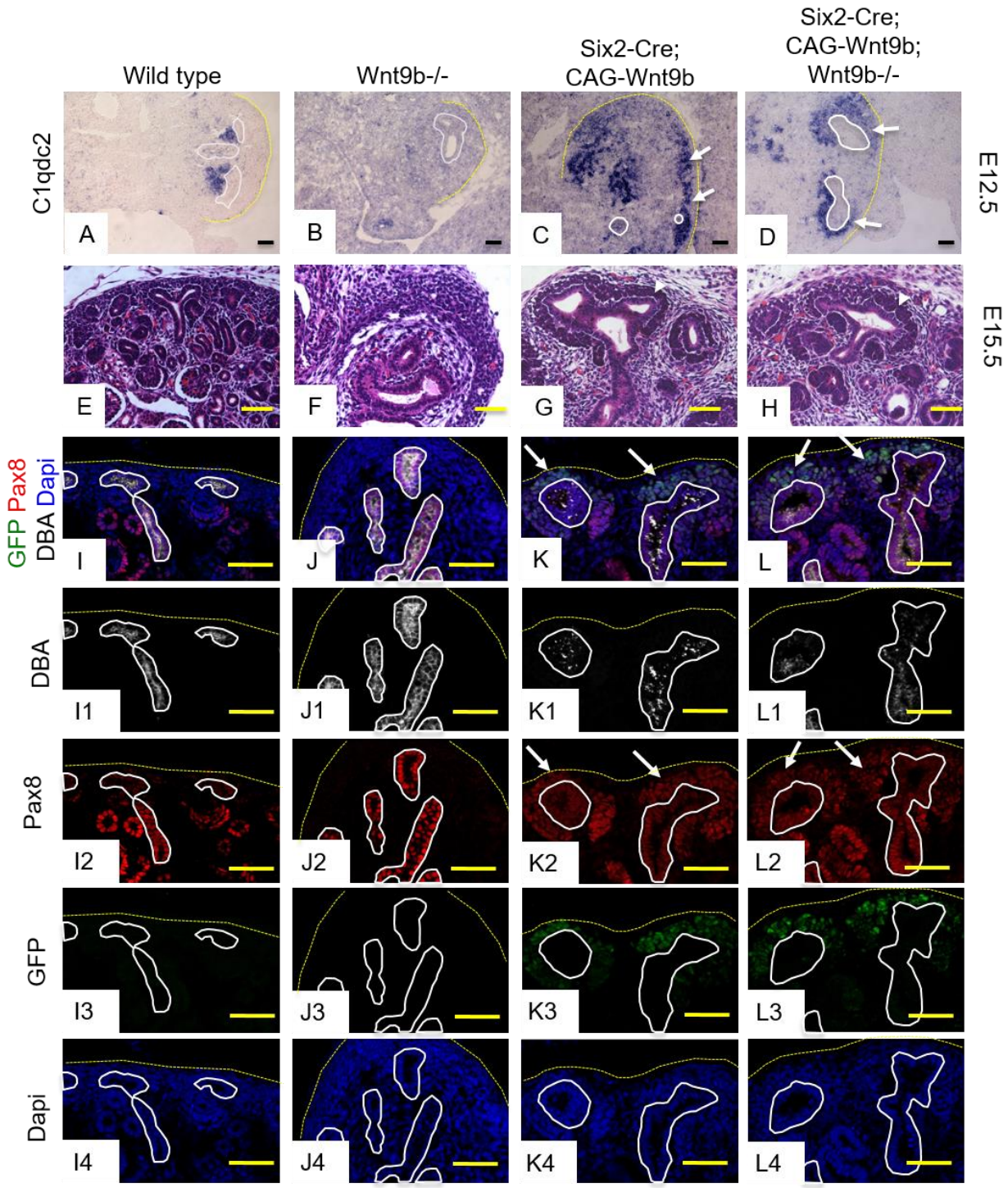


Fig. 3.9. Homogenous expression of Wnt9b in the NPCs induces differentiation but not renewal. A-L4: Sections of E12.5 (A-D), E15.5 (E-L) or E16.6 (I-L4) wildtype (A, E, I, I1, I2, I3, I4), Wnt9b$^{-/-}$ (B, F, J, J1, J2, J3, L4), Six2-Cre;CAG-Wnt9b (C, G, K, K1, K2, K3, K4) or Six2-CAG-Wnt9b;Wnt9b$^{-/-}$ kidneys (D, H, L, L1, L2, L3, L4) hybridized with

antisense probe to the Wnt9b differentiation target C1qdc2 (A-D), stained with Hemotoxylin and Eosin (E-H) or immunostained (I-L4) with antibodies to collecting duct marker DBA (grey in I1-L1),Wnt9b differentiation target Pax8 (red in I2-L2) and GFP (I3-L3). The ureteric bud is outlined in white and cortex of kidney is outlined in yellow. Arrows point to ectopic C1qdc2 or Pax8 expression, arrowheads indicate ectopic PTAs. Scale bars are 100uM for E-H and 50uM for the rest.

To determine whether the CAG-Wnt9b allele was processed and secreted by the NPCs, we generated clones expressing the transgene using the Six2-CreERT2 line and a low dose of tamoxifen. Expression of the Wnt9b-dependent differentiation marker Pax8 was observed (Fig. 2.10.C) adjacent to the Wnt9b expressing cells. Unexpectedly, we also observed expression of the Wnt9b-dependent renewal marker Cited1 in the Six2-CreERT2;CAG-Wnt9b;Wnt9b$^{-/-}$ kidneys (Fig. 2.10.F). This data suggests that while uniform activation of Rosa-Wnt1 in the NPCs results in progenitor renewal and uniform activation of CAG-Wnt9b results in differentiation, mosaic activation of CAG-Wnt9b in these cells results in both renewal and differentiation.

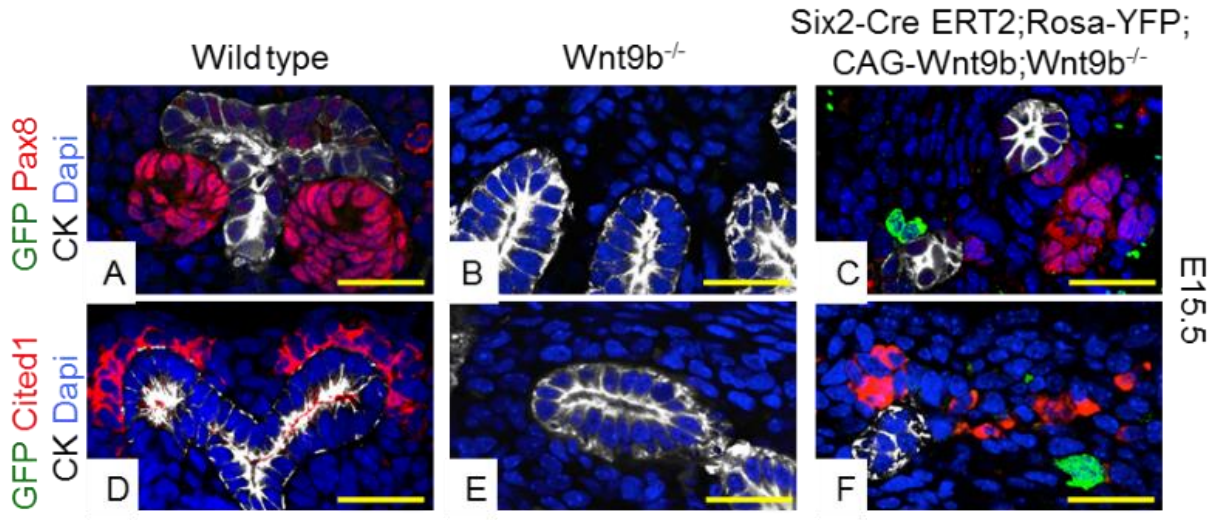


Fig. 3.10. CAG-Wnt9b expressing NPCs secrete a functional Wnt protein. Immunofluorescence staining on sections of E15.5 wildtype (A,D), Wnt9b$^{-/-}$ (B,E) and Six2-CreERT2;CAG-Wnt9b;Rosa-YFP;Wnt9b$^{-/-}$ (C,F) kidneys. Antibodies are to GFP (green), pan-cytokeratin (white), renewal target gene Cited1 (red in D-F) and differentiation target gene Pax8 (red in A-C). All sections are counterstained with DAPI. Scale bars are 50uM.

Different levels of beta-catenin activity induce different cell fates

The data presented to this point produce a paradox. While uniform activation of Rosa-Wnt1 promotes renewal and uniform activation of CAG-Wnt9b promotes differentiation, activation of either transgene in the adjacent UB or mosaically within the NPCs promotes both renewal and differentiation. While it is likely that the phenotype induced by Six2Cre;CAG-Wnt9b is the result of Wnt/beta-catenin gain of function, why Rosa-Wnt1 cannot induce a similar phenotype is unclear? One possibility is that the two ligands may trigger differential levels of beta-catenin activation and that different levels of beta-catenin could be promoting different cell fates within the NPCs as has been proposed in other progenitor cell types [106, 108, 110].

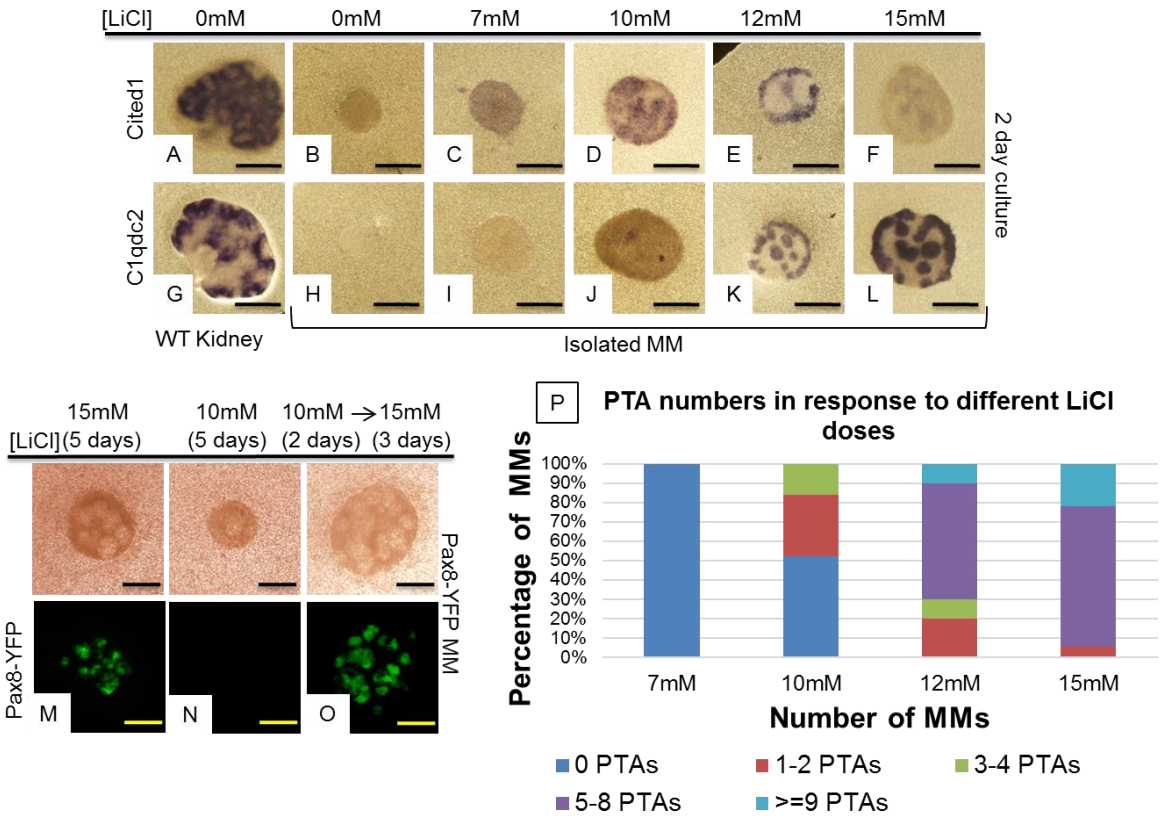


Fig. 3.11. Distinct thresholds of beta-catenin activity determine NPC fate. Wildtype (A-L) or Pax8-YFP (M-O) E11.5 kidneys (A, G) or isolated metanephric mesenchymes (B-F, H-O) were cultured in media containing 0mM (A, B, G, H), 7mM (C, I), 10mM (D, J, N), 12mM (E, K), 15mM (F, L, M) or 10 and 15mM (O) concentrations of the Gsk3 antagonist LiCl for 48 hours (A-L) or 120 hours (M-O). Treatments were constant except for (O) where tissues were cultured in 10mM LiCl for 2 days then shifted to 15mM for 3 days. Cultured tissues were hybridized with anti-sense probes for the Wnt9b renewal target gene Cited1 (A-F), the Wnt9b differentiation target gene, C1qdc2 (G-L) or imaged for Pax8-YFP expression (Q-S). P: Quantification of PTAs in 7mM, 10mM, 12mM and 15mM treated wildtype MMs. P-values are * = 6.06E-04 and # = 3.99E-07. Scale bars are 200uM.

To determine if different levels of beta-catenin activation promoted distinct responses in NPCs, isolated MMs were treated with various doses of the Gsk3 inhibitor

LiCl (0, 7, 10, 12 and 15mM) and assayed by in situ hybridization. Although LiCl can have pleiotrophic effects, previous studies have shown that within the NPCs, it induces Wnt9b target gene expression in a beta-catenin dependent manner [114]. As expected,isolated wild type MM did not survive beyond 48 hours of culture and failed to express either class of Wnt9b targets (N= 7/7, Fig. 2.11.B and 2.11.H). MMs treated with a 7mM concentration of LiCl survived but rarely expressed the renewal target Cited1 (N=2/8) and failed to express differentiation target C1qdc2 (N= 5/5, Fig. 2.11.C and 2.11.I). Upon treatment with 10mM LiCl, 19/26 MMs assessed showed strong expression of the renewal target Cited1 while 12/25 showed expression of the differentiation target C1qdc2, although this expression was usually confined to 2 or fewer aggregates (Fig. 2.11.D, 2.11.J and 2.11.P). Treatment with 12mM LiCl also resulted in survival in all instances (18/18). 8/8 MMs assayed expressed renewal markers and 10/10 expressed differentiation targets (Fig. 2.11.E, 2.11.K). In contrast to what was observed with 10mM, the majority of 12 mM treated MMs had greater than 3 PTAs per MM. MMs treated with 15mM LiCl survived and 18/18 assayed expressed differentiation target Clqdc2 (Fig. 2.11.L, 2.11.P). These MMs also frequently expressed low levels of renewal target Cited1 (N=16/35, Fig. 2.11.F). To determine if this staining marked a remaining progenitor population, we stained sections with an antibody to Cited1. However, we were unable to detect Cited1 protein in these tissues (not shown).

Similar results were found with additional differentiation targets Wnt4 and Pax8 (Fig. 2.11.M). However, analysis of additional renewal targets Fam19a5 and Pla2g7 showed expression under all conditions. This most likely represents the fact that expression in contrast to Cited1, which is expressed only in the renewing NPCs, both of

these genes are expressed in both the renewing NPCs and the differentiating PTAs. Thus, this data is consistent with the hypothesis that low blockade of Gsk3 results in NPC renewal and high level blockade results in differentiation.

Although low levels of LiCl promoted the expression of renewal target genes, whether these MMs were actually competent NPCs was not clear. To test this, we generated a Bac transgenic mouse line expressing YFP under the control of the Pax8 regulatory elements to serve as a live indicator of differentiation. Pax8-YFP expression mirrored the expression of Pax8 mRNA in wild type embryonic urogenital systems and showed a similar reliance on Wnt9b/beta-catenin activity suggesting it was a faithful Pax8 reporter (Fig. 2.12. This transgene will be further described at a later date). MMs from Pax8YFP E11.5 kidneys were isolated, placed in culture and assessed for YFP expression. As expected, treatment with 15mM LiCl resulted in robust YFP expression (N=2/2) while MMs treated with 10mM concentration did not activate detectable levels of YFP (N=5/6, Fig. 2.11.M-2.11.N). However, isolated MMs treated with 10mM LiCl for 2 days then switched to 15mM activated high levels of Pax8YFP indicating that low levels of LiCl maintained the NPCs in a differentiation competent state (N=6/7, Fig. 2.11.O). These data suggest that differential levels of Gsk3 activity can promote differential response in isolated MMs.

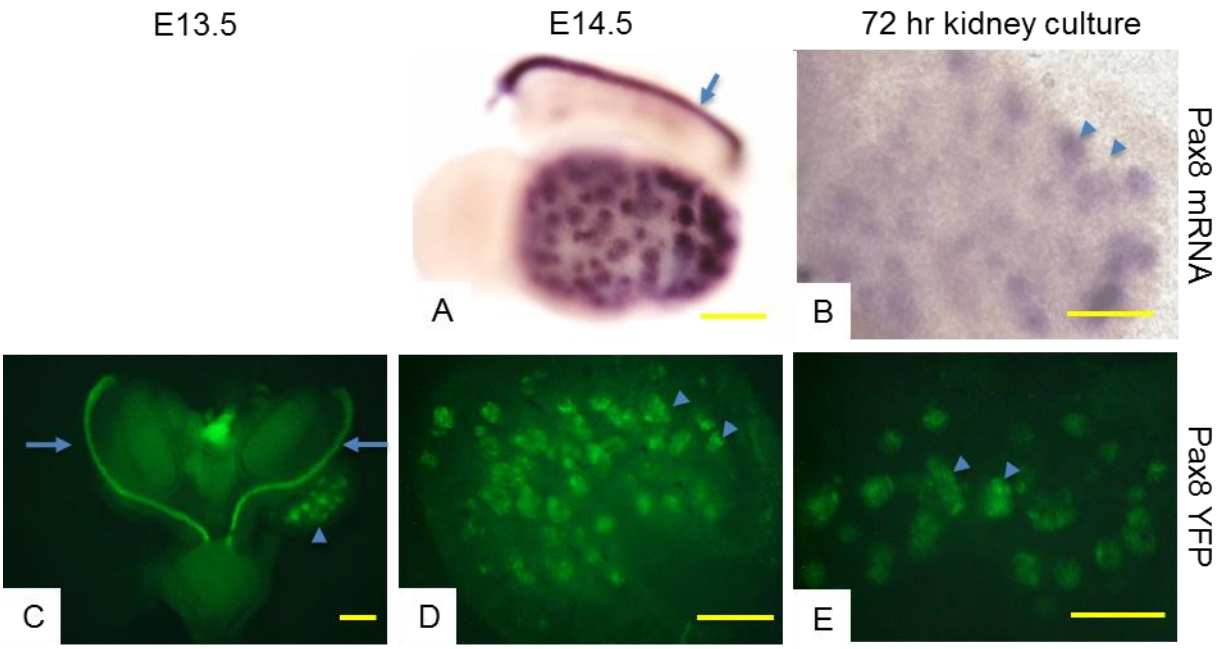


Fig. 3.12. Pax8-YFP expression in mouse UGS. Whole mount in-situ hybridization (A-B) or fluorescent images (C-E) evaluating the expression of Pax8 mRNA (A-B) and Pax8-YFP (C-E) in E13.5 (C) or E14.5 (A,B,D,E) isolated kidneys or intact urogenital systems. B and E are images of kidneys isolated at E11.5 and cultured for 72 hours ex-vivo. Note that YFP expression replicates the expression of Pax8 mRNA. The arrows indicate Mullerian duct and arrow heads point to pre-tubular aggregates/renal vesicles. Scale bars are 200uM (A,B,D) and 250uM (C,E).

Differential fates promoted by uniform activation of Rosa-Wnt1 and CAG-Wnt9b are the result of differential levels of ligand activity.

To this point, we have shown that low level inhibition of Gsk3 induces NPC renewal while high level inhibition induces differentiation. Further, uniform activation of Rosa-Wnt1 elicits only the renewal response while that of CAG-Wnt9b promotes differentiation. The simplest explanation for these results is that Rosa-Wnt1 induces low level beta-catenin activation throughout the mesenchyme resulting in NPC renewal and CAG-Wnt9b

induces high level beta-catenin activity resulting in differentiation. If this is the case, one would predict that Six2-Cre;Rosa-Wnt1 and low level LiCl would cooperate to induce differentiation. To test this, we supplemented Six2-Cre;Rosa-Wnt1 MMs with a dose of LiCl (10mM) and then assessed expression of both renewal and differentiation target genes. Although MMs expressing either Six2-Cre;Rosa-Wnt1 (Fig. 2.1.C and 2.1.F) or wild type MMs treated with 10mM LiCl (Fig. 2.11.D and 2.11.J) form few if any PTAs but express high levels of renewal marker Cited1, the 10mM LiCl treated Six2-Cre;Rosa-Wnt1 MMs form high numbers of PTAs as assessed by the expression of differentiation target C1qdc2 (N=3/3, Fig. 2.13.F-2.13.G and 2.13.M-2.13.N).

To test if the differentiation response of NPCs induced by homogenous activation of CAG-Wnt9b was due to relatively high Wnt ligand levels (compared to Rosa-Wnt1), we added IWP2 or IWPL6, inhibitors of the o-acyl-transferase Porcupine, to the culture media of Six2-Cre;CAG-Wnt9b isolated MMs. Porcupine is necessary for production of an active ligand and we previously showed that treatment of wildtype kidneys with a high dose (5 uM of IWP2 or 50 nM of IWPL6) resulted in complete loss of both differentiation and renewal targets[57, 114]. Further, we found that the high dose also prevented survival in the Six2-Cre;CAG-Wnt9b MMs (N=6/6, Fig. 2.14.D). However, treatment of Six2-Cre;CAG-Wnt9b MMs with a low dose of the porcupine inhibitors IWP2 at 500nM (N=4/4) or IWPL6 at 10nM (N=8/11) rescued expression of the NPC renewal target Cited1 although the differentiation target C1qdc2 (N=3/3 at 1uM, N=2/2 at 500nM) was also expressed (Fig. 2.13.D, 2.13.K, 2.14.B, 2.14.F and not shown).

Since attenuation of Wnt activity in the Six2-Cre;CAG-Wnt9b isolated MMs resulted in rescued expression of renewal target Cited1, we next asked whether

attenuation of beta-catenin would have the same effect. Six2-Cre;CAG-Wnt9b MMs were treated with a low dose of the beta-catenin antagonist IWR1. Once again, high dose treatment with this drug completely blocked Wnt response[114]. However, similar to what was observed with the Porcupine inhibitors, treatment of Six2-Cre;CAG-Wnt9b MMs with a low dose of IWR1 (500nM) rescued expression of the renewal target Cited1 (Fig. 2.13.E and 2.13.L). In sum, while uniform expression of Wnt1 in NPCs results in renewal target expression, this response can be shifted to differentiation by repressing Gsk3 levels. Reciprocally, while uniform expression of a Wnt9b transgene in the NPCs results in differentiation, this response can be shifted to renewal by lowering the levels of either the Wnt ligand or beta-catenin. These data support the hypothesis that NPCs respond to different Wnt/beta-catenin activity levels with low levels promoting renewal and high levels promoting differentiation.

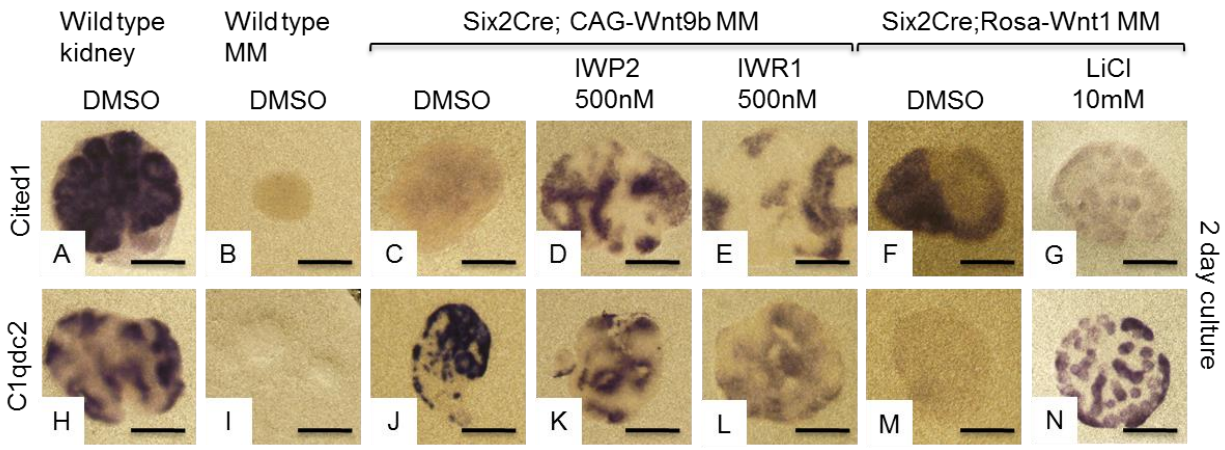


Fig. 3.13. Progenitor renewal can be promoted in Six2-Cre;CAG-Wnt9b isolated metanephric mesenchymes by antagonizing Wnt or Gsk3 activity. Wildtype intact kidneys (A, H), wildtype isolated metanephric mesenchyme (MM) (B, I), Six2-Cre;CAG-Wnt9b isolated MM (C-E and J-L) or Six2-Cre;Rosa-Wnt1 isolated MM (F-G, M-N) were analyzed by whole mount in-situ hybridization with probes to Wnt9b renewal target gene,

Cited1 (A-G) or Wnt9b differentiation target gene, C1qdc2 (H-N). Analysis was performed after 48 hours of culture in media supplemented with DMSO (A-C, F, H-J, M), a low dose (500nM) of the Porcupine inhibitor IWP2 (D, K), (a low dose 1uM) of the tankyrase inhibitor IWR1 (E, L) or low dose (10mM) of LiCl (G, N). Scale bars are 200uM.

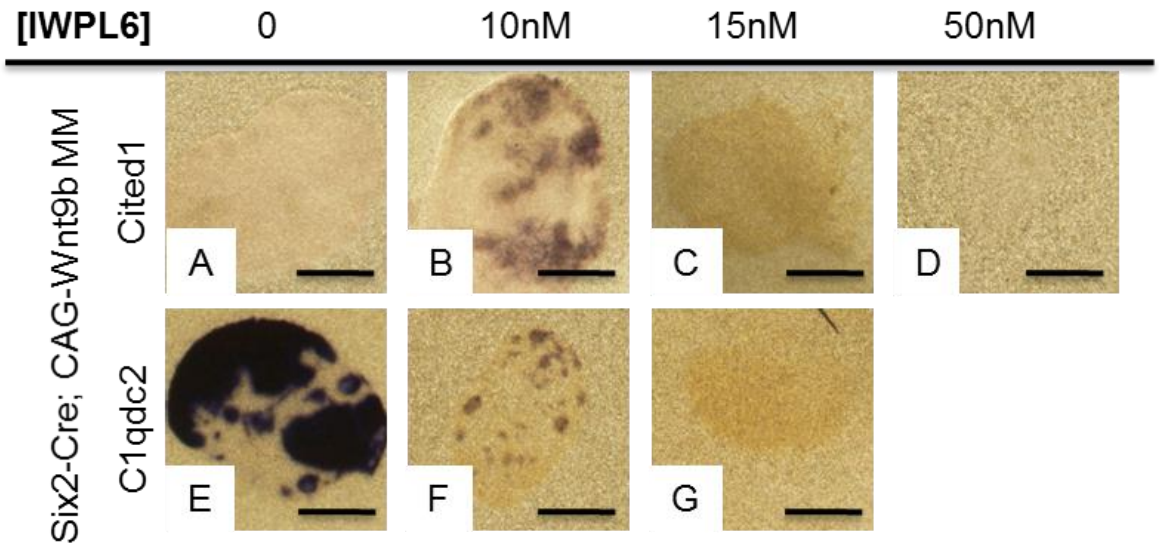


Fig. 3.14. Response of NPCs to antagonists of Wnt. Whole mount in-situ hybridization evaluating the expression of Wnt9b differentiation target genes C1qdc2 (E-G) or renewal target gene Cited1 (A-D) in E11.5 Six2-Cre;CAG-Wnt9b isolated MM (A-G) cultured in media containing DMSO (A,E), 10nM (B,F), 15nM (C,G), or 50nM (D) of Wnt inhibitor IWPL6 for 48 hours. Scale bars are 100uM (A-G).

Morphology of the Wnt source does not provide instructional cues for NPC fate determination

The data presented to this point suggest that NPC fate can be dictated by different levels of Wnt/beta-catenin signaling. We next asked the fundamental question of how differential beta-catenin activity is established within the NPCs. As Wnts are secreted factors, the simplest hypothesis is that a gradient of the ligand exists across the NPC field with different levels of ligand producing distinct responses. However, it is difficult to envision how this could occur in the developing kidney as the expression domains of the renewal and differentiation targets of Wnt9b do not appear to correlate with the relative distance of the receiving cells to the Wnt9b source, the ureteric bud (UB). Instead, response correlates with the spatial relationship between the UB and the NPC with renewing cells on the cortical side of the UB tip and differentiating cells on the medullary side (Fig. 2.18).

One possible explanation for the differential spatial response to Wnt ligand is that the ligand is secreted at higher levels from the medullary UB tips. To test this hypothesis, we took advantage of the fact that the Rosa-Wnt1 allele encodes a GFP fusion protein. As the Hoxb7-Cre;Rosa-Wnt1 mouse rescues both renewal and differentiation (and expression of both classes of target genes, Fig. 2.5), if a difference in secretion levels underlies these two processes, we should see spatial differences in GFP protein levels in responding cells in Hox7Cre-Rosa-Wnt1 kidneys. GFP signal was detected throughout the UB of Hoxb7-Cre;Rosa-Wnt1 kidneys at both E11.5 and E12.5 (Fig. 2.15.A). The signal was most intense in the cell membranes however there was no detectable differences between staining on the cortical and medullary sides of the UB tips (Fig.

2.15.A′). We also did not detect signal above background in the responding NPCs. Interestingly, GFP expression was much more intense in the distal-most regions of the Hoxb7Cre lineage, the ureter, although signal was not detected beyond the extracellular matrix (ECM). It is possible that increased levels of Wnt ligand secreted from ureter is sufficient to amplify beta-catenin signal and promote differentiation of NPCs. To test this possibility we activated Rosa-Wnt1 in Wnt9b null kidneys using Ksp-Cre [118], a driver that is only active in the stalk of the UB (and not the tips) from E11.5 through 15.5 (after E15.5, this line becomes active in the UB tips and distal nephrons). Ksp-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ mutants activate both renewal and differentiation targets of Wnt9b starting from E11.5 (Fig. 2.15.C-J). However the kidneys formed in these mutants are small and hypoplastic (relative to wildtype and Hoxb7-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ kidneys) (compare Fig. 2.15.N to 2.15.K and 2.5.D) indicating that expression in the distal/medullary collecting ducts alone is not sufficient to completely rescue Wnt9b activity. We previously showed that ablation of Wnt9b using Ksp-Cre had no effect on expression of renewal or progenitor targets prior to E17.5 although it did affect tubule morphogenesis [118]. Taken together, these data suggest that there is no difference in Wnt ligand secretion between the cortical and medullary sides of the UB tips and that increased secretion of Wnt ligand from the distal medullary portion of the collecting duct does not play a role in NPC fate.

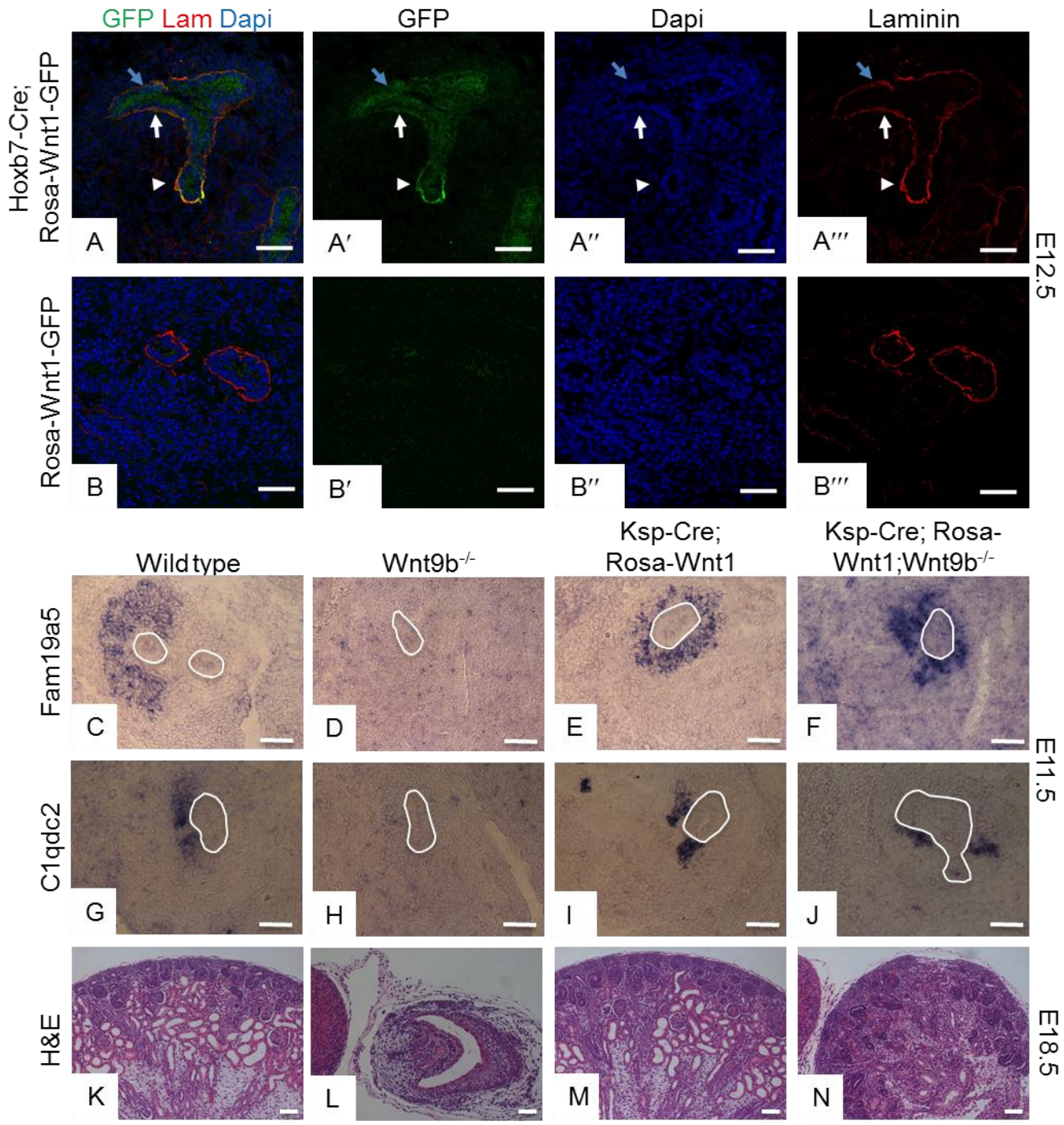


Fig. 3.15. Expression of Rosa-Wnt1 in the UB and response to NPCs to Rosa-Wnt1 expression in the UB stalk. A-B: Immunofluorescence staining of Hoxb7-Cre;Rosa-Wnt1-GFP (A) or Rosa-Wnt1-GFP (B) E12.5 kidney sections with antibodies to GFP(green in A′, B′), basement membrane marker Laminin (red in A‴, B‴) and counterstained

with Dapi (blue in A′′, B′′). Blue arrow points to the cortical side of the UB and white arrow points to the medullary side. The white arrowhead indicates the distal portion of the UB stalk. C-N: In-situ hybridization or Hematoxylin and Eosin staining on E11.5 (C-J) or E18.5 (K-N) wildtype (C,G,K), Wnt9b$^{-/-}$ (D,H,L), Ksp-Cre;Rosa-Wnt1 (E,I,M) or Ksp-Cre;Rosa-Wnt1;Wnt9b$^{-/-}$ (F,J,N) kidney tissue sections. Tissues were hybridized with anti-sense probes to the Wnt9b renewal target gene, Fam19a5 (C-F) or the Wnt9b differentiation target gene, C1qdc2 (G-J). The UB is outlined in white. Scale bars are 50uM.

During kidney development, the differentiating cells sit at the concave cleft of the Wnt9b expressing UB tip/stalk while the NPCs surround the convex tip of the UB. Previous modeling studies predict that secreted molecules will be present at higher levels in the cleft region of the branched structures, which corresponds exactly with the location of differentiation target gene expression [119]. Thus, it seemed plausible that the shape of the UB could affect the levels of Wnt ligand that the NPCs were exposed to. To test if shape and morphology of the Wnt source can influence cell fate decision, we activated Wnt expression within the stromal cells of the MMs and analyzed the response of NPCs with respect to the different shapes that the stroma forms.

Isolated Foxd1-Cre;Rosa-Wnt1;Rosa-YFP MMs were cultured and assessed for the expression of C1qdc2 (differentiation gene) and Cited1 (renewal gene). In contrast to isolated wildtype MMs which died within 48 hours, the Foxd1-Cre;Rosa-Wnt1 MMs survived and expressed both renewal and differentiation targets (N=26/26, Fig. 2.16.A-2.16.B). Irrespective of the morphology of the Wnt source relative to the receiving NPCs (concave or convex), the Foxd1-Cre;Rosa-Wnt1 MMs expressed the differentiation target gene C1qdc2 directly abutting the source of Wnt1 (N=11/11) and the renewal target

Cited1 at a distance from the source (N=8/9, Fig. 2.16.A', A'', B' B''). This finding rules out the hypothesis that morphology of the source dictates ligand levels and response. However, this data suggests that either a gradient of active ligand dictates fate or that the stroma produces a signal(s) that influences fate.

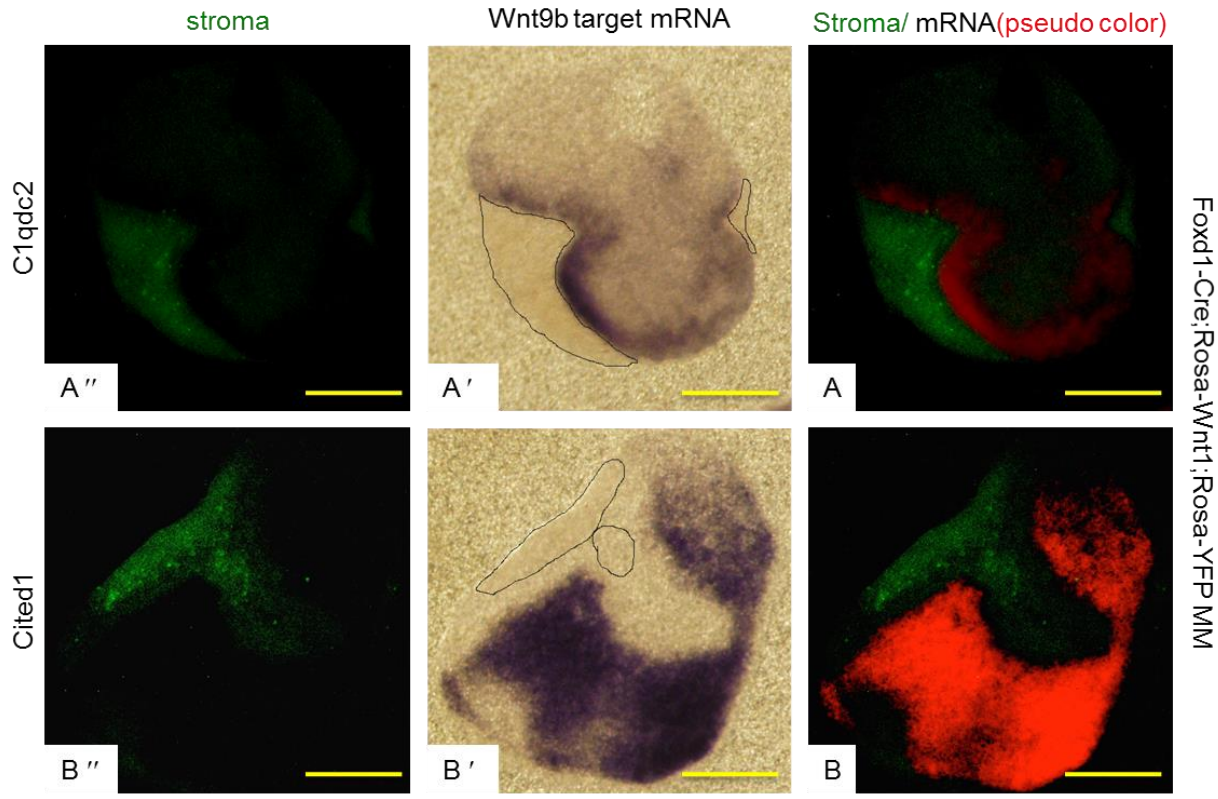


Fig. 3.16. Proximity to the stroma affects NPC fate. (A,B) E11.5 Foxd1-Cre;Rosa-Wnt1; Rosa-YFP isolated metanephric mesenchyme were cultured for 2 days and subjected to in-situ hybridization for Wnt9b renewal target gene Cited1 (B′) or Wnt9b differentiation target gene C1qdc2 (A′) followed by fluorescent antibody stain for GFP (A′′, B′′). In A and B, the fluorescent image and a pseudo-colored version of the in-situ image were merged. Note that the differentiation target C1qdc2 is expressed immediately adjacent to the Wnt source while the renewal target is expressed at a distance from the source of the Wnt. Scale bars are 200uM.

We know from previous work that signals produced by the stroma can influence NPC fate [12, 47, 120]. The stroma can either be providing signals that act in parallel with Wnt9b to dictate fate or the stroma could be providing signals that influence Wnt/beta-catenin activity. To determine if differential levels of Wnt/beta-catenin activity alone are sufficient to dictate response in NPCs, we isolated and cultured a purified population of these cells in the absence of the stroma [121]. As previously shown, wild type NPCs did not renew unless the media was supplemented with 1.5uM CHIR (a GSK-3 antagonist/beta-catenin agonist) (Fig. 2.17.A-2.17.D). Interestingly, supplementation with 3uM CHIR induced expression of the differentiation marker Lef1 in 2D culture and MET in 3-D culture (Fig. 2.17.K-2.17.N, 2.17.I). Further, NPCs isolated from Six2-Cre;Rosa-Wnt1 kidneys could be maintained and passaged in a Six2+/Lef1- progenitor state without supplementation with CHIR (Fig. 2.17.E-2.17.F). Supplementation of these cells with CHIR stimulated expression of Lef1 in 2D culture and induced MET in 3D organoid culture (Fig. 2.17.O-2.17.R, 2.17.J). In contrast, NPCs purified from Six2-Cre;CAG-Wnt9b kidneys expressed both Six2 and Lef1 but could not be passaged whether they were supplemented with 1.5 uM CHIR or not (Fig. 2.17.G-2.17.H, 2.17.S-2.17.T and not shown). These data suggest that in the absence of stromal signals, altering Wnt/beta-catenin activity alone is sufficient to determine NPC fate. In other words, the stroma does not produce additional instructive signals that dictate NPC response. Instead, our data suggest that NPC fate is affected by beta-catenin levels (Fig. 2.18).

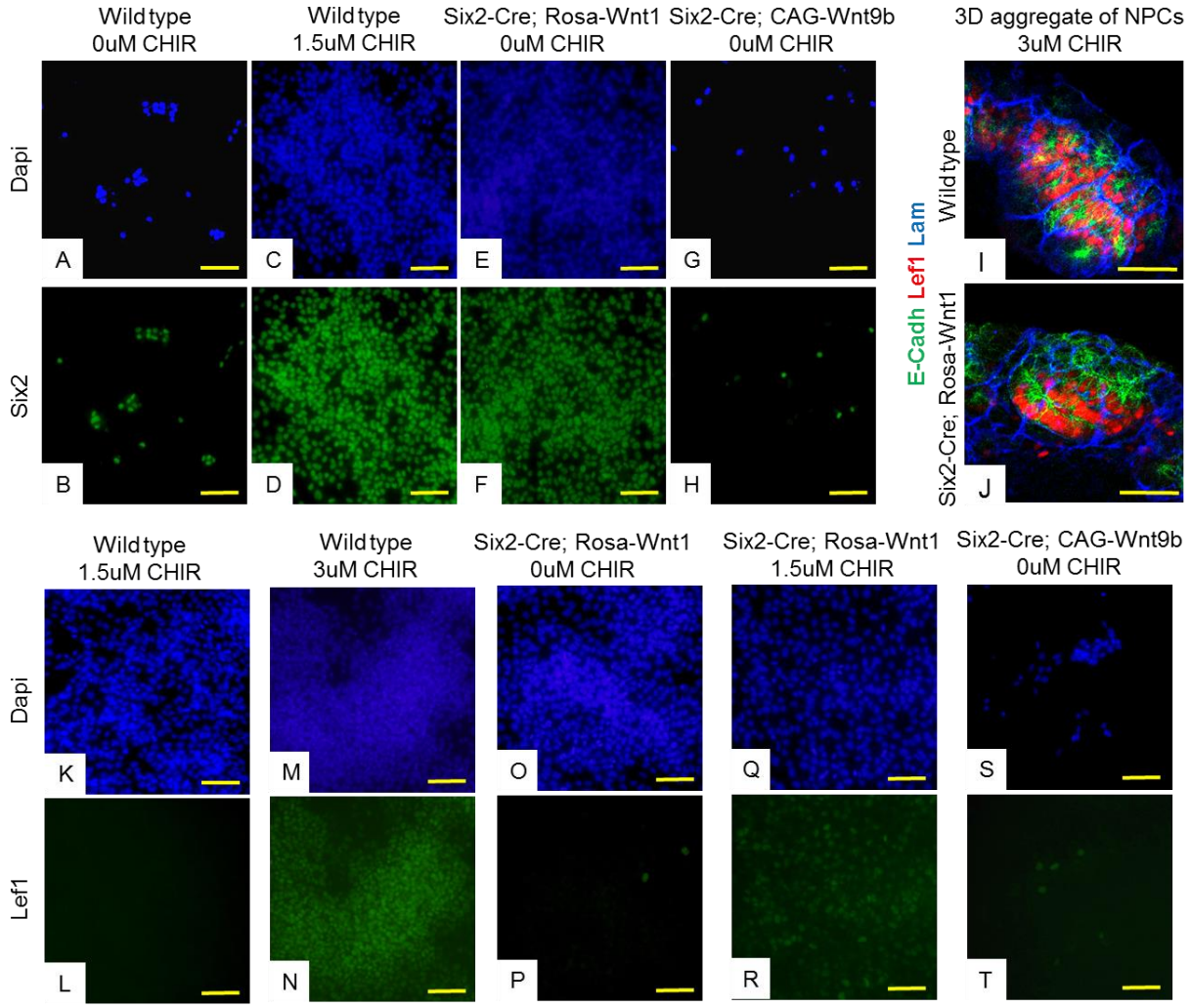


Fig. 3.17. Distinct beta-catenin activity levels induce distinct cell fates on isolated, pure NPC cultures. Wildtype (A-D, I, K-N), Six2-Cre;Rosa-Wnt1 (E-F, J, O-R) or Six2-Cre;CAG-Wnt9b (G-H,S-T) purified NPCs were cultured in media with 1.5uM CHIR (C-D, K-L, Q-R), 0uM CHIR (A-B, E-H, O-P, S-T) or 3uM CHIR (I-J and M-N) and stained with antibodies to the NPC marker Six2 (green) (B-H), differentiation gene Lef1 (green in L-T and red in I-J), epithelial tubule marker E-cadherin (green in I,J), or basement membrane marker Laminin (blue in I,J). In I and J, cells were aggregated and grown at the air media interface for two days. All images are counterstained with DAPI (blue). Scale bars are 100uM.

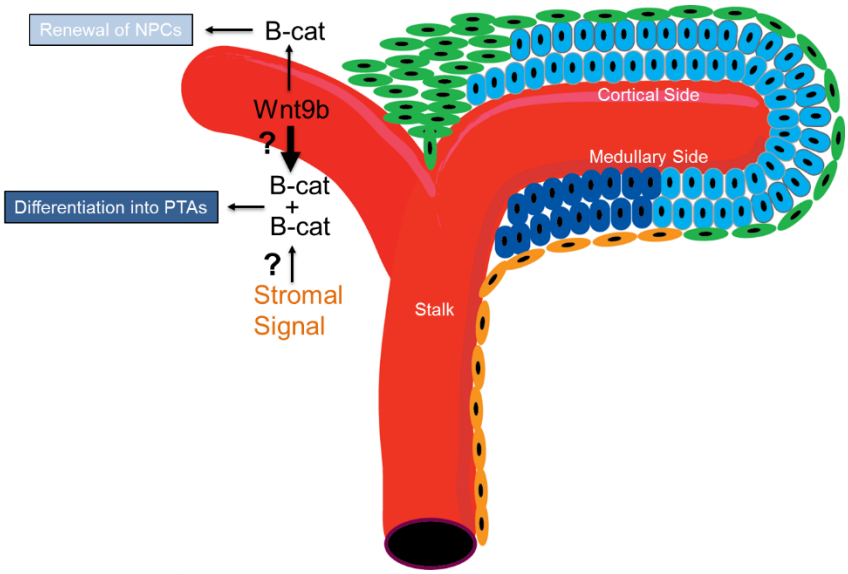


Fig. 3.18. Model for Wnt/beta-catenin signaling in NPCs. Schematic representation of the embryonic kidney indicating the Wnt9b-expressing ureteric bud epithelium in red, the renewing NPCs that express Wnt9b renewal targets in light blue, the differentiating NPCs or pre-tubular aggregates (PTAs) that express Wnt9b differentiation targets in dark blue, the cortical stromal cells in green and the medullary stromal cells in orange. Wnt9b ligand is produced by the ureteric bud and secreted to the surrounding MM where it stimulates a low/baseline level of beta-catenin activity, which promotes renewal. High levels of beta-catenin activity which promote differentiation are a result of unknown beta-catenin amplifying signal(s), perhaps emanating from the medullary stroma and/or higher active Wnt ligand in the medullary NPCs.

Discussion

We previously showed that Wnt9b was necessary for both renewal and differentiation of NPCs in the embryonic kidney [40]. Here we demonstrate that the disparate responses of NPCs to Wnt9b can be mimicked by modulating Wnt/beta-catenin levels. Treatment of isolated MMs with different doses of the Gsk3 antagonist LiCl elicits different responses within isolated MMs. Further, two different Wnt transgenes can elicit opposite responses when expressed homogeneously in the NPC population. Although it is not clear why the different transgenes are able to elicit different response, the fact that the response to each transgene can be transposed by modulating Wnt/beta-catenin activity levels suggests that they stimulate different levels of beta-catenin activation. This may be due to differences in mRNA levels driven by the different promoters, differeneces in protein levels/stability or the strength of receptor ligand interactions in the different genetic backgrounds. But together, our data show that distinct levels of Wnt/beta-catenin activity are sufficient to direct the decision of a progenitor to engage in self renewal versus differentiation.

Work done in multiple tissues including the epiblast, hair follicles and the gut [106-110] show pleiotropic roles for Wnt/beta-catenin signaling. Frequently, gradients for the Wnt ligand are invoked in establishing graded beta-catenin signaling in the development of various tissues [122-124]. The simplest models for morphogen gradients propose ligands diffusing freely from their source resulting in protein concentrations decreasing with distance from the source. Such a model for Wnt signaling has been disfavored, as studies have shown that the ligand is tightly associated with the membranes of both the producing and receiving cells. Indeed, using the GFP tag on the Hoxb7-Cre;Rosa-Wnt1

allele, we are unable to detect tagged Wnt protein beyond the ureteric bud ECM casting doubt on whether a freely diffusible ligand is present. Most recently, a model where the Wnt gradient is created by dilution of the ligand as the source and receiving cells divide, was proposed for the intestinal stem cell niche. This model also does not appear feasible for the embryonic kidney. First, in the developing kidney, the oldest (differentiating) NPCs require the highest levels of beta-catenin, not the lowest as would exist in the dilution model. Secondly, as mentioned above, the renewing and differentiating cells are positioned at the same distance relative to the Wnt9b source [78, 119]. We can also rule out the possibility that activation of the Wnt9b target gene Wnt4 within the responding cells is the amplifying mechanism as Wnt9b differentiation targets do not require Wnt4 for their expression [40]. Further, recent evidence suggests that the role for Wnt4 in kidney development is beta-catenin independent [41]. Although we cannot rule out the possibility that the ureter/distal collecting ducts produce the highest levels of Wnt protein and this creates a gradient, the fact that expression of Rosa-Wnt1 transgene in these cells does not rescue a Wnt9b mutant nor does ablation of Wnt9b from these cells affect the balance of renewal vs differentiation casts doubt on this model [40]. Another possibility is that although we do not detect differences in the absolute levels of the Wnt1-GFP fusion protein secreted from the cortical vs. medullary tips of the UB, there may be spatial differences in post-translational modifications of the Wnt ligand that affect its activity.

Based on our data and previous work by others, we favor a model wherein signals from the progenitor cell niche non-autonomously regulate beta-catenin levels in the responding cells independent of Wnt9b ligand concentration. Specifically, we propose that the stroma produces a signal that modulates beta-catenin activity. Although a model

wherein stromally produced signals antagonize beta-catenin activity is also consistent with the data produced in this study, our previous studies showed that ablation of the stroma resulted in NPC renewal without differentiation [12]. In other words, without the stroma, the program associated with low level beta-catenin activity is observed, which is inconsistent with the hypothesis that the stroma produces a Wnt/beta-catenin antagonizing signal. The identity of the beta-catenin agonizing signal is still unknown. Although other Wnt ligands are candidates, we feel this is unlikely for several reasons. First, although the stroma does express several Wnts (including Wnt4, Wnt5a, Wnt11), all are expressed at later stages of development and in the deeper medullary stroma. Thus, they are not expressed in the right place or at the right time to play a role. In addition, gene ablation studies are inconsistent with such a role. Further, it is important to note that in the absence of the ureteric bud or Wnt9b, neither class of beta-catenin target genes is expressed. If stroma simply produced another Wnt that acted additively with Wnt9b to amplify beta-catenin levels, one might predict that upon removal of the UB, we would see renewal only. This is not the case. Although we previously showed that the stromally produced factor Fat4 could modulate Wnt/beta-catenin response in the NPCs, we have no evidence that this factor works as a beta-catenin agonist [12].

We propose that the stroma produces an agonist that amplifies beta-catenin but only in response to a Wnt ligand. One candidate for such a signal is a member (s) of the R-spondin family of beta-catenin agonists. Interestingly, Rspo3 is expressed in the stroma at the earliest stages of kidney development. Unfortunately, Rspo3 mutants do not survive past E10.5 due to vascular defects preventing us from analyzing kidney development. However, addition of recombinant Rspo3 to Six2-Cre;Rosa-Wnt1 NPCs did not induce

differentiation (data not shown) suggesting that this may not be the agonist. Identification of the stromal agonist will be of great interest in future studies focused on nephron endowment, repair, engineering and diseases that affect beta-catenin activity within the NPCs such as Wilms' tumors.

CHAPTER FOUR

Methods and Materials

Mouse strains:

The mouse alleles Wnt9b$^{+/-}$, Hoxb7-Cre, Rosa-Wnt1 (Carroll et al., 2005), Six2-CreERT2, Six2-Cre, Foxd1-Cre (Kobayashi et al., 2008), Ksp-Cre (Shao et al., 2002), CAG-Wnt9b (Kiefer et al., 2012), Numb$^{floxed/floxed}$ [98], Numblike$^{floxed/floxed}$ [125] and Rosa-YFP are all previously described. With the exception of the CAG-Wnt9b line, all have been deposited at the Jackson labs for distribution.

To generate the Pax8-YFP Bac Transgenic (Pax8-YFP^{tg}), a 166 kb C57BL/6J mouse bacterial artificial chromosome (BAC) from the RPCI23 library was identified that encompassed the entire Pax8 locus. This BAC clone was modified by targeting a Yellow Fluorescent Protein (YFP) coding sequence into the ATG start site of the Pax8 locus. This inserted a YFP fusion protein coding sequence, SV40 polyA signal, and a FRT-flanked Kanamycin resistance gene cassette. Once correctly targeted clones were identified and the selection cassette was removed using FLP recombination. This final modified BAC clone was linearized and prepared for microinjection into CD-1 zygotes. Transgenic founders were obtained and bred to C57BL/6 mice to establish the Pax8-YFP-tg colony. The BAC clone backbone vector (pBACe3.6) was not removed prior to microinjection and thus is retained in the transgenic animals. The Chloramphenicol Resistance (CmR) gene present in the vector is used for genotyping mice carrying the Pax8YFP transgene.

Ex-vivo culture:

Organ culture, metanephric mesenchyme isolation at E11.5 and small molecule treatments (LiCl, CHIR, IWR1 and Iwp2, IwpL6) were performed as previously described

(Carroll et al., 2005, Karner et al., 2011). Metanephric mesenchyme of E11.5 kidneys were isolated away from the UB by treating the kidneys with Tyrode's solution (Vize P.D., Woolf A.S., Bard J.B.L.) at room temperature for 70sec and then with Fetal Bovine Serum on ice for 10min followed by surgical micro dissection using a stereomicroscope. The isolated mesenchyme or the intact kidneys were cultured on an air-media interface using a transmembrane filter and regular DMEM media with or without the addition of small molecules for 48 hours or 5 days. LiCl was used at concentrations 7mM, 10mM, 12mM and 15mM diluted in sterile water. IWR1 and Iwp2 or Iwpl6 containing media were changed every 12 hours and 24 hours respectively. All assays were repeated 5 or more times with at least 3 individual kidneys/isolated mesenchymes from 3 distinct embryos per replicate. After culture, the tissue with the transmembrane filter still attached was fixed in 4% PFA overnight at 4degrees and washed in PBS 3X5minutes before proceeding with further analysis.

In-situ Hybridization:

The samples were prepared the exact same way samples for immunofluorescence were prepared. Following sucrose treatment the samples were dehydrated in an ethanol series (30%, 50%, 70%) and stored at a -20 degrees freezer if they could not be subjected to in situ hybridization right away. Whole-mount and section in-situ hybridization were performed as previously described (Karner et al., 2011,[12]). Antisense probes used were C1qdc2, Cited1, Fam19a5, Wnt4. Restriction enzyme and transcription enzymes are as described in the above-cited articles.

PTA Quantification of LiCl treated MM:

LiCl treated MMs were stained with an antisense probe to C1qdc2 and distinct domains of C1qdc2 expressing cells were counted. Large domains of expression (presumably representing 2 or more PTAs fused together) were counted as two PTAs irrespective of their size. Student's t-test with two tailed distribution and two samples with unequal variances was used to determine significance. T-test was run between 10mM and 12mM samples and, 10mM and 15mM samples as two different tests.

Histology/Immunofluorescence on tissue section:

Hematoxylin and Eosin staining and immunofluorescence on all tissue sections except Fig. S7A-B were performed on kidneys sections as described[12]. Tissues in Fig. S7A-B were fixed in 4% PFA for 30min at room temperature and subjected to immunofluorescence as mentioned above. Cultures of isolated mesenchyme or intact kidneys following fixation were cryopreserved in 30% Sucrose in PBS overnight and frozen in OCT (Tissue Tek). Tissue blocks were sectioned to 10um thick sections and subjected to the same immunofluorescence protocol. The antibodies against Six2, Cited1, E-Cadherin, Pan-cytokeratin, GFP, Lotus tetragonolobus lectin (LTA), Lef1, Laminin were used as previously described [12]. Pax8 antibody (Proteintech, 10336-1-AP for Fig. 3 and Ab189249 for Fig. S4) at 1:700, Numb antibody (ab14140) at 1:700 through tyramide amplification, Caspace-3 (Cell Signaling, #9662) at 1:500, WT-1 (sc-393498) at 1:200 and Nephrin (ab58968) at 1:750 dilutions were used following above mentioned protocol.

Following immunostaining the sections were treated with Dapi or sytox for 10min at RT and mounted using 50% glycerol in PBT.

Immunofluorescence on NPCs:

The cells were fixed in 4% PFA for 10 min at RT. After fixation, the following steps were performed to immunostain the cells: 3 washes with PBST (PBS without calcium magnesium salts + 0.1% TritonX 100) for 5 min each. Blocking for an hour in PBST+10%FBS block. Incubation with primary antibodies (Six2 at 1:100, Lef1 at 1:200) for 2 hours. 3 washes with PBST. Incubation for an hour with Alexa fluor secondary antibodies. 3 washes with PBST. Incubation of Dapi at 1:1000 for 10 min. 3 washes with PBST.

Cell Division Angle Measurement:

E11.5 kidney tissues were dissected, processed, sectioned at 30uM thickness and immunostained with antibodies to Six2, Laminin or Entactin and Sytox dye following above mentioned protocol. Dividing NPCs at Anaphase were identified by the distinct positions and captured as Z-stacks. The images were processed in Imaris. The chromosomes of a dividing cell were marked, and the midpoint measured and marked. The shortest perpendicular line from the midpoint to the ureteric bud was drawn. Then, a tangent to the UB that intersects this perpendicular line was drawn. The co-ordinates of the endpoints of the tangent and the two chromosomes of the dividing cell were noted.

Using these four points, the angle of intersection was calculated in Microsoft Excel sheet using the formula

Wnt1 or CAG-Wnt9b mosaic expression analysis:

Pregnant mothers carrying potential Six2-CreERT2; Rosa-Wnt1; $Wnt9b^{-/-}$ or Six2-CreERT2; CAG-Wnt9b; Rosa-YFP; $Wnt9b^{-/-}$ were gavaged with 1.5mg/40gm body weight tamoxifen at E10.5 of pregnancy. At E15.5, kidneys of these embryos were dissected, sectioned at 10uM each and performed H&E or immunostained with antibodies against Cited1, GFP, Pan-Cytokeratin or Pax8.

NPC isolation, & culture:

NPCs from E17.5 kidneys of wild type, Six2-Cre;Rosa-Wnt1 or Six2-Cre;CAG-Wnt9b kidneys were isolated and cultured as per published protocol [121]. Cells were cultured in 24 well plates at 100K/well seeding density and passaged or set up for differentiation at 70% confluency.

Glomerular Counts:

Serial sections where every 3 sections are skipped were taken and stained with Hematoxylin and Eosin. Images at 10x magnitude were taken of the entire kidney sections. The glomeruli were manually counted from the images. Student's t-test was used to obtain p-value.

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