BETA-CATENIN AND DEVELOPMENT OF THE UROGENITAL SYSTEM

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Dedication

I would like to dedicate my thesis to Marie Kinman a.k.a. Grammy.

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BETA-CATENIN AND DEVELOPMENT OF THE UROGENITAL SYSTEM

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The urogenital system is composed of the kidneys, gonads, urinary and reproductive tracts. Components of the urogenital system play many important roles in the body; kidneys function by regulating the body's fluid volume, acidity and mineral composition, while the reproductive tract is necessary for propagation of our species. Therefore, the study of urogenital system development is important in the understanding of disorders associated with both the kidneys and the reproductive tract and their treatment.

Urogenital system development begins with the formation of an epithelial tube, called the Wolffian duct. From the Wolffian duct forms a ureteric bud, which, along with the metanephric mesenchyme, will undergo a series of morphogenetic changes, eventually giving rise to the adult kidney. The Wolffian or Müllerian ducts, along with the bipotential gonads, will develop into the male or female reproductive tracts, respectively. Although many signals are involved in development of the urogenital system, canonical Wnt/beta-catenin signaling is known to play a significant role.

To better understand the role Wnt signaling plays in reproductive tract development, we conditionally removed beta-catenin from the Wolffian duct using a HoxB7cre line of mice. We determined that beta-catenin is necessary for Müllerian duct formation. Additionally, removal of beta-catenin from the Wolffian duct leads to premature differentiation, preventing degradation of the Wolffian duct in females and inhibiting proper formation of the Wolffian duct into components of the male reproductive tract. In addition to our mouse model, we validated the efficacy of small molecule inhibitors of Wnt signaling in embryonic kidney culture. Functional small molecule Wnt inhibitors will provide an important tool for the continued study of urogenital system development along with the potential treatment of diseases associated defective Wnt signaling.

Table of contents

List of Publications

Grote, D., et al., 2008. Gata3 acts downstream of beta-catenin signaling to prevent ectopic metanephric kidney induction. PLoS Genet. 4**,** e1000316.

Marose, T. D., et al., 2008. Beta-catenin is necessary to keep cells of ureteric bud/Wolffian duct epithelium in a precursor state. Dev Biol. 314**,** 112-26.

Merkel, C. E., et al., 2007. Molecular regulation of kidney development: is the answer blowing in the Wnt? Pediatr Nephrol. 22**,** 1825-38.

List of Figures

List of Abbreviations

APC	adenomatous polyposis coli
Aqp	aquaporin
BAT-gal	beta-catenin activating transgene
CamKII	calmodulin-dependent protein kinase II
CD	collecting duct
CE	convergent extension
CK1	casein kinase 1
dact/frd	dapper/frodo
Dgo	diego
Dkk	dickkopf
Dpl	daple
Dsh/Dvl	dishevelled
Fmi/Stan	flamingo/starry night
Fz	frizzled
GSK3	glycogen synthase kinase 3 beta
HIG2	hypoxia-inducible protein-2
Inv	inversin
IW	Inhibitor of Wnt signaling
IWP	inhibitor of Wnt production
IWR	inhibitor of Wnt response
JNK	c-Jun N-terminal kinase

LiCl	lithium chloride
Lif	leukemia inhibitory factor
Lrp	lipoprotein receptor–related protein
MD	Müllerian duct
MDCK	Madin-Darby canine kidney
MET	mesenchymal to epithelial transition
MIS	Müllerian inhibitory substance
MISR	receptor of Müllerian inhibitory substance
MM	metanephric mesenchyme
nkd	naked cuticle
NPNII	nephronophthisis type II
PCP	planar cell polarity
Pk	prickle
PKC	protein kinase C
PKD	polycystic kidney disease
RCC	renal cell carcinoma
RV	renal vesicle
sFRP	secreted frizzled-related protein
SRY	sex-determining region Y
Stbm/Vang	strabismus/van gogh
TCF/LEF	T-cell factor/lymphoid enhancing factor
TGFbeta	transforming growth factor beta
UB	ureteric bud

WD	Wolffian duct
Wif	Wnt inhibitor factor
ZO1	zonula occludins

Chapter 1:

Introduction

During embryonic development, three sets of kidneys form within the mammalian intermediate mesoderm (Vize, 2003). The three kidney types form in a temporal as well as anterior-to-posterior sequence. The first to form (and the most anterior) is the pronephros. Although in mammals this organ appears to be non-functional from a physiological standpoint, it is essential for the development of other tissues and cell types, including the mesonephric kidney. The mesonephros appears to be physiologically functional in that its tubules are vascularized and it does produce urine; however, this function is completely dispensable for normal embryogenesis in mice. The situation is slightly different in humans, where mesonephric function plays an important role in maintaining amniotic fluid, which is essential for the proper development of other organs such as the lungs (Duenhoelter and Pritchard, 1977). In addition, the mesonephros is required for formation of the male and female reproductive tracts in all vertebrates. The last kidney to form is termed the metanephros, and this organ will become the functional adult kidney in mammals.

The metanephric kidney serves an essential role in tissue homeostasis by regulating the balance of water and electrolytes in the plasma. It also excretes metabolic waste products and regulates the production of certain hormones. As defects in the development of the urinary system constitute some of the most common human birth defects, a better understanding of the genes required for formation of this organ is of great interest. Here, we will review data demonstrating that the Wnt pathway plays critical roles in multiple cellular events that occur during the development of the mammalian kidney.

Kidney development

Development of the metanephros begins on embryonic day (E)10 in mice (approximately E32 in humans), when a caudal portion of the Wolffian duct (WD) adjacent to the hindlimbs branches dorsally and invades a population of pre-specified mesenchyme known as the metanephric mesenchyme (MM) (figure 1-1a,b). This epithelial bud, known as the ureteric bud (UB), will continue to branch within the expanding MM throughout the embryonic period and, perhaps, for a short period after birth. Ultimately, the UB derivatives will form the collecting duct system and extra-renal ureter.

Shortly after invasion by the UB, a group of mesenchymal cells within the MM will condense and form a cap around the tips of the UB (figure 1-1c). A subset of cells within the condensate will aggregate and undergo a mesenchymal-to-epithelial transition, forming the renal vesicles (RVs) (figure 1-1d). The majority of the nephron, from the renal corpuscle through the distal tubule, is derived from the RV. The RVs will undergo morphogenesis, forming comma- then S-shaped bodies (figure 1-1e). A capillary will invade the cleft of the proximal part of the S-shaped body and begin to form the glomerulus. The distal portion of the S-shaped body will fuse to the UB, forming one continuous lumen. Non-epithelial portions of the MM will contribute to the smooth muscle, stroma and, perhaps, to the microvasculature of the kidney (Kobayashi et al., 2005b). The processes of UB branching, RV formation, growth and morphogenesis reiterate themselves during development until, depending on the organism, the kidney takes on its final form and size. In the mouse, this is approximately 1 cm in rostral/caudal

length and 10,000–20,000 nephrons, while the average adult human kidney is 10–12 cm in length and contains 500,000–1,000,000 nephrons.

Embryological studies have shown that kidney development depends on inductive interactions between the UB and the MM for the survival, proliferation and differentiation of the MM and the reiterative branching of the UB (Barasch et al., 1996; Basson et al., 2005; Grobstein, 1953; Grobstein, 1955; Grobstein, 1957; Kispert et al., 1998; Kispert et al., 1996; Mori et al., 2003; Sainio et al., 1997). Identifying the ligands and receptors regulating these processes has been a major emphasis in the field over the past 20 years, and substantial progress has been made.

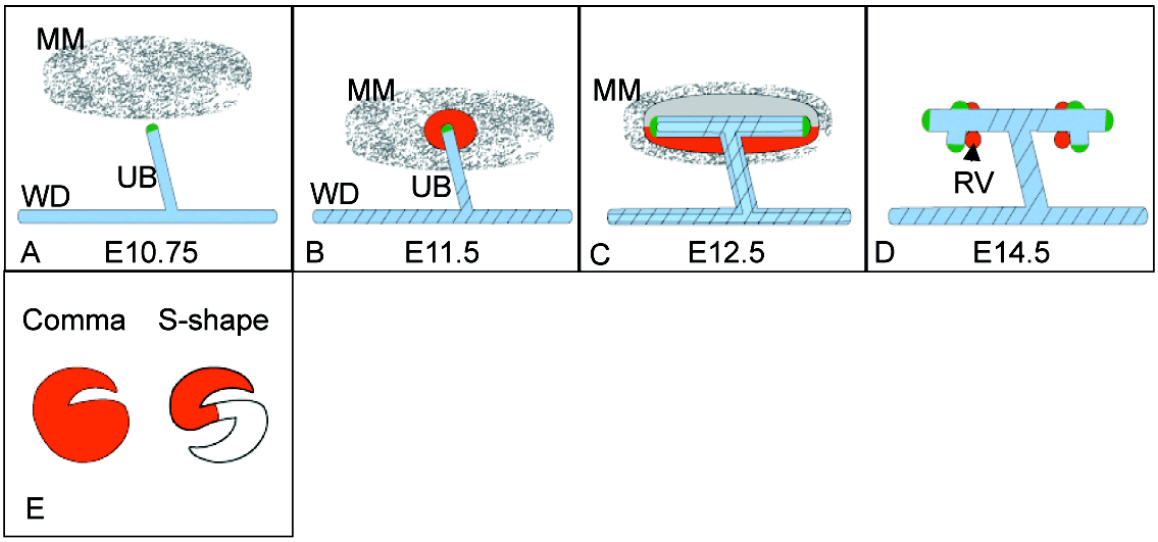


Figure 1-1. Schematic of kidney development. (A) At E 10.75, the UB forms from the WD. (B) The UB invades the MM at E 11.5. (C) The UB forms a T-bud, and the MM condenses. (D) Branching morphogenesis of the UB takes place, and the RVs begin to form. (E) The RVs will then become comma- and S-shaped bodies, and the branching UB forms the collecting duct. The S-shaped body will fuse to the collecting duct and undergo further morphogenesis to become the nephron (not shown). (A–E) Expression of Wnts in the developing kidney are indicated as follows: green Wnt11, blue Wnt9b, red Wnt4, diagonal lines Wnt7b. UB=ureteric bud, WD=Wolffian duct, MM=metanephric mesenchyme, RV=renal vesicle.

Reproductive tract development

Proper development of the male and female reproductive tracts is necessary for continuation of our species. Reproductive tract development begins with formation of the WD from the intermediate mesoderm around E9.0. The WD is an epithelial tube that forms from the intermediate mesoderm and extends caudally until it reaches the cloaca by E10.5. After the WD has fully formed, a mesoepithelial tube, called the Müllerian duct (MD), forms parallel to the WD from E11.5 to E13.5. Before sexual differentiation takes place, both males and females are sexually dimorphic, forming both the WD and MD along with bipotential gonads. In males, the gonads differentiate into testes and the WD gives rise to portions of the male reproductive tract. The gonads in females differentiate into ovaries with the MD differentiating into portions of the female reproductive tract.

Development of the MD is considered biphasic. The first phase involves invagination of the coelomic epithelium near the anterior portion of the WD. After the initial invagination, the MD tip then elongates parallel to the WD until it reaches the cloaca. Many studies have shown that the WD is necessary for this elongation phase and ultimately, formation of the MD (Gruenwald, 1941). In both Lim1 and Pax2 mutant mice, the WD degrades and MD formation is initiated but fails to form fully (Kobayashi et al., 2005a; Miyamoto et al., 1997; Torres et al., 1995). In addition to acting as a physical guide for MD elongation, there is also evidence of a molecular interaction between the WD and the MD. In Wnt9b mutants, MD formation is initiated but the duct does not fully elongate (Carroll et al., 2005). Wnt9b is expressed in the WD epithelia but the WD is not affected in Wnt9b mutants. These results support a role for the WD interacting with the MD at a molecular level.

Previously, it was unknown whether the second phase, MD elongation, occurred due to proliferation and migration or due to a MET. To determine which process is necessary for the elongation phase, Dr. Carroll cultured wild-type E11.5 urogenital systems with one MD tip surgically removed. After culturing the explant for three days, Dr. Carroll visualized the MD and WD by staining for Pax8 mRNA (data not shown). A MD does not form when the tip is removed. In addition, Pax8 mRNA expression shows that the WD did not degrade. These results indicate that the MD forms due to migration and not MET. Furthermore, Orvis et al. performed additional studies showing that the MD elongates due to proliferation and extension of the distal tip of the MD (Orvis and Behringer, 2007).

Gonadal differentiation of the male requires the sex-determining region Y (sry) gene, located on the Y chromosome. Production of the SRY protein, or testis determining factor, initiates sex determination of the male, causing differentiation of the gonad into the testis. During differentiation into the testes, Sertoli cells form and produce Müllerian inhibitory substance (MIS). Production of MIS will lead to the degradation of the MD. Male gonads will further differentiate and form Leydig cells, which make up the interstitial cells of the testis. Leydig cells are necessary for production of androgens. Secretion of testosterone prevents degradation of the WD, allowing it to differentiate into such portions of the male reproductive tract as the epididymis, vas deferens and seminal vesicle.

The female reproductive tract is thought to be the default state. This theory is due to the fact that in the absence of SRY, the gonads differentiate into ovaries. Furthermore, the ovaries neither produce MIS nor do they produce testosterone. In the absence of

testosterone, the WD is degraded whereas in the absence of MIS, the MD is maintained. The MD can then differentiate into portions of the female reproductive tract such as the oviducts, uterus and upper vagina.

Several Wnts are expressed in the developing mouse urogenital system. Subsequent studies using misexpression and functional deletion have shown distinct roles for this pathway in organ formation and disease. This introduction will cover the known and hypothesized roles for Wnt signaling in development of the mouse kidney and reproductive tract.

Wnt signaling

The Wnts make up a family of secreted glycoproteins that have been implicated in embryonic induction, cell polarity generation, and cell fate specification in metazoan species from hydra to humans (Miller, 2002; Wodarz and Nusse, 1998). The mammalian genome contains 19 individual Wnt ligands that elicit distinct subcellular events based on the environment in which the signal is received. In the majority of cases, signaling is initiated upon ligand binding to the cysteine-rich, extra-cellular domain of a frizzled (Fz) seven-pass transmembrane receptor and, in some cases, the low-density lipoprotein 5 and 6 co-receptors (Lrp5 and Lrp6 in mammals, arrow in flies) (Bhanot et al., 1996; Bhanot et al., 1999; Chen and Struhl, 1999). Upon binding of the ligand to its receptor(s), there are several distinct signal transduction cascades utilized by the Wnts that can be roughly grouped as the canonical/beta-catenin pathway and the non-canonical/beta-catenin independent pathways.

The canonical pathway

The canonical pathway utilizes beta-catenin as a transcriptional regulator. In the absence of ligand/receptor interactions, beta-catenin levels are regulated by a destruction complex consisting of two scaffolding proteins, axin and adenomatous polyposis coli (APC), and two serine/threonine kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) (figure 1-2a) (Giles et al., 2003; Logan and Nusse, 2004). beta-catenin binds to the complex, where it is subsequently phosphorylated by CK1 and GSK3, targeting it for ubiquitination and destruction by the proteasome. The binding of Wnt to Fz activates the cytoplasmic protein disheveled (Dsh/Dvl). Activation of Dvl leads to disruption of the beta-catenin destruction complex (through recruitment of axin to the membrane, where it interacts with the cytoplasmic tail of an Lrp) (figure 1-2b). Disruption of the destruction complex results in the accumulation of beta-catenin in the cytoplasm. Stabilized, cytoplasmic beta-catenin translocates into the nucleus, where it competes with members of the groucho family of co-factors for interactions with the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family of transcription factors. When complexed with groucho and certain other co-factors, the Lef/Tcfs act as transcriptional repressors. It is generally thought that, when complexed with β-catenin, the Lef/Tcfs act as transcriptional activators, although there is an increasing body of data suggesting that certain isoforms of the Lef/Tcfs may act as repressors, even when bound to beta-catenin (Arce et al., 2006).

It should be noted that, in addition to its crucial role in mediating canonical Wnt signaling, beta-catenin also plays a role in establishment of the adherens junctions and interacts with the actin cytoskeleton by binding membrane bound E-cadherin and

cytoplasmic α-catenin. It has been suggested that Wnt signaling, in part, functions through maintaining a balance between cytoplasmic/nuclear and junctional beta-catenin (Nelson and Nusse, 2004; Perez-Moreno and Fuchs, 2006).

The non-canonical pathway

Wnt signaling also appears to trigger β-catenin-independent signaling events. The two most frequently discussed non-canonical/β-catenin-independent pathways are the Ca2+-releasing pathway and the planar cell polarity/convergent extension (PCP/CE) pathway. In the Ca2+-releasing pathway, Wnt binding stimulates calcium flux and activates calcium-sensitive factors such as protein kinase C (PKC) and calmodulin-dependent protein kinase II (CamKII) (Sheldahl et al., 1999; Veeman et al., 2003a). This pathway may work in a manner analogous to the Wnt/beta-catenin pathway by targeting calcium-responsive transcription factors [26]. There is some disagreement as to whether the calcium pathway signals through Dvl or even the Fz receptors.

Another non-canonical pathway identified is the planar cell polarity (PCP)/convergent extension (CE) pathway, so-named because of the cellular processes the pathway is thought to mediate (figure 1-2c). PCP describes the polarization of cells within the plane of the tissue, while convergent extension is a morphogenetic process that takes place during gastrulation, neurulation and organ formation and describes the intercalation of cells in an epithelial sheet to form a longer and narrower strip of tissue (Fanto and McNeill, 2004; Karner et al., 2006b).

Components of the PCP/CE pathway were first identified in Drosophila mutants and affected the orientation of cuticular bristles, wing hairs and the ommatidia in the eye. Some of the molecules identified had previously been implicated in Wnt signal

transduction, including Fz and Dvl, thus leading to the speculation that this may be a Wnt-mediated process. In flies, a molecular readout of PCP is the localization of the proteins strabismus/van gogh (Stbm/Vang), and prickle (Pk) on the proximal side of the cell and diego (Dgo), Fz and Dvl on the distal side. Another protein, flamingo/starry night (Fmi/Stan) is also required for PCP, but its localization is not polarized.

Over the past several years, it has become clear that orthologs of the fly PCP/CE genes are essential for various aspects of vertebrate embryogenesis, including gastrulation movements, pelage development and inner-ear formation. Importantly, within the sensory epithelium of the mouse inner ear, Vangl2 (a flamingo homolog), Fz-3 and Fz-6 proteins are asymmetrically localized within the plane of the epithelium, suggesting that the sub-cellular localization, and perhaps function, of at least some of the core PCP components is conserved in mammals (Montcouquiol et al., 2006; Wang et al., 2006b).

Signaling through the PCP/CE pathway activates alternative downstream elements of Dvl, such as the GTPases Rho, Rac and Cdc42 and the c-Jun N-terminal kinase, JNK (Bejsovec, 2005; Karner et al., 2006b; Veeman et al., 2003a). Ultimately, the activation of these molecules orients the cytoskeleton and its associated organelles and protein complexes (Karner et al., 2006a). This pathway has been implicated in oriented cell migration and oriented cell divisions that affect tissue growth and morphogenesis.

How Wnt signaling mediates PCP is unclear. No mutation of a Wnt ligand has been associated with PCP defects in Drosophila, causing some speculation that this is a Wnt-independent event. However, there is evidence of Wnt involvement in PCP establishment in both C. elegans and vertebrates (Veeman et al., 2003a). In the C. elegans

early embryo, the Wnt Mom-2 is both necessary and sufficient for spindle orientation and Fz localization in a transcription-independent manner, and the relative location of the Wnt appears to establish the orientation of the polarity, suggesting that the ligand may play an instructive role in establishing planar cell polarity (Goldstein et al., 2006). Wnt5a and Wnt11 regulate PCP and CE movements during organogenesis and gastrulation in zebrafish and Xenopus embryos, although the evidence suggests that, in this system, the ligands may play permissive roles (Bejsovec, 2005; Heisenberg et al., 2000; Tada and Smith, 2000).

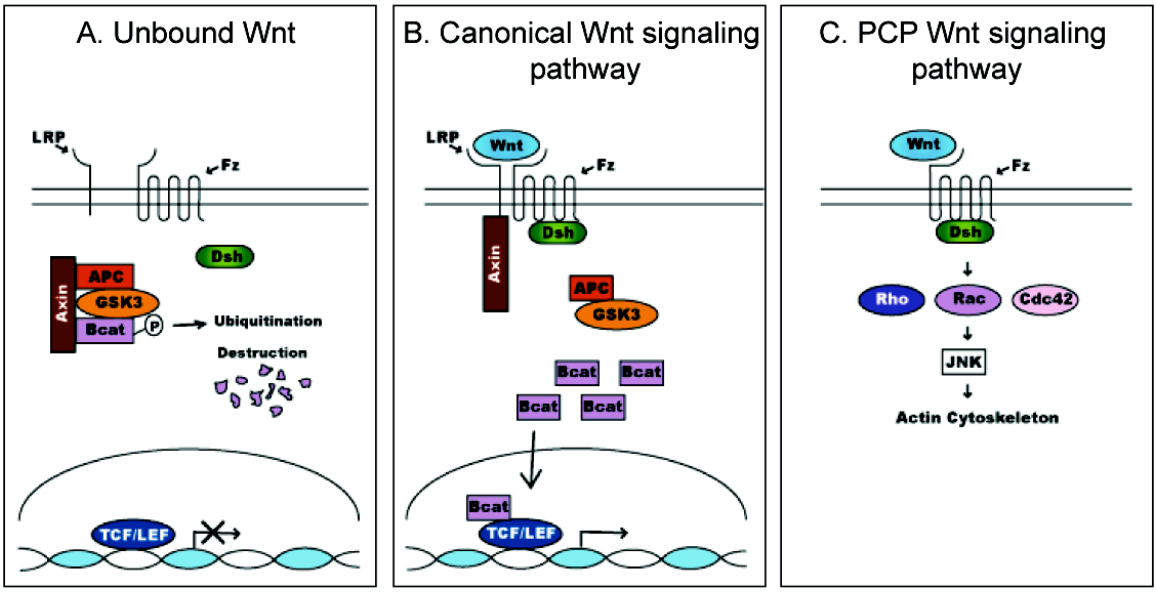


Figure 1-0. Summary of Wnt signaling. a In the absence of bound Wnt ligand, β-catenin is degraded, due to phosphorylation by GSK-3 beta and binding to the destruction complex. b In canonical signaling, binding of a Wnt to its Fz receptor and Lrp co-receptor results in inactivation of the destruction complex. This allows β-catenin to accumulate in the cytoplasm and translocate into the nucleus, where it activates transcription of Wnt target genes in cooperation with Lef/Tcf co-factors. c In the planar cell polarity (PCP) pathway, Rho, Rac and Cdc42 act downstream of Dsh/Dvl and function to rearrange the actin cytoskeleton and establish cell polarity. In vertebrates, this is thought to be a Wnt-dependent process

Determination of pathway specificity

What determines pathway usage in vivo is still not well understood. Historically, it was thought that pathway specificity was determined by the ligand itself. The Wnts were grouped into canonical or non-canonical classes based on their ability to transform C57MG cells, induce secondary axes in Xenopus embryos or induce tubule formation in isolated kidney mesenchyme. Wnts that could act positively in these assays were considered canonical Wnts, while those that could not were considered non-canonical. Although these rough classifications were used for many years, it was soon apparent that not all Wnts could be neatly grouped into one class or the other. Although Wnt1, Wnt3a and Wnt8 always seemed to trigger the canonical event, and Wnt5a always appeared to act non-canonically, other Wnts acted in a canonical manner in some assays and a non-canonical manner in others. For instance, Wnt4 cannot transform C57s or induce a secondary axis in Xenopus (putting it in the non-canonical class), but it does induce tubule formation in isolated mouse mesenchyme. Wnt11 does transform C57s but does not induce a secondary axis or induce tubules. Much of this discrepancy could be explained if there was binding specificity between ligands and various receptors and that either affinity for the receptor determined pathway usage, or that different receptors signaled specifically through the canonical or non-canonical pathways. In support of the former hypothesis, He et al. showed that Wnt5a was capable of acting canonically (inducing a secondary axis in Xenopus) if its mRNA was co-injected with that of Fz5. They hypothesized that the inability of Wnt5a to act canonically was due to the absence of its "canonical receptor" from the Xenopus early embryo.

Subsequent studies have shown that, in fact, Wnt5a, and other so-called non-canonical Wnts, actively inhibit canonical signaling (Topol et al., 2003). In some cases, this ability to repress canonical signaling is mediated by a distinct class of previously orphan, tyrosine kinase-like receptors known as the Rors (Mikels and Nusse, 2006). Ror2 activity appears to activate Jnk, and active Jnk has been shown to prevent accumulation of nuclear beta-catenin (Liao et al., 2006; Oishi et al., 2003). Thus, a potential model is that, if a Ror is present, Wnt5a binds to it and inhibits canonical signaling through activation of Jnk. If Ror2 is absent, Wnt5a can bind to a frizzled receptor and activate canonical signaling.

A further level of regulation is most likely determined by the cytoplasmic environment in which the signal is received. Various intracellular inhibitors of the canonical pathway have been identified, and several of these appear to work on the Dvl protein, acting as switches that divert signaling through the non-canonical pathway (Brott and Sokol, 2005; Wharton, 2003). In addition, there are several known co-factors for Lef/Tcf that may compete with beta-catenin for binding and thus block canonical pathway activation (Arce et al., 2006).

The role of Wnts in kidney development

Analysis of mouse embryos by in situ hybridization has revealed the expression of six Wnts in the developing kidney: Wnt2b, Wnt4, Wnt6, Wnt7b, Wnt9b, and Wnt11 (Carroll et al., 2005; Herzlinger et al., 1994; Itaranta et al., 2002; Kispert et al., 1996; Lin et al., 2001; Stark et al., 1994). Another eight Wnts have been shown to be present by sequencing of urogenital system cDNAs, with only Wnt5b identified as being specifically

located in the kidney. In this section we will cover reported and hypothesized roles for several Wnts that have been implicated in various aspects of kidney development.

Wnts in renal vesicle formation

An interest in Wnt signaling in the process of renal vesicle formation arose in 1994, when it was discovered that Wnt1 could substitute for the UB as an inducer of tubulogenesis (Herzlinger et al., 1994). Wnt1 is not expressed in the developing kidney, suggesting that it mimics the activity of another Wnt that fulfills the role in vivo. Since this initial observation, it has been discovered that several Wnts are expressed in the developing UB, including Wnt6, 7b, 9b and 11. Both Wnt6 and 9b are expressed throughout the WDs and the ureteric bud/ proximal collecting duct system from E10.5 through birth, although expression levels are lower in the ampullary tips (figure 1-3c,g,k,o and (Carroll et al., 2005; Itaranta et al., 2002)). Wnt7b is weakly expressed in the WD and the stalk of the UB but not in the vertical portion or tips of the bud at E11.5 and E12.5 (figure 1-3b and f) (Carroll et al., 2005). At later stages, Wnt7b is expressed in the ureter and distal collecting duct system (figure 1-3j and n). Wnt11 is expressed in the WD at E9.0 (Kispert et al., 1996). As the UB invades the MM, Wnt11 is confined to the branching tip of the bud, with no expression in the stalk or the cranial WD (Kispert et al., 1996). When the UB undergoes its first bifurcation at E11.5, Wnt11 expression splits, and Wnt11 is expressed only at the tips of the T bud (figure 1-3d, (Kispert et al., 1996)). Wnt11 continues to be expressed at the distal tips of the ureter from E12.5 to E18.5 (figure 1-3h,l,p).

The expression of these ligands in the developing ureteric bud suggested that they could be playing a role in tubule induction. To test the sufficiency of each of these factors

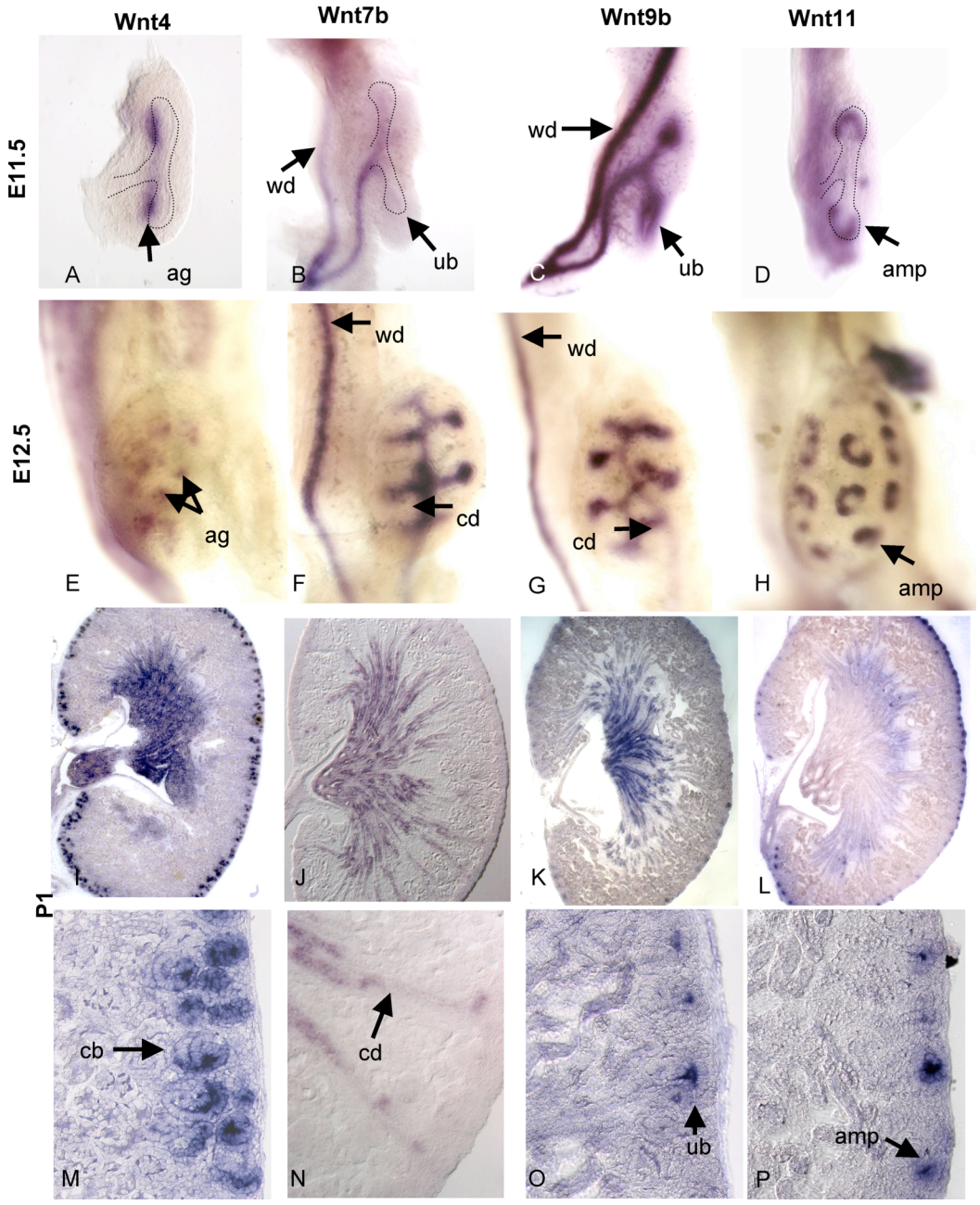


Figure 1-3. Wnt expression in the developing kidney. The expression pattern of Wnt4 (A,E,I,M), Wnt7b (B,F,J,N), Wnt9b (C,G,K,O), and Wnt11 (D,H,I,P) are shown in the developing kidney by whole-mount in situ hybridization at E 11.5 (A–D) and E 12.5 (E–H). Expression of the Wnts at P1 is shown by section in situ hybridization (I-P). M-P are high-magnification views of the cortex of kidneys shown in I–L. All hybridizations were performed with previously characterized probes and techniques [42]. Wnt7b P1 images provided by Jing Yu. ag aggregate, wd Wolffian duct, ub ureteric bud, amp ureteric bud ampullae, cd collecting duct, cb comma-shaped bodies

to induce tubules, cells expressing the individual ligands were co-cultured with isolated E11.0 metanephric mesenchyme. Wnt6, Wnt7b and Wnt9b can all induce tubulogenesis, while Wnt11 cannot (Carroll et al., 2005; Kispert et al., 1998). Of the "inducing" ureteric bud-expressed ligands, in subsequent genetic studies, only Wnt9b was supported for a role in tubule induction.

Wnt9b −/− mice die within 24 h of birth due to agenesis of the kidneys (Carroll et al., 2005). In the Wnt9b −/− mutants, the UB invades the MM, but the UB fails to induce the expression of several pre-tubular aggregate markers, including Wnt4, in the adjacent MM. Wnt4 has also been shown to play an essential role in renal vesicle formation (Stark et al., 1994). However, unlike Wnt9b, Wnt4 is required in the target cells, the so-called

pre-tubular aggregates. Wnt4 mRNA is expressed in the aggregates as early as E11.0 and continues to be expressed in the RVs and the comma- and S-shaped bodies (figure 1-3a,e,i,m). Expression of Wnt4 is lost in wild-type tubules after the S-shaped body fuses to the collecting duct (Stark et al., 1994).

Similar to Wnt9b mutants, Wnt4 −/− pups also lack kidneys and die within 24 h of birth. In the Wnt4 null embryonic kidneys, some branching morphogenesis of the UB takes place, but the mesenchyme fails to convert into epithelial structures by E12.5 (figure 1-4b and (Stark et al., 1994)). At E14.5 a few newly formed RVs can be found in the mutant kidneys, suggesting that another Wnt, perhaps Wnt6 (see below), may be able to compensate for Wnt4 at later stages (Kobayashi et al., 2005b).

Like Wnt9b, Wnt4 is also sufficient to induce RV formation in isolated wild-type MM. Interestingly, Wnt4 can also induce RV formation in Wnt9b mutant MM, suggesting that a major defect underlying the Wnt9b mutant phenotype is the failure to

induce expression of Wnt4 (Carroll et al., 2005). Wnt9b, on the other hand, cannot rescue Wnt4 mutant mesenchyme, suggesting that the two ligands signal through different receptors and/or branches of the pathway. However, Wnt6 can rescue the Wnt4 mutants, and Wnt6 expression levels are increased in Wnt4 mutants, presenting the possibility that it compensates for Wnt4 at later stages in Wnt4 null kidneys (Itaranta et al., 2002).

The current data indicate that, in wild-type mice, UB-produced Wnt9b is necessary for tubule formation, at least in part through its activation of Wnt4 expression in the adjacent mesenchyme. These data suggest that a continuum of Wnt signaling plays a major role in conversion of mesenchyme to epithelium (figure 1-4c).

The precise mechanism for Wnt function in tubule formation is still not known. It has been suggested that survival and/or proliferation of the mesenchyme is necessary, and perhaps even sufficient, for tubulogenesis. Because a relatively normal mesenchymal population is maintained in Wnt9b (and Wnt4) mutant kidneys through at least E14.5 and there is no significant change in the rate of apoptosis in E11.5 Wnt9b mutants, a defect in survival in either of these mutants seems highly unlikely ((Carroll et al., 2005) and data not shown). Although certain survival factors, such as Fgf8, are not expressed in Wnt9b mutant mesenchyme, this is most likely a secondary consequence of a failure to induce pre-tubular aggregate formation and most likely does not play a causal role in the phenotype. In fact, the co-culturing of Wnt9b mutant mesenchyme with recombinant Fgf8 protein does not induce tubule formation or Wnt4 expression (our unpublished observations).

Whether defects in cell proliferation play a role in the Wnt9b mutant phenotype is less clear. Although mesenchymal cells in mutant kidneys survive, they do not appear to

increase in number. Thus, it is possible that the failure to form tubules is secondarily caused by a failure to reach some critical cell number. However, the mutant metanephric anlage expresses a number of genes associated with its specification and it is of normal size at E11.0, even though it does not express Wnt4 or Pax8. The only apparent defect at E11.0 is the failure to induce pre-tubular aggregate markers, suggesting that the mutant phenotype manifests itself prior to any changes in cell number (Carroll et al., 2005). The simplest explanation for the phenotype is that Wnt9b (and Wnt4) are directly involved in the process of mesenchymal-to-epithelial transition and that this secondarily affects cell proliferation but not cell survival.

There are several ways in which Wnts could play a direct role in mesenchymal-to-epithelial transition. One possibility is that Wnt signaling directly regulates cell adhesion.

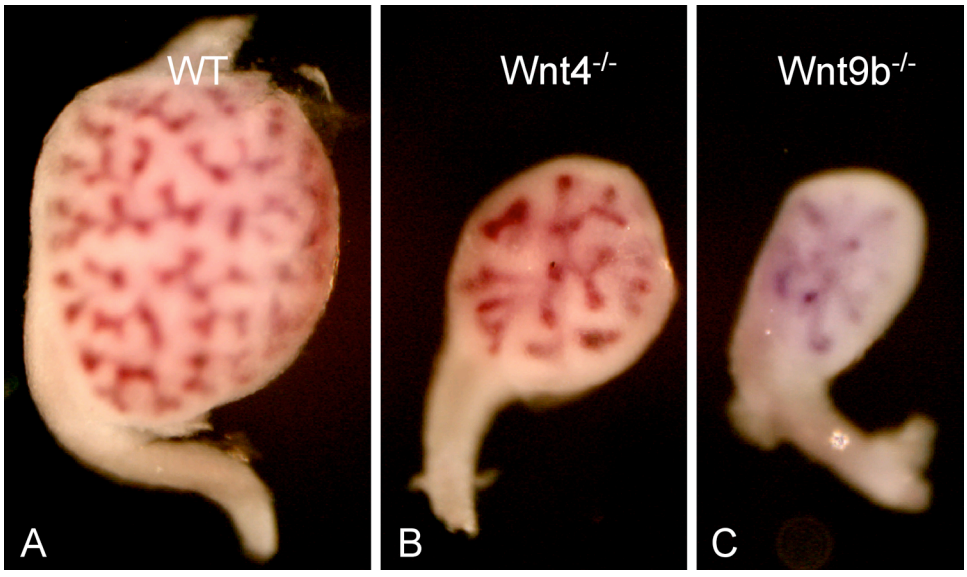


Figure 1-4. Wnt knockout phenotypes. E 14.5 wild-type (WT) (A), Wnt4$^{-/-}$ (B), and Wnt9b$^{-/-}$ (C) kidneys stained with Wnt9b for visualization of the collecting ducts. (B) In Wnt4$^{-/-}$ kidneys, decreased UB branching occurs. (C) In Wnt9b$^{-/-}$ kidneys, there is a more severe defect in UB branching than in Wnt4 mutants

Such a role has been suggested for Wnt4, whose over-expression results in an increase in the expression of the cell adhesion molecule E-cadherin (Vainio et al., 1999b). Alternatively, Wnt signaling could be regulating other sub-cellular processes, such as cell polarization or vesicular trafficking, that are indirectly necessary for cell adhesion and epithelium formation. A better understanding of the role of Wnt signaling in tubule formation will require a more detailed molecular and cellular analysis of Wnt9b and Wnt4 mutants.

Another question that remains is how Wnt signaling relates to the activity of other factors that appear to be sufficient for kidney tubule formation, including the leukemia inhibitory factor (Lif) and transforming growth factor beta (TGFβ) (Barasch et al., 1999; Plisov et al., 2001). Previous studies have shown that, similarly to Wnt9b and Wnt4, both of these factors weakly induce canonical Wnt signaling in isolated mesenchyme. Therefore, it is possible that Lif (or other members of the Il-6 family of cytokines) and/or Tgf-beta signaling function upstream of, or in some way mimics, Wnt activity in the mesenchyme. How this relates to the normal in vivo situation is unclear, as genetic analysis has so far not revealed a direct role for either of these signaling pathways in kidney tubule formation.

Another possibility is that Lif and/or TGFβ function by inducing survival and/or proliferation in cells that have already been weakly induced and that this is sufficient to trigger tubulogenesis. Yang et al. showed that isolated rat mesenchymes already expressed Wnt4 prior to their treatment with Lif, suggesting that Lif was either necessary to maintain the Wnt4 expressing cells in the absence of the bud or was working through a parallel pathway that is required along with Wnt signaling (Yang et al., 2002). The fact

that Wnt4 expression in the rat kidney appears to be independent of the ureteric bud may partially explain why Lif is sufficient to induce tubulogenesis in the rat but not in the mouse, where Wnt4 appears to be dependent on a signal from the bud. It is interesting to note that canonical Wnt signaling appears to be sufficient to induce tubulogenesis in mesenchyme isolated from mouse mutants that do not form a caudal WD or ureteric bud (Gata3 and Gdnf mutants, respectively) and, therefore, have never been exposed to any other duct/bud derived signals (Kuure et al., 2007). These data are suggestive that canonical Wnt signaling acts alone in tubule formation and that other sufficient factors are either acting through the Wnt pathway or through some other mechanism.

The role of Wnt signaling in branching morphogenesis

Several Wnts are expressed in or around the developing ureteric bud. As mentioned, the expression patterns of Wnt6, Wnt7b, Wnt9b and Wnt11 are all consistent with roles in development of this structure. In addition, at later stages of development, Wnt4 and Wnt11 are both expressed in the medullary stroma, possibly signaling back to the adjacent collecting ducts, although it appears that, at least for Wnt4, the medullary expression is actually mediating the formation of smooth muscle cells (Itaranta et al., 2006). However, for Wnt9b and Wnt11, the hypothesized roles in ureteric bud formation are supported by genetics.

The Wnt11 mutant phenotype in mice is variably penetrant, with some mutants dying in utero and others dying shortly after birth (Majumdar et al., 2003). In null embryos that survive until birth, the kidneys are histologically normal but are smaller, with 36% fewer glomeruli than in their wild-type littermates. Gdnf expression is significantly down-regulated in the mutant mesenchyme. GDNF is the ligand for the Ret

receptor tyrosine kinase, which is expressed in the ureteric bud tips. Both Gdnf and Ret are required for normal branching of the UB (Moore et al., 1996; Shakya et al., 2005). When Ret +/− mice are crossed with $Wnt11^{+/-}$ mice, the resulting kidneys of the double heterozygotes are 52% smaller than those of wild-type. $Ret^{+/-}$;$Wnt11^{-/-}$ mice have kidneys 67% the size of those of $Ret^{+/-}$;$Wnt^{+/-}$ mice. These studies indicate that Wnt11 acts in parallel with the Ret/Gdnf pathway to regulate branching of the ureter (Majumdar et al., 2003).

In addition to defects in formation of the renal vesicles, Wnt9b mutants also have defects in branching of the ureteric bud (Carroll et al., 2005). Although the UB undergoes the first bifurcation normally, branching after the T stage is disrupted (Compare figure 1-4a and c). In the $Wnt9b^{-/-}$ mutants, expression of the branching regulators Wnt11 and GDNF is down-regulated prior to the appearance of any morphological signs of branching defects. In addition, the ureteric buds of Wnt9b mutants branch significantly less than those of Wnt4 mutants, suggesting that the phenotype is not a secondary effect from failure to form the renal vesicles but instead a direct role for Wnt9b in regulation of secondary branching of the ureteric bud (figure 1-4b). As discussed above, Wnt11 and the Ret/Gdnf pathways normally regulate UB branching. At this point it is unclear whether the cellular target of Wnt9b in branching is the Gdnf-expressing mesenchyme or the Wnt11-expressing ureteric bud.

Signals from the mesenchyme are necessary for the survival and branching of the ureteric bud. Treatment of isolated ureteric buds with the Wnt agonist LiCl sustains branching of isolated ureteric buds. Although numerous Wnts that are expressed in and around the developing bud could mediate this phenotype, Wnt2b appears to be sufficient

to fulfill this role. Wnt2b is weakly expressed from E11.5 to E13.5 in the perinephric cells surrounding the differentiating mesenchyme (Lin et al., 2001). The co-culturing of cells expressing this gene with isolated mesenchyme has no effect; however, co-culture with isolated ureteric buds leads to survival and branching of the bud epithelium. These data suggest that Wnt2b may function to support the initiation of branching of the UB, although there are no genetic data to support this hypothesis.

Pathway usage in the kidney: canonical or non-canonical?

As mentioned, the branch of the pathway utilized by an individual ligand is dependent on the cellular environment in which the signal is received. The specific pathway used by each of the Wnts involved in the development of the metanephric kidney is unknown. In fact, examination of the expression of pathway components and target genes suggests that the canonical, PCP and calcium branches are all active during metanephric kidney development (Doble and Woodgett, 2003; Kispert et al., 1998; Lyons et al., 2004; Osafune et al., 2006; Simons and Walz, 2006). The challenge now is identifying the ligands that mediate signaling through each branch and the cellular processes they regulate.

It was generally thought that the canonical pathway was involved in tubule induction and the non-canonical pathway in ureteric bud branching. This was based on the observations that stabilization of beta-catenin in the mesenchyme and co-culture of mesenchyme with “canonical” Wnts, such as Wnt1 and Wnt3a, induced tubule formation, while mutation of “non-canonical” Wnts, like Wnt11, led to branching defects. This classification also seemed to fit with the established roles for canonical signaling in cell proliferation and adhesion and non-canonical signaling in cell movement/migration.

However, recent data suggest that the situation is significantly more complex and that interplay between both branches of the pathway may be necessary for normal renal development.

Pathway usage in renal vesicle formation

Studies making use of a canonical pathway reporter in mice, in which multimerized Lef/Tcf binding sites control expression of the β-galactosidase reporter (the so-called Bat-Gal mouse), as well as examination of the mRNA for a general beta-catenin target gene, Axin2, showed little, if any, beta-catenin activity in the mesenchyme, while there was high activity in the UB and collecting ducts (Davies and Garrod, 1995; Jho et al., 2002; Maretto et al., 2003) (figure 1-5). These observations suggested that canonical signaling was not directly involved in development of the mesenchyme or the renal vesicles. In fact, work performed by Osafune et al. suggested that the PCP pathway was required in the mesenchyme downstream of Wnt4 during tubule formation and that canonical signaling actually inhibited Wnt4 activity (Osafune et al., 2006). This was consistent with results obtained by Cai et al., showing that both Wnt4 and Wnt11 could stimulate Jnk activity and that this activated Pax-2, a transcription factor required for multiple aspects of kidney development (Cai et al., 2002).

These results were seemingly in direct opposition to those in the previous studies on canonical pathway activity in tubule formation. This contradiction could be rectified if one assumed that Wnt9b acted in a canonical manner to stimulate Wnt4 expression, which subsequently induced tubulogenesis through the PCP pathway. However, studies using Madin–Darby canine kidney (MDCK) cells have shown that Wnt4 can activate a β-catenin/LEF/TCF luciferase reporter and Wnt4 can stabilize beta-catenin in isolated MM,

suggesting that it signals through the canonical branch (Carroll et al., 2005; Lyons et al., 2004). Therefore, an alternative model is that the canonical Wnt signaling pathway, mediated by Wnt9b and/or Wnt4, is involved in the early stages of nephrogenesis, driving cell adhesion and/or cell proliferation (This hypothesis supposes that axin2 and Bat-Gal are not faithful reporters of canonical pathway activity in the MM.). Once the pre-tubular aggregates or renal vesicles have formed, Wnt signaling is shifted, through an unknown mechanism, to a non-canonical role, mediating tubule growth/extension. This model suggests that a fine balance between canonical and non-canonical signaling must exist in the developing kidney.

Pathway usage during branching morphogenesis

Examination of both Bat-Gal and axin2 expression indicates high levels of canonical activity in the branching tips of the ureteric bud and lower levels in the stalk, suggesting that this branch of the pathway may play an important role in ureteric bud/collecting duct development. This fits with the data showing that lithium, an agonist

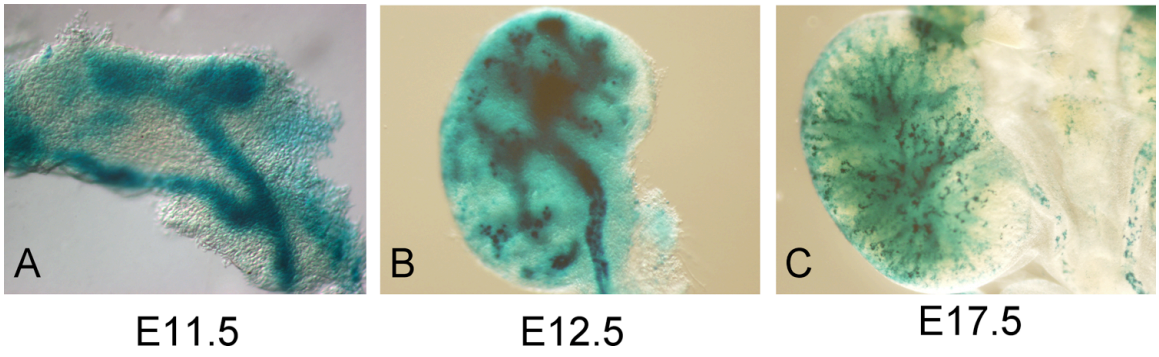


Figure 1-5. Canonical Wnt signaling in the developing urogenital system. beta-galactosidase staining shows canonical Wnt signaling in the developing kidneys of Bat-gal mice. (A) Staining shows activated beta-catenin in the Wolffian duct and ureteric bud at E 11.5. At (B) E 12.5 and (C) E 17.5, canonical Wnt signaling is found at high levels in the developing collecting ducts and, perhaps, at lower levels in the metanephric mesenchyme

of the canonical pathway, supports branching of isolated buds (Lin et al., 2001). What exactly the canonical pathway is doing in the ureteric bud is unknown, and a better understanding may have to wait for tissue-specific ablation studies. One potential role is mediation of the adhesive state of cells in the bud tips. In other models of branching morphogenesis, it has been suggested that the epithelia reverts to a mesenchymal state during branching (Affolter et al., 2003). Canonical signaling could be mediating such a transition in the kidney, although this would be contrary to other data, where a loss of beta-catenin is correlated with epithelial-to-mesenchymal transition (Cox et al., 1996). It is possible that Wnt signaling is simply mediating differential adhesiveness between the tips and the stalk, perhaps through regulating the expression or activity of a cell adhesion molecule. Interestingly, the L1Cam gene has been identified as a direct transcriptional target of β-catenin, and L1Cam has been implicated in mediating normal branching of the UB (Debiec et al., 2002; Gavert et al., 2005). Another possibility is that canonical signaling could be maintaining the cells of the ureteric bud in a precursor/undifferentiated state, allowing them to continue dividing and/or branching. Such a role has previously been established for canonical signaling in multiple tissues (Alonso and Fuchs, 2003; Pinto and Clevers, 2005; Reya et al., 2003; Zechner et al., 2003).

The ligand mediating canonical signaling in the bud tips is unknown. It could be one of the mesenchymally expressed Wnts or one of the bud-expressed Wnts. Candidates for a mesenchymally expressed Wnt include Wnt2b acting redundantly with another Wnt such as Wnt4 (our unpublished observations rule out a role for mesenchymally expressed Wnt4 alone). Wnt9b or Wnt11 would seem to be good candidates for the bud-expressed Wnts, given their expression patterns and their role in branching morphogenesis, but

neither appears to be necessary for axin2 or Bat-gal expression on its own (our unpublished observations). A third possibility is that reporter activation is Wnt independent. Recent data suggest that a number of non-Wnt factors are able to activate the beta-catenin pathway, including the secreted molecules norrin, dickopf2, Sfrp1 and the R-spondins (Kazanskaya et al., 2004; Mao and Niehrs, 2003; Uren et al., 2000; Wu et al., 2000; Xu et al., 2004). Although several of these molecules are expressed in the embryonic kidney, their roles in the development of this organ are unclear, although sFrp-1 has been suggested to have an inhibitory role in tubule formation (Yoshino et al., 2001).

There is no direct evidence indicating a role for the non-canonical pathway in the development of the ureteric bud/collecting duct system. As mentioned, in the Bat-Gal mouse β-galactosidase is expressed throughout the bud/ducts through birth. If this reporter represents a faithful readout of pathway activity and if only one pathway is activated in a specific cell type at a particular point in time, then one can conclude that the non-canonical pathway is not playing a major role in the prenatal development of the collecting duct system. However, if axin2 transcripts represent a more faithful readout, then the canonical pathway is not active in the distal collecting ducts, and it is possible that any Wnts expressed in or around this tissue (including Wnt6, Wnt7b and Wnt9b) or the Wnts expressed in the medullary stroma (Wnt4 and Wnt11) may be signaling to the distal collecting ducts through the non-canonical pathway. However, conclusive proof of the involvement of the non-canonical branch will require identification of the receptors for each of the kidney-expressed Wnts as well as a more careful examination of the expression and functional roles of the canonical and non-canonical pathway determinants

during renal development. It is tempting to speculate that the processes of convergent extension and/or planar cell polarity are involved in the directional growth of the collecting ducts, and evidence will be presented below suggesting that this is indeed the case.

The role of Wnts in reproductive tract development

Many Wnts are expressed in the developing reproductive tract. Among these Wnts, only four exhibit a reproductive tract phenotype when ablated: Wnt4, Wnt5a, Wnt7a, and Wnt9b. We will discuss the roles they play in development of both the male and female reproductive tracts.

Along with its expression in the MM of the developing kidney, Wnt4 is expressed starting at E9.5 in the mesenchyme along the length of the mesonephros as well as in the coelomic epithelium in the presumptive gonad (Vainio et al., 1999a). The gonads form around E11.0 at which time Wnt4 is expressed in the mesenchyme of the bipotential gonads. When sex specific differentiation of the gonads is initiated at E11.5, Wnt4 expression is downregulated in the male gonad but maintained in the female gonad.

Multiple reproductive tract defects are present in mice that are homozygous null for Wnt4 (Vainio et al., 1999a). First, a loss of Wnt4 results in a significant decrease in the number of oocytes. Wnt4 mutant ovaries contain less than 10% the number of oocytes in wild-type ovaries. These results indicate that Wnt4 acts to regulate oocyte development in females. In addition to defects in oocyte development, the MD fails to form in both males and females. The consequence for lacking a MD is only observed in mutant females since they cannot form female reproductive tracts without the MD. As mentioned earlier, MD formation is biphasic. Mouse knock out studies determined that

Wnt4 regulates the first phase of MD development, invagination from the coelomic epithelium. Along with defects in MD formation, the WD is maintained in mutant females. In the absence of Wnt4 in female gonads, steroidogenesis is initiated in the ovary, leading to maintenance of the WD. Both the sex specific expression of Wnt4 in the differentiated gonads and the ectopic androgen synthesis in Wnt4 mutant ovaries are indicative of a role for Wnt4 in suppression of Leydig cell formation in the female gonad. Therefore, in the absence of Wnt4, the female gonad becomes masculinized, leading to the production of male hormones and resulting in a partial sex reversal phenotype.

In addition to playing an important role in kidney development, Wnt9b is also necessary for development of the reproductive tract. During reproductive tract development, Wnt9b is expressed throughout the WD epithelium from E9.5 until E14.5 (Carroll et al., 2005). At birth, mice that are homozygous null for Wnt9b form normal testis and ovaries but are missing oviducts, uteri, and upper vaginas in females and epididymis and vas deferens in males. Analysis of the female embryonic Wnt9b phenotype identifies the elongation phase of MD development as defective since in Wnt9b mutants, MD formation is initiated but it fails to extend. To determine through which pathway Wnt9b functions in MD development, Carroll et al. ectopically expressed Wnt1 throughout the WD in Wnt9b mutants. Wnt1 is expressed in the embryonic brain and spinal cord and is thought to primarily signal through the canonical pathway (Herzlinger et al., 1994). Expression of Wnt1 rescues the MD phenotype, inducing elongation of the MD to the cloaca. Therefore, not only is Wnt9b necessary for elongation of the MD, but this study implies that it is signaling though the canonical Wnt pathway.

The expression of Wnt7a is restricted to the MD epithelia from E12.5 to E14.5 (Parr et al., 2001). The MD regresses in males after E14.5 along with loss of expression of Wnt7a. While Wnt7a expression is lost in males, it continues to be expressed in females throughout development. Mice that are homozygous null for Wnt7a exhibit multiple reproductive tract phenotypes rendering both males and females infertile. In wild-type males, MIS produced by Sertoli cells binds its receptor, MISR, leading to the degradation of the MD. Expression of MISR, located in the mesenchyme surrounding the MD, is lost in Wnt7a mutants, causing mutant males to maintain their MD. The ectopic MD in mutant males prevents the vas deferens from properly connecting at its distal end, disrupting sperm flow and causing infertility. Maintenance of the MD in males is also found in MIS and MISR mutants (Behringer et al., 1994; Mishina et al., 1996). However, in embryos mutant for either MIS or MISR, the MD differentiates into female reproductive tract organs. In Wnt7a mutants, the MD fails to differentiate properly in both males and females.

Female reproductive tract structures derived from the MD remain relatively undifferentiated until after birth. In addition to activating MISR, Wnt7a, along with Wnt5a, is necessary for patterning of the MD (Mericskay et al., 2004; Miller and Sassoon, 1998). After birth, Wnt7a mutants exhibit defects in oviduct coiling along with the uterus having a thinner and less muscular wall. In both Wnt7a and Wnt5a mutants, the uterine glands fail to form. Furthermore, in the absence of Wnt5a, mutant females show defects in formation of the cervix and vagina. Therefore, Wnts are not only important for embryonic development of the reproductive tract but they are also necessary for proper differentiation of MD structures after birth.

Support that development of the reproductive tract is regulated by canonical Wnt signaling comes from studies removing beta-catenin from the MD mesenchyme both after birth and embryonically (Arango et al., 2005; Deutscher and Hung-Chang Yao, 2007). An anti-Müllerian hormone receptor 2 (Amhr2, also known as MISR) cre line was used to embryonically remove beta-catenin starting at E15.5 (Deutscher and Hung-Chang Yao, 2007). Removal of beta-catenin from the MD mesenchyme resulted in disruption of oviduct and uterus differentiation, recapitulating the Wnt7a female reproductive tract phenotype. In addition, the expression pattern of both Wnt5a and Wnt7a remained unaffected in Amhr2cre;Bcat knockouts indicating that the mutant phenotype is due to removal of beta-catenin, and not loss of the Wnt ligands. From this study, it appears that Wnt7a is signaling through beta-catenin during MD differentiation, further supporting a role for canonical Wnt signaling in reproductive tract development.

Kidney tubule formation and growth; a balancing act?

For years it has been known that improper regulation of the canonical Wnt pathway correlated with and caused various human diseases, including cancers. More recently it has been suggested that a fine balance between the canonical and non-canonical branches of the Wnt pathway is essential for the development and homeostasis of multiple tissues, including the kidney. One of the clearest examples of such a balance comes from functional analysis of the inversin (Inv) gene in mice. Inv encodes an ankyrin repeat domain encoding protein that has homology to diversin, the vertebrate ortholog of the fly PCP protein, diego. Mutations in Inv cause nephronophthisis type II (NPNII), an autosomal recessive disorder characterized by extensive renal cysts (Simons et al., 2005). Mutation of inv in mice results in renal cysts and situs inversus (Otto et al., 2003).

Studies performed by Simons et al. suggest that inversin plays an important role in regulating Wnt signaling and the choice between the canonical and non-canonical signaling pathways during kidney development (Simons et al., 2005). Misexpression of inversin in cultured cells resulted in reduced levels of stabilized cytoplasmic β-catenin. Inv and Dvl physically interacted, and co-expression of Inv mRNA repressed the formation of secondary body axes that were induced by Dvl (but not by β-catenin) in Xenopus embryos, suggesting that Inv binds to and inhibits the ability of Dvl to mediate canonical Wnt signaling (Simons et al., 2005). This study also showed that morpholino-mediated knockdown of Inv in zebrafish resulted in increased expression of canonical Wnt targets, and defects in CE movements, a process dependent on non-canonical Wnt signaling (Simons et al., 2005). Thus, Inv appears to promote a switch from the canonical pathway to the non-canonical pathway. In Inv mutants, the canonical pathway is enhanced, while the non-canonical pathway is abrogated, leading to cyst formation.

It appears that perturbation of the Wnt pathway may play a general role in kidney cystogenesis. Increased levels of beta-catenin are strongly correlated with cyst formation in humans and over-expression of beta-catenin or c-Myc, a downstream target of β-catenin, in mice gives rise to cystic kidneys (Cowley et al., 1991; Cowley et al., 1987; Harding et al., 1992; Qian et al., 2005; Romagnolo et al., 1999; Saadi-Kheddouci et al., 2001; Trudel et al., 1998; van Adelsberg, 2000). Further, deletion of the beta-catenin destruction complex protein APC from the kidney epithelia results in increased nuclear beta-catenin and formation of cystic tubules (Qian et al., 2005). Presumably, over-expression of beta-catenin results in increased cell proliferation and perhaps other defects in differentiation, cell adhesion or polarity that contribute to cyst formation.

As the canonical and non-canonical pathways are antagonistic toward each other, over-expression of beta-catenin should also antagonize the non-canonical pathway (although this has not been shown in the kidney). Planar cell polarity/convergent extension processes have been shown to be involved in orienting the plane of cell division and/or cell intercalation during the growth of organs such as the heart and the pancreas (Karner et al., 2006b). Defects in either of these two processes could contribute to the formation of cystic tubules. For example, when the orientation of cell division is randomized by mutation of the PCP gene dachsous, the shape of the Drosophila wing becomes shorter and wider (Baena-Lopez et al., 2005). By extrapolation, a change in the orientation of cell division during kidney growth could lead to an increase in tubule diameter, perhaps at the expense of tubule length, and thus contribute to cyst formation. Recently, just such an observation was made in the Hnf1β mutant mouse and pck rat models of polycystic kidney disease (PKD). Fischer et al. showed that the orientation of cell division is randomized in these mutants prior to cyst formation, suggesting a causal role in cystogenesis (Fischer et al., 2006). Although neither of these genes has been directly tied to non-canonical Wnt signaling, defects in the planar cell polarity branch of the pathway would presumably lead to similar defects in the orientation of cell division that would contribute to the cystic phenotype.

Another process shown to be dependent upon planar cell polarity that could contribute to cystogenesis is the intercalation of cells during convergent extension movements (Heisenberg et al., 2000; Matsui et al., 2005; Wallingford et al., 2002; Wallingford et al., 2000). Removal of Dvl, Vangl and prickle orthologs results in convergent extension defects during neurulation, cochlear formation and gastrulation,

indicating that PCP signaling is required for these morphogenetic movements in vertebrates (Carreira-Barbosa et al., 2003; Ciruna et al., 2006; Darken et al., 2002; Takeuchi et al., 2003; Veeman et al., 2003b; Wang et al., 2006a; Wang et al., 2005). Cell intercalation has not been demonstrated during the development of the mouse kidney tubules although it does occur during the development of the fly Malpighian tubules (Jung et al., 2005). So, it seems plausible that defects in PCP signaling could result in defects in the orientation of cell division and, possibly, in the intercalation of cells during kidney growth, resulting in tubules with an increased diameter. It is currently unclear whether a defect in either branch of the Wnt pathway (activation of the canonical or inactivation of the non-canonical) alone would be sufficient to cause cyst formation or, as the two are mutually antagonistic, whether it is even possible to get one without the other.

Given the extensive data implicating mis-regulation of the Wnt pathway in human disease, there has been intense investigation into the molecular nature of the factors necessary for regulation in vivo. Some of this regulation occurs at the level of the ligand, affecting its ability to interact with its receptors or co-receptors (Kawano and Kypta, 2003). Such antagonists include the secreted frizzled-related proteins (sFRPs), the Wnt inhibitory factors (Wifs), and the dickkopfs (Dkk) (Kawano and Kypta, 2003). In the case of the sFRPs, there is some evidence that they play a role in the development of the kidney. Both sFRP-1 and sFRP-2 are expressed in the developing kidney but in distinct patterns. While sFRP-2 expression overlaps the expression of Wnt4 in the condensed mesenchyme and epithelial bodies, sFRP-1 expression is limited to the interstitial mesenchyme (Lescher et al., 1998; Yoshino et al., 2001). In explant studies, rat metanephroi were treated with sFRP-1, sFRP-2, or both (Yoshino et al., 2001). sFRP-1

exhibited an inhibitory effect on the formation of tubules, while sFRP-2 treatment had no effect. However, when sFRP-2 was combined with sFRP-1, the inhibitory effect of sFRP-1 was decreased. These results suggest that sFRP-2 may function to promote Wnt4 activity (possibly by repressing Wnt9b?), while sFRP-1 assures that tubulogenesis is restricted to the aggregate mesenchyme. sFRP-2 appears to be a target of Wnt-4 activity, as expression is ablated in Wnt4 mutants (Yoshino et al., 2001). How these results relate to the in vivo situation is unclear. Recent studies indicate that sFrp1 and 2 play redundant roles during embryogenesis (Satoh et al., 2006). Embryos lacking both genes die in utero with anterior–posterior patterning defects, a phenotype that is similar to that of Wnt5a null embryos. Wnt5a does not interact with the sFrps and is generally considered a non-canonical Wnt. These data lend support to the hypothesis that repression of the canonical pathway is necessary for activation of the non-canonical pathway. At this point, there has been no published characterization of the kidneys in the sFRP double mutants.

A second type of regulation appears to be through the production of intercellular antagonists of the signal transduction pathways. As one might expect, several proteins have recently been identified that appear to interact with Dvl to regulate pathway or branch utilization, including the previously discussed Inv, naked cuticle (nkd), daple (Dpl) and dapper/frodo (dact/frd) proteins, although, with the exception of Inv, no role in kidney development has been established for any of these factors (Brott and Sokol, 2005; Oshita et al., 2003; Simons et al., 2005; Wharton, 2003). Although the Inv mutant kidneys are slightly smaller than normal and cystic ones, much of early nephrogenesis occurs normally. This suggests that either the shift from one branch to the other is not

essential during early embryonic stages or that there is another factor that fulfills this role at earlier time points.

And, finally, there are several proteins that affect the ability of beta-catenin to function as a transcriptional activator including groucho, kaiso, cited1 and specific isoforms of the Lef/Tcfs (Arce et al., 2006; Plisov et al., 2005). Outside of cited1, there is little known of the expression of these genes during kidney development. However, cited1 is expressed in the condensed kidney mesenchyme, and over-expression of this gene in cultured kidneys blocks tubule formation, suggesting that inhibition of beta-catenin activity does play an important role in tubulogenesis.

In this dissertation, I will describe my studies using a small molecule Wnt inhibitor in an organ culture model and a mouse model with beta-catenin conditionally removed from the WD to understand the role beta-catenin plays in development of the urogenital system. My specific aims are as follows:

1) Validate the efficacy of small molecule inhibitors of Wnt signaling in embryonic kidney culture.

2) Determine the cause of Wolffian duct maintenance in beta-catenin mutant females.

3) Determine the cause of a lack of Müllerian duct derivatives in the P1 beta-catenin mutant females.

Chapter 2

The effect of small molecule Wnt antagonists on kidney development

2.1 Abstract

Constitutive activation of Wnt signaling has been implicated in playing a causal role in certain kidney disorders such as renal cell carcinomas and polycystic kidney disease. Thus, targeted inhibition of Wnt signaling could be useful for treating these diseases. Two classes of small molecule antagonists of Wnt signaling were identified from a high throughput screen performed by Lawrence Lum's group at University of Texas Southwestern. These Inhibitors of Wnt signaling (IWs) could be a useful tool for studying kidney development and Wnt signaling. Since the IWs inhibit Wnt signaling in a dosage dependent manner, we will be able to look at the effect of varying amounts of Wnt inhibition during kidney development. In addition, due to the specificity of one of the classes of IWs, Inhibitors of Wnt Response (IWR), we can look at the effect of inhibiting only canonical Wnt signaling. First, we tested functionality of the IWs in organ culture. Branching morphogenesis and renal vesicle formation are processes known to be regulated by Wnt signaling. Using these processes as a measure of inhibition of Wnt signaling in embryonic kidney cultures, we showed that the IWs do function in organ culture. Future tests of the efficacy of the IWs *in vivo* may reveal therapeutic uses for Wnt inhibitors in the treatment of polycystic kidney disease.

2.2 Introduction

Embryonic development of the mouse urogenital system begins around E9.5 when an epithelial tube called the Wolffian duct (WD) forms from the intermediate mesoderm and extends caudally until it reaches the cloaca. The WD gives rise to the three embryonic kidneys: the pronephros, mesonephros, and metanephros. The metanephric

kidney develops into the functional adult kidney. Development of the metanephric kidney begins at E10.0 when the ureteric bud (UB) sprouts dorsally from a caudal portion of the WD. The UB invades a population of cells known as the metanephric mesenchyme (MM). The UB signals to the MM, causing it to undergo a mesenchymal to epithelial transition (MET) to form renal vesicles. The renal vesicles undergo morphogenetic changes to form comma- then s-shaped bodies and eventually develop into nephrons. At the same time that the UB signals to the MM, the MM is signaling back to the UB, causing the UB epithelia to undergo several rounds of branching morphogenesis. These branches differentiate to form the collecting ducts. These feedback pathways demonstrate how the kidney is formed due to reciprocal interactions between the UB epithelium and the MM. Although several signaling pathways contribute to kidney development, Wnt signaling plays a key role.

Wnts are secreted glycoproteins that function through two main Wnt signal transduction pathways: canonical (beta-catenin dependent) and non-canonical (beta-catenin independent). During canonical Wnt signaling, a Wnt ligand, bound to its Frizzled receptor (Fz) and lipoprotein receptor–related protein (Lrp) co-receptor, results in the activation of dishevelled (Dvl). Dvl then acts to inhibit the beta-catenin destruction complex which consists of Axin, APC, CK1, and GSK3beta. Inhibition of the destruction complex prevents destruction of beta-catenin, allowing it to translocate into the nucleus and function as a transcriptional activator of Wnt target genes.

The non-canonical Wnt signal transduction pathway functions independently of beta-catenin. There are two main branches of non-canonical signaling: the Ca^{2+}-releasing pathway and the planar cell polarity (PCP)/convergent extension (CE) pathway. In the

Ca^{2+}-releasing pathway, active Wnt signaling stimulates an intracellular release of Ca^{2+} in a G-protein dependent manner (Kuhl et al., 2000; Sheldahl et al., 1999). The release of Ca^{2+} then leads to the activation of Ca^{2+} sensitive enzymes such as protein kinase C (PKC) and Ca^{2+}-calmodulin-dependent protein kinase II (CamKII). Another branch of non-canonical Wnt signaling functions to regulate PCP and CE. PCP is the organization of cells within the plane of an epithelium whereas CE is a process during which a group of cells intercalate to become longer and narrower. The PCP/CE pathway functions through Dvl to activate the JNK pathway and GTPases such as Rho, Rac, or CDC42 (Karner et al., 2006b). Activation of these proteins leads to a rearrangement of the actin cytoskeleton.

Two families of small molecule inhibitors of Wnt signaling (IWs) were identified by Lum's group in a high throughput screen (Tang et al., 2008). One family of Wnt inhibitors functions in Wnt producing cells (IWPs) by inhibiting a protein called porcupine (Porc). A transmembrane protein localized to the endoplasmic reticulum, Porc binds to and stimulates the N-glycosylation of all Wnts (Tanaka et al., 2002; Tanaka et al., 2000). Porc dependent posttranslational modification is necessary for Wnt secretion. Therefore, inhibition of Porc prevents Wnt secretion, thus inhibiting all Wnt signaling. The second family acts upon Wnt responding cells (IWRs) by stabilizing Axin, a member of the beta-catenin destruction complex. By stabilizing Axin, the destruction complex is also stabilized, leading to the destruction of beta-catenin. Therefore, IWRs act specifically to inhibit canonical Wnt signaling.

2.3 Results

IWs inhibit branching morphogenesis and renal vesicle formation in cultured embryonic kidneys

Two families of IWs were identified in a high throughput screen assaying the ability of a library of small molecules to inhibit the activity of a Wnt signaling reporter in mouse L cells (Tang et al., 2008). In addition to inhibiting Wnt signaling in L cells, the IWs have also been shown to function in zebrafish in a regeneration assay (Chen et al., 2009). As previously mentioned, the IWs could potentially serve as a cancer therapeutic. Before IWs can be explored as a possible cancer drug, an assay is needed to test the efficacy of the small molecule Wnt inhibitors in an *in vivo* mammalian system. Many aspects of kidney development are regulated by Wnt signaling (Merkel et al., 2007). In addition, embryonic kidneys can successfully be cultured, allowing for *ex vivo* treatment with the small molecule inhibitors. Therefore, we propose to test the efficacy of the IWs by treating cultured embryonic kidneys and assaying different aspects of Wnt regulated kidney development.

Among the processes of kidney development that are regulated by Wnt signaling is branching morphogenesis of the UB (Merkel et al., 2007). Therefore, we used branching as a measure of IW activity. In order to visualize branching UB tips, we stained the treated explants for expression of E-cadherin protein, which is restricted to the epithelium. Even though there are two families of IWs, we initially tested three compounds: IWP1, IWP2, and IWR1. When embryonic urogenital systems are treated for two days with 50uM of IWP1, IWP2, or IWR1, branching of an E11.5 explanted urogenital system was significantly decreased (figure 2-1). When treated with a dosage

curve of IWP2, from 0.5uM to 50uM, the number of branching tips decreases with increasing concentration of drug (figure 2-2). Furthermore, the decrease in branching tips is statistically significant, as analyzed by the student's t-test, for all treatments as compared to the control explant (0.5uM IWP2, n=7 p=0.02, 5uM and 50uM IWP2, n=7 and 6, $p<0.001$). Therefore, not only does treatment with the IWs inhibit branching morphogenesis in organ culture, but the effect of IWP2 on branching is dosage dependent.

To better understand the effect of IWP2 on branching morphogenesis, we looked at the expression of Wnt11, a marker of branching. Wnt11 is expressed at the branching tips of the UB and acts in a positive feedback loop to regulate expression of GDNF, a ligand for the RET tyrosine receptor, in the surrounding mesenchyme (Majumdar et al., 2003). Together with the GDNF/RET pathway, Wnt11 is necessary for branching of the UB. We treated E11.5 urogenital systems with a dosage curve of IWP2 and stained for Wnt11 mRNA expression. Wnt11 expression is downregulated in urogenital systems treated with the lowest dose of IWP2, 0.5uM, and completely inhibited at 5uM and 50uM IWP2 (figure 2-3). Since Wnt11 is necessary for UB branching, loss of Wnt11 indicates that treatment with IWP2 affects the pathway involved in branching of the UB.

In addition to branching morphogenesis of the kidney, Wnt signaling also plays a role in renal vesicle formation. To visualize renal vesicles, we used a Pax8YFP line of transgenic line of mice. Pax8 is not only expressed in renal vesicles,

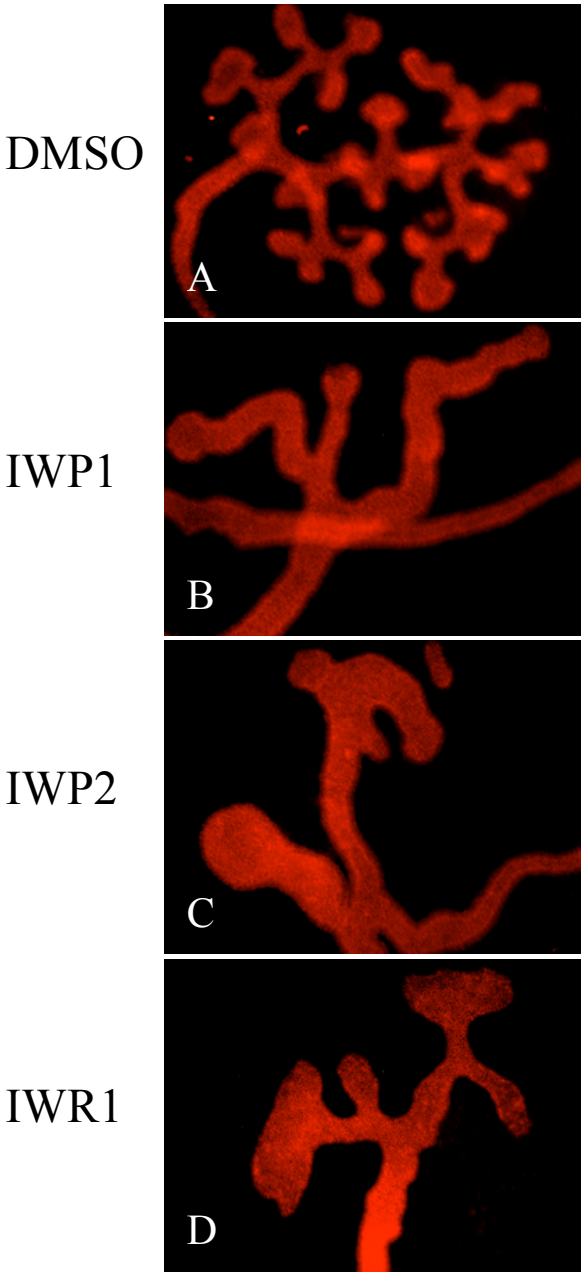
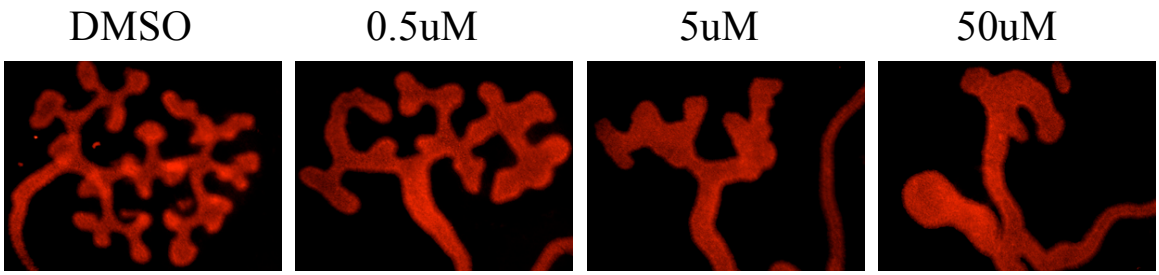


Figure 2-1. IW compounds inhibit branching in embryonic kidney culture. Embryonic urogenital systems were dissected out at E11.5. They were cultured for two days in the presence of (A) a DMSO control, (B) 50uM IWP1, (C) 50uM IWP2, or (D) 50uM IWR1. Wholemount antibody staining of E-cadherin allows for visualization of the UB branches. Compared to the DMSO control, branching is greatly inhibited during treatment with either IWP1, IWP2, or IWR1.



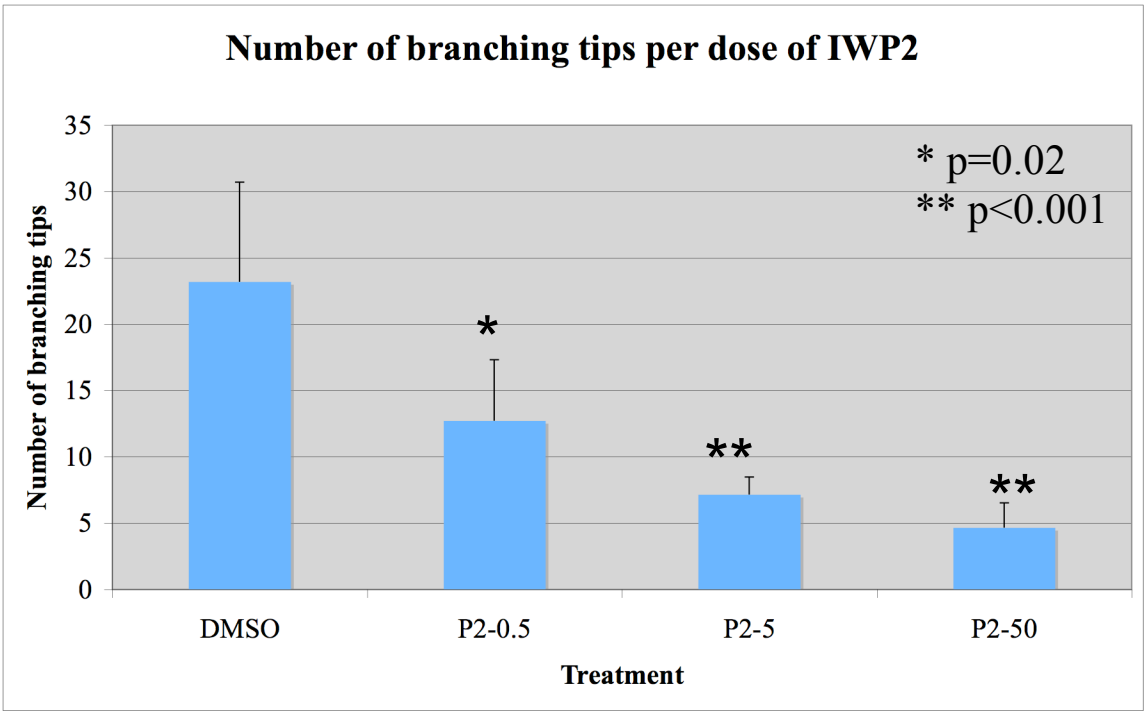


Figure 2-2. IWP2 inhibits branching in a dose dependent manner. E11.5 urogenital systems were cultured for two days in the presence of IWP2 at a concentration of either 0.5uM, 5uM, or 50uM IWP2. The cultured urogenital systems were stained with E-cadherin antibody for visualization of the UB branches. As compared to the DMSO control, each dose of IWP2 led to inhibition of branching. The number of branching tips for an n of 5 was measured for each treatment. Not only does IWP2 exhibit a dosage dependent inhibition in the number of branching tips but the decease in tips is statistically significant for all doses as compared to the DMSO control.

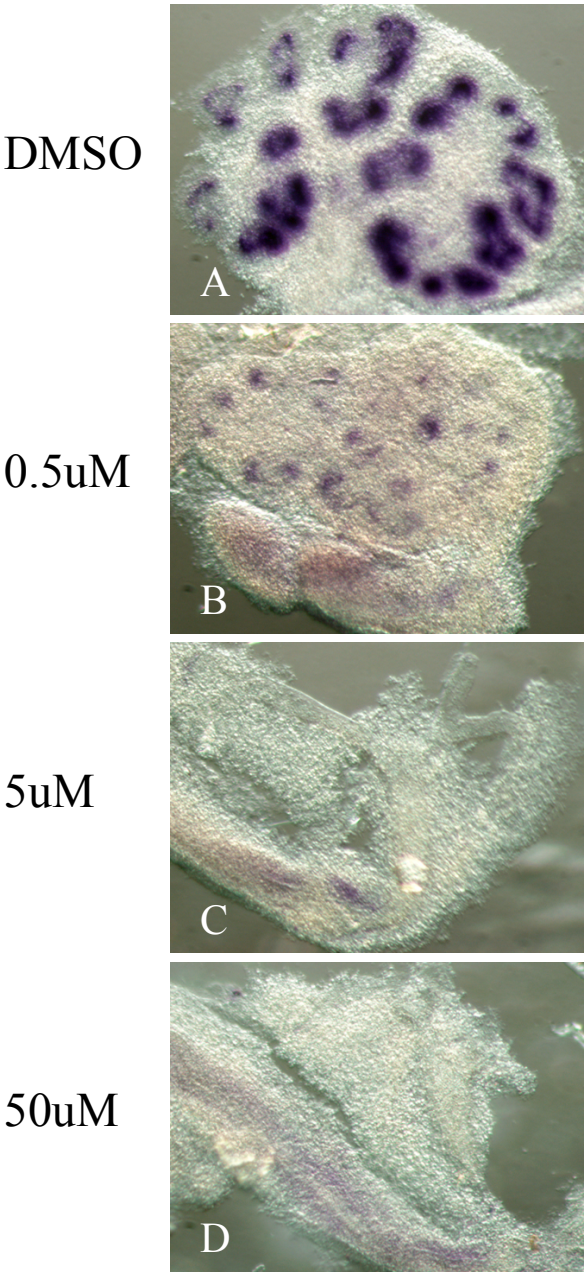


Figure 2-3. IWP2 inhibits Wnt11 mRNA in a dose dependent manner. E11.5 urogenital systems were treated with (A) a DMSO control, (B) 0.5uM IWP2, (C) 5uM IWP2, or (D) 50uM IWP2. After two days of treatment, wholemount in situ hybridization was performed on the tissue using a probe for Wnt11 mRNA. Wnt11 mRNA is expressed at the branching tips of the UB. At a dose 0.5uM IWP2, Wnt11 expression is greatly decreased and at 5uM and 50uM IWP2, Wnt11 expression is completely gone.

but is also a direct target of canonical Wnt signaling (Ohyama et al., 2006; Schmidt-Ott et al., 2007). When cultured for two days, control explants form several renal vesicles (figure 2-4). However, treatment with either 5uM or 50uM of IWP2 completely inhibits renal vesicle formation (figure 2-4). These results are further supported by in situ hybridization of IWP2 treated urogenital systems to visualize Pax8 mRNA expression. In 0.5uM IWP2 treated mesenchyme, Pax8 mRNA expression decreases. However, similar to the Pax8YFP data, Pax8 mRNA is visibly absent at doses of 5uM and 50uM of IWP2 (figure 2-5). Therefore, treatment with IWP2 inhibits Pax8 positive renal vesicles. Furthermore, since Pax8 is a target of canonical Wnt signaling, these results imply that IWP2 also inhibits canonical Wnt signaling.

Axin2 expression downregulated in IWP2 treated embryonic kidneys

To further characterize the effect IWP2 has on canonical Wnt signaling, we looked at the mRNA expression of Axin2, a direct transcriptional target of beta-catenin (Jho et al., 2002). Axin2 expression levels are similar in 0.5uM treated urogenital systems as compared to the control (figure 2-6). However, at the two highest doses of IWP2, 5uM and 50uM, Axin2 expression is greatly decreased (figure 2-6). Therefore, these results imply that IWP2 can inhibit beta-catenin transcriptional activity in mouse embryonic kidney culture.

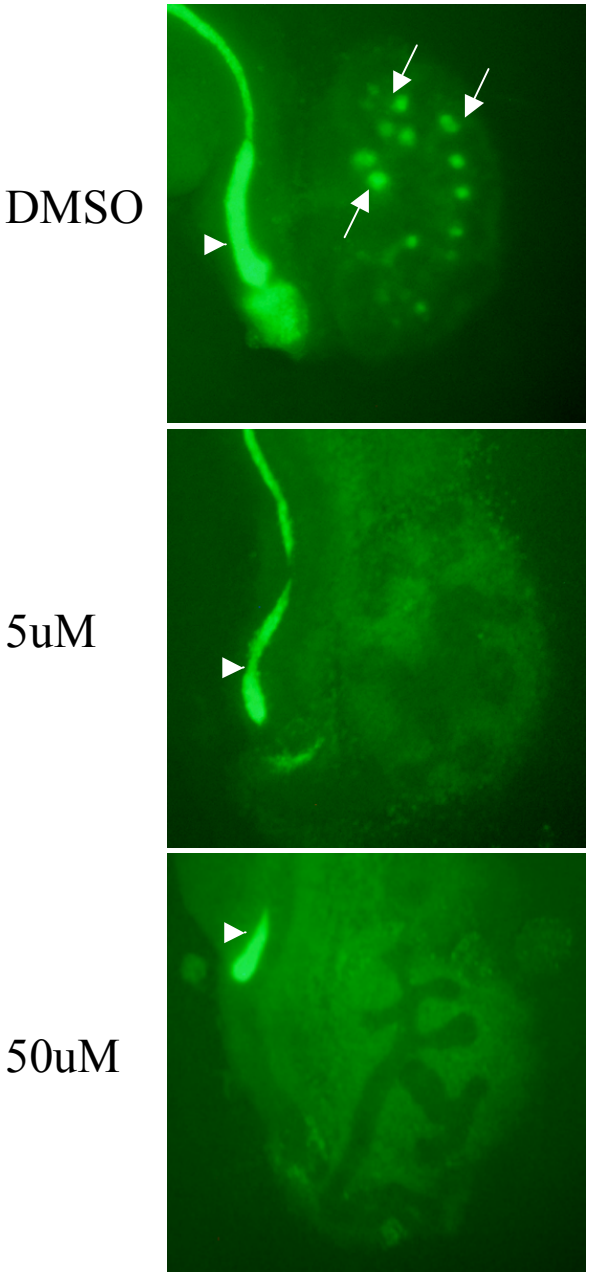


Figure 2-4. IWP2 inhibits renal vesicle formation. Urogenital systems were dissected out from Pax8GFP mice at E11.5. These explants were then treated with DMSO as a control, 5uM IWP2, or 50uM IWP2. Pax8 is expressed in the renal vesicles along with the MD. After two days of culture, renal vesicles are represented by Pax8GFP protein expression (arrows). Treatment with both 5uM and 50uM of IWP2 completely inhibit renal vesicle formation. Furthermore, treatment with 50uM IWP2 inhibits MD elongation (arrowhead).

Effects of IWP2 due to inhibition of Wnt signaling and not due to drug toxicity

We have shown that IWP2 inhibits the Wnt signaling pathway as measured by decreased branching morphogenesis and inhibited renal vesicle formation. However, since the highest doses are the most effective, it is possible that these processes are inhibited due to drug toxicity rather than Wnt inhibition. Previously, we have shown that removing beta-catenin from the WD leads to premature differentiation (Marose et al., 2008). To further test the effect IWP2 has on canonical Wnt signaling, it was important to determine if IWP2 treated embryonic urogenital systems exhibited a similar prematurely differentiated phenotype. After two days of culture with 50uM IWP2, we sectioned and stained the control and IWP2 treated urogenital systems for expression of ZO1a+ protein, a marker of tight junctions expressed only in mature epithelia. In vivo, ZO1a+ is not expressed in mouse embryonic kidney epithelia until E14.5 (Marose et al., 2008). Similarly, E11.5 explants treated for two days with DMSO as a control do not express ZO1a+ (figure 2-7). However, after two days of treatment with 50uM IWP2, precocious expression of ZO1a+ is present in WD epithelia (figure 2-7). These results indicate that, similar to beta-catenin mutants, treating E11.5 urogenital systems with 50uM IWP2 leads to premature differentiation. Premature differentiation in the IWP2 cultured urogenital systems is the likely cause of branching inhibition. Furthermore, precocious expression of ZO1a+ and thus premature differentiation indicates that impeded development of the cultured kidneys is due to Wnt inhibition and not drug toxicity.

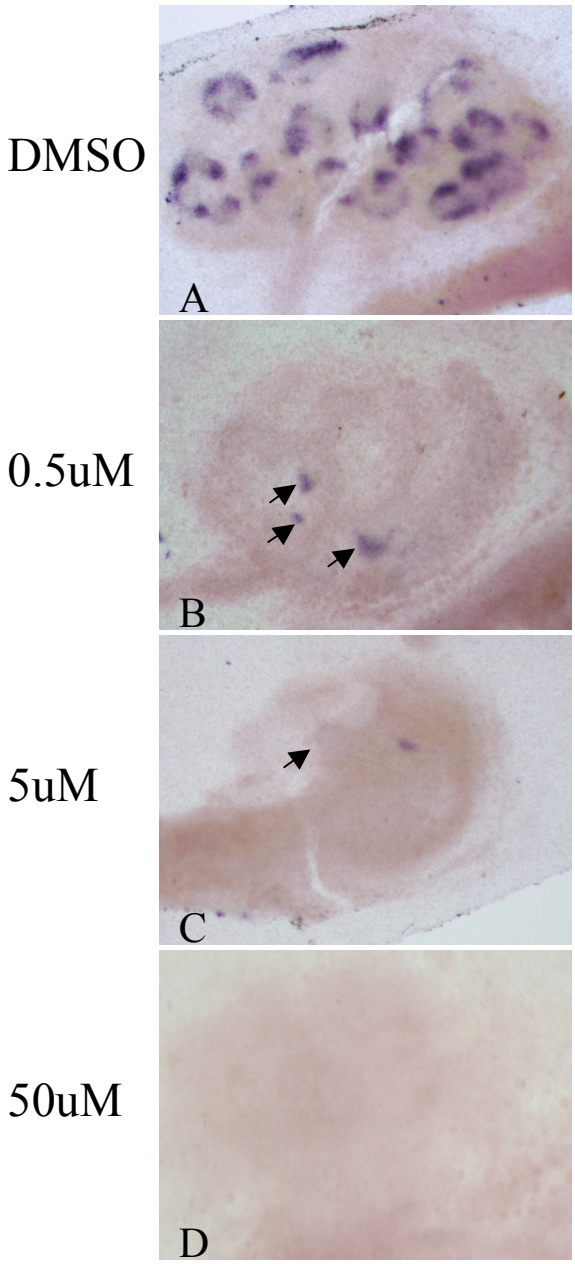


Figure 2-5. Pax8 mRNA expression is inhibited by IWP2 in a dose dependent manner. As previously mentioned, Pax8 is a marker of renal vesicles. E11.5 urogenital systems were cultured and treated with a dosage curve of IWP2. (A) After two days of culture and based on Pax8 expression, many renal vesicles have formed in the DMSO control. (B) However, only three Pax8 labeled renal vesicles form (arrows) when treated with 0.5uM IWP2. (C) At a dose of 5uM, there appears to be one Pax8 labeled renal vesicle (D) while there is no Pax8 mRNA expressed at a dose of 50uM IWP2.

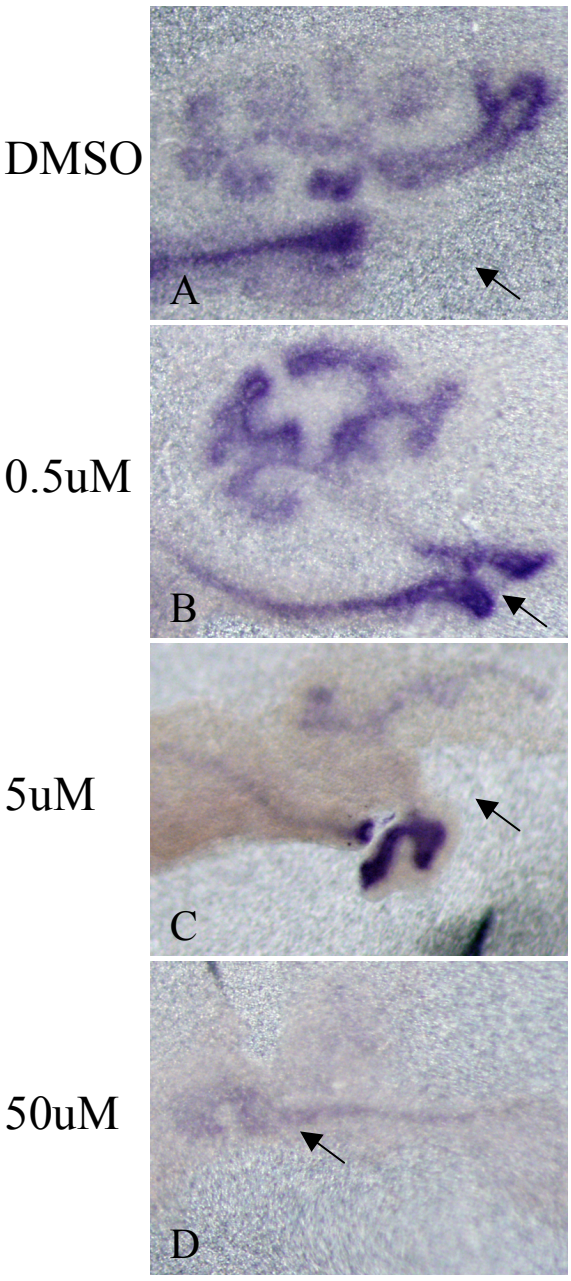
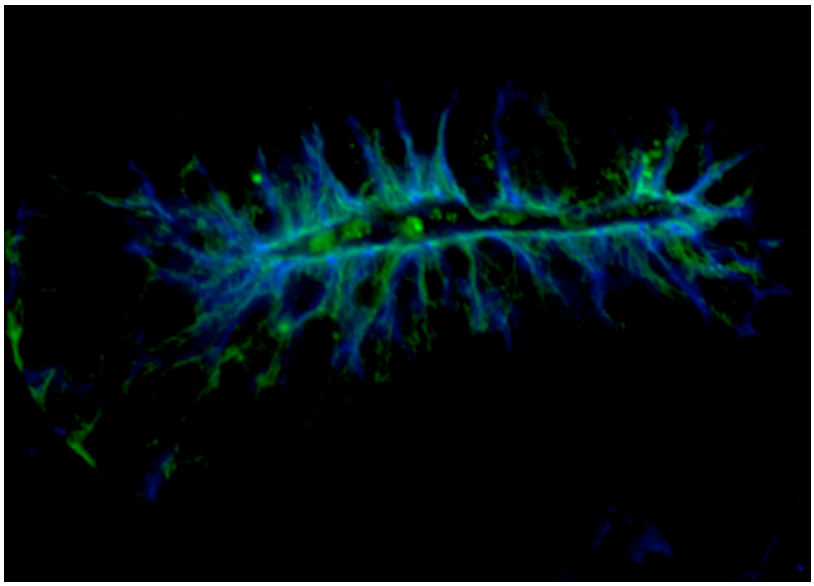


Figure 2-6. Axin2 mRNA expression is inhibited in a dose dependent manner by IWP2. Axin2 is a transcriptional target of canonical Wnt signaling. Therefore, Axin2 mRNA expression can be used as a readout for canonical Wnt signaling activity. E11.5 urogenital systems are treated for two days with either (A) a DMSO control, (B) 0.5uM IWP2, (C) 5uM IWP2, or (D) 50uM IWP2. (A,B) The Axin2 mRNA expression level is comparable in the DMSO control and the 0.5uM IWP2 treatment. (C,D) However, Axin2 mRNA expression is greatly reduced when treating with either 5uM IWP2 or 50uM IWP2 (arrow=WD)

IWP2 inhibits Wnt secretion in culture embryonic kidneys

In tissue culture, IWP2 functions by inhibiting Porc, a protein necessary for Wnt secretion. To determine whether or not IWP2 functions in a similar manner in organ culture, we used a RosaWnt1GFP line of mice to visualize Wnt secretion (Carroll et al., 2005). In RosaWnt1GFP mice, the expression of a Wnt1GFP fusion protein is under the control of the Rosa promoter. There are no antibodies for mammalian Wnt proteins that function in immunofluorescent assays and so the Wnt1GFP fusion protein allows for easy visualization of Wnt1. Since the Rosa promoter is only activated in areas of cre activity (Soriano, 1999), crossing RosaWnt1GFP to a HoxB7cre line of mice results in Wnt1GFP expression throughout the WD and UB starting at E9.5. We cultured E11.5 HoxB7cre; RosaWnt1GFP urogenital systems for two days, treating them with 50uM IWP2. Wholemount expression of GFP in the branching UB and WD verified that Wnt1GFP is expressed in culture (figure 2-8). To determine the cellular localization of Wnt1GFP, we sectioned the cultured urogenital systems before staining for Wnt1GFP expression. Upon secretion, Wnts predominantly localize to the extracellular matrix (ECM) (Bradley and Brown, 1990; Reichsman et al., 1996). In the control sample, Wnt1 protein is localized basolaterally in the UB epithelia. (figure 2-9). A basolateral localization suggests that Wnt1 is located in the ECM. When treated with 50uM IWP2, Wnt1 protein does not localize basolaterally (figure 2-9). It is likely that Wnt1 is localizing to the endoplasmic reticulum in IWP2 treated UBs but we need to co-stain with an endoplasmic reticulum marker to determine whether or not this is the case. Regardless, these results suggest that treatment with IWP2 alters the subcellular localization of Wnt1 and inhibits Wnt secretion.

DMSO



50uM IWP2

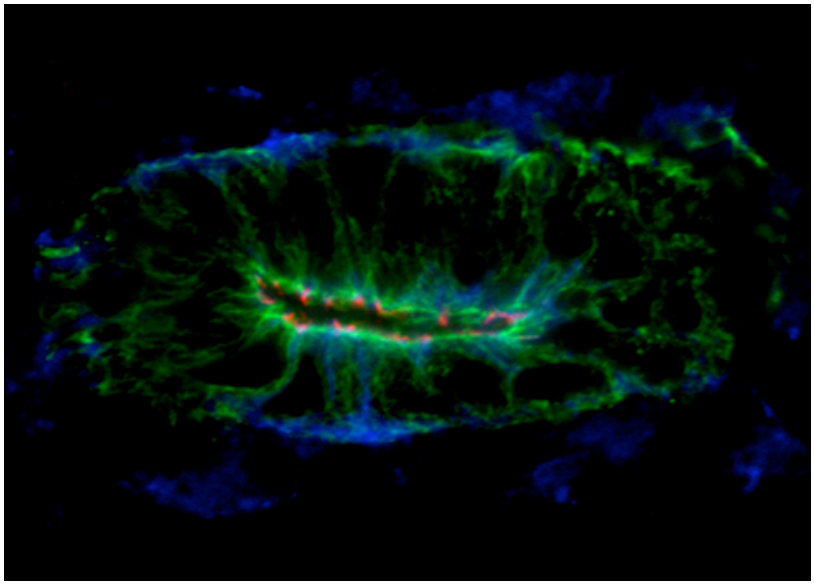


Figure 2-7. Treatment with IWP2 causes premature differentiation. Urogenital systems were cultured for two days starting at E11.5. The cultured tissue was then section and stained for ZO1a+, red, a tight junction protein expressed in only mature epithelia. Cytokeratin, green, was used as an epithelial marker and beta-catenin, blue, marks adherens junctions. ZO1a+ is not expressed in the DMSO control treated tissue. However, the apically located ZO1a+ protein is expressed in the 50uM IWP2 treated tissue, indicating premature differentiation.

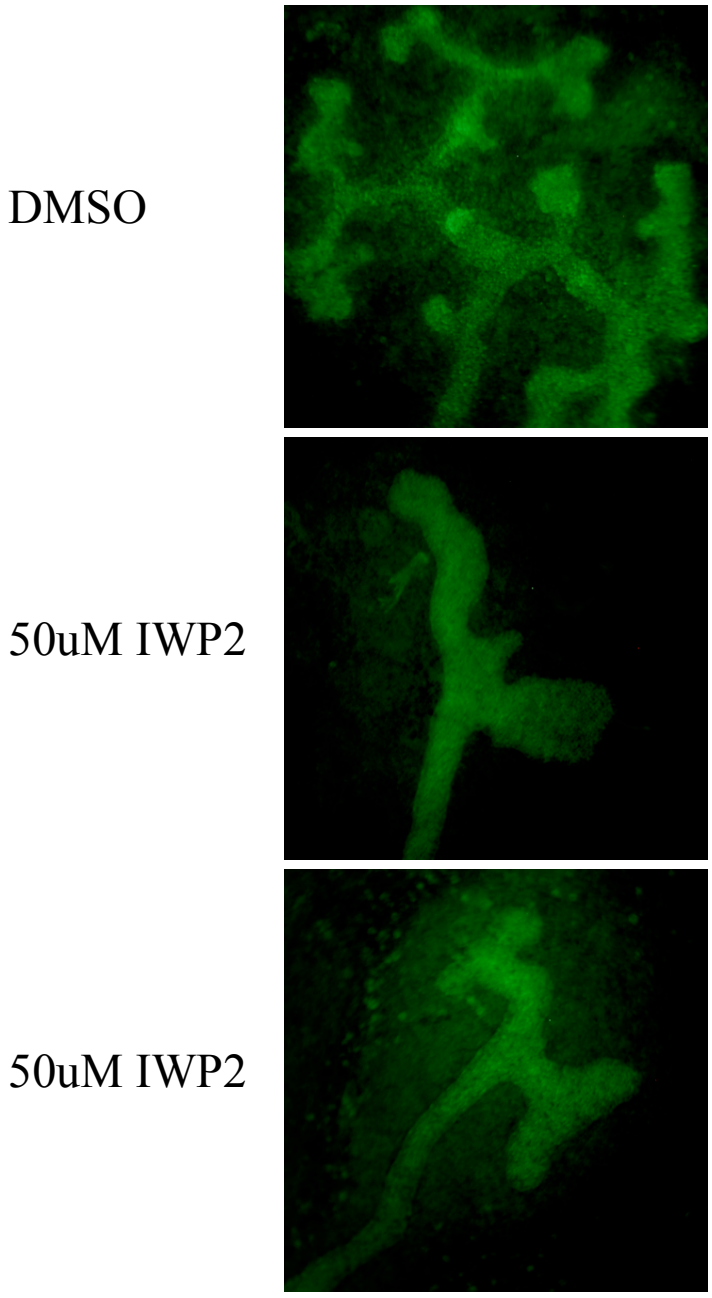
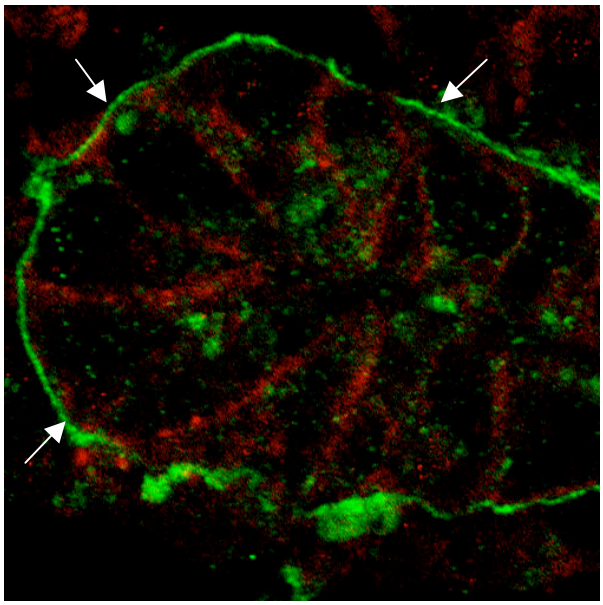


Figure 2-8. Cultured RosaWnt1GFP urogenital systems. Urogenital systems from mice expressing HoxB7cre; RosaWnt1GFP were cultured in the presence of DMSO as a control or 50uM IWP2 starting at E11.5. After two days of treatment, wholemount images were taken of the cultured kidneys looking for GFP expression determined that RosaWnt1GFP is expressed in culture.

DMSO



50uM IWP2

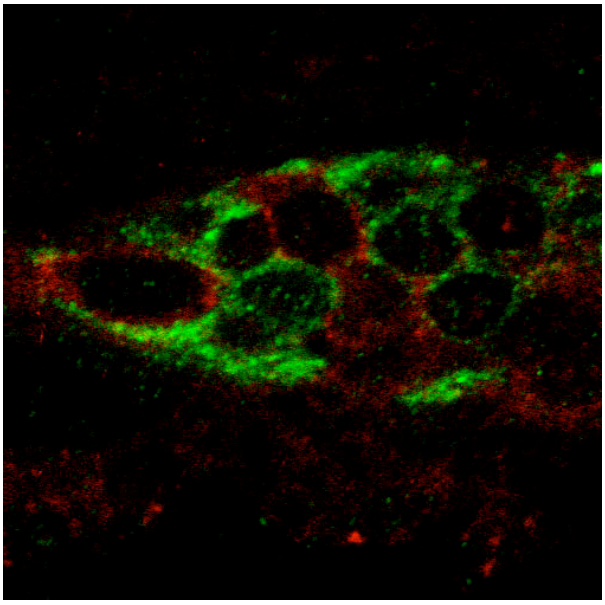


Figure 2-9. Wnt1 subcellular localization disrupted by treatment with IWP2. The cultured explants from figure 2-8 were sectioned and stained with antibodies for GFP, green, and beta-catenin, red. GFP staining shows Wnt1 expression. The arrow in the DMSO treated control indicates basolaterally located Wnt1 in the UB. This basolateral localization is lost in UBs treated with IWP2.

The expression patterns of Wnt reporters in developing urogenital systems

There are several known canonical Wnt signaling reporters in mammals, such as Axin2 mRNA expression and BAT-gal transgenic mice. Axin2 mRNA expression is directly regulated by beta-catenin transcriptional activity (Jho et al., 2002). The BAT-gal transgene contains multiple TCF binding sites that, in the presence of beta-catenin transcriptional activity, lead to the expression of beta-galactosidase (Maretto et al., 2003). However, both reporters only show canonical Wnt signaling activity in the WD and UB epithelia even though there is proof of canonical Wnt signaling activity in the mesenchyme as well (Deutscher and Hung-Chang Yao, 2007). We hypothesized that beta-catenin is differentially expressed in the urogenital system and controls the expression of different genes in a dosage dependent manner. Therefore, we wanted to identify additional Wnt signaling reporters with different expression patterns.

In Drosophila, naked cuticle (nkd) gene expression is directly regulated by armadillo, the Drosophila homolog of beta-catenin (Zeng et al., 2000). Nkd functions as an inhibitor of Dsh, thus inhibiting canonical Wnt signaling. There are two Nkd proteins in mice and humans, Nkd1 and Nkd2 (Wharton et al., 2001). Even though Nkd has been linked to beta-catenin in Drosophila, not much is known about the role the Nkds play in mammalian Wnt signaling. We were given $Nkd1^{Lacz}$ and $Nkd2^{Lacz}$ mice from Dr. Keith Wharton (Zhang et al., 2007). For both mouse strains, the exon encoding for the Dvl binding site was replaced with an IRES-lacZ/neomycin cassette. First, we needed to determine whether or not Nkd1 and/or Nkd2 are expressed in the developing urogenital system. Nkd1 is expressed as early as E11.5 in the developing urogenital system whereas Nkd2 is not expressed until after E11.5 (figure 2-10). The expression of both Nkd1 and

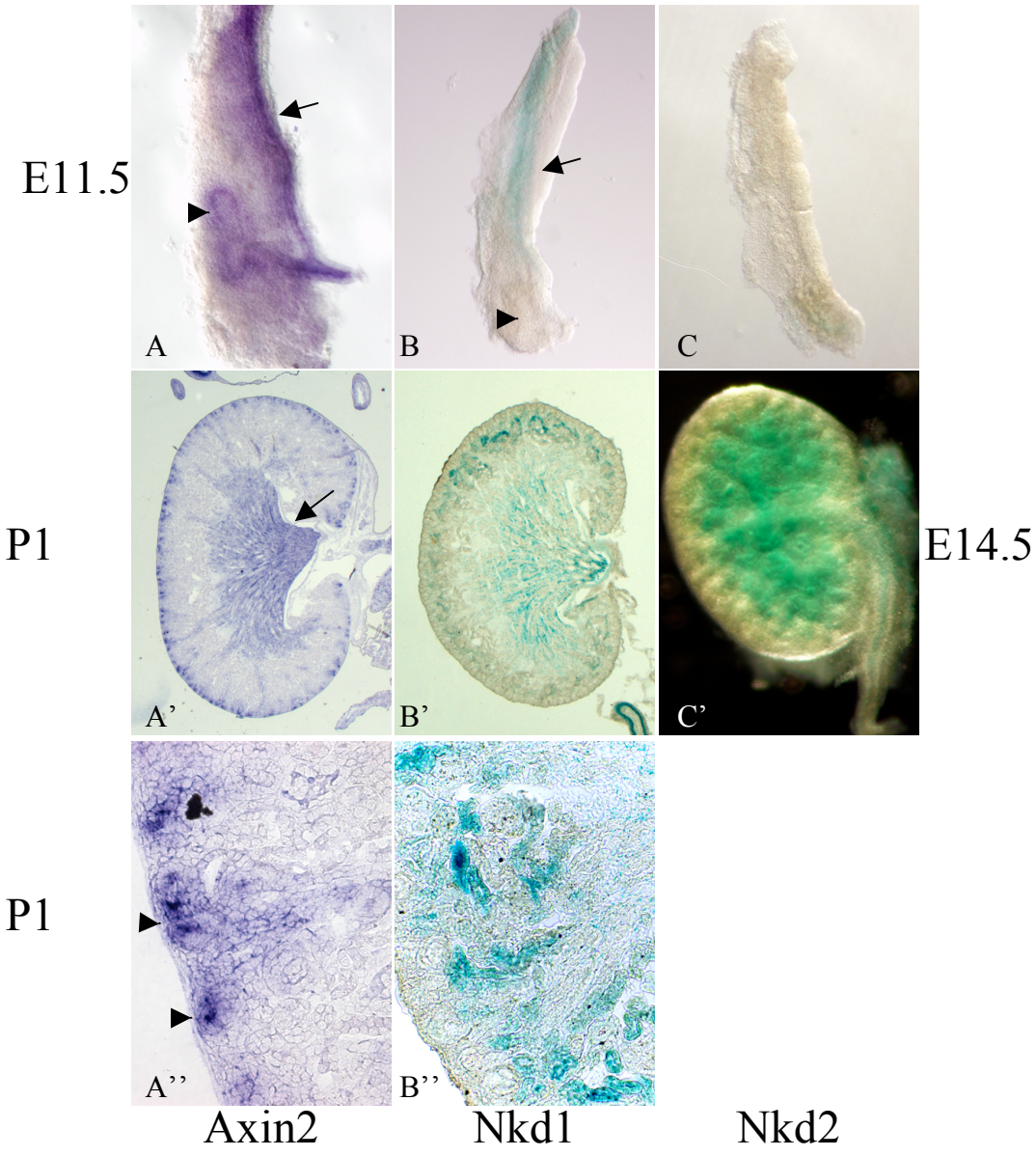


Figure 2-10. Expression patterns of Wnt signaling reporters. (A) In situ hybridization shows Axin2 mRNA expressed in the WD (arrow) and UB (arrowhead) epithelia at E11.5. (A',A'') In the kidney at P1, Axin2 is expressed in the medulla (arrow in A') as well as the branching UB tips (arrowhead in A''). LacZ staining indicates Nkd expression in urogenital systems from either a (B,B',B'') Nkd1^{LacZ} or (C.C'') Nkd2^{LacZ} embryo. (B) Nkd1 expression is initiated by E11.5 (arrow=WD, arrowhead=UB). (B',B'') In a P1 kidney, Nkd1 expression appears to be located in the mesenchymally derived nephron. (C) Nkd2 is not expressed in the urogenital system at E11.5 (C') but is expressed in the embryonic kidney by E14.5.

Nkd2 is restricted to the mesenchyme surrounding the WDs and MDs and appears to be expressed in the mesenchyme of the developing kidney (figure 2-10).

Nkd expression is affected by IWP2

If Nkds are reporters of canonical Wnt signaling in mammals, then it is interesting that the expression patterns of Nkd1, Nkd2, Axin2, and Bat-gal differ spatially and temporally. Axin2 and BAT-gal are expressed in the WD and UB epithelia starting at least as early as E11.5 (figure 2-10) whereas Nkd1 and Nkd2 are mesenchymally expressed and Nkd2 expression does not begin until after E11.5. Since Nkd1 and Nkd2 are directly regulated by beta-catenin transcriptional activity in Drosophila, we explored whether Wnt signaling in mammals affects the expression of the Nkds.

To determine the role Wnt signaling has on the expression of the Nkds, we dissected out urogenital systems from either Nkd1 or Nkd2 embryos. We cultured the Nkd urogenital systems starting at E12.5 since Nkd2 expression does not turn on until E14.5. We then treated the cultured urogenital systems with a dosage curve of IWP2 from 0.5uM to 50uM. After two days of culture, we were able to visualize Nkd expression by staining for lacZ (figure 2-11). The expression of both Nkd1 and Nkd2 was completely gone when treated with 50uM IWP2. Since IWP2 inhibits all Wnt signaling, these results indicate that the expression of both Nkd1 and Nkd2 is activated in the presence of Wnt signaling. However, an effect with IWP2 does not indicate which Wnt signal transduction pathway induces Nkd expression.

Treatment with IWP2 demonstrates that Nkd expression is induced by Wnt signaling. However, we wanted to identify which Wnt pathway induces Nkd expression in order to determine if mammalian Nkds are regulated in a similar manner to that of their drosophila homolog. IWR1 specifically inhibits beta-catenin transcriptional activity so we treated E12.5 nkd1 and nkd2 urogenital systems with 50uM IWR1 for two days. Nkd1 expression appeared to be downregulated when treated with 50uM IWR1 (figure 2-12). However, preliminary results show that treatment with 50uM IWR1 had no effect on the expression of Nkd2 (figure 2-12, N=3). Even though it might appear that Nkd2 expression is not affected by inhibition of canonical Wnt signaling, it was recently determined that the potency of IWR1 is approximately 100-fold weaker than IWP2 ((Chen et al., 2009) and personal communication with Courtney Karner). Therefore, 50uM IWR1 is equivalent to 0.5uM IWP2. Since 0.5uM IWP2 has a lesser effect on Nkd2 expression than on Nkd1 expression (figure 2-11), it would appear a stronger dose of IWR1 would be needed to show any effect on Nkd2 expression. Therefore, even though the results indicate that Nkd1 and Nkd2 expression is induced by Wnt signaling, we have not yet determined which pathway regulates Nkd2 expression. It appears that Nkd1 expression is regulated by canonical Wnt signaling. However, in order to identify the pathway necessary for Nkd2 expression, we will have to look at the effect that treatment with concentrations greater than 50uM IWR1 has on Nkd2 expression.

2.4 Discussion

When the IWs were identified, it was demonstrated that they are effective inhibitors of Wnt signaling in tissue culture. In order to determine whether or not these

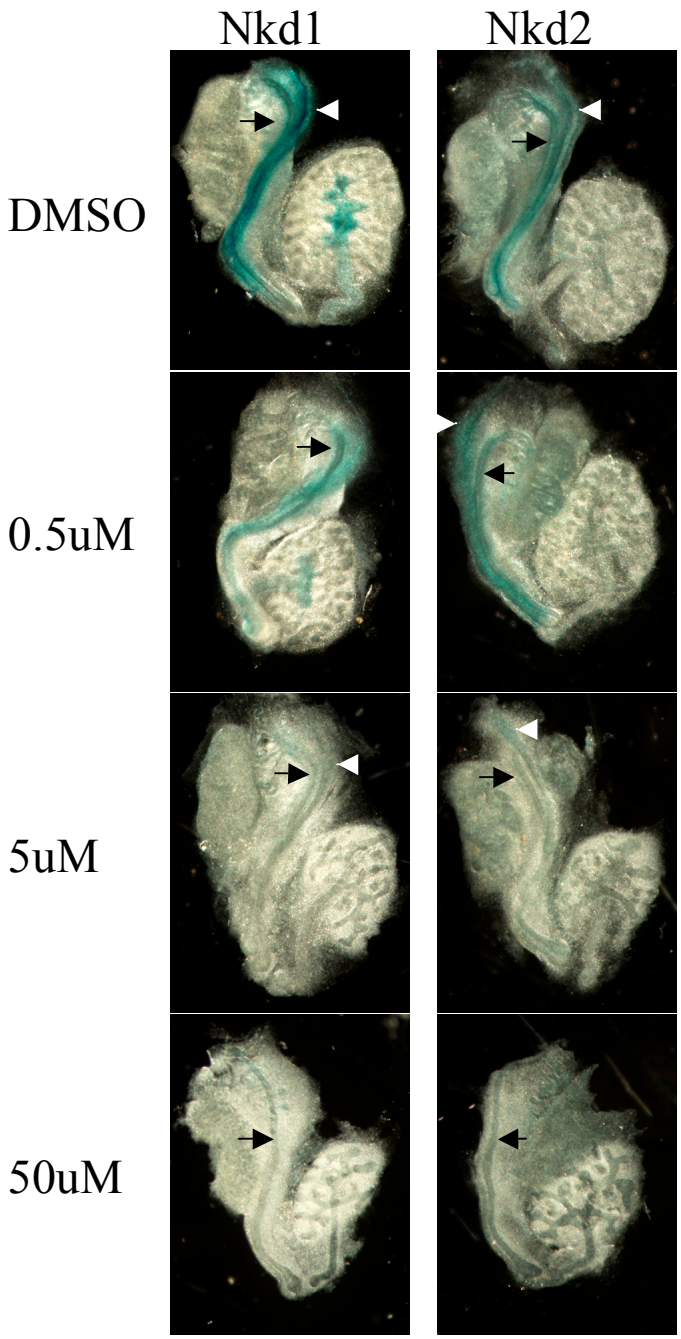


Figure 2-11. Expression of Nkd1 and Nkd2 is inhibited by IWP2. Nkd1^{LacZ} and Nkd2^{LacZ} urogenital systems were cultured, starting at E12.5, for two days with a DMSO control, 0.5uM IWP2, 5uM IWP2, or 50uM IWP2 and then stained for LacZ. The expression of both Nkd1 and Nkd2 decreases when treated with 0.5uM IWP2 and is completed lost with treatments of either 5uM or 50uM IWP2 indicating that Wnt signaling induces Nkd1 and Nkd2 expression. (black arrow=WD, white arrowhead=MD)

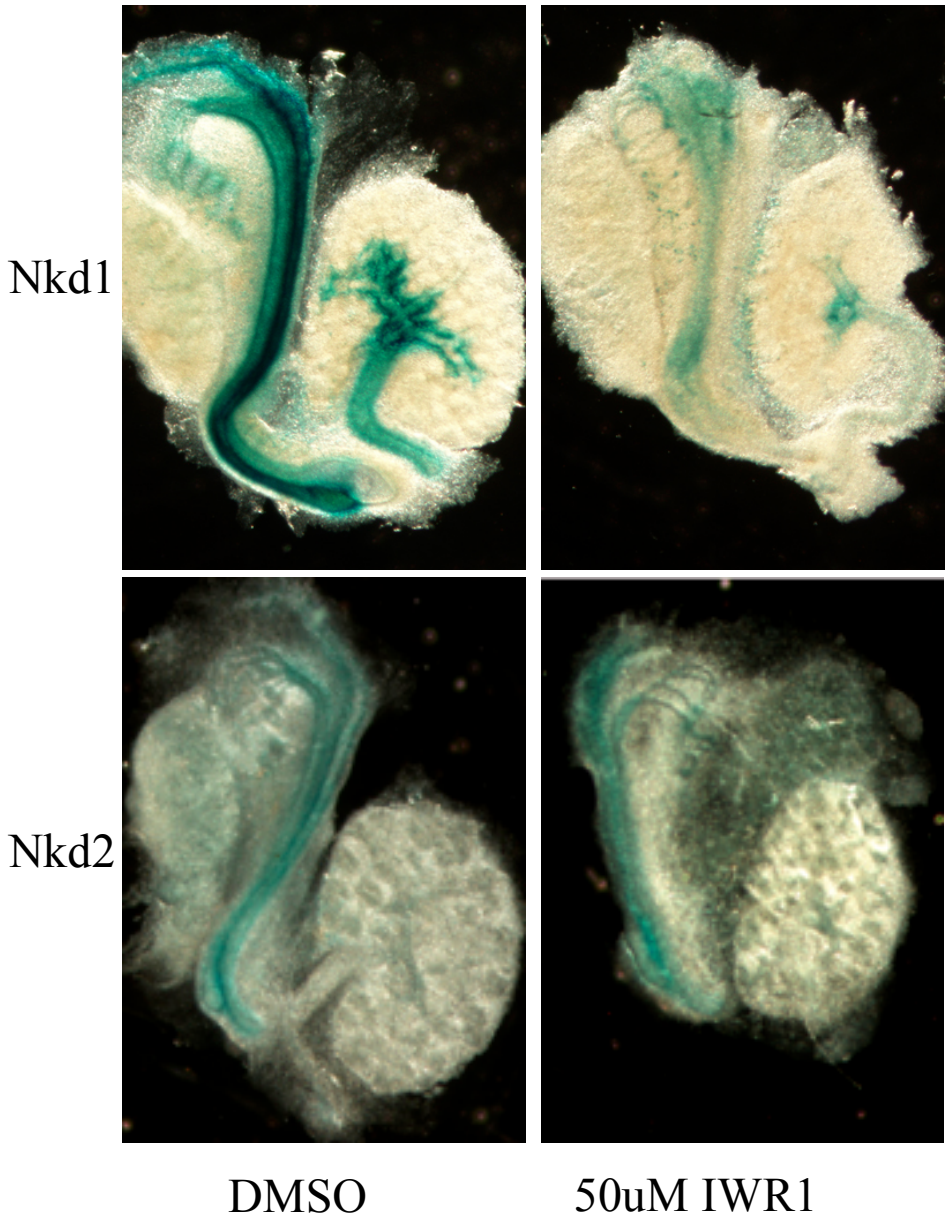


Figure 2-12. Effect of IWR1 on Nkd1 and Nkd2 expression. E12.5 Nkd1 and Nkd2 urogenital systems were treated with either DMSO as a control of 50uM IWR1. After two days of treatment, explants were stained for LacZ to visualized Nkd expression. IWR1 treatment causes a decrease in Nkd1 but has no effect on Nkd2 expression.

small molecule Wnt inhibitors function through multiple layers of cells, we treated cultured embryonic kidneys. Since certain aspects of kidney development are regulated by Wnt signaling, culturing embryonic kidneys in the presence of the IWs provided an easy assay to measure the efficacy of these drugs. Both branching morphogenesis and renal vesicle formation, Wnt regulated processes, are inhibited by the IWs.

The highest dose of IWP2 has the greatest effect on branching morphogenesis and renal vesicle formation. Branching appears to be inhibited almost completely with no renal vesicles forming. That observation brought into question whether IWP2 is toxic at higher doses or if it is inhibiting these processes by disrupting Wnt signaling. In addition to exhibiting the similar phenotype of WD premature differentiation to that of a beta-catenin mutant (figure 2-7), the expression of certain genes, such as Pax2 (figure 2-13), are still present in treated kidneys. Regardless, markers of proliferation and apoptosis should be examined to rule out a toxic effect of the small molecules. Furthermore, a microarray comparing IW treated to wild-type would give a more comprehensive view of the molecular impact the IWs have on the developing kidney.

Interestingly, there are obvious morphological differences between IWP2 and IWR1 treated urogenital systems, with IWP2 treated urogenital systems showing a more severe phenotype. IWR1 inhibits only canonical Wnt signaling while IWP2 inhibits both canonical and noncanonical Wnt signaling. A more severe phenotype in IWP2 treated urogenital systems as compared to IWR1 treated urogenital systems could be due to the fact that IWP2 inhibits all Wnt pathways as opposed to IWR1 only inhibiting canonical signaling. However, we have only compared urogenital systems treated with equal doses of both IWP2 and IWR1. Since these two drugs vary in potency, we need to compare

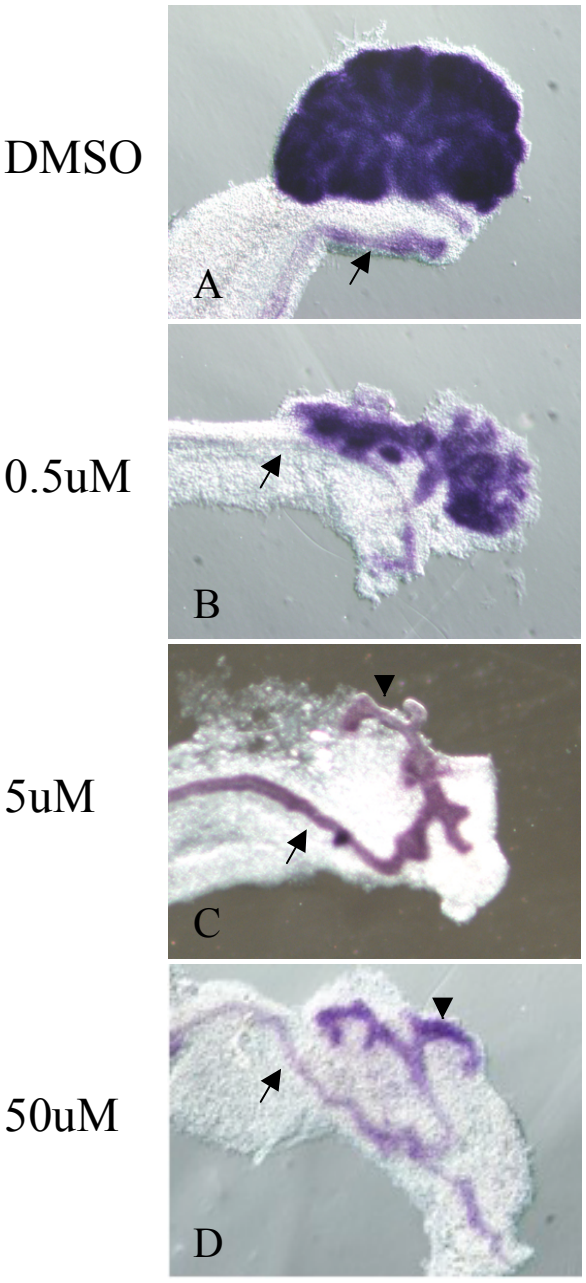


Figure 2-13. Pax2 expression is downregulated in IWP2 treated urogenital systems. E11.5 urogential systems were cultured for two days in the presence of (A) a DMSO control, (B) 0.5uM IWP2, (C) 5uM IWP2, or (D) 50uM IWP2. In wild-type, and the DMSO contol, Pax2 is expressed in the WD, UB and portions of the MM that will give rise to nephrons. Treating with 0.5uM IWP2 leads to decreased expression of Pax2 in the mesenchyme. Both 5uM and 50uM doses completely inhibit mesenchymal expression of Pax2. However, Pax2 is still express in the WD and the UB. (arrow=WD, arrowhead=UB)

them at equivalent doses. Furthermore, an examination of processes regulated by non-canonical signaling would provide evidence that IWP2 inhibits non-canonical as well as canonical Wnt signaling in embryonic kidney culture. If the morphological differences are indicative of the inhibition of the different pathways, further examination of these morphological differences between IWP2 and IWR1 treated urogenital systems could better identify which pathway regulates what developmental processes.

There are currently no known transcriptional readouts of non-canonical Wnt signaling in mammals. The IWs could be used to identify downstream targets of noncanonical Wnt signaling. A microarray could be performed on IWP2 versus IWR1 treated tissue to identify differences in gene expression caused by the two treatments. Transcriptional targets of non-canonical Wnt signaling could be identified from such a screen. A transcriptional readout of non-canonical Wnt signaling would be a very important tool to study the role non-canonical Wnt signaling plays in development.

Even though the testing of these drugs is in its preliminary stages, the ability of IWs to function through multiple cell layers in organ culture suggests potential success *in vivo*. Improper regulation of Wnt signaling is thought to play a causal role in several kidney diseases such as Wilms' tumor, polycystic kidney disease (PKD), renal cell carcinoma (RCC), and diabetic nephropathy (Merkel et al., 2007; Pulkkinen et al., 2008).

Wilms' tumor is a fetal renal malignancy that often leads to removal of an entire kidney. Unfortunately, Wnt signaling is overactive in Wilms' tumor due to beta-catenin gain-of-function mutations (Koesters et al., 1999). Since both families of IWs function upstream of beta-catenin, neither would serve as an effective therapeutic for Wilms' tumor.

Nephropathy, a progressive kidney disease resulting in damage to the glomerulus, is another condition associated with Wnt signaling. There are several causes of nephropathy such as diabetes, high blood pressure, and long term exposure to certain drugs. Studies have shown Wnts as playing a role in high glucose-induced apoptosis in nephropathy (Lin et al., 2006). High glucose leads to the downregulation of Wnts 4 and 5a as well as increased levels of GSK3beta, subsequently inhibiting canonical Wnt signaling. High glucose-mediated cell apoptosis can be reduced by inhibiting GSK3beta and thus upregulating Wnt signaling. Therefore, Wnt inhibitors would only worsen high glucose mediated nephropathy. However, in addition to high glucose, studies have shown that a condition called nephrogenic diabetes insipidus can be acquired as a side effect to certain medications, most notably lithium (Christensen et al., 1985). LiCl acts as a Wnt agonist. This would imply that over activation of canonical Wnt signaling is a cause of diabetic nephropathy. Therefore, treatment with small molecule Wnt inhibitors would prove useful in treating lithium induced diabetic nephropathy.

RCCs consist of adult malignancies of the kidney. Several components of the Wnt pathway are mutated in RCCs, causing aberrant activation of canonical Wnt signaling (Pulkkinen et al., 2008). Hypoxia-inducible protein-2 (HIG2), an early onset diagnostic marker of RCC, functions by binding to Fzd10, activating several genes associated with canonical Wnt signaling, and causing increased rates of proliferation (Togashi et al., 2005). Furthermore, the TCF/beta-catenin complex binds and activates the HIG2 promoter. Currently, surgery is most efficient treatment for RCC. Treating the cancer molecularly would be less invasive and possibly a better option than surgery.

PKD is the most common inherited disease in the United States and the fourth leading cause of kidney failure. As described in the introduction, both canonical and non-canonical Wnt signaling pathways are implicated in cystogenesis (Otto et al., 2003; Qian et al., 2005; Saadi-Kheddouci et al., 2001; Simons et al., 2005). Therefore, both RCC and PKD are examples of diseases that small molecule Wnt inhibitors could potentially target as a therapeutic. After the efficacy of the IWs in embryonic kidney culture has been completely characterized, the next step is to test the drugs in mice. Eventually, these small molecule Wnt inhibitors could be useful therapeutic agents for a variety of diseases involving improper regulation of Wnt signaling.

2.5 Materials and methods

Organ culture

E11.5 (wild-type) or E12.5 (Nkd1, Nkd2) urogenital systems were dissected out and cultured for 48 h in DMEM with 10% FBS, 1% Pen/Strep and either IWP1, IWP2, IWR1, or DMSO as a negative control. The treated media was changed every 12 hours. After 48 h, tissues were rinsed in PBS and were either fixed for β-galactosidase staining, in situ hybridization or antibody staining.

Wholemount expression analysis

Rosa reporter activity was assessed as previously described (Soriano, 1999). For in situ hybridization, tissues were post-fixed in 4% paraformaldehyde for 24 h at 4° prior to hybridization with antisense, digoxygenin labeled mRNA probes. Probes examined were Wnt7a (cut with SalI and transcribed with T7 polymerase), Pax8 (XbaI, T7), Wnt9b (see Carroll et al., 2005), Wnt11 (see Carroll et al., 2005), Axin2 (EcoRI, T3), Pax2 (see

Carroll et al., 2005). Hybridized embryos were cleared in 80% glycerol and photographed using a Nikon digital still camera (DXM1200) on a Nikon stereomicroscope (SMZ1500).

Mice

Wnt1GFP was ectopically expressed in the WD with mice doubly heterozygous for Hoxb7-Cre (Yu et al., 2002) and Rosa26 Wnt1EGFP (Carroll et al., 2005). Nkd1 and Nkd2 protein expression was identified by the expression of beta-galactosidase from either $Nkdl^{LacZ}$ or $Nkd2^{LacZ}$ mice (Zhang et al., 2007). Pax8YFP mice were used to visualize renal vesicles and the MD.

Immunohistochemistry

E11.5 urogenital systems cultured for two days in either 50uM IWP2 or DMSO were fixed in 4% paraformaldehyde at 4 °C overnight, washed 3 times in PBS and either put into a blocking solution for a wholemount antibody stain or equilibrated in 30% sucrose then imbedded in OCT and flash frozen on dry-ice. Frozen tissue was sectioned at 10 uM on a cryostat and sections were stored at − 80 °C until usage. Thawed sections were boiled for 10 min in 10 mM Tris, pH 8.0 and 5 mM EDTA then incubated with primary antibodies to beta-catenin (rabbit, 1;500, Chemicon, AB19022 and mouse, 1:500, Sigma c7207), ZO1a+ (rat, 1:200, Santa Cruz, sc-33725) cytokeratin (mouse, 1:200, Sigma, C2562), GFP(rabbit, 1-;1000, abcam, ab290). The secondary antibodies were Alexafluor 488, 568 and 633 (Molecular Probes). Tissues were mounted in VectaShield with Dapi (Vector labs). Sections were viewed and photographed on a Zeiss LSM510 Axioplan inverted confocal microscope.

Chapter 3:

Beta-catenin and development of the reproductive tract

3.1 Abstract

Due to the strong expression of a Lef/TCF/beta-catenin reporter in the Wolffian duct (WD) and its derivatives, we created a line of mice in which beta-catenin was removed from these tissues by Cre recombinase expressed under the control of the Hoxb7 promoter. Surprisingly, in mutant females, we saw a partial sex reversal: no Müllerian duct (MD) derivatives formed and the WD was maintained. Maintenance of the WD is independent of ectopic androgen synthesis in the gonads. Normally the MD forms through invagination of the coelomic epithelium and subsequent extension toward the cloaca. In the beta-catenin mutant females, the MD invaginates but never reaches the cloaca. This appears to be due to the absence of a migratory signal normally produced by the WDs. Interestingly, Wnt9b, a gene that we have previously shown is essential for caudal extension of the MDs, appears to be absent in the beta-catenin mutant cells. We are currently testing whether the absence of Wnt9b is causal for the mutant phenotype. Finally, improper maintenance of the mutant WD appears to be the result of premature differentiation of this structure. These data not only further our knowledge of the molecular regulation of the reproductive system but also show for the first time that the WD can be maintained in the absence of gonadal androgens.

3.2 Introduction

Proper development of the male and female reproductive tracts is required for continuation of the species. In mice, the somatic portions of the reproductive system arise from the intermediate mesoderm. In males, the epididymides, vas deferentia and seminal vesicles are derived from the WDs. In females the oviducts, uterus, cervix and upper-

vagina are derived from the MDs. Previous studies have shown that in mice, the MD is induced to form by the WD between E11.5 and E13.5. At E13.5, prior to undergoing sexual differentiation, both male and female mammalian embryos have bipotential gonads possessing both the WD and MD. In an embryo that is genotypically male, the gonads differentiate into testes and produce Müllerian inhibiting substance (MIS) and androgens such as testosterone. In response to MIS, the MD is degraded while the WD is maintained by testosterone. In embryos that are genotypically female, the gonads differentiate into ovaries and since no testosterone or MIS is produced, the WD is degraded and the MD is maintained. Since the absence of MIS and testosterone results in the degradation of the WD, it was thought that formation of the female reproductive tract was the default state.

Reproductive tract development is dependent on two processes: sex specification and sex differentiation. In mammals, sex specification is a process in which genes specific to either a male or female specify their sexual morphology. Sex differentiation is the process in which the bipotential gonads differentiate into either testes or ovaries which will then produce hormones that cause the WDs or MDs to differentiate into the male or female reproductive tracts, respectively. Although the molecular regulation of sex specification is well understood (Sekido and Lovell-Badge, 2009), we know surprisingly little about the molecules regulating sex differentiation. Interestingly, sex specification birth defects are extremely rare whereas sex differentiation defects constitute some of the most common birth defects seen in humans. Therefore there is great interest in discovering factors that lead to defects in the differentiation of sex organs.

Wnts constitute a family of secreted glycoproteins that are essential for the development of all multi-cellular organisms thus far examined. The Wnt signal can be transduced down one of several different pathways roughly divided into the canonical and non-canonical pathways. There are several Wnts expressed in and around the developing reproductive tract including Wnt4, Wnt5a, Wnt7a, and Wnt9b. Ablation of any of these genes results in distinct defects of the female reproductive tract. The cellular targets of these Wnts and the pathway through which they function in reproductive tract development are unknown. The following research is focused on the role of the canonical/beta-catenin dependent signal transduction branch of the pathway. To investigate the role of this branch in urogenital development, we have taken advantage of several tools that are available including conditionally inactive and activated mouse lines as well as small molecule antagonists of the canonical pathway.

3.3 Results

Conditional removal of beta-catenin from the WD results in partial sex reversal

We previously characterized canonical Wnt activity in the developing urogenital system using a BAT-gal line of transgenic mice and the expression of Axin2 mRNA. As was mentioned, the BAT-gal transgene contains multiple TCF binding sites that, in the presence of beta-catenin transcriptional activity, drive the expression of beta-galactosidase. Also, Axin2 is a direct transcriptional target of beta-catenin. At E11.5, both reporters indicate that canonical Wnt signaling is active in both the WD and UB (Marose et al., 2008). Canonical Wnt signaling remains active in the WD and WD

derivatives until birth (figure 3-1). Even though reporters show canonical Wnt activity in the WD, the WD is not affected in any of the published Wnt knockouts. Therefore, we were interested to determine what role canonical Wnt signaling plays in this tissue.

To determine the role of canonical Wnt signaling in development of the reproductive tract, we removed beta-catenin conditionally in areas of active canonical Wnt signaling with a HoxB7cre line of mice. Hoxb7cre is expressed throughout the WD starting at E9.5 as well as the UB once it forms (Yu et al., 2002). Beta-catenin is removed mosaically in the Hoxb7cre; $Bcat^{c/-}$ mice with the amount of beta-catenin removed variable between mice (Marose et al., 2008). Regardless, mice with beta-catenin conditionally removed from the WD and UB die within 24 hours of birth due to kidney failure.

Beta-catenin mutants exhibit multiple defects of the urogenital system. The kidneys are either aplastic or cystic/dysplastic with dilated ureters (Marose et al., 2008). In addition to kidney defects, mutant females exhibit a partial sex reversal phenotype (figure 3-2). Mutant females form histologically normal ovaries but maintain WD derivatives. HoxB7cre is expressed in the WD and WD derivatives but is not expressed in the MD. Using the Rosa Reporter, we were able to identify the WD and WD derivatives in HoxB7cre;$Bcat^{c/-}$;Rosa26 mutant mice by evidence of LacZ expression. Both B7cre;$Bcat^{+/-}$;Rosa26 males and B7cre;$Bcat^{c/-}$;Rosa26 females, but not B7cre;$Bcat^{+/-}$;Rosa26 females, showed LacZ expression in the WD derivatives (figure 3-2), indicating that mutant females maintain their WD. Along with LacZ staining proving that the WD is

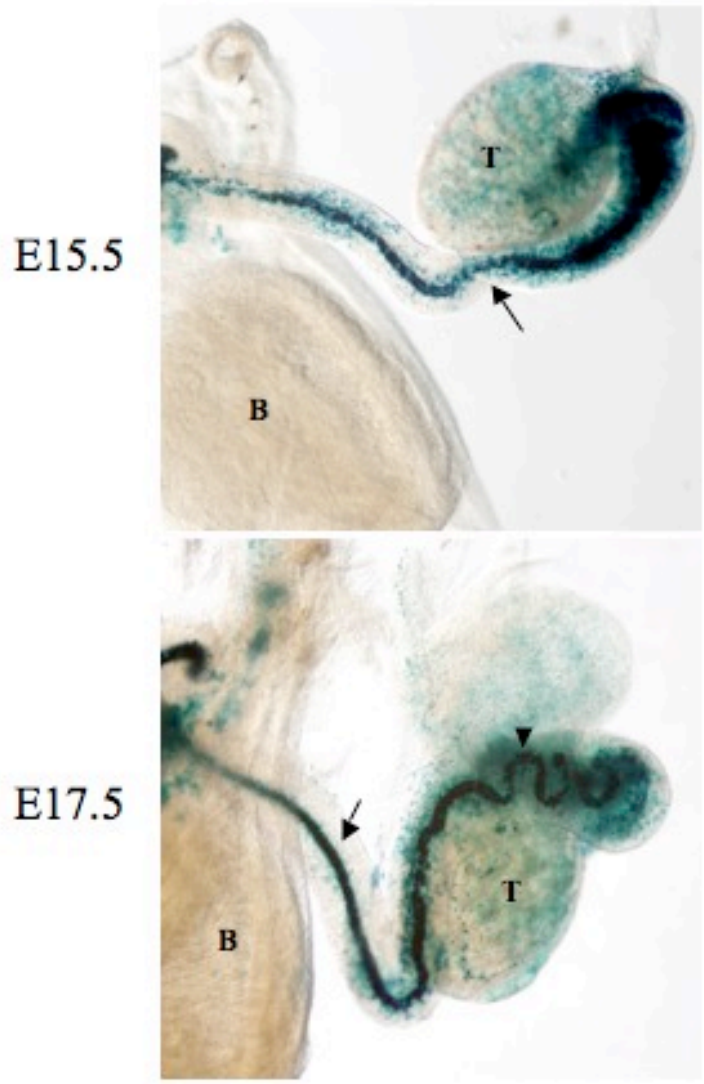


Figure 3-1. Beta-catenin activity in developing male reproductive tract. We used BAT-gal mice as a reporter of canonical Wnt signaling activity. LacZ staining is indicative of areas of active canonical Wnt signaling. At E15.5, the MD has regressed in males and canonical Wnt signaling remains active in the WD (arrow). As the WD undergoes differentiation in the male reproductive tract, canonical Wnt signaling activity continues throughout the WD and the WD derivatives in males until birth (arrow=vas deferens, arrowhead=epididymis). (T=testis, B=bladder)

maintained, the mutant WD appears to have coiled anteriorly, near the ovary, reminiscent of an epididymis. Furthermore, in addition to maintenance of the WD, mutant females do not form oviducts, uteri, an upper vagina or any MD derivatives.

Similar to the beta-catenin mutants, Wnt4 mutants exhibit partial sex reversal phenotype, exhibiting maintenance of WD derivatives but lacking MD derivatives. A partial sex reversal phenotype is commonly associated with defects in sex determination. For example, Wnt4 is expressed in the ovaries and removal causes masculinization of the ovary and thus ectopic expression of androgens. However, beta-catenin expression is not affected by excision with HoxB7cre in the gonads. However, in addition to expression in the WD, HoxB7cre is also expressed in portions of the neural crest that give rise to the adrenal glands. Since adrenal glands are hormone-producing organs, removal of beta-catenin from the adrenal glands could lead to the ectopic expression of androgens. To determine which tissue is responsible for the beta-catenin mutant phenotype, we conditionally removed beta-catenin with two additional cre lines. Wnt1cre is expressed in portions of the neural crest that give rise to the adrenal gland but is not expressed in the WD. Pax2cre is expressed in the WD but not in the neural crest. Removal of beta-catenin in the adrenal gland with Wnt1cre results in normally forming female reproductive tracts (figure 3-2). However, when beta-catenin is removed from the WD with Pax2cre, mutant females exhibit maintenance of the WD and a lack of MD derivatives (figure 3-2). Since removal of beta-catenin from the WD results in the same reproductive tract phenotype as HoxB7cre; $Bcat^{c/-}$ females, we can conclude that it is removal of beta-catenin from the WD, and not the adrenal glands, that causes the partial sex reversal phenotype.

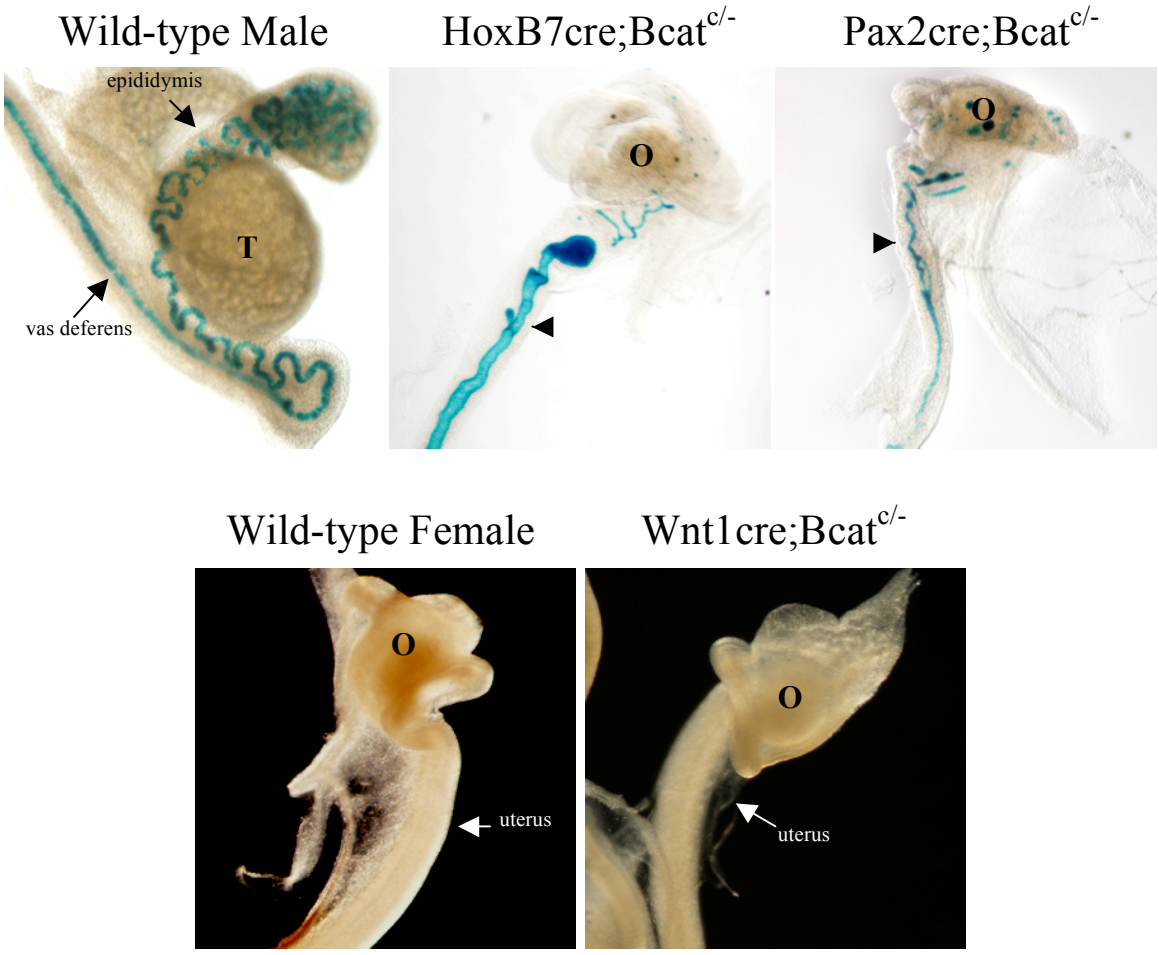


Figure 3-2. P1 beta-catenin female mutant phenotype. Beta-catenin was conditionally removed using either HoxB7cre, Pax2cre, or Wnt1cre. LacZ staining by use of the Rosa Reporter represents areas of HoxB7cre and Pax2cre activity. Both HoxB7cre and Pax2cre are expressed in the WD but not in the MD. Females with beta-catenin removed in areas of HoxB7cre expression or Pax2cre expression exhibit the same female reproductive tract phenotypes: loss of MD derivatives and maintenance of WD, as indicated by LacZ staining in comparison to the wild-type male. Removal of beta-catenin from the neural crest by use of Wnt1cre results in a phenotypically normal female reproductive tract as compared to a wild-type female. (O=ovary, T=testis, arrowheads=WD derived structure)

Beta-catenin mutant females do not become masculinized

As mentioned, Wnt4 mutant females ectopically express androgens. Even though beta-catenin is still expressed in the ovaries in HoxB7cre;Bcat$^{c/-}$ mice, a signal from the WD could be lost, leading to masculinization of the mutant ovaries. To determine if beta-catenin mutant ovaries ectopically express androgens, we looked for the expression of certain differentiated testes markers. Differentiated testes form two main cell types: Leydig and Sertoli cells. Leydig cells produce testosterone, which leads to maintenance of the WD, whereas Sertoli cells produce MIS, which leads to degradation of the MD. Both differentiated testes (figure 3-3) and Wnt4 mutant ovaries express Cyp17alpha, a gene encoding an enzyme necessary for testosterone biosynthesis, 3betaHSD, a Leydig cell marker, and MIS, a Sertoli cell marker (Vainio et al., 1999a). However, none of these differentiated testes markers are expressed in beta-catenin mutant ovaries (figure 3-3). Wnt4 mutant ovaries, in addition to expressing testis differentiation factors, become rounded in shape and develop a few sex cords, reminiscent of a testis. However, beta-catenin mutant ovaries look histologically normal which, along with the expression data, indicates that beta-catenin mutant ovaries are not masculinized.

WD maintenance due to premature differentiation

It has been shown in other tissues that beta-catenin acts to maintain cells in a precursor-like state. For example, removal of beta-catenin in CNS results in larger ratio of differentiated cells to precursor cells (Brault et al., 2001). In addition, active Wnt/beta-

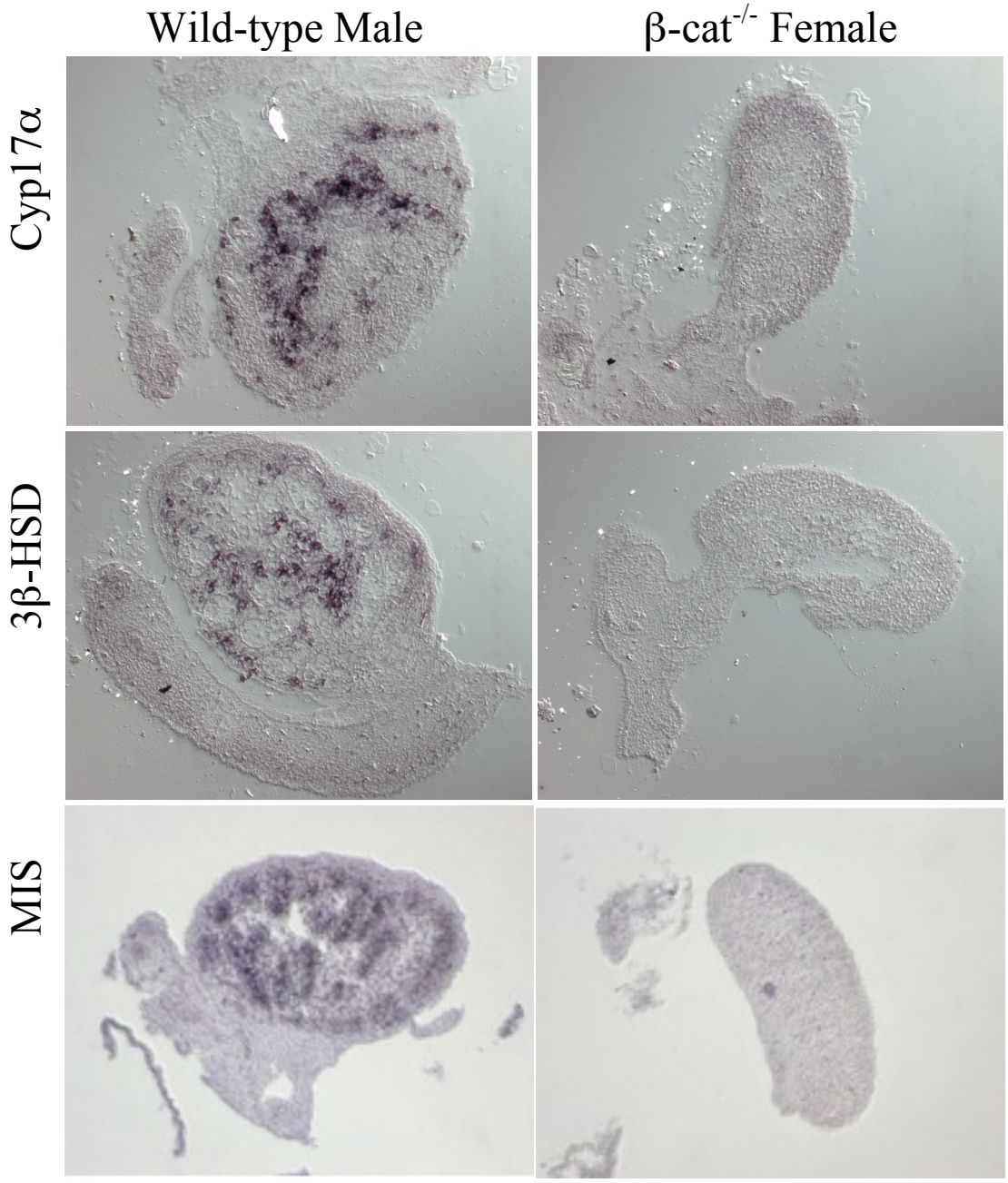


Figure 3-3. Gonads are specified correctly in beta-catenin mutants. In the top panel, an E14.5 wild-type testis and a β-catenin mutant ovary are stained by in situ hybridization for 17α-hydroxylase (Cyp17α) mRNA expression, a key enzyme in the synthesis of male hormones. The middle panel shows in situ staining with 3β-hydroxysteroid dehydrogenase (3β-HSD) expression, a Leydig cell marker. The bottom panel shows staining for Mullerian inhibiting substance (MIS) expression. All three markers of the differentiated testis are expressed in the wild-type male but not in the beta-catenin mutant ovaries.

catenin signaling acts to maintain intestinal and epidermal stem cells (van de Wetering et al., 2002; Zhu and Watt, 1999). We previously showed that beta-catenin is necessary to keep the cells of the WD in an undifferentiated state (Marose et al., 2008). We hypothesized that maintenance of the WD in females is not due to defects in sex determination but rather due to premature differentiation of the WD. To test this, we stained for markers of differentiation: ZO1a+ and Aqp3. ZO1a+ is a tight junction protein expressed only in mature epithelia. Aqp3 is a water channel protein expressed in the collecting duct and the collecting tubule of the mature kidney as well as in the basal cells of the proximal portion of the epididymis. At E11.5, neither ZO1a+ nor Aqp3 is expressed in the embryonic WD. However, in WDs mutant for beta-catenin, we show that both ZO1a+ and Aqp3 are prematurely expressed at E11.5 (Marose et al., 2008). Precocious expression of differentiation markers indicates that beta-catenin mutant WDs are prematurely differentiating. We hypothesize that this premature differentiation is preventing the WD from becoming degraded in mutant females, independent of androgens.

Since mutant WDs precociously express Aqp3, which is expressed in both the mature kidney and male reproductive tract, we wanted to determine into what the WD is differentiating at P1. The male reproductive tract is composed of the epididymis, vas deferens and seminal vesicles. The epididymis and vas deferens can be divided into multiple molecularly distinct regions: the caput (head), corpus (body), and cauda (tail) epididymis, and the proximal and middle vas deferens. Multiple Aqps are patterned throughout the male reproductive tract. Even though several of these Aqps are also expressed in the mature kidney, Aqp9 expression is specific to the male reproductive

tract. Aqp9 is expressed in the principal cells of all segments of both the epididymis and vas deferens. We wanted to examine the expression of Aqp9 in our mutants to determine whether or not they are differentiating into male reproductive tracts or kidneys. We developmentally profiled the expression of Aqp9 in wild-type male reproductive tracts and determined that it is absent at P1 but expressed at P7 (figure 3-4). However, neither mutant males nor mutant females express Aqp9 at P1 (figure 3-4). Even though we hypothesized that the mutant WDs are prematurely differentiating, a lack of Aqp9 expression in both mutants indicates that the WDs are not prematurely expressing Aqp9. Since mutants die within 24 hours of birth, we cannot determine whether or not Aqp9 is expressed by P7. Therefore, we needed to look at the expression of another water channel protein.

Aqp2, an apical marker of kidney collecting ducts and principal cells in the middle vas deferens, is expressed at P1 in both the kidney and male reproductive tract (figure 3-5). In beta-catenin mutant males but not females, Aqp2 is located apically in the mutant WD. Sex specific expression of Aqp2 would indicate that the mutant WD is differentiating into the male reproductive tract and not the kidney., Even though the WD does not regress in female mutants, the fact that female mutant WDs do not express Aqp2 indicates that females are missing factors necessary for differentiation of the WD. Therefore, it appears that We currently do not know whether premature differentiation of the WD or loss of factors downstream of beta-catenin plays the causative role in the retardation of male reproductive tract development in the beta-catenin mutants.

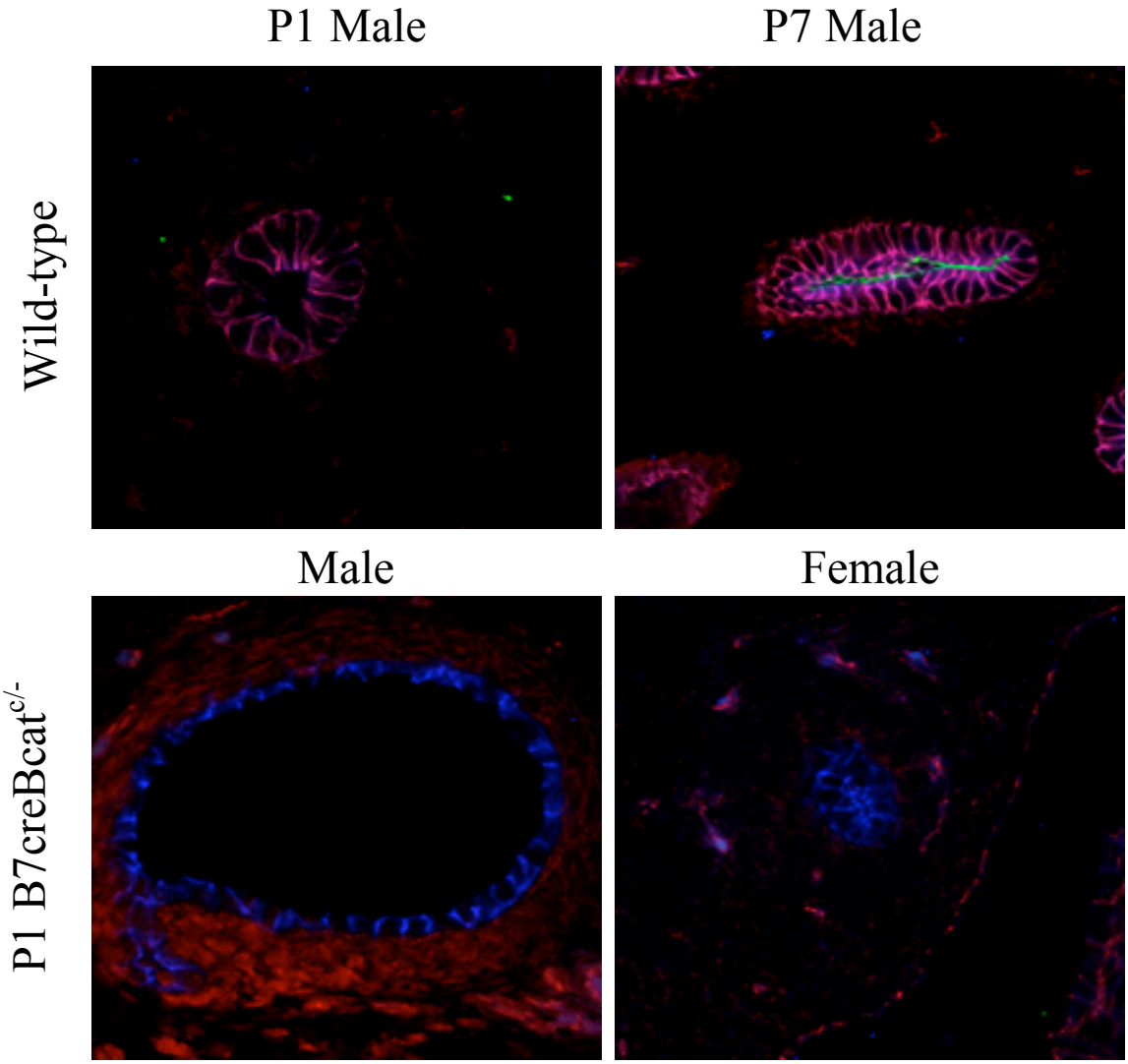


Figure 3-4. Aqp9 is not expressed in beta-catenin mutant Wolffian ducts. Wild-type and mutant WD derivatives are stained with Aqp9 (green), beta-catenin (red), and E-cadherin (blue). Aqp9 is a water channel protein expressed at P7 but not P1 in the wild-type male reproductive tract. At P1, neither beta-catenin mutant males or females, identified by lack of beta-catenin staining, express Aqp9.

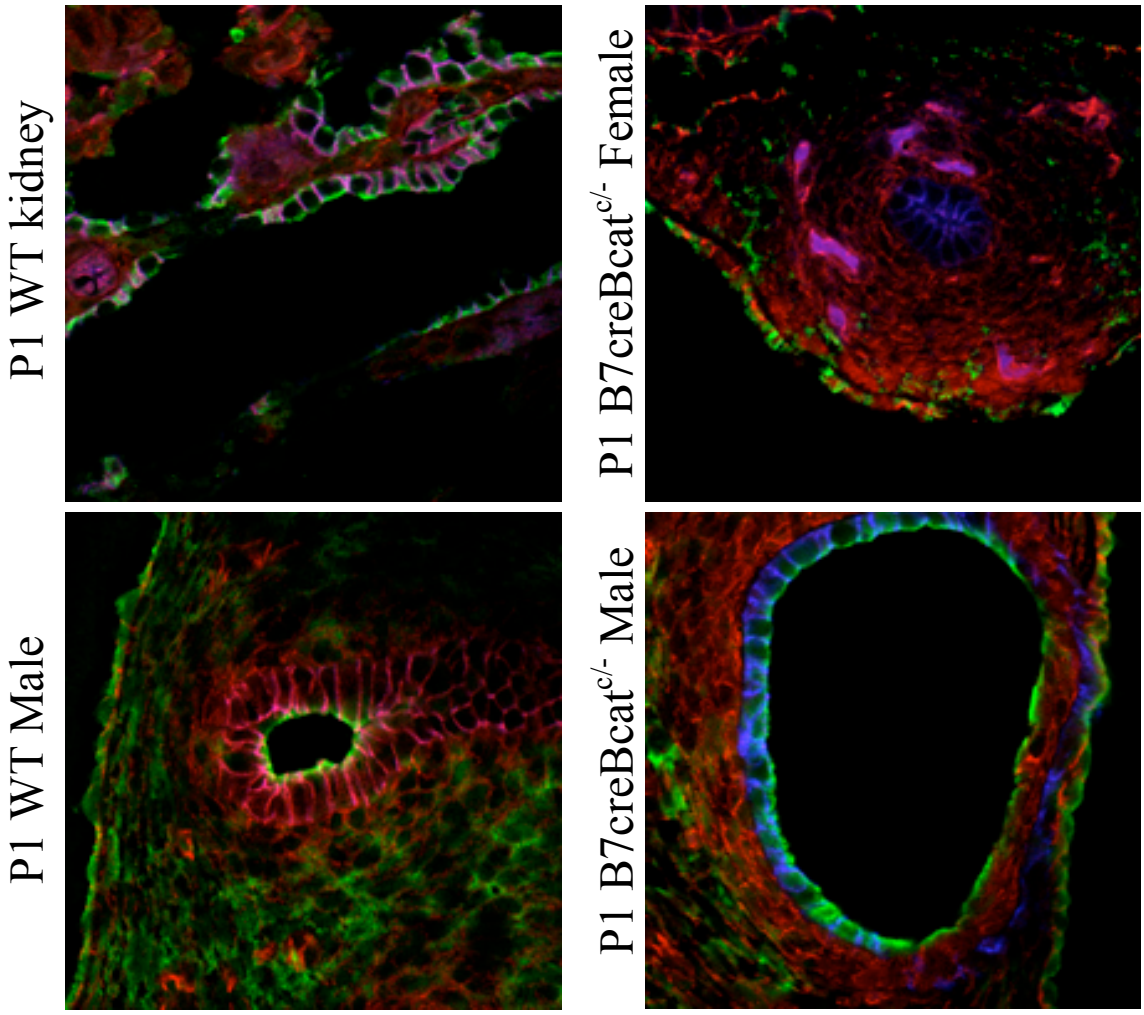


Figure 3-5. Aqp2 expression in mutant Wolffian ducts. Aqp2 (green) is expressed apically in the collecting ducts in P1 kidneys and in the principal cells of the middle vas deferens in P1 male reproductive tracts. Aqp2 is expressed in male mutant WDs in the absence of beta-catenin (red) but is not expressed in female mutant WDs. Green=Aqp2, Red=beta-catenin, Blue=E-cadherin.

Even though mutant males appear to be differentiating, they do exhibit a male reproductive tract phenotype. The male reproductive tract does not form normally, specifically in areas of beta-catenin activity such as the epididymis and vas deferens (figure 3-6). The epididymis fails to coil properly and in portions, become extremely dilated. In addition, the vas deferens sometimes becomes dilated as well. Dilation of the epididymis and vas deferens could be due misexpression of a water channel protein. These results indicate that beta-catenin is necessary for the proper formation of the male reproductive tract.

Further support of a role for beta-catenin in proper development of the male reproductive tract comes from the fact that canonical Wnt signaling is active throughout development of the male reproductive tract (figure 3-1). We hypothesized that beta-catenin is necessary to maintain the WD in an undifferentiated state during development after which beta-catenin activity subsides and the male reproductive tract can differentiate. Therefore, in addition to preventing the degradation of the WD, removing beta-catenin from the WD prevents the normal formation of the male reproductive tract.

MD formation defect during the elongation phase

MD formation occurs in two phases: first by invagination of the coelomic epithelium and then by elongation to the cloaca. Several studies on MD development indicate that the elongation phase is dependent on signals from the WD. Since the WD is maintained in beta-catenin mutants, we hypothesized that premature differentiation of the

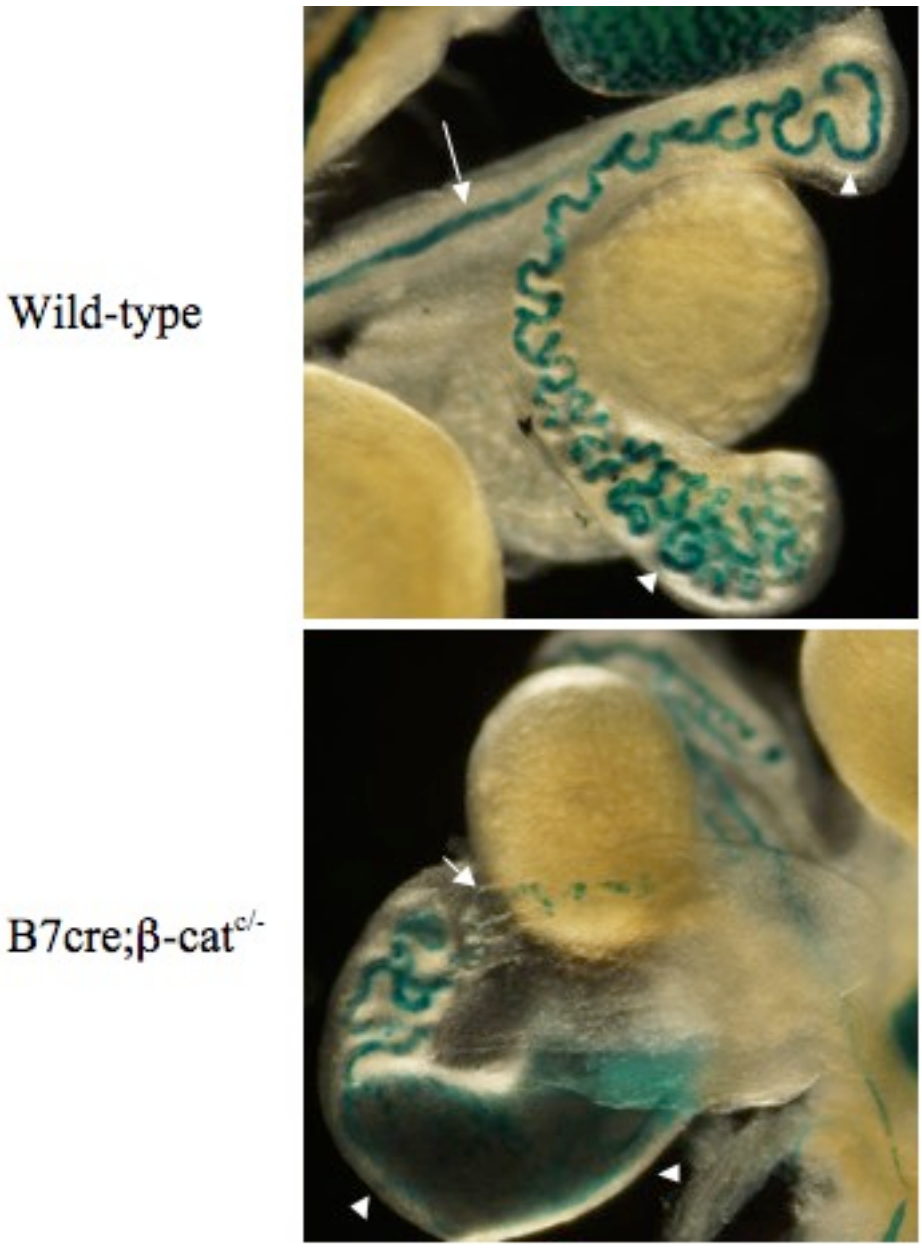


Figure 3-6. P1 Beta-catenin mutant male reproductive tract phenotype. Male reproductive tracts are stained with LacZ in WD derivatives (areas of HoxB7cre activity). In wild-type, arrow=vas deferens, arrowheads=epididymis. Removal of beta-catenin from the WD prevents proper formation of the epididymis and vas deferens. The mutant epididymis fails to coil properly (arrow) and becomes dilated (arrowheads). In several mutants the vas deferens also become dilated.

mutant WD prevents proper formation of the MD. At E14.5 the MD has extended all the way to the cloaca in wild-type embryos. To determine during which phase is there a defect in the beta-catenin mutants, Dr. Carroll staining E14.5 wild-type and beta-catenin mutants with either Pax8 or Wnt7a mRNA to visualized the MD. Mutant MD formation is initiated but does not extend all the way to the cloaca (figure 3-7). These results indicate that the elongation phase of MD development is defective in beta-catenin mutants.

The MD fails to fully elongate in beta-catenin mutants but observations of the mutant MD tips indicate it continues to proliferate. The mutant tip appears to be coiling and indicates instead a loss of a migration cue (figure 3-7). To more closely study this development, we used a Pax8YFP line of mice to visualize the MD in culture. Pax8 is expressed in the MD, renal vesicles, and weakly in the WD. With the Pax8YFP mice, we were able to dissect out and culture urogenital systems starting at E11.5. Culturing Pax8YFP urogenital systems allows for visualization of the MD in real time. Cultured urogenital systems were imaged every 3 hours until E13.5. Even though we were not able to visualize any coiling MD tips, we did observe inhibition of MD elongation in mutants whereas the wild-type MDs continued to extend to the cloaca (figure 3-8). Furthermore, the mutant MD tips appear to thicken, indicating that the mutant tips are continuing to proliferate (figure 3-8).

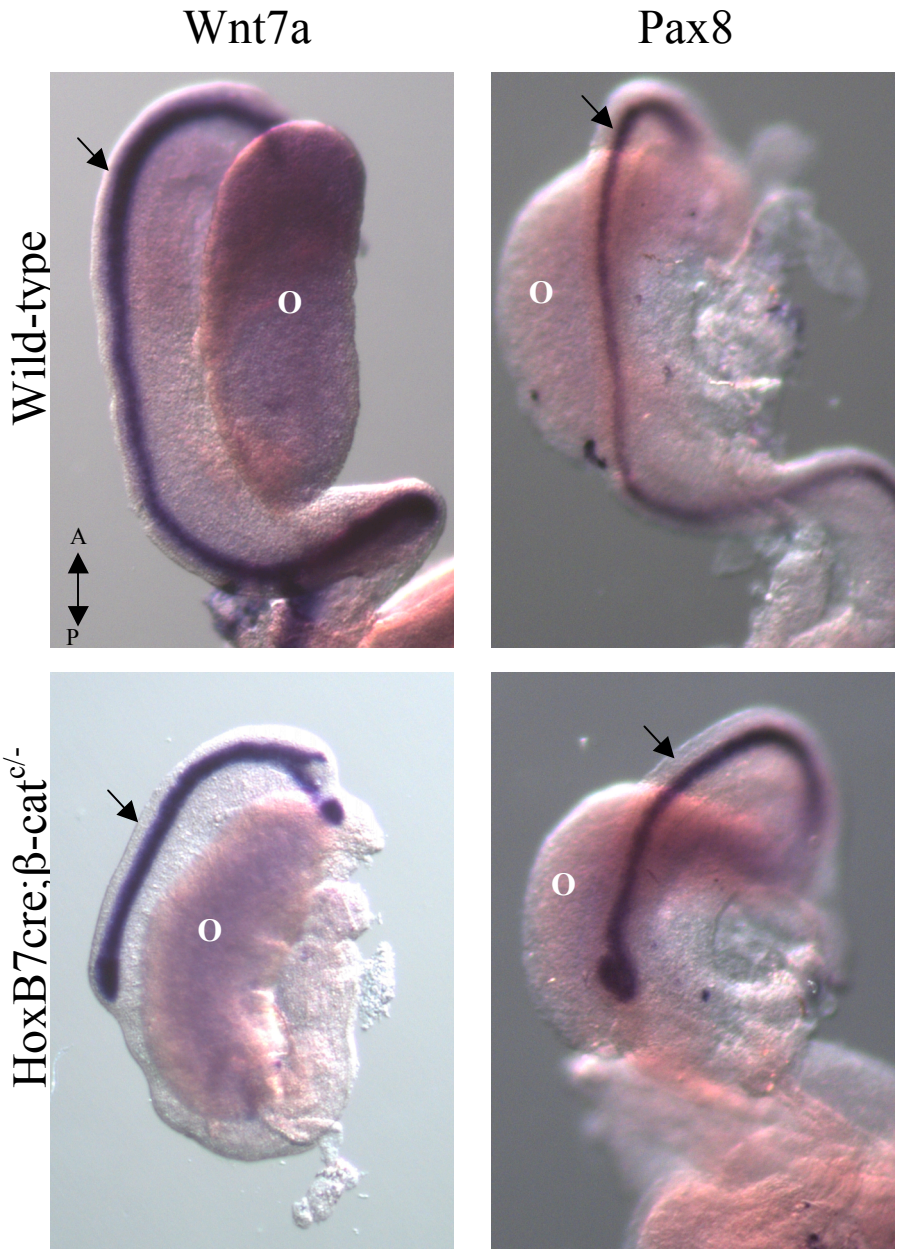


Figure 3-7. The Müllerian duct does not fully form in beta-catenin mutants. E14.5 ovaries are stained for either Wnt7a (left panel) or Pax8 (right panel) by in situ hybridization. The Müllerian duct (arrow) elongate fully in the wild-type ovary but has not migrated completely posteriorly in the beta-catenin mutant. Furthermore, the mutant MD tip in the bottom right panel appears to continue proliferating but is coiling upon itself, indicative of loss of a migratory cue. (O=ovary, data produced by Dr. Thomas Carroll)

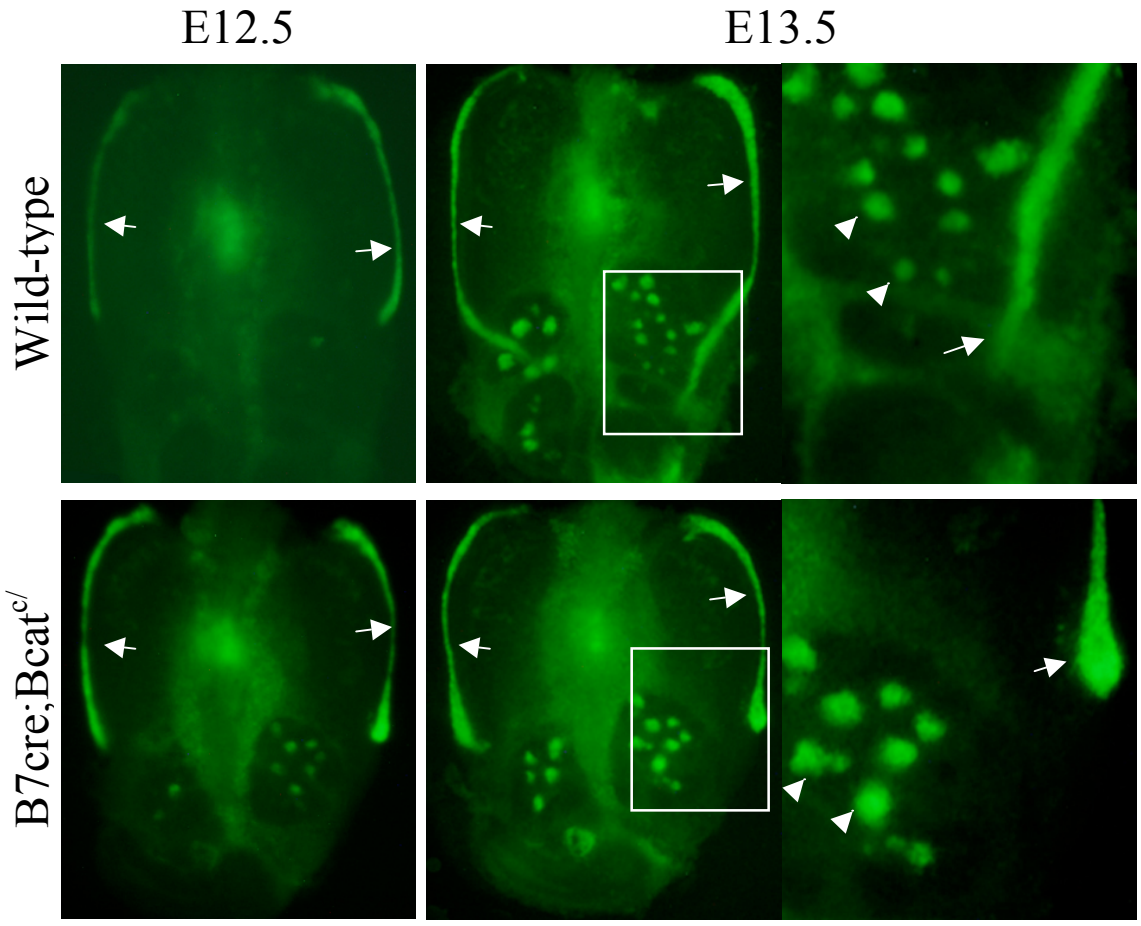


Figure 3-8. Beta-catenin mutant Müllerian duct migration in culture. Urogential systems from both wild-type and HoxB7cre;Bcat$^{c/-}$ mice expressing a Pax8YFP transgene were dissected out and cultured. Since Pax8 is expressed in the MD (arrows), the Pax8YFP fusion protein allowed us to visualize the MD during culture by expression of GFP. At E12.5, the MD has elongated about halfway to the cloaca. 24h later, the mutant MD has stopped migrated and formed a bulge of cells at the tips (see right panels for magnified images, arrows=MD tip, arrowheads=RVs). The bulge of cells at the mutant tip could be indicative of continued proliferation but loss of a migratory cue.

Wnt9b not sufficient to rescue MD phenotype

Similar to the beta-catenin mutants, Wnt9b mutants do not form a MD (Carroll et al., 2005). The defect in formation occurs during the elongation phase since MD formation is initiated. Considering that Wnt9b mutants have a similar defect in MD extension, it is possible that a loss of Wnt9b from the WDs of beta-catenin mutants (due to premature differentiation) is contributing to the MD phenotype. Dr. Carroll examined Wnt9b expression in beta-catenin mutants and determined that Wnt9b mRNA is lost in a mosaic pattern along the length of the WD (figure 3-9). The loss of Wnt9b mRNA is coincident with beta-catenin mutant cells. These results indicate that beta-catenin functions upstream of Wnt9b.

Carroll et al demonstrated rescue of the MD phenotype in Wnt9b mutants by expressing Wnt1 driven by the Rosa promoter in areas of beta-catenin mutant cells. It has been shown that Wnt1 can rescue the MD phenotype in Wnt9b null mice (Carroll et al., 2005). E14.5 urogenital systems were collected and the MD was visualized by staining for Wnt7a mRNA. Both wild-type and B7cre;RosaWnt1 MD extended fully however, B7cre;RosaWnt1;Bcatc/- MD failed to elongate, similar to the B7cre;Bcatc/- (figure 3-10). This result indicates that Wnt1 is not sufficient to rescue the beta-catenin MD phenotype. Furthermore, these results imply that even though loss of Wnt9b might be playing a role in formation of the MD, another factor is missing in the beta-catenin mutants.

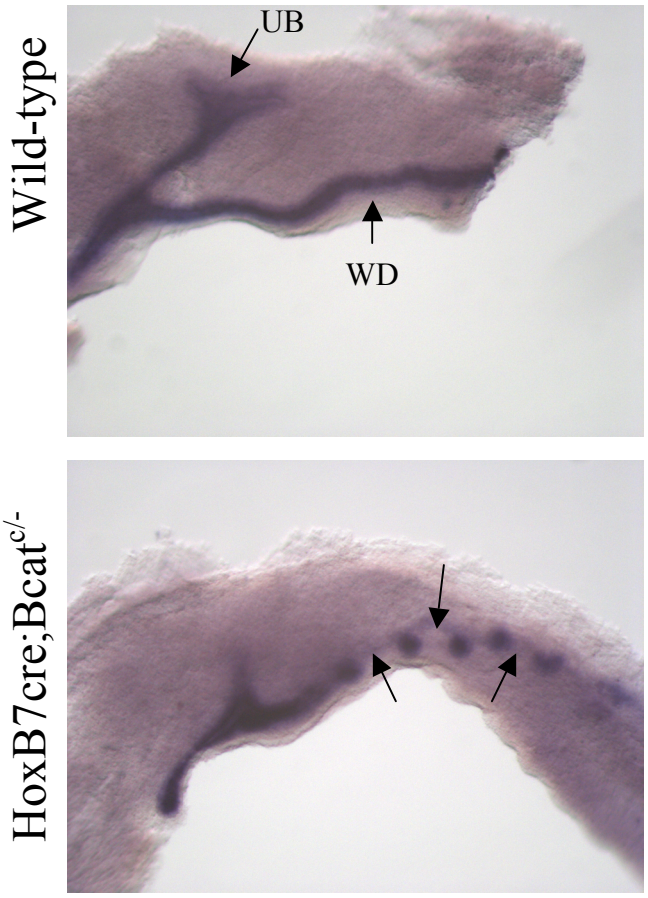


Figure 3-9. Loss of beta-catenin leads to a loss of Wnt9b. E11.5 embryos express Wnt9b mRNA in the Wolffian duct and ureteric bud. Previous studies have shown that Wnt9b is necessary for the formation of the posterior portions of the Müllerian duct. In the beta-catenin mutant, Wnt9b expression is patchy along the Wolffian duct suggesting loss of this gene may be responsible for the lack of Müllerian duct migration. Arrows point to areas of mutant Wolffian duct where Wnt9b expression is absent. (data producted by Dr. Thomas Carroll)

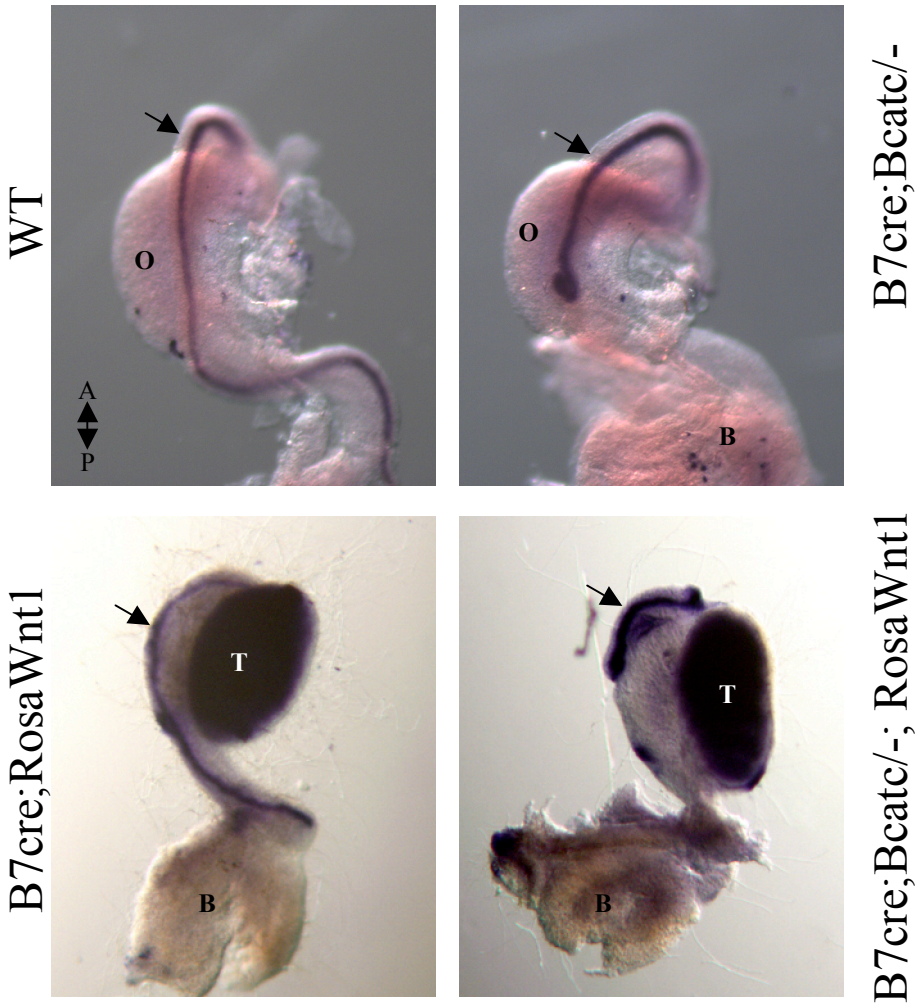


Figure 3-10. Ectopic expression of Wnt1 is not sufficient to rescue Müllerian duct phenotype. Carroll et al. demonstrated that ectopic expression of a canonical Wnt, Wnt1, is sufficient to rescue the Wnt9b mutant MD phenotype. Since HoxB7cre;Bcat$^{c/-}$ embryos exhibit the same MD phenotype along with loss of Wnt9b mRNA expression, we ectopically expressed Wnt1 in the WD using a RosaWnt1 line of mice. MDs in E14.5 reproductive tracts were visualized by in situ hybridization of Pax8. MD forms normally when ectopically expressing RosaWnt1 (bottom left panel). However, when expressed in a beta-catenin mutant, the MD still fails to form .(data in top two panels produced by Dr. Thomas Carroll) (B=bladderT=testis, O=ovary, arrow=MD)

3.4 Discussion

To address the role of beta-catenin in development of the reproductive tract, we conditionally removed beta-catenin from the WD using a HoxB7cre line of mice. HoxB7Cre; $Bcat^{c/-}$ mutant females exhibit a partial sex reversal phenotype. The WD is maintained in mutant females independent of ectopic androgen synthesis in the gonad but due to premature differentiation of the WD. Partial sex reversal is a fairly common human birth defect that is typically caused by sex determination defects. However, beta-catenin mutants exhibit no defects in sex determination.

Even though the WD is maintained in mutant females, it does not differentiate into a mature male reproductive. However, beta-catenin mutant male WDs appear to differentiate into the male reproductive tract. These results would imply that beta-catenin mutant females are lacking factors necessary for differentiation of the WD into the male reproductive tract. Regardless of the fact that mutant male WDs express markers of the differentiating male reproductive tract, beta-catenin mutant males fail to form a normal male epididymis or vas deferens. The male mutant phenotype could be due to ectopic expression of a water channel protein. It is also possible that patterning of the mutant male WDs is defective. Male reproductive tract organs, such as the epididymis and vas deferens, are divided into molecularly different sections. As was mentioned, there are several Aqps expressed throughout the different segments of the male reproductive tract. Therefore, it would be useful to better characterize the expression of these Aqps in all segments of the mutant WD derivatives. Furthermore, there are several Hox genes expressed in the reproductive tract. We looked at the mRNA expression of Hoxa9, Hoxd9, and Hoxa10 at E11.5 and E14.5 in wild-type embryos (figure 3-11). They are all

located more distal in the reproductive tract. Regardless, a more in depth analysis of patterning genes and their expression patterns in beta-catenin mutant reproductive tracts would help determine if removal of beta-catenin produces patterning defects. Since Wnt signaling is active throughout the development of the wild-type male reproductive tract and since beta-catenin mutant males failure to form a normal male reproductive tract indicates that beta-catenin plays a role in proper formation of the WD into the male reproductive tract.

Another phenotype observed in beta-catenin mutants is failure of the MD to form. The MD phenotype appears to be due to loss of a signal(s) from the WD, preventing proper migration of the MD. Based on expression data for various canonical Wnt signaling reporters, it appears that canonical Wnt signaling is active not only in the WD but also in the MD and the mesenchyme surrounding it. We tested the efficacy of two families of small molecule inhibitors of Wnt signaling (IWs) in embryonic kidney culture. We were interested determining if treating cultured urogenital systems with the IWs would recapitulate the reproductive tract phenotype. We treated E12.5 urogenital systems expressing the Pax8YFP transgene with 50uM of either inhibitor of Wnt production 2 (IWP2) or inhibitor of Wnt response 1 (IWR1). The Pax8YFP fusion protein is expressed in the MD, allowing for live imaging. Images of the treated urogenital systems at E12.5, right after dissection, show the MD has migrated almost halfway to the cloaca (figure 3-12). Two days later, the control treated MD has extended all the way to the cloaca whereas elongation of both the IWP2 and IWR1 treated MDs is halted before reaching the cloaca. Even though the IW treated MD has not reached the cloaca by E14.5 in culture, we do not know if MD elongation is being inhibited or if it is just

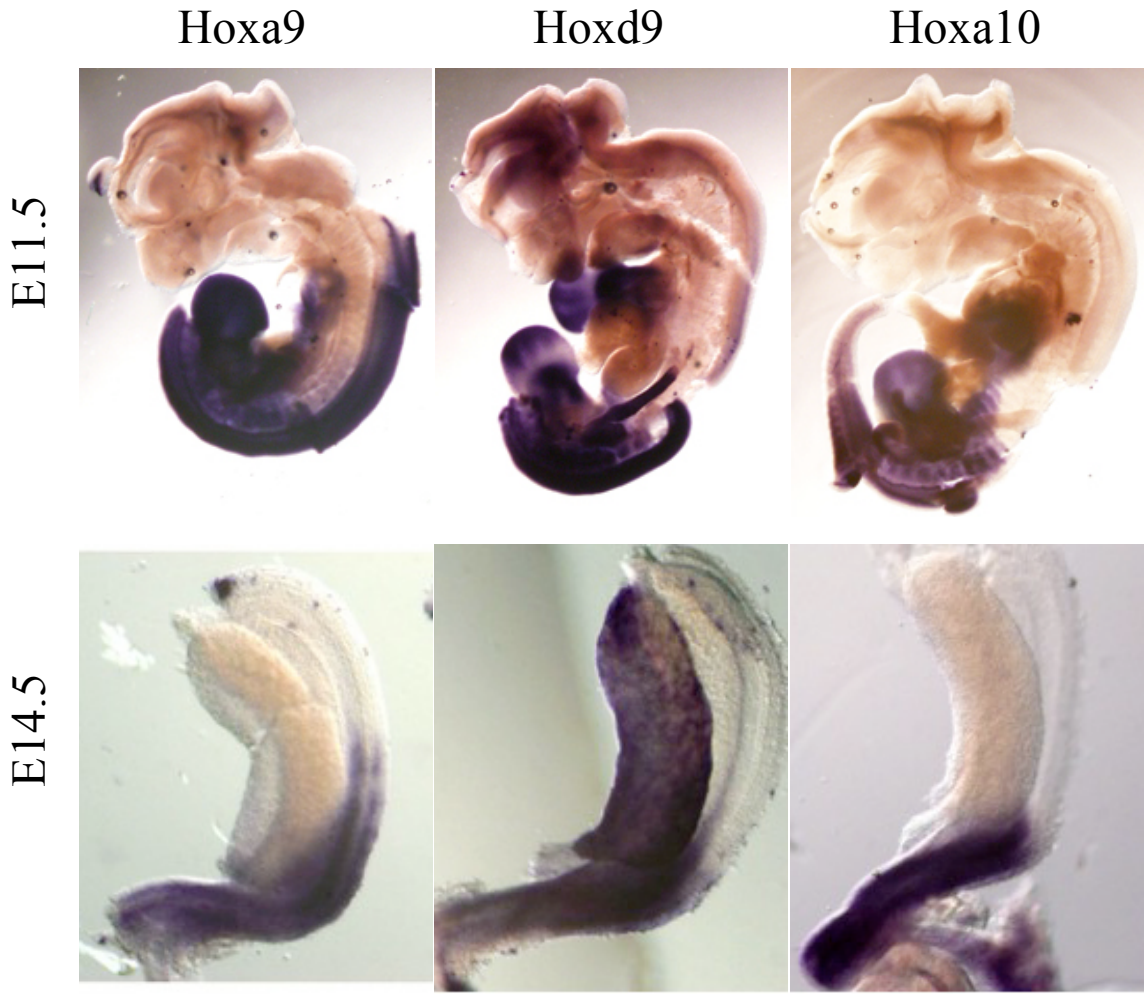


Figure 3-11. Hox genes in patterning of the reproductive tract. The expression of (A) Hoxa9, (B) Hoxd9, and (C) Hoxa10 was examined by in situ hybridization was performed on both E11.5 embryos and E14.5 reproductive tracts, at which time both the WD and MD are fully formed. All of the Hox genes looked at are expressed in the posterior portion of the whole E11.5 embryo and distally in the E14.5 reproductive tract. In addition to expression in the sex duxts, Hoxd9 also appears to be expressed in the gonad.

developmentally delayed. The IWs are inhibiting Wnt signaling is all cell type whereas beta-catenin is only removed from the WD in the HoxB7cre;Bcat$^{c/-}$ mutants. Furthermore, there could be a window of time during which Wnt signaling plays a more crucial role in MD development and culturing urogenital systems starting at E12.5 is past the point where Wnt signaling has already initiated a developmental program. Regardless, more experiments, such as starting and stopping at different time points along with different dose of drugs, should be performed to better understand these results.

We hypothesized that instead of androgens causing maintenance of the WD in beta-catenin mutants, premature differentiation is preventing the epithelial tube from degrading. However it is possible that androgens are produced below the levels of sensitivity by in situ hybridization. The only way to rule out a role for androgens in the beta-catenin WD maintenance phenotype is to treat mutant embryos with flutamide, an androgen blocking. If the WD maintenance is independent of androgens, treatment with flutamide would not prevent the beta-catenin mutant phenotype. If the WD is maintained due to premature differentiation, this premature differentiation could be playing a larger role than just causing WD maintenance and could also be preventing the cross talk between the WD and the MD. Furthermore, since beta-catenin is a transcriptional activator, removing it from the WD most likely inhibits the expression of genes necessary to signal between the WD and MD. We observed a loss of Wnt9b mRNA expression in the mutant WD, coincident with loss of beta-catenin. Since Wnt9b expression is lost in beta-catenin mutants and Wnt9b mutants exhibit a similar loss of MD derivatives phenotype, Wnt9b could be involved in this cross talk. We demonstrated that an ectopically expressed canonical Wnt could rescue the MD phenotype, indicating that a

Wnt9b-like signal is not sufficient to rescue the beta-catenin mutant MD phenotype. Therefore, the expression of additional genes necessary for MD development is likely lost in beta-catenin mutants. To identify these additional signals, a microarray should be performed. Unfortunately, performing a microarray on beta-catenin mutant WDs could prove difficult since the mutants have variable mosaicism for beta-catenin removal. Either laser capture or FACS sorting

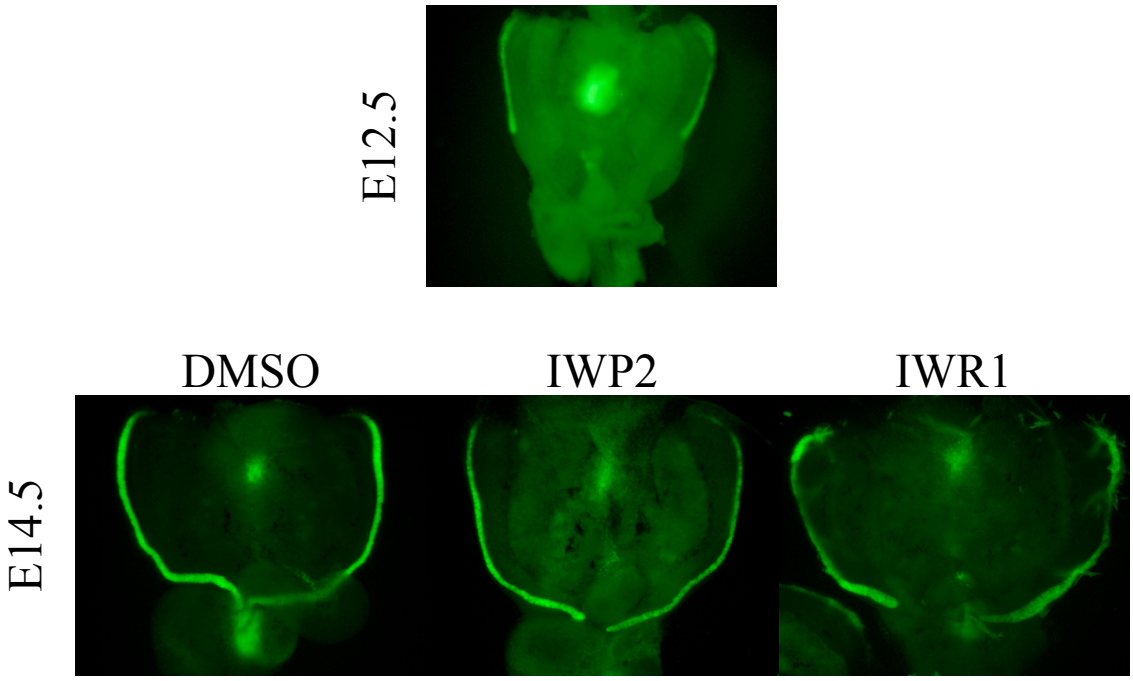


Figure 3-12. IWP2 and IWR1 treatment inhibits Müllerian duct elongation. Urogenital systems from mice expressing the Pax8YFP transgene were dissected out and cultured starting at E12.5. Pax8 expression marks the MD. At E12.5, MD formation has been initiated and at the time of dissection has extended halfway towards the cloaca. The urogenital systems were treated with either a DMSO control, 50uM IWP2 or 50uM IWR1. After two days of treatment, MDs in the DMSO control has extend fully to the cloaca. However, it appears that MD elongation is either halted or delayed when treated with IWP2 or IWR1.

techniques could be used to separate out mutant cells for the microarray. Another option would be to treat wild-type WDs with IWR1 to inhibit canonical Wnt signaling. Treated versus untreated WDs could be compared in a microarray. From the microarray we could gain a better understanding of genes regulated by beta-catenin in reproductive tract development. Taken together, our results indicate that canonical Wnt signaling plays an important role in reproductive tract development.

3.5 Materials and Methods

Wholemount expression analysis

Rosa reporter activity was assessed as previously described (Soriano, 1999). For in situ hybridization, tissues were post-fixed in 4% paraformaldehyde for 24 h at 4° prior to hybridization with antisense, digoxygenin labeled mRNA probes. Probes examined were Wnt7a (cut with SalI and transcribed with T7 polymerase), Pax8 (XbaI, T7), Wnt9b (see Carroll et al., 2005), Hoxa9 (SalI, T7), Hoxd9 (SalI, T3), Hoxa10 (Xho1, T7). Hybridized embryos were cleared in 80% glycerol and photographed using a Nikon digital still camera (DXM1200) on a Nikon stereomicroscope (SMZ1500).

Section ISH

E14.5 reproductive tracts were fixed in 4% paraformaldehyde at 4 °C overnight, washed 3 times in PBS, equilibrated in 30% sucrose then imbedded in OCT and flash frozen on dry-ice. Tissue was sectioned at 10 uM on a cryostat and sections were stored at − 80 °C until usage. Sections were hybridized with antisense, digoxygenin labeled mRNA probes. Probes examined were Cyp17alpha, 3beta-HSD, MIS. Hybridized sections were mounted

with histomount and photograph using a Zeiss Axioplan MRc5 camera on a Zeiss Axioplan2 compound microscope.

Flutamide

Pregnant dams were dosed daily by oral gavage with flutamide (F9397, Sigma-Aldrich Corp., St. Louis, MO; 100 mg/kg/d) in 1 ml/kg corn oil/2.5% dimethylsulfoxide (Sigma) from at E10.5 until birth.

Immunohistochemistry

e12.5 embryos were fixed in 4% paraformaldehyde at 4 °C overnight, washed 3 times in PBS, equilibrated in 30% sucrose then imbedded in OCT and flash frozen on dry-ice. Tissue was sectioned at 10 uM on a cryostat and sections were stored at − 80 °C until usage. Thawed sections were boiled for 10 min in 10 mM Tris, pH 8.0 and 5 mM EDTA then incubated with primary antibodies to beta-catenin (rabbit, 1;500, Chemicon, AB19022 and mouse, 1:500, Sigma c7207), Ki-67 (rabbit, 1:1000, Novo-Castra, NCL-Ki67p), Pax2 (rabbit, 1:500, Covance, PRB-276P), E-cadherin (Rat, 1:200, Zymed, 13-1900), Aqp9 (rabbit, 8ug/mL, Alpha Diagnostic International, Cat. #AQP91-A), Aqp2 (rabbit, provided by Mark Knepper). The secondary antibodies were Alexafluor 488, 568 and 633 (Molecular Probes). Tissues were mounted in VectaShield with Dapi (Vector labs). Sections were viewed and photographed on a Zeiss LSM510 Axioplan inverted confocal microscope.

Mouse strains

WD/UB specific deletion of beta-catenin was accomplished by crossing mice triply heterozygous for Hoxb7-Cre (Yu et al., 2002) or Pax2cre (Rowitch et al., 1999), beta-catenin (Brault et al., 2001), and the Rosa reporter (Soriano, 1999) to mice homozygous

for the conditionally inactive allele of beta-catenin (ctnnb1tm2Kem). Neural crest specific deletion of beta-catenin was accomplished by crossing mice doubly heterozygous for Wnt1-Cre (Danielian et al., 1998) and beta-catenin. Additionally, beta-catenin reporter mice, BAT-gal (Maretto et al., 2003), were used to look at active canonical Wnt signaling. Wnt1 was ectopically expressed in the WD with mice triply heterozygous for Hoxb7-Cre, beta-catenin, and Rosa26 Wnt1EGFP (Carroll et al., 2005), to mice homozygous for the conditionally inactive allele of beta-catenin (ctnnb1tm2Kem). Pax8YFP mice were used for visualization of the MD during culture. Noon on the day of vaginal plug detection was considered E0.5.

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