Distinct roles of retinoid signaling in the lower urinary tract

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Abstract

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Retinoic acid (RA)-signaling is involved in a broad spectrum of cellular processes, including formation of most embryonic tissues, epithelial differentiation, and is a critical regulator of stem cell differentiation in vitro. Studies from our lab have focused on the role of RA-signaling in the urinary tract, where we find that it plays multiple roles. By inducing expression of a floxed dominant-negative mutant Rar receptor, termed RaraDN, in the bladder, we find that RA-receptor signaling from the bladder epithelium plays distinct roles during urinary tract development; it is required for establishing mature ureter-bladder connections and for differentiation of the bladder epithelia.

Congenital abnormalities of the kidney and urinary tract (CAKUT) characterize a range of lower urinary tract defects such as kidney and ureter agenesis, hydronephrosis, and vesicoureteral reflux. Development of the lower urinary tract, which consists of the kidneys, ureters, bladder, and urethra, is crucial for removal of toxic substances from the blood and depends on patent connections between the ureter and the bladder. Impaired vitamin A signaling, either by maternal vitamin A deficiency in mice, or deleting RA-synthesizing enzymes and RA-receptors, leads to syndromic urinary tract abnormalities similar to those seen in humans. Our previous studies have suggested that proper ureter-bladder connections depend on signals derived from the bladder. By selectively inhibiting RA-signaling in the bladder epithelium, we show that RA-receptor signaling from the
bladder is required for nephric duct (ND) insertion into the cloacal epithelium, CND maturation, and late-stage ureteral apoptosis in part through Ret. In addition, we find that RA acts independently of Ret where it regulates bladder growth and epithelial differentiation.

The bladder epithelium, or urothelium, is a stratified epithelium that lines the major portion of the lower urinary tract and provides a crucial barrier between urine and blood. It contains basal, intermediate, and umbrella cells that synthesize and traffic uroplakin proteins to its apical surface. Vitamin A has been shown to be necessary for preventing keratinization of the bladder epithelia, and in vitro, it can induce the differentiation of endodermal ES cells into populations of cells that express markers of the urothelium. Recent studies suggest that Shh-expressing population in the adult bladder contains progenitors that can repopulate the urothelium after damage. Here we report that RA-receptor-dependent signaling temporally regulates Shh-expressing urothelial progenitors and is required for formation of intermediate and umbrella cells during early development. Furthermore, we find that in the absence of RA-signaling, Shh-progenitors undergo a fate change, down-regulating uroplakins and up-regulating squamous markers, suggesting that RA is normally required for either positively regulating urothelial differentiation or negatively suppressing squamous differentiation.
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Chapter I

Literature Review

I. Overview

Vitamin A and its active derivative retinoic acid (RA) are essential for formation of most embryonic tissues, and for testis, eye, and epithelial differentiation in adults (1). RA is also a critical regulator of stem cell differentiation \textit{in vitro} (2). The enormous number of cellular effects of RA is due to transcriptional regulation. In some cases, RA exerts these effects through epigenetic mechanisms, while in other cases it regulates \textit{Hox} genes that mediate numerous functions required for patterning and organogenesis (2).

Our studies have focused on the role of RA-signaling in the urinary tract, where retinoids are required for kidney, ureter and bladder formation, as well as for generating connections between these tissues. In this case, RA acts in large part by regulating expression of the \textit{Ret} gene, a master regulator of epithelial cell behavior that is required for branching morphogenesis in the kidney and ureter insertion into the bladder. In these studies, we find that RA-dependent signals from the bladder regulate ureter insertion at distinct stages during development, for ND insertion and later stages of ureter maturation, and these steps may depend on Ret. We also find that RA acts independently of Ret during bladder formation, where it regulates outgrowth of the urogenital sinus. Strikingly, we also observe a role for retinoids as regulators of a novel progenitor population that is required for formation of the urothelium, a specialized epithelium that lines the urinary outflow tract that functions as a barrier preventing exchange of water and nitrogenous substances between urine and the blood. In this chapter, I will provide literature review
pertaining to RA-signaling, its role in the lower urinary tract, and its role in other
developmental processes as it pertains to my studies.

II. Introduction to Nuclear Receptors

Nuclear receptors (NRs) constitute a super-family of ligand-regulated
transcription factors that affect an array of processes such as development, metabolism,
reproduction, and cell differentiation (3). NRs are highly conserved proteins found in
several species, including arthropods, vertebrates, sponges, and echinoderms. The
structure of a NR is defined by six regions of varying homology designated A-F. These
domains are conserved through evolution and between members of the NR superfamily
(Fig. 1.1a,b) (4). The two most evolutionary-conserved regions, domains C and E,
correspond to the zinc-finger-based DNA binding domain (DBD) and a C-terminus
ligand-binding domain (LBD). The DBD is composed of two C4 zinc fingers: CI that
provides DNA-binding specificity; and CII, which contains a weak dimerization interface
that allows DBDs to dimerize with other NRs (5). DBD specificity towards target gene
activation was experimentally shown by substituting the DNA-binding domain of the
human estrogen receptor (hER) with that of human glucocorticoid receptor (hGR). The
resulting chimeric receptor switched its template specificity and activated glucocorticoid-
regulated genes instead of estrogen-regulated genes (6). While some NRs, such as
steroidogenic factor (SF-1), function as monomers and bind to a single response element
on the DNA, others function as homo- or heterodimers through two half-sites that can be
arranged as direct repeats or inverted repeats. These half-sites contain dimer-specific
spacing that further contributes to receptor binding specificity.
The less conserved domain of the two, the LBD, is composed of eleven to thirteen alpha-helices that form a hydrophobic pocket allowing the LBD to bind lipophilic molecules. This domain also contains a weak dimerization interface that allows different NRs to form dimers, thereby expanding transcriptional diversity. A ligand-dependent AF-2 activation domain is located at the C-terminus end of the LBD. Structural and molecular studies have shown that upon ligand binding, the LBD undergoes a conformational change that creates a surface allowing recruitment of coactivator complexes (members of the p160 co-activator family) through AF-2 (7,8). This recruitment activates transcription. A hinge region D provides protein flexibility allowing concurrent receptor dimerization and DNA binding. In addition, NRs also contain an N-terminus A/B domain that contains a ligand-independent transactivation function 1 (AF-1). Genetic evidence for a functional role for the AF-1 domain comes from analysis of truncated mutants lacking this domain. Deletion of the hGR AF-1 domain results in a 10- to 20-fold reduction of transcriptional activity (9). The function of domain F is not known; however this region is phosphorylated in some NRs and may modulate the activation functions of AF-1 and AF-2 (10).

Depending on how they transduce signals into a transcriptional response, NRs can be divided into two major families: Type I and Type II receptors. Type I receptors, such as the steroid receptors ER and GR, undergo nuclear translocation upon ligand binding and bind as homodimers on inverted repeat DNA half-sites to initiate transcriptional activation. Type II receptors, including thyroid hormone receptors (TR) and Retinoic Acid Receptors (RAR), among others, are often found in the nucleus regardless of the presence of a ligand. These receptors usually form heterodimers with Retinoic X
Receptor (RXR) to direct repeats called retinoic acid response elements (RAREs) (11,12,13). In the absence of a ligand, some of these receptors, such as TR-RXR and RAR-RXR heterodimers, function as transcriptional repressors by interacting with co-repressors. Ligand binding to the receptors results in release of corepressor complexes and recruitment of coactivator proteins that initiate a cascade of events activating downstream gene transcription (Fig. 1.2). Examples of Type I and Type II receptors and their ligands are shown in Table 1.1.

III. Nuclear Retinoic Acid Receptors are the functional mediators of RA-dependent signaling

A. RAR structure and regulation of transcription

Retinol (Vitamin A) and its active derivative retinoic acid (RA) are essential for development, organogenesis, and homeostasis of most body tissues through the effect of RA on proliferation, apoptosis, cellular differentiation, and epigenetic mechanisms (1,2,14). Apart from vision, the retinoids act through regulation of retinoid-dependent gene expression. More than 500 genes respond to RA, either directly or indirectly through intermediary transcription factors. These genes encode growth factors, transcription factors, protein kinases and phosphatases, etc. (Table 1.2) (15,16). In vitro, RA has been shown to inhibit differentiation of tumor cells and increase transcription of genes (17). RA may also be a natural morphogen that provides positional cues in the developing limb buds (18,19).

The receptor for RA was discovered based on the assumption that it may be a member of the ligand-dependent NR super-family that shares homology with conserved
domains of other NRs. As mentioned in Section II, two evolutionary-conserved functional domains, C and E, are common to all members of the NR family. With more than 50% sequence identity, Domain C, or the DBD, is the more conserved region of the two and is responsible for the specificity of transcriptional activation of target genes (5,6). Strategies to identify a receptor for RA exploited the discovery that domain C, that is highly conserved, can be swapped between receptors to alter receptor specificity. For example, the DBD of the hGC and hER can be interchanged to form functional chimaera receptors. This hybrid receptor recognizes the glucocorticoid-responsive element of the MMTV-LTR vector but stimulates transcription upon addition of oestrogen (6). In the search for the RA receptor, a similar assay system was established; the DBD of an unknown hormone receptor (X) was swapped with that of hGR (20,21). Transfected cells were then exposed to a string of candidate ligands, including RA. Adding RA elicited a dramatic increase in transcriptional induction as measured by chloramphenicol acetyltransferase (CAT) activity. This response labeled receptor X as a retinoid-responsive receptor. The identification of retinoic acid receptor (RAR) was followed by the discovery of several other RARs and their isoforms, such as RARα, RARβ, and RARγ; and by the discovery of retinoid X receptors (RXRsα,β,γ) and their differential ligands (Table 1.3) (13,22,23,24,25,26).

RARs and RXRs share a high degree of sequence similarity between themselves and with other members of the human nuclear receptor family (13). RARs and RXRs exhibit the conserved structure of nuclear receptors, suggesting that retinoids function in the body in a manner that is very similar to steroid hormones and thyroid hormones. Like other NRs, the structure of the retinoid receptors can also be divided into six regions (A-
(Fig. 1.3a) (27). The DBD, or domain C, is highly conserved between the three RAR
types (94-97%) and between the three RXR types (91-97%) (Fig. 1.3b). Domain E,
which contains the LBD, ligand-dependent transcriptional activation function AF-2 and a
weak dimerization interface, is also conserved between the retinoid receptors (Fig. 1.3a).
Interestingly, domain D or the hinge region is more highly conserved between species
than between the RAR and RXR types. The variability in the N-terminal domain (A/B)
gives rise to the major RAR and RXR isoforms (28). While the three RARs can
heterodimerize with the three RXRs, RXRs can form homodimers with each other and
can also heterodimerize with other nuclear receptor family members such as vitamin D
receptor (VDR), the peroxisome proliferator activated receptors (PPARs), and the thyroid
hormone receptors (TR) (Table 1.4) (14,29).

The form of retinoid that controls retinoid-dependent transcriptional activation is
retinoic acid (see Section B). A number of studies suggest that in vitro, 9-cis RA, a
natural isomer of all-trans RA, may also be a mediator of RA-receptor signaling.
Whereas 9-cis RA only binds to RXRs, all-trans RA can bind to and activate each of the
three RARs (RARα, RARβ, RARγ) and each of the three RXRs (RXRa, RXRβ, RXRγ),
(Table 1.3) (29,30). At supraphysiological levels in vitro, 9-cis RA can activate and affect
the activity of RXR homodimers and other nuclear receptors (31,32). However, there is
discrepancy in the literature whether or not 9-cis RA is an active ligand for retinoid-
mediated transcription in vivo. Although the AF-2 region of RXRα has been shown to be
functionally important for mouse development, it does not need to bind 9-cis RA to
function (33). Furthermore, all-trans RA but not 9-cis RA is sufficient to rescue lethal
defects in RA synthesis created by mutations in Raldh2, the major RA-synthesizing
Although all-trans RA is detectable in many mammalian tissues, detection of 9-cis RA has been reported but not confirmed in vivo. However, since 9-cis RA is a natural isomer of all-trans RA, this is not surprising. Thus, these studies suggest that only the RAR partner needs to be liganded in order for the heterodimeric complex to be active; the RXR partner does not necessarily need RA or 9-cis RA binding.

Transcriptional regulation of RA-dependent genes relies on the binding of nuclear receptor complexes to DNA response elements. While most steroid receptors bind as symmetrical homodimers on inverted repeats with a 3bp spacer (IR3), RAR/RXR heterodimers recognize well defined cis-acting response elements-termed retinoic acid response elements (RAREs) and retinoid X response elements (RXREs)-that are found in the promoter regions of retinoid-responsive genes (13). The most frequent spacing between the repeats is 5bp (DR5; e.g., in the RARb2 and RARa2 genes), but DR1 and DR2 have also been found in some genes (Fig. 1.4a). On DR2 and DR5 sites, RXR partner occupies the 5’ motif while on DR1 sites, RXR partner occupies the 3’ motif (Fig. 1.4b) (13,35).

RAR/RXR heterodimers can bind to RAREs while RXR/RXR homodimers can bind to RXREs (13). Activation of retinoid signaling through RARs and RXRs follows a similar mechanism of other ligand activated NRs (7). In the absence of a ligand, RAR/RXR heterodimers and co-repressor complexes-including a histone deacetylase (HDAC)/Sin3 complex-are bound to RARE sites in the promoter of target genes. This corepressor complex is proposed to be recruited by corepressors NCoR and SMRT-proteins that maintain the chromatin in a condensed and thus transcriptionally-repressed state (36). In the presence of a ligand, RAR/RXRs undergo a conformational change that
allows dissociation of corepressors and recruitment of coactivators possessing histone
deacetyltransferase (HATs such as p300/CBP complex), methyltransferase (HMTs such
as CARM-1), kinase or ATP-dependent (SWI/SNF) chromatin remodeling enzymatic
activities (37,38,39) (Fig. 1.5). The ability of these receptors to bind retinoic acid as a
ligand and to alternatively bind corepressors and coactivators provides a mechanism
through which gene transcription can be activated or repressed by retinoids. RA-receptors
are widely expressed in the embryo and adult, in non-overlapping patterns, and are
evolutionarily conserved (Table 1.5) (40,41,42,43). Based on this, it was proposed that
the broad collection of functions mediated by retinoids was due to differential gene
activation by combinations of RAR and RXR heterodimers (28). However, things appear
to be more complicated than previously thought (Section B).

B. Retinoids are synthesized from retinol taken up in the diet

Retinoic acid (all-tans, 9-cis, 13-cis), can be synthesized by plants, but cannot be
synthesized de novo by humans or other vertebrate species, and therefore must be
acquired from the diet (13,44,45). Within the diet, two types of retinoids are available:
preformed retinoid and proretinoid carotenoids. Preformed vitamin A consumed in meat
and dairy products consists of retinol and retinyl esters. Provitamin A carotenoids such as
β-carotene, α-carotene, and β-cryptoxanthin, are found in dark greens and yellow fruits
and vegetables such as mangoes, carrots, papaya (44). Once provitamin carotenoids are
converted to retinol within the intestine and other tissues, it is no longer functionally
indistinguishable from retinoid obtained from the diet as preformed vitamin A. Within
the intestine, all retinol is converted to retinyl ester by one of two enzymes,
lecithin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase. Upon conversion, retinyl esters are incorporated into nascent chylomicrons for co-secretion with dietary lipids into the lymphatic system (45). Approximately 66-75% of dietary retinoids (processed as chylomicrons and chylomicron remnant retinoid) are taken up by the liver and stored in fat globules in hepatic stellate cells (HSCs) while peripheral tissues clear the remaining dietary retinoids (25-33%) (46).

C. Retinoid and retinol transport

Within the liver both in embryos and adults, retinyl esters are converted to retinol by one or more retinyl ester hydrolases and are subsequently stored. Retinol that is secreted by the liver is bound to retinol-binding protein ($Rbp$), a lipocalin-family member, and transported in the blood where it is made available to tissues (47). $Rbp$ is necessary in adults in embryos for mobilization of retinol from the liver. $Rbp$-deficient mice that are fed regular dietary retinoid are physiologically normal, albeit with lower than normal serum retinol levels (47,48,49). In this case, chylomicron-remnant postprandial retinoid delivery pathways can deliver RA to tissues in the absence of $Rbp$. These findings suggest that $Rbp$ may be more important during conditions when the diet is poor, for mobilization of stored retinol and transport to tissues. During gestation, embryos obtain retinol and RA from the maternal blood. Maternal $Rbp$ is thought to deliver retinol to the yolk sac where it crosses the maternal-fetal barrier by an unknown mechanism. During development, as in post-natal stages, retinol is stored in the fetal liver and transported to tissues by fetal $Rbp$. 
**Strab**, a retinoic acid inducible gene, is a membrane receptor that has recently been shown to be a receptor for Rbp whose expression is important for the uptake of retinol (50). Strab mutations in humans and animals result in a number of defects observed in vitamin A deficiency and RAR-knockouts, including eye, diaphragm and the heart, as well as mental retardation.

Other retinol and retinoid binding proteins include cellular retinol binding protein (Crbp) and cellular retinoic acid binding protein (Crabp). These proteins were proposed to be important regulators of RA-signaling, as they are able to bind retinol and RA in cell extracts in cells *in vitro*. However, gene knockout studies suggest that their function *in vivo* is limited (51,52).

**D. RA-synthesis and degradation regulates RA-receptor signaling**

In the adult, excess or insufficient RA can lead to a variety of congenital abnormalities (53,54). The availability of RA in tissues is controlled in large part by spatially expressed RA-synthesizing and RA-degrading enzymes. Once Rbp delivers retinol to a cell, its conversion to RA occurs in a two-step process. First, retinol is oxidized to retinaldehyde, a reaction catalyzed by two enzyme families, the cytosolic alcohol dehydrogenases (Adh1, Adh3, and Adh4) and microsomal retinol dehydrogenases (Rdh1 and Rdh10) (Fig. 1.6) (53,54). Recent data suggests that this first step is carried out predominantly by Rdh10; since, unlike mutations in the Adh genes which cause few RA-deficiency like defects, a point mutation in Rdh10 impairs RA synthesis and causes embryonic abnormalities resulting in lethality (Table 1.6) (55,56,57). The data also
suggests that $Rdh10$ is necessary to produce sufficient levels of retinaldehyde to serve as a substrate for the second step in RA synthesis. This first step is reversible as $Rdh12$ and some aldo-keto reductases such as $Akr1b10$, can catalyze the reduction of retinaldehyde back to retinol (58,59).

The second and final step in the pathway is irreversible and generates all-trans RA (that from hereon will be referred to as RA). Three different type of retinaldehyde dehydrogenases have been described in the literature that are able to catalyze the oxidation of retinaldehyde to RA ($Raldh1$, $Raldh2$, and $Raldh3$) (53,54,60). However, $Raldh1$ appears not to be specific for RA-synthesis nor is it required for RA-synthesis in vivo (61,62). $Raldh3$ mutants die after birth due to breathing defects caused by nasal passage blockage, but with the exception of eye defects, display few other phenotypes, suggesting that this enzyme is unlikely to be required independently for RA-synthesis (Table 1.6) (63,64).

A strong body of evidence suggests that $Raldh2$ generates the major source of RA in the embryo; mutations in $Raldh2$ generate embryonic phenotypes that recapitulate those in VAD and $Raldh2$ expression correlates closely with RA-signaling activity (54,65,66,67,68,69,70). Niederreither et al. used a Rare-LacZ RA-reporter mouse that harbors 3 copies of a RA-response element from $Rarb2$ gene fused to $LacZ$, to map the distribution of RA-receptor signaling activity (71). In cells that have physiological quantities of RA, Rare-promoter activity is induced, driving expression of $LacZ$. In wild type embryos, Rare-LacZ activity and $Raldh2$ expression correlated closely, while in mutants lacking $Raldh2$, LacZ activity was dramatically down regulated (72). The newly
synthesized RA can diffuse over a few cell layers where it generally acts via paracrine signaling. However, the mechanism of RA-release has not been clarified.

RA-receptor signaling is also controlled by localized degradation mediated by oxidative enzymes such as cytochrome P450 (Cyps), a family of enzymes that convert RA to more polar and more readily excreted 4-hydroxy and 4-oxo metabolites (73). The Cyp26 family includes Cyp26a1, Cyp26b1, and Cyp26c1. Cyp26A1 has been proposed to play a major role in formation of 4-hydroxy and 4-oxo metabolites of RA in many tissues such as the skin, lung, and liver (73). Consistent with this, inactivation of Cyp26 genes results in ectopic activation of RA-signaling, generating defects also seen in maternal RA excess and thus demonstrates a requirement for localized degradation during organogenesis as well as in post-natal stages (74,75,76).

In summary, most cells in the embryo and in the adult express RA-receptor family-members. Specificity of RA-signaling appears to be generated in large part by spatio-temporal expression of RA-synthesizing and RA-degrading enzymes. These opposing activities can generate boundaries of RA-signaling that are crucial for patterning tissues including the heart, pancreas and hindbrain (Section F).

E. Vitamin A-deficiency syndromes are recapitulated in RA mouse models

Retinoids play a crucial role for many developmental processes such as patterning of early embryonic structures, cell proliferation, differentiation, and apoptosis. Evidence for this was first shown in the early part of the twentieth century when it was found that vitamin A deficiency (VAD) resulted in human abnormalities or ocular malformation in newborn pigs (77,78). In rats, deprivation of vitamin A results in growth retardation,
blindness, and sterility (79). The most characteristic feature of VAD animals is widespread keratinization of a number of epithelia including the respiratory tract, the alimentary tract, the genito-urinary tract, and the cornea (80,81). Further studies revealed that retinoids are also important for embryonic development. Embryos from VAD dams exhibit a spectrum of developmental malformations, including the heart, the urogenital tract, and the respiratory tract (82,83,84). Addition of vitamin A to the diet reverses most of the congenital malformations; further confirming that vitamin A is the dietary nutrient responsible for many of these developmental processes (85).

The discovery of NRs Rars and Rxrs (Section A), which contains 14 different family members with distinct expression patterns, suggested that the diversity in RA-functions may reflect differential and site-specific gene regulation (28,43,86,87). Analysis of Rar single knockout mutants, surprisingly, revealed few developmental defects. Rarb mutants survived to adulthood, and appeared to be anatomically normal. \textit{Rarg} mutants displayed squamous metaplasia of the seminal vesicles and prostate and \textit{Rara} mutants failed to thrive after birth, and displayed testis degeneration (88,89,90). Inactivation of multiple Rars and Rxrs however, generated embryonic defects that together mirrored those observed in VAD animal models suggesting that Rars and Rxrs were the major conductors of RA-signaling \textit{in vivo} during embryonic development (13,91,92,93,94,95,96) (Table 1.7). Rar double mutants also displayed phenotypes that were not observed in VAD syndromes such as skeletal defects (Table 1.8) (95).

However, these syndromes were reproducible in rodents lacking members of the RA signal transduction pathway, such as \textit{Raldh2}, \textit{Rdh10}, and \textit{Stra6}, suggesting that the full spectrum of RA effects may not be achievable through the classic VAD animal model.
The role of Rxr in retinoid signal transduction was also deduced through knockout mouse models. While \(Rxrb\) and \(Rxrg\) null mutant mice with one allele of \(Rxra\) (\(Rxrb/g^-/Rxra^{+/+}\)) are viable and do not display any overt abnormalities related to VAD defects- \(Rxra^{+/+}\) mutants die in utero around embryonic age E14.5 due to hypoplastic ventricular myocardium and ocular abnormalities (97,98). These abnormalities indicate that \(Rxra\) is functionally the most important Rxr during morphogenesis of the embryo; however because double \(Rxra/Rxrb\) null mutants are embryonic lethal at an earlier age (E9.5), like Rars, there may be some functional redundancy amongst Rxs. Compound \(Rara^{+/+}/Rxra^{+/+}\) mutants displayed many developmental defects that were found in single and double \(Rar\) mutants, suggesting that \(Rar/Rxr\) heterodimers are the functional dimmers responsible for transducing a majority of the RA-dependent processes (99). No synergistic effect was observed in \(Rara^{+/+}/Rxrb^{+/+}\) or \(Rara^{+/+}/Rxrg^{+/+}\), suggesting that \(Rxra\) is the primary heterodimeric binding partner of \(Rars\). The AF-2 (ligand-dependent activation) domain of \(Rxra\) was demonstrated to be important for mouse development. AF-2 mutants, \(Rxraaf2^o\), die at birth and exhibit a subset of abnormalities previously observed in \(Rxra^{+/+}\) mutants. Furthermore, compound \(Rar(a,b, or g)^{+/-}/Rxraaf2^o\) mutants reveal many defects that were observed in \(Rar/Rxra\) and \(Rar\) double mutants (33), suggesting that the AF-2 domain of \(Rxra\) is indispensible for developmental functions mediated by \(Rar/Rxr\) heterodimers (Table 1.9).

F Paracrine and autocrine modes of RA-mediated signaling

As described in Section A, retinoids regulate major embryonic growth and patterning processes by signal transduction through \(Rar/Rxr\)-family of receptors. There
are two modes of RA-dependent signaling: paracrine signaling and autocrine signaling. Under paracrine signaling, RA generated by \textit{RalDh} enzymes in one cell is released and travels to another cell for gene regulation. Alternatively, under autocrine signaling, a \textit{RalDh}-expressing cell generates RA to influence its own gene expression (54,65). While there are many examples of paracrine modes of RA signaling-some of which will be discussed in this section-no such examples of cell-autonomous signaling exist (Table 1.8). However, this form of RA-dependent signaling cannot be ruled out.

1. Hindbrain patterning

Hindbrain patterning depends on RA generated by \textit{Raldh2} in the presomitic mesoderm that travels rostrally along the neuroectoderm up to the rhombomere r3/r4 boundary regulating expression of a number of genes including \textit{Hoxb1}, which is required for pattern induction (100,101). A major function of RA signaling in the hindbrain involves regulation of \textit{Hox} family of genes. Many members of Hox genes, such as \textit{Hoxb1}, are direct targets of RA signaling, and contain RAREs in their promoter region (102). \textit{Hox} genes are differentially expressed along the antero-posterior axis of the developing hindbrain and are involved in rhombomere formation and specification (100). Interestingly, another RA-responsive gene, \textit{Hnf1b}, is required for repression of \textit{Hoxb1} in r3 and r5. RA-dependent gene expression is also tightly regulated in r3 and r5 boundaries through induction of \textit{Cyp26a1} (103). This induction degrades RA (see \textbf{Section D}) generating a boundary that limits \textit{Hoxb1} expression to r2 and r4. Consistent with this, knockout of \textit{Cyp26a1} in mouse and zebrafish results in an anterior extension of \textit{Hoxb1} expression into an area that normally develops into midbrain or forebrain, while knockout of RA-receptor X results in defective rhombomere patterning (53,104,105).
2. Regulation of heart patterning

Another important role of RA signaling is for heart development. *Raldh2* is expressed in the presomitic mesoderm but during somitogenesis, its expression is restricted to the lateral plate mesoderm just posterior to the cardiac crescent (106). RA generated by *Raldh2* travels into posterior heart mesoderm where it is thought to restrict the cardiac progenitor pool by activating transcription in myocardial progenitors. In the absence of *Raldh2* or RA-receptor signaling, uncommitted lateral mesoderm cells adopt a myocardial progenitor fate instead of other fates such as forming paryngeal or pancreatic progenitors (107). Studies on mice lacking *Raldh2* suggest that the effect of RA is through repression of *Fgf8* expression in the posterior region of the heart (108). The heart is one of many examples where retinoid signaling coordinates with other signaling pathways such as *Bmp4*, *Fgf* and *Shh* to regulate growth and limit the extent of stem and/or progenitor cell populations. As is the case with heart patterning, this often happens in an antagonistic manner with *Fgf* signaling pathway.

3. Pancreas induction

RA regulates patterning of a number of endodermal derivatives, including the pancreas, where RA acts at distinct stages. RA signaling in this case is mediated by *Raldh2*, which is expressed in the lateral plate mesoderm generating RA that activates RA-receptor signaling in nearby gut endoderm (109). Studies in the mouse indicate that RA-deficiency or defective RA-receptor signaling at early stages inhibits outgrowth of the pancreas primordia. In this case, *Pdx1*-expressing pancreatic progenitors fail to form (110). Recent results for the Prince lab indicate that RA also acts at later stages,
controlling endocrine progenitor fate via *Mnx1* (111). Consistent with these observations, in ES cell studies, RA plays a similar role regulating differentiation of *Pdx1* endocrine progenitors towards insulin-producing cells (112).

**G. Role of retinoids in stem cell specification**

One of the functions of RA-dependent signaling is to trigger specification and differentiation events or to limit the extent of progenitor populations during organogenesis. Here are examples of the varying roles of retinoid signaling in stem cell differentiation and specification during development.

1. **Role of retinoid signaling in motor neuron specification**

Many studies have suggested that retinoids acts at many different stages in progenitor cell differentiation and specification of motor neuron (MN) subtype identity. Retinoid receptor activation has been demonstrated to be important for neurogenesis, ventral neural tube patterning, and specifying late-stage MN subtypes *(Fig. 1.7)* (113).

Initial studies defined a role for retinoids in promoting the first step of neurogenesis. In the early stages of a developing embryo, *Fgfs* are expressed in the presomitic mesoderm while *Raldh2*, the enzyme responsible for RA synthesis, is expressed in somitic mesoderm. The antagonistic relationship between these two signaling networks is suggested to promote or inhibit neurogenesis (113). The receptors for RA are expressed in the spinal cord (Rara, Rab, Rxra ) and in the caudal neural plate (Rarg and Rxrg) therefore suggesting that RA signaling may be involved in neuronal differentiation. To test the ability of RA to induce neurogenesis, Diez del Corral et al.
(2003) exogenously applied RA to neural plate explants and found that it blocked \( Fgf8 \) expression and increased the numbers of \( NeuroM \)-positive cells-a proneural class bHLH transcription factor expressed by newly differentiating neurons (114). They also found that vitamin A deficient quail embryos had fewer numbers of neurons. Thus, these initial studies defined a role for retinoids in promoting the first step of neurogenesis.

Diez del Corral et al. and Novitch et al. (2003) subsequently showed that retinoid receptor activation directs the differentiation of neural progenitor cells to specific ventral neuronal subtypes (114,115). As the neural tube forms, downregulation of \( Fgf8 \) expression and upregulation of RA signaling promote onset of class I and II homeodomain (HD) proteins, respectively. Class I and II HD proteins define progenitor domains but do not mark onset of neuronal subtype-restricted progenitors. Novitch et al. showed that retinoid-mediated transcriptional activation of \( Olig2 \) expression facilitates the transition from a progenitor domain to MN progenitors in the ventral spinal cord (115).

\( Olig2 \)-positive MNs that arise from the progenitor domain in the ventral spinal cord are later organized into distinct columns that are anatomically and molecularly different. The subclasses of MNs can be defined as follows: 1. Body wall muscle-innervating MNs form a median motor column (MMC) along the spinal cord, 2. Limb muscle-innervating MNs form lateral motor columns (LMC) at brachial (forelimb) and lumbar (hindlimb) levels, and 3. Sympathetic neuron-innervating MNs form the column of Terni (CT) only at thoracic levels (116). Jessell’s group sought to determine what signals are involved in organizing MNs into distinct subclasses considering that they all originate from the same progenitor domain. Sockanathan et al. proposed that because
*Raldh2* is expressed in mesoderm adjacent to the brachial spinal cord at higher levels than in thoracic or lumbar spinal cord, RA might be the signal that specifies brachial LMCs. In support of this hypothesis, they found that blocking RA signaling prevented post-mitotic MNs from acquiring brachial LMC identity (116).

MNs that are generated at the branchial levels of the spinal cord can be further subdivided into two subclasses: medial LMC neurons that enervate ventrally-derived limb muscles, and lateral LMC neurons that project to dorsally-derived limb muscles (116). MNs that are destined to form the medial LMC leave the cell cycle before lateral LMC neurons. In addition to their distinct migratory patterns, lateral motor neurons are also molecularly distinguishable from other motor neurons by their expression of LIM homeobox genes. Initially, all somatic motor neurons express *Isl1* and *Isl2* and maintain their expression; however, lateral LMC neurons repress *Isl1* expression and begin to express *Lim1*. Based on the migratory patterns of the LMC neurons, Sockanathan et al. speculated that the fate of lateral LMC neurons might be regulated by retinoid signals from early medial LMC neurons. They found that medial LMC MNs express *Raldh2*, and that RA can induce later-born LMC MNs to express the lateral LMC specific marker *Lim1*.

Taken together, these studies demonstrate that RA plays a crucial role in sequentially defining many of the steps in MN differentiation into a specific neuronal type. RA synthesis - first from the presomitic mesoderm, then from the paraxial mesoderm and later from the motor neurons themselves - helps establish the transcriptional identity of motor neuron subtypes.
2. Role of retinoid signaling in pancreas induction and cell-type specification

Like motor neuron specification, retinoid-mediated transcriptional activation is also required at various stages of pancreas development. As reported in F3, RA-synthesized in the mesoderm by Raldh2 is necessary for specification of the pancreas (109). Studies in zebrafish with disrupted RA signaling either by a pan-RA receptor antagonist, or the zebrafish neckless (nls) mutants that lacks Raldh2, indicate that retinoid-signaling is necessary for specification of endocrine progenitors. Mutant zebrafish embryos failed to express markers of endocrine progenitors such as Pdx1 or islet1, and markers of the liver, suggesting that inhibition of RA signaling prevents differentiation of a hepatopancreatic progenitor population (112). Similarly, mouse embryos carrying a null mutation of Raldh2 lack expression of Pdx1 and Prox1 in the dorsal endoderm but not in the ventral endodermal pancreatic progenitors (110,117). Dorsal Pdx1 expression is rescued with low dose maternal RA supplementation. Expression of liver specific marker Hex, however, is not affected, indicating that retinoids are not required for liver specification in mice. Thus, retinoid receptor activation, like motor neuron differentiation, is also necessary for the first step in the development of the dorsal pancreatic lineage.

Pdx1-positive endodermal pancreatic progenitors differentiate into Neurogenin 3 (Ngn3)-positive endocrine progenitor cells (118,119). Ngn3-positive endocrine progenitors then give rise to different endocrine cell types at multiple stages of mouse embryonic development that are defined by expression of distinct transcription factors (120). For example, glucagon-positive (Glu) α-cells appear by E9.5 whereas insulin-positive (Ins) appear by E14.5 and onwards. To determine signals involved in pancreatic
cell-type specification, Ostrom et al. (2008) showed that Raldh1 is expressed in mouse pancreatic epithelium at E13.5, around the time of β-cell differentiation (121). Ex vivo pancreatic explant cultures were used to assess the role of retinoid-dependent signaling for β-cell differentiation. In explants cultured in the presence of RA, there was a 3-fold increase in the number of Ins-positive cells, but there was no difference in the number of Glu-positive cells. These results suggest that RA-signaling is necessary for differentiation of Ngn3-positive endocrine progenitors into β-cells but not into α-cells. Furthermore, Ostrom et al. showed that RA is also required for induction of Ngn3-positive endocrine progenitor cells, thus establishing a sequential role for RA-dependent signaling during pancreatic cell type differentiation (Fig. 1.8). Stage and cell-type specific inhibition of RA-signaling in vivo might provide a clearer understanding of the role of RA in pancreatic differentiation.

IV. Introduction to Urinary Tract Development

During embryogenesis, many independently formed organs organize into complex systems that conduit air, food, and waste into and out of the embryo. The development of the urinary tract is one such example. The urinary tract consists of an upper portion (kidneys and ureter) and lower portion (bladder and urethra) that form independently and assemble during mid-gestation to give rise to an organ system that allows passage of waste through the embryo (Fig. 1.9) (122). The kidneys, ureter, and mesonephric ducts or Wolffian ducts (WDs) are derived from tissue that lies between the lateral plate and the paraxial mesoderm called the intermediate mesoderm, whereas the bladder and urethra
are derived largely from cloacal endoderm and peri-cloacal mesenchyme (Section G) (123,124).

The primary functions of the urinary system are to maintain body homeostasis by regulating electrolyte balance of the blood and by controlling excess water and waste. The urinary tract in mammals has evolved a number of mechanisms that enable efficient urine removal from the bladder in order to prevent urine accumulation in the kidney. Renal filtrate generated by nephrons in the kidney normally collects in the pelvis, where it is propelled to the ureter via the ureteral-pelvic junction. Like the ureters, the ureteropelvic junction is lined with muscle, and contains pacemaker cells that are critical for controlling peristaltic waves that propel urine to the bladder. Once in the ureters, myogenic peristalsis via the ureter muscle coat moves urine to the bladder. In the bladder, an anti-reflux valve mechanism located at the ureterovesicular junction prevents backflow to the ureter and kidney. All of these events depend on a patent connection between the upper (kidney and ureter) and lower (bladder and urethra) urinary tract compartments, which if abnormal, can cause hydronephrosis and subsequent damage to the kidney. (125).

In mice, urinary tract development is initiated at E8.5 by the formation of the nephric duct (ND, also called mesonephric duct, and WD) in the intermediate mesoderm (125,126). At approximately E9, the WDs continue to extend along the anterior-posterior axis and open into the cloaca, an endodermal organ that eventually becomes partitioned by the urorectal septum into the urogenital sinus (ventrally) and the hindgut (dorsally). From E11.5 to E14.5, the urogenital sinus differentiates cranially into the bladder, and caudally into the urethra (bladder development is discussed in more detail in Section G).
At E10.5, an epithelial diverticum called the ureteric bud (UB) branches from the WD at a stereotypical position just above the cloaca. Signals from the neighboring metanephric mesenchyme (MM) induce the ‘tip’ of the UB to undergo a series of characteristic t-shaped branching, ultimately generating the collecting duct system of the kidney (126,127). The portion of the UB lying outside of the metanephros, called the ‘trunk’, differentiates into the ureter. Thus, while the MM surrounding the UB tip undergoes mesenchymal-epithelial transition to form nephrons, the mesenchyme surrounding the UB trunk differentiates into smooth muscle cells (128).

At this time, the upper and lower compartments are still in an immature configuration, as the proximal ureter is connected to the kidneys and the lower (distal) ureter is separated from the urogenital sinus by a terminal segment of the WD called the common nephric duct (CND). Direct connections between the ureters and bladder are established between E10.5 and E13.5 (129). During ureter maturation, the CND undergoes remodeling as the ureters separate from the WD and shifts its orifice to the base of the bladder. Defects in any of the steps such as abnormal outgrowth position of the UB, or defective CND remodeling can result in ectopic location of the ureter orifice in the bladder (Section A).

Paramesonephric or the Mullerian ducts (MDs) form from invagination of the coelomic epithelium of the mesonephros between E11.5 and E12.5 in mice, and between 6 and 7 weeks of gestation in humans (130,131). They migrate caudally with the WDs and connect to the dorsal side of the urogenital sinus. The presence of the WD seems to be essential for MD formation as mice mutants that lack Emx2 fail to develop WD and MD derivatives (132). The gonads form near the kidneys and differentiate into the testes.
for males and ovaries for females. By E17.5, males and females are completely sexually distinguishable. In males, the MDs degenerate between E15.5 and E17.5 and the WDs develop into the epididymis, vas deferens and seminal vesicles. In females, the WDs regress but the MDs differentiate and form the oviduct, uterus, cervix, and the upper portion of the vagina.

Due to many conserved genes and signaling pathways between human and mice, development of the urinary tract is very similar. For example, mutations in Ret in humans and rodents generate similar sets of urinary tract defects, including renal hypoplasia, agenesis, vesicoureteral reflux and physical obstruction. The developmental stage comparison for the urinary tract is shown in Table 1.11.

A. CAKUT and classical models of urinary tract abnormalities

As mentioned in the previous section, the upper (mesodermal origin) and lower (endodermal origin) urinary tract compartments form independently but are connected through sequential processes during gestation to yield a functional outflow tract. Defects in any of the steps can lead to lower urinary tract abnormalities.

Congenital anomalies of the kidney and urinary tract (CAKUT) include a spectrum of defects that affect as many as 1% of human newborns (133). These include kidney anomalies such as kidney aplasia, hypoplastic kidneys, vesicoureteral reflux (abnormal flow of urine from the bladder to the upper urinary tract), and ectopic ureters that terminate in a location other than the bladder (133,134). For example, if the UB fails to separate from the WD in males, it will end in WD derivatives such as the ejaculatory
duct, the vas deferens, or the seminal vesicle. However, in females, its termination site may be the vagina or the caudal portion of the urogenital sinus or the urethra.

Many of these obstructions have been reproduced in rats and mice by a number of teratogens (135). In the early 20th century, Wilson and Warkany (1948) found that maternal vitamin A deficiency resulted in ectopic ureters in 37 out of 42 fetuses, with a third of them either retaining connection with the WD, or joined to the urethra (84). Other studies have shown that maternal folic acid deficiency can result in a similar ectopic ureter phenotype in rat fetuses (135).

A number of theories have been postulated to try to explain the mechanism or cause of urogenital tract anomalies (136). In the late 1800s, Weigert and Meyers hypothesized that in the case of duplicated ureters, when two separate UBs form from the same WD, the ureter from the lower pole separates from the WD earlier and inserts at its normal position (137). The upper ureter, however, enters the bladder at an abnormally medial and caudal site, which leads to obstructions. In the 1970s, Mackie and Stephens expanded on the Weigert-Meyer rule by postulating the Ureteric Bud Theory (138). Based on analysis of duplicated ureters from human embryos and animals, they proposed that the final insertion site of the ureter could be correlated to the original sprouting site of the UB. For example, if the UB sprouts cranially to the normal site, it remains attached to the WD or joins the urogenital sinus at a more posterior position than normal such as the urethra, causing an obstruction. On the other hand, if the UB sprouts more caudally to the normal site, the ureter joins the bladder more lateral to the normal insertion site in the trigone, a defect that can lead to reflux. Ectopic ureter insertion at any of these sites can
lead to the formation of an ureterocele, or a cystic dilation at the intra-vesicular ureteral segment that can result in vesicoureteral reflux and renal damage (Fig. 1.10) (139).

Furthermore, to explain how abnormal UB sprouting position can affect the final ureter insertion position, Mackie and Stephens proposed a mechanism by which normal ureter maturation occurs. According to their theory, the CND, the portion of the WD that separates the UB from the urogenital sinus at E11, expands and differentiates into the trigone, the component of the bladder that is located between the bladder head and the urethra. This expansion enables the ureter to separate from the WD and translocate to its final insertion site in the trigonal base. The trigone is a triangular-shaped region located at the base of the bladder. The trigonal base is surrounded by a muscular sheath that encircles the intravesicular ureter and prevents backflow of urine to the ureter and kidneys (140). The principle events of urinary tract development based on Mackie-Stephens hypothesis are shown in Fig. 1.11.

However, because the structures of the trigone and the bladder are histologically indistinguishable, this theory was never tested. Also, it is not clear from the Weigert-Meyer and Mackie-Stephens models as to why the upper ureter remains attached to the WD but the lower ureter is integrated at the correct site. The proceeding sections highlight advances that have been made in urinary tract development from use of mouse models and through discovery of conserved genes and signaling pathways between mice and men.

B. Mouse models for urinary tract development
The availability of \textit{Hoxb7-Gfp} and \textit{Hoxb7-cre} transgenic lines enabled researchers to test the Mackie-Stephens ureter maturation theory. In these mouse lines, CND cells are selectively labeled with green fluorescent protein (GFP) or \textit{Cre} recombinase, thus allowing the fate of CND cells to be followed during ureter maturation (141,142). From E11 to E13, the CND descends and grows laterally as a wedge against the dorsal epithelium of the urogenital sinus (\textbf{Fig. 1.12a-d}). Between E12 and E14, there is flattening of the CND wedge as the ureter and WD separate. By E14 and onwards, Gfp-positive ureter and WD cells were visible, but \textit{Hoxb7-Gfp} reporter expression was not detected in the bladder trigone, where the CND wedge had previously expanded and flattened. This suggests that the CND does not differentiate into the trigone but may be a transient structure that undergoes remodeling as the new ureteral orifice is formed (\textbf{Fig. 1.12d-f}) (129). Closer analysis of \textit{Hoxb7-Gfp} embryos costained with laminin, a component of the basal laminin, revealed that at E11, the CND joins the dorsal side of the urogenital sinus. By E13, as CND cells regress, the ureteral orifice merges with the bladder epithelium (\textbf{Fig. 1.12g-l}). Use of the \textit{Hoxb7-cre; R26R-lacZ} transgenic mouse line to permanently label cells expressing \textit{Hoxb7-cre} and all the daughter cells with lacZ, showed a similar pattern of activity; lacZ was expressed throughout the ureters, WDs and CND but was restricted in the trigonal region (129). Furthermore, staining \textit{Hoxb7-Gfp} embryos with TUNEL and with antibodies against activated Caspase-3-both which label apoptotic cells-revealed many TUNEL and activated Casp-3-positive cells in the CND but few in the WD farther away from the urogenital sinus epithelium, suggesting that bladder-derived signals or intercalation of CND and bladder epithelial cells may be important for rearranging CND cells to establish mature ureter-bladder connections.
During these stages, as the ureter is separating from the WD, the urogenital sinus is growing and expanding into the future bladder and the urethra (Section G).

Programmed cell death (PCD) is also observed at later stages when the ureteral orifice opened into the bladder. Analysis of Hoxb7-Gfp embryos stained with activated Caspase-3 revealed many apoptotic bodies in the caudal ureter at E15 that were absent at E16, suggesting that PCD in the distal ureter may be important for ensuring unobstructed urine flow to the bladder (129). Cell death of the caudal ureter is also proposed to play a role in degeneration of Schwalla’s membrane (Fig. 1.13) (134).

C. Role of Ret signaling in lower urinary tract development

One essential component of urinary tract development is glial cell-derived neurotrophic factor GDNF, its receptor tyrosine kinase Ret and its GPI-linked co-receptor Gfra1 (143). Binding of GDNF ligands to Gfra1 coreceptor activates Ret receptor tyrosine-kinase-dependent signaling.

Ret and Gfra1 are first expressed throughout the WD while Gdnf is expressed in the MM surrounding the caudal portion of the WD from which the UB sprouts. Once the UB begins to differentiate into the mature kidney, Ret expression is downregulated in the WD and UB stalk, and becomes restricted to the UB tips. The expression pattern of Gfra1 largely mimics that of Ret; however, Gfra1 is also found in Ret-negative domains such as the mesenchyme and developing nephrons (144,145,146).

The Weigert-Meyer rule states that the site of the initial outgrowth of the UB is a critical step; if it sprouts more cranially or caudally, the final insertion site of the ureter is ectopic which can lead to ureterocele and vesicoureteral reflux (137). Studies in mice
reveal that Gdnf-Ret signaling pathway is important for regulating the initial UB outgrowth. Mice null for Gdnf, Ret, or Gfra1 reveal defects in the development of the enteric nervous system (ENS) and defects in the urinary tract; renal agenesis or hypodysplasia was caused by a failure of the UB to sprout from the WD or to differentiate normally (147,148,149). However, while the majority of Ret null mutants (~70-30%) display renal agenesis due to failure in UB outgrowth, the remaining mutants that do form ureters display ectopic ureter insertion or hydronephrosis (150,151). Studies showed that the formation of a single UB at the right place depends on spatially restricted expression of Gdnf. For example, aberrant expression of Gdnf throughout the WD in vivo or application of Gdnf-soaked beads next to a WD in an organ culture causes numerous ectopic UB to sprout (152,153). Thus, regulation of Gdnf signaling by other signaling mechanisms is necessary to ensure UB growth at the right location (126). For example, mutations in Foxc1 and Foxc2 or Slit2 or its receptor Robo2 result in an expansion of the Gdnf expression domain and the induction of supernumerary UBs and ectopic ureters (126,154).

In humans, mutations in the Ret gene can lead to hereditary cancer syndromes: Hirschsprung’s disease (intestinal aganglionosis), and CAKUT (155). Ret activation results in phosphorylation of key docking tyrosine (Y) residues that interact with adaptor proteins. These interactions activate downstream signaling cascades such as Raf/Ras, PKC, PI3K/AKT, and MAPK, which regulate cell proliferation, apoptosis, migration, and differentiation (Fig. 1.14) (156). Mutations in RetY1015 (PLCγ pathway) and RetY1062 (PI3K/MAPK pathway) reveal distinct roles in the urinary tract; while disruption of PLCγ
pathway results in supernumerary UBs, disruption of Pi3K/MAPK pathway leads to
development of a rudimentary kidney.

In addition to regulating the evagination of the UB from the WD and branching
morphogenesis, Ret-Gdnf signaling also plays crucial roles at other stages of urinary tract
development. A variety of studies have shown that addition of Gdnf to kidney cultures
and expression of activated forms of Ret in the WD induces ectopic UBs and abnormal
branching, suggesting that Gdnf/Ret signaling may be a chemoattractive cue that the UB
differentiates towards. In the ENS, Gdnf acts as a chemoattractant that guides migration
of progenitor enteric neural crest cells (PENCCs) to the foregut and along the length of
the bowel (157,158). Similarly, Ret-Gdnf signaling was also found to be important for
mediating migration of the ND in the urinary tract; in amphibians, pronephric duct (PND)
will migrate towards a Gdnf-soaked bead but will fail to migrate if it is expressed
uniformly down the migrating path (159). In the absence of Ret in mice, NDs fail to reach
the cloaca resulting in ectopic ureters that join the bladder abnormally. Interestingly, like
ENCCs, ND tip cells also display axon-like projections that are absent in Ret⁻/⁻ mutants,
suggesting that mesenchymal Gdnf may act as a chemoattractant that guides Ret-
expressing ND cells towards the cloaca (160).

Altogether, these results suggest that Ret-Gdnf signaling plays multiple roles
during urinary tract development; Ret is required for ND extension and insertion with the
cloaca, UB induction, and for kidney growth. Defects in any of these processes can lead
to urinary tract defects such as ectopic ureters, ureterocele, and vesicoureteral reflux.

D. Role of retinoid signaling in lower urinary tract development
Like Ret, there is also a sequential requirement for retinoids during urinary tract development. Ret<sup>−/−</sup> mutants exhibit widespread urogenital tract abnormalities—such as renal hypoplasia, renal agenesis, ureter ageneis and ectopic ureters—that are also observed in embryos from VAD dams (84,146). As previously stated, retinoid signaling is transduced by nuclear retinoic acid receptors and retinoid X receptors; signal transduction is dependent on the availability of retinoic acid, which is synthesized from retinol by retinol and retinaldehyde dehydrogenase family of enzymes. Within the lower urinary tract, expression of Rars and Rxrs are widespread. Compound mutant mice lacking multiple Rar isoforms are embryonic lethal and display severe malformation in the urinary tract that mirror those observed in VAD embryos such as defects in kidney development, ureter ectopia or agenesis, and hydronephrosis (84,91,92,99). One of the defects observed in Rarab2<sup>−/−</sup> mutants (null for Rara and Rarb2) is defective branching with undetectable Ret expression in the UB (161,162). Forced expression of Ret in UB cells of Rarab2<sup>−/−</sup> mutant rescues kidney abnormalities suggesting that a major function of RA during renal development is in regulating Ret (162). Furthermore, directly blocking RA-dependent transcription in UB cells by expressing a dominant-negative RA receptor, RaraT403 (from hereon referred to as RaraDN, Section V) driven by HoxB<sup>7</sup>Cre promoter results in phenotypes similar to those seen in VAD and Rarab2<sup>−/−</sup>-embryos, suggesting that RA generated by Raldh2<sup>+</sup> stromal cells acts directly on Rars in UB cells to maintain Ret expression (Table 1.12) (163).

Analysis of Rarab2<sup>−/−</sup> mutants also revealed ectopic termination sites of the ureters. In comparison to wild-type embryos, where the ureters join the bladder and the vas deferens joins the urethra, in Rarab2<sup>−/−</sup>-males and females have ureters joined to the
vas deferens, the uterus, and the vagina, instead of the bladder (164). This obstruction caused ureter dilation at onset of urine production. During normal ureter maturation (Section A), ureters are displaced from their initial position in the caudal WD to their final insertion site in the bladder. Mackie and Stephens hypothesized that the distal portion of the WD or the CND, differentiates into the trigone and enables the ureter to move away from the WD and reposition the ureteral orifice to the base of the bladder (138). However, use of HoxB7-Gfp and HoxB7Cre; R26LacZ transgenic mouse models to lineage-trace CND cells suggests that the bladder trigone does not derive from the CND but that the CND undergoes apoptosis instead enabling the ureter to separate from the WD (129). The process of normal ureter maturation can be divided into three stages: UB formation, vertical displacement, and lateral displacement. Using HoxB7-Gfp transgenic embryos, Batourina et al. found that after UB outgrowth, which takes place between E10.5-E11.5, the CND regresses, bringing the ureters into close contact with the urogenital sinus. From E12-E14, the CND undergoes lateral displacement, probably resulting from cellular rearrangements and integration of CND cells with urothelial cells that expand and flatten its morphology, followed by apoptosis. As this occurs, ureters separate from the WD and fuse with the urogenital sinus epithelium. Expansion and growth of the urogenital sinus from E11.5-E14.5 into the mature bladder then positions the ureteral orifice at the base of the bladder.

In Rarab2- mutants, the UB sprouted from the WD at the appropriate location and the CND had descended to the level of the urogenital sinus, but had failed to undergo lateral displacement and apoptosis, suggesting that RA-signaling around or from the bladder epithelium is necessary for late-stage ureter maturation. Furthermore, RA-
dependent Ret expression was also found to be important for epithelial expansion of the CND. In Rarab2- mutants, Ret expression was downregulated in the distal portion of the WD and the dorsal region of the urogenital sinus. Analysis of Ret−/− mutants revealed that distal ureters also terminate in the uterus or in the vas deferens, suggesting that Ret signaling is important not only for UB outgrowth and morphogenesis, but also important for ureter maturation. Indeed, forced expression of Ret using HoxB7-Ret transgenic mice in Rarab2- mutants rescues distal ureter abnormalities suggesting that vitamin A controls lateral displacement, apoptosis, and ureter maturation through Ret.

According to the Weigert-Meyer rule, when two UBs branch from the WD, the lower UB that forms closer to the bladder inserts at the normal position in the bladder. However, the upper UB that is farther away from the bladder epithelium, fails to join normally. Batourina et al. were able to recapitulate a duplicated ureter system by exposing embryos to retinoic acid during the stage of UB formation. They showed that CND1 (from UB1 that sprouted close to the urogenital sinus) underwent apoptosis; whereas CND2 (from UB2 that formed farther away from the urogenital sinus) remained in an immature configuration with few apoptotic cells. The fact that apoptosis occurs close to and within the bladder epithelium suggests that bladder-derived signals may be important for inducing programmed cell death (129). My first project (Chapter II) is based on these observations.

Like Ret, retinoid signaling is also required for caudal migration of the ND towards and insertion with the cloacal epithelium. RA signaling is largely regulated by Raldh2, the major RA synthesizing enzyme. During early embryogenesis, Raldh2 is expressed in the mesenchyme surrounding the ND, while the receptors for RA are
expressed in the ND epithelium. Studies by Chia et al. demonstrated that defects observed in \textit{Raldh2}^{-/-} embryos such as ectopic ureters were a result of delayed ND insertion (160). While NDs of wild-type embryos had elongated to the posterior of the embryo and turned to fuse with the cloaca, NDs of \textit{Ret}^{-/-} mutants and \textit{Raldh2}^{-/-} mutants had migrated along the anterior-posterior axis but had failed to turn towards the cloaca. Thus ND insertion with the cloaca establishes the primary connection between the upper and lower urinary tracts. Interestingly, ND and bladder-related defects were not observed in \textit{Rarab2}^{-/-} mutants, but were present in other classes of RAR knockouts such as \textit{Rarag}^{-/-}, suggesting that signals emanating from the bladder may play a role in ureter maturation. It would also be interesting to see if RA signaling regulates apoptosis of cloacal epithelium for initial insertion of the ND.

The severity of embryonic defects depends on the extent of inhibition of RA-signaling and in the case of RA-receptor mutants, on the distribution of receptors and their transcriptional specificity. Graded levels of vitamin A deficiency results in progressively more severe embryonic phenotypes, in the urinary tract, this was observed in the kidney and bladder, both of which became increasingly hypoplastic as maternal vitamin A levels decreased. \textit{Rar} compound mutants display a large number of defects that vary in severity and recapitulate those observed in VAD. For example, \textit{Rarab2}^{-/-} mutants display renal hypoplasia, CND defects and squamous metaplasia of the vagina and uterus. \textit{Rarag}^{-/-} mutants display generally more severe phenotypes, including renal agenesis, bladder agenesis and hypoplasia, agenesis of the caudal ND and squamous metaplasia of the seminal vesicles and prostate. All of these defects were observed in models of RA-deficiency.
Taken together, these studies demonstrate that RA regulation of Ret is necessary for many stages of urinary tract development such as branching morphogenesis of the UB, ureter maturation, and ND guidance. The current model of urinary tract formation based on these studies is shown in Fig. 1.15.

E. Pax2/Pax8, Gata3, and Lim1 regulation of the ND

Urinary tract development can be divided into three major categories: WD formation, ureter budding and nephrogenesis, distal ureter maturation, and bladder development. Although most lower urinary tract anomalies have been linked to defects in UB induction, ureter maturation and ND insertion with the cloaca may also be equally important (165). The ND plays an important role in urinary tract development; it is required for kidney formation and for establishing the initial link between the upper and lower urinary tracts. Other signaling pathways, besides Ret and RA, have also been shown to play a role in ND morphogenesis.

Chia et al. discovered that ND insertion depends on Ret; Ret expression in turn depends on RA (160). In the lower urinary tract, Gata3 is expressed at E9.5 in the ND of the mesonephros, and remains restricted to the ureter tips. In Pax2-/- Pax8+/− embryos, Gata3 expression is undetectable, suggesting that Pax2 or Pax8 are upstream regulators of Gata3 in the ND and that Gata3 may be an important effector of Pax gene function (166). Similar to Ret−/− and Raldh2−/− mutants, NDs in Gata3−/− embryos fail to extend caudally toward the cloaca and follow a misguided path towards the surface ectoderm. In this mutant, defects in ND guidance were also due to downregulation of Ret, which is thought to be involved in axon-like guidance in NCCs and in the ND.
Another gene that is necessary for ND morphogenesis is *Lim1*, a homeobox gene required for head and urogenital development in the mouse. In the urinary tract, it is expressed in the intermediate mesoderm, the nephric duct, the ureteric bud and in the developing Mullerian ducts. Since *Lim1*-deficient mutants die by E10, *Lim1* was conditionally removed in ND cells using the floxed allele of *Lim1* and Pax2-cre (167). *Lim1* conditional postnatal mice had renal hypoplasia, unilateral hydronephrosis, and megaureter. Analysis at earlier stages revealed that the ND had failed to extend caudally and UB morphogenesis was stunted. Additionally, although Ret, Pax2, Gata3, and Emx2 expression were not altered, Wnt9b and E-cadherin were downregulated.

The homeobox gene *Emx2* is also essential for dorsal telencephalon and urogenital tract development. *Emx2* is expressed in the WDs and mesonephric tubules, and *Emx2* mutant mice lack kidneys, ureters, gonads, and genital tracts (168,169).

**F. Role of apoptosis in lower urinary tract development**

Apoptosis is important for morphogenesis of many organs during embryonic development, including during limb bud formation, development of the nervous system, and for opening of the vagina during reproductive tract development (170,171,172). During urinary tract development, apoptosis is important during nephrogenesis as well as for establishing mature ureter connections with the bladder.

Apoptosis can be characterized by cell shrinkage, nuclear condensation, and DNA fragmentation (173). The apoptotic pathway can be classified into two categories: the intrinsic and extrinsic pathways. The intrinsic pathway of apoptosis is mitochondrial-mediated and involves pro- and anti-apoptotic Bcl2-family members. Bcl2, an anti-
apoptotic protein, prevents cell death by inhibiting pro-apoptotic proteins such as Bak, Bax, and BH3 (174). Apoptotic signals stimulate Bak and Bax to oligomerize, open the outer mitochondrial membrane, and release proteins such as cytochrome c, apoptosis-inducing factor (AIF), and EndoG (175). While AIF and EndoG facilitate nuclear condensation and DNA fragmentation, cytochrome c acts with apoptotic protease activating factor-1 (APAF-1) and caspase 9, through effector caspases 3, 6, and 7, to induce cell death (Fig. 1.16) (176). The extrinsic pathway is receptor-mediated and is initiated when proapoptotic ligands bind death receptors such as Fas or tumor necrosis factor (TNF), to initiate recruitment of adaptor proteins and pro-caspase 8 to form the death-inducing signaling complex (DISC) (177). Pro-caspase 8 then cleaves the executioner caspase 3 for apoptosis (Fig. 1.16b).

G. Development and anatomy of the urinary bladder

As mentioned in Section C, the urinary tract consists of the kidneys, ureter and the bladder. Waste filtered by nephrons in the kidneys is propelled through a conduit called the ureter and stored in the bladder until excretion through the urethra. The mammalian urinary bladder is derived from the cloaca, an endodermal sac that between E11-E14 partitions into the hindgut and anus dorsally and the urogenital sinus ventrally. At the same time, the urogenital sinus differentiates into the mature bladder cranially and the urethra caudally (Fig. 1.17) (178). The main function of the bladder is to store urine. Structurally (from outside to inside), the bladder is made up of the serosa, detrusor muscle, muscularis mucosa, lamina propria, and the epithelium (Fig. 1.18a) (179). The detrusor muscle contracts to simultaneously expel urine and close the ureter.
intravesicular junction to prevent urine backflow to the kidneys (vesicoureteral reflux).

The bladder epithelium, also called the urothelium, lines the major portion of the lower urinary tract, including the renal pelvis, ureters, bladder, and urethra (180). It allows the bladder to accommodate large changes in urine volume while maintaining an impermeable barrier that prevents toxic exchanges between blood and urine. This task is largely accomplished by highly specialized umbrella cells that line the apical layer of the urothelium (Section 1). Furthermore, the urothelium can transmit information from its lumen to other parts of the bladder and is also involved in active ion transport (181). In the proceeding sections, I’ll focus on the advancements that have been made in bladder research.

1. Bladder urothelium

The urothelium is one of the slowest cycling epithelia in the body with a turnover rate of about 6 months to a year (182,183,184). It is composed of basal, intermediate, and umbrella cells of varying morphology; basal cells are thought to differentiate into intermediate cells that in turn differentiate to form umbrella cells (Fig. 1.18b) (181). However, this hypothesis has never been tested in vivo.

Basal cells are mononucleate and approximately 5-10 \( \mu \)m diameter. Intermediate cells are approximately 10-15 \( \mu \)m diameter and the number of cellular layers in the intermediate zone varies between species: in rodents, the intermediate layer is one to two layers thick, while in humans it can be anywhere from one to five layers thick (185,186). Umbrella cells are large cells that line the apical layer of the urothelium. Their morphology depends on whether the bladder is filled or not; in an unfilled state, umbrella
cells are cuboidal in shape, while in a filled state, they become flat and squamous in appearance, covering up to 20 intermediate layer of cells that lie below them.

The high-resistance barrier function of the urothelium is generated by umbrella cells. Umbrella cells are responsible for producing and secreting a family of transmembrane proteins called uroplakins (UPs) including UPIa, UPIb, UPII, and UPIIIb that arrange into asymmetric unit membranes (AUM) plaques on the apical membrane of umbrella cells (187). An additional feature of umbrella cells is the presence of fusiform- and cuboidal-shaped vesicles (DFV) that are responsible for delivery of UPs to the apical surface of umbrella cells (180). The superficial umbrella cells are connected through tight junctions that also aid in barrier function.

Initial isolation of urothelial plaques identified four major proteins of varying sizes, UPIa (27kDa), UPIb (28kDa), UPII (15kDa) and UPIIa (47kDa) that form UPIa/II and UPIb/III heterodimers (184,186,188,189). Data suggests that these uroplakin proteins are necessary for maintaining the impermeability barrier function of the urothelium. Knockout of UPIIIa gene in mice leads to a 70% loss of urothelial plaques whereas ablation of UPII leads to a hyperplastic bladder with complete loss of plaques, umbrella cells, and fusiform vesicles (186,190,191). UPIIIa homozygous null-mutant mice also display hydronephrosis due to large ureteral orifices at the intravesicular junction (190). This data suggests that both uroplakin heterodimers (UPIa/II and UPIb/III) are required for plaque formation.

Early in development (E15), the apical surface of superficial cells is characterized by microvilli. By E18, the microvilli are replaced by distinctive AUM plaques that occupy most of the surface. In addition to uroplakin, the urothelium is also characterized
by cytokeratin marker expression (192). While cytokeratin 20 (Krt20) expression is restricted to umbrella cells at E18 and in the adult, cytokeratin 7 (Krt7) is thought to be expressed in all 3 cell types.

Urothelial basal and intermediate cells also express p63, a homologue of p53 tumor suppressor gene (193). p63 is necessary for development of squamous epithelia; mice null for p63 have defects in epithelial differentiation (194). In the urogenital sinus epithelium (UGE), p63 is expressed at E12 and onwards (195). Basal and intermediate cells are p63-positive, whereas umbrella cells are p63-negative. Previous studies have suggested that p63 is necessary for urothelial differentiation (193,194). Interestingly though, urothelia from mice null for p63 still contain a single layer of cells that resemble umbrella cells and express UPIII, a marker of superficial cells. The observation that umbrella cells can form in the absence of p63+ basal and intermediate cells suggests that these cells do not represent umbrella cell stem cell population (196). Conversely, though, in UPIII and UPII null embryos, the bladder contains a thicker than normal layer of cells that are UP-negative but cytokeratin-positive (187).

2. Signaling pathways involved in urothelial differentiation

Recent advances in bladder research have been aimed at understanding the transcriptional signaling pathways that are involved in urothelial differentiation. In the skin, the Grainyhead transcription factor Get1/Grhl3 is responsible for terminal differentiation of epidermal cells and for maintaining epidermal barrier function in the mouse (197,198). Get1 is expressed in the umbrella cells at E16.5, E18.5 and in adults, suggesting that Get1 might play a role in umbrella cell differentiation. To determine the
role of Get1 in urothelial differentiation, Yu et al. generated Get1\(^{-/-}\) mice. In addition to neural tube and epidermal barrier defects, Get1\(^{-/-}\) also exhibited abnormalities in umbrella cell differentiation (199). While wild type bladders had plaques on their superficial surface, Get1\(^{-/-}\) urothelia was covered with microvilli. Furthermore, Get1 was found to be a direct transcriptional regulator of UPII. Yu et al. hypothesized that Get1 is important for differentiation of intermediate cells into umbrella cells. However, there is no evidence in the literature that suggests that umbrella cells are derived from intermediate or basal cells.

Peroxisome proliferator-activated receptor g (PPARg), a member of the nuclear receptor superfamily, is also expressed in the urothelium (200,201). PPARg signaling pathways is activated by heterodimerization with RXR that then bind to peroxisome proliferator response elements (PPRE) in the promoters of target genes (202). To study the role of PPARg in urothelial differentiation, an *in vitro* model using normal human urothelial (NHU) cells was used. PPARg was found to induce expression of uroplakin protein through induction of the intermediate transcription factors *Foxa1* and *Irf-1* (203,204). PPARg-dependent signaling is also important for regulating tight junction development (205). Thus, PPARg signaling may be important for maintaining urothelial barrier function through expression of uroplakins and by regulating tight junction development.

Another important regulator of urothelial maintenance is Vitamin A. Wolbach and Howe originally reported that vitamin A deficiency induces widespread keratinizing squamous metaplasia in a number of epithelia, including esophagus, corneal and conjunctival, and the bladder epithelia (80,81,206). Recent studies by Liang et al. suggest that squamous metaplasia is accompanied with changes in keratin and uroplakin
expression (207). Interestingly, there was a sharp border between regions of keratinizing and normal epithelia. It’s presently unclear whether changes in the urothelium occur in existing cells or by expansion and replacement of invading cells. As mentioned before, RA is important in a number of systems as regulators of progenitor cell fate, including islet cells and motor neurons (Section G). Similarly, RA can also induce ES cells to differentiate along an endodermal pathway into populations of cells expressing UP but not Krt5, a marker of basal cells (208).

3. Damage-induced bladder injury models

Urothelial umbrella cells build an extraordinary barrier in the bladder that can be compromised by persistent damage by interstitial cystitis, inflammation, chronic urinary tract infection (UTI), and toxins (180,181). However, because of the unique properties of umbrella cells, epithelial cells from other organs, such as the gut and skin, are unable to reconstitute lost or damaged umbrella cells (180,181). Recent research in bladder development has focused on identifying the mechanisms involved in urothelial repair.

In the lower urinary tract, Shh is required for bladder development and is expressed in the urothelium. Shh-signaling in the bladder is thought to play a role in bladder muscle development. Recent data by Shin et al. suggests that Shh expression in the adult urothelium is restricted to basal cells (209). However, in response to bacterial (uropathogenic strain of Escherichia coli) and chemical damage (protamine sulphate), Shh-expressing cells were present in all cell layers, including the superficial cell layer, suggesting that the Shh-expressing population in the mature bladder has the capacity to regenerate umbrella cells. In response to injury, Mysorekar et al. also showed that unlike
the normal adult urothelium, which has a slow rate of proliferation, the urothelium undergoes rapid division (210). In the absence of Bmp4, a signaling molecule expressed in the bladder mesenchyme, urothelial regeneration is impaired, suggesting that signaling from subepithelial-mesenchymal compartment may be important for umbrella cell formation.

V. Dominant-negative RARaT403

RARaDN is a truncated form of human Rara that lacks the C-terminus AF-2 transactivation domain at amino acid 403, which is normally required for RA-dependent transcriptional activation. As discussed in Section II and III, NRs are closely-related structurally and functionally. In the absence of a ligand, the TR suppresses activity of a responsive promoter. Addition of thyroid hormone however, relieves this repression and stimulates transcription. The oncogenic derivative of TR, v-erbA, functions as a repressor; when it is coexpressed with TR, it blocks endogenous activation by thyroid hormone. The constitutive negative phenotype of v-erbA is due to mutations in the C-terminus of TR (211). The nuclear receptors TR, v-ErbA, hER, and the three distinct isoforms of Rars (a,b, and g), share a high degree of homology in their C-terminus LBD AF-2 region. Due to this similarity, Damm et al. developed mutant Rars that are truncated at the C-terminus AF-2 and resemble v-ErbA and unliganded TR in that they constitutively repress basal transcriptional level even in the presence of a ligand (212). Thus, dominant-negative Rars retain their ability to heterodimerize with Rxrs, and can still bind to RA, but are unable to undergo the conformational change necessary for coactivator recruitment and transcriptional activation (Fig. 1.19).
A number of studies have shown that RaraDN inhibits endogenous RA-mediated transcription of target genes and produces phenotypes similar to those observed in VAD embryos (Table 1.12) (213,214,215). Sockanathan et al. found that electroporation of the neural tube of chick embryos with RaraDN blocks specification of LMC identity (116). Blockage of retinoid signaling in the mouse skin under the keratin-14 (Krt14) promoter resulted in inhibition of mature suprabasal cells, suggesting that one of the roles of RA signaling in epidermal differentiation is to promote commitment of basal cells to keratin-1/10 (Krt1/10) suprabasal cells (215). In the pancreas, driving expression of RaraDN under the control of Pdx1 promoter results in complete agenesis of dorsal and ventral pancreas; however in Raldh2−/− mutant mice only dorsal pancreatic development is affected (121).

There is discrepancy in the literature regarding the use of dominant negative RAR. RaraDN is a potent inhibitor of RA-signaling due to the fact that it can still bind its ligand RA, can still bind to the promoter region of target genes, and can also heterodimerize with Rxr. Because Rxr is also required for heterodimerization with other steroid receptors such as PPARg, TR, and VDR for signaling, the effects of RaraDN may be through a non-canonical pathway. Specifically, sequestering Rxr may prevent transcriptional activation of other signaling pathways (216). However, in the epidermis, signal transduction via VDR or TR is not affected in RaraDN mutant embryos, suggesting that expression of RaraDN does not interfere with other Rxr-dependent signaling pathways (217).
Figure 1.1 Nuclear receptors are evolutionary conserved and share domain homology (a) Structural organization of nuclear receptors. The DBD contains two zinc finger motifs necessary for DNA binding specificity and dimerization. Region D, the hinge region, provides flexibility for DNA binding and for dimerization. Domain E contains the LBD, dimerization interface, and a ligand-dependent activation function. (b) Domains C and E are highly conserved amongst nuclear receptors. Percent amino acid homology is indicated for each region in the boxes in relation to hGR. The AF-2 activation core is the region with most conservation within the LBD. Adapted from (4,13).
Figure 1.2 Mechanisms of nuclear receptor regulation. This diagram illustrates modes of regulation by Type I and Type II nuclear receptors. (a) Ligand-binding to Type I receptors in the cytosol results in translocation to the nucleus, where the dimers bind to hormone response elements (HRE) where they activate transcription. (b) Type II receptors on the other hand, are present in the nucleus regardless of ligand binding status and bind to the nucleus as heterodimers. In the absence of a ligand the heterodimer is bound to co-repressor complexes.

* Taken from http://en.wikipedia.org/wiki/Nuclear_receptor.
### Table 1.1 Nuclear Receptor Superfamily. Summary of Type I and Type II Receptors and their ligands. Adapted from (11,12,13).

<table>
<thead>
<tr>
<th>Type I</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen</td>
</tr>
<tr>
<td>AR</td>
<td>androgen</td>
</tr>
<tr>
<td>MR</td>
<td>mineralcorticoid</td>
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</table>

<table>
<thead>
<tr>
<th>Type II</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₃R</td>
<td>thyroid</td>
</tr>
<tr>
<td>RAR</td>
<td>all-trans RA</td>
</tr>
<tr>
<td>VDR</td>
<td>1,25-(OH)₂-VD₃</td>
</tr>
<tr>
<td>PPARα</td>
<td>fatty acids</td>
</tr>
<tr>
<td>PPARγ</td>
<td>fatty acids</td>
</tr>
<tr>
<td>LXR</td>
<td>oxysterol</td>
</tr>
</tbody>
</table>
Table 1.2 Partial list of genes regulated by RA.

<table>
<thead>
<tr>
<th>Developmental processes</th>
<th>Key target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoid-related enzymes</td>
<td>-Alcohol dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>-Aldose reductase</td>
</tr>
<tr>
<td></td>
<td>-Cytochrome P450 (CYP26A1)</td>
</tr>
<tr>
<td>Nuclear receptors</td>
<td>-Retinoic acid receptors (RARα,RARβ)</td>
</tr>
<tr>
<td></td>
<td>-Vitamin D receptor (VDR)</td>
</tr>
<tr>
<td></td>
<td>-Peroxisome proliferator-activated receptor (PPARα,PPARγ)</td>
</tr>
<tr>
<td>Membrane receptors</td>
<td>-Stimulated by retinoic acid (Stra6 also known as RBP receptor)</td>
</tr>
<tr>
<td></td>
<td>-Class B scavenger receptor family of cell surface protein (CD36)</td>
</tr>
<tr>
<td>Formation of early somites</td>
<td>-Pdx1</td>
</tr>
<tr>
<td>Spinal cord motor neuron differentiation</td>
<td>-Pax6</td>
</tr>
<tr>
<td></td>
<td>-Olig2</td>
</tr>
<tr>
<td>Kidney formation</td>
<td>-Ret</td>
</tr>
<tr>
<td>Hindbrain anteroposterior patterning</td>
<td>-Hoxa1, Hoxb1, Hoxa3, Hoxd4, vHnf1</td>
</tr>
<tr>
<td>Lung induction</td>
<td>-Hoxa5, TGF-β1</td>
</tr>
</tbody>
</table>
Table 1.3 Retinoic acid (RAR) and Retinoic X (RXR) receptors.
Adapted from (13)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Major Isoforms</th>
<th>Ligand</th>
</tr>
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<tbody>
<tr>
<td>RARα</td>
<td>α1, α2</td>
<td>all-trans RA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and 9-cis RA</td>
</tr>
<tr>
<td>RARβ</td>
<td>β1, β2, β3, β4</td>
<td></td>
</tr>
<tr>
<td>RARγ</td>
<td>γ1, γ2</td>
<td></td>
</tr>
<tr>
<td>RXRα</td>
<td>α1, α2</td>
<td>9-cis RA</td>
</tr>
<tr>
<td>RXRβ</td>
<td>β1, β2</td>
<td></td>
</tr>
<tr>
<td>RXRγ</td>
<td>γ1, γ2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.3 Structure of retinoid receptors and homology with other NRs. (a) Structural organization of RAR receptor. This diagram illustrates domains of RAR receptor with high degree of sequence conservation in the DBD and LBD. Sequence homology in the AF-2 Core activation domain between NRs. (b) DNA sequences in the DBD corresponding to highly conserved region of 24 nucleotides. Adapted from (13,27).
Table 1.4 RXR heterodimeric partners.

<table>
<thead>
<tr>
<th>Retinoic acid receptor (RAR)</th>
<th>Vitamin D3 receptor (VDR)</th>
<th>Peroxisome proliferator-activated receptor (PPAR)</th>
<th>Thyroid hormone receptor (TR)</th>
<th>Farnesoid X receptor (FXR)</th>
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Figure 1.4 (a) The classical retinoid response element (RARE) is a direct repeat of the motif 5’-PuG(G/T)TCA-3’ spaced by DR1, DR2, and DR5. The response elements from target RA-genes are shown. (b) On DR2 and DR5 sites, RXR partner occupies the 5’ motif. (c) On DR1 sites, RAR/RXR heterodimers bind with reverse polarity or as RXR homodimers. * Taken from (13).
Figure 1.5 Retinoid-mediated signaling. (a) In the absence of RA, the RAR/RXR heterodimer is bound to DNA and co-repressors. Transcriptional repression is induced by through histone deacetylation. (b) Binding of a ligand, relieves transcriptional repression and induces a conformational change resulting in binding of coactivators, histone acetylation and methylation and transcription. * Taken from (65).
Table 1.5 Summary of RAR gene expression patterns in the developing organs.
Adapted from (40).

<table>
<thead>
<tr>
<th></th>
<th>Rara</th>
<th>Rarb</th>
<th>Rarg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>+ (r4 and r7, hypothalamus, hippocampus)</td>
<td>+ (r7, hypothalamus)</td>
<td>-</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>+ (ventricular neuroepithelium)</td>
<td>+</td>
<td>+ (early neural plate)</td>
</tr>
<tr>
<td>Eye</td>
<td>+ (neural retina)</td>
<td>+</td>
<td>+ (ocular/periocular mesenchyme)</td>
</tr>
<tr>
<td>Skin</td>
<td>+</td>
<td>-</td>
<td>+ (epidermis)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>+</td>
<td>+ (mesenchyme)</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>+ (proximal bronchi)</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>+ (liver capsule)</td>
<td>-</td>
</tr>
<tr>
<td>Intestine</td>
<td>+</td>
<td>+ (epithelium, outer mesenchyme)</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>+</td>
<td>+ (stroma)</td>
<td>-</td>
</tr>
</tbody>
</table>
**Figure 1.6** Regulation of retinoid signaling. Vitamin A from the diet is converted to RE and stored in the liver. *
Adapted from (65).
Table 1.6 Mouse mutants from targeted disruption of murine genes encoding proteins of the retinoid signaling pathway. Adapted from (55,56,57).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme Activity</th>
<th>Mutant phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adh1</td>
<td>Oxidation of retinol to retinaldehyde</td>
<td>Nonlethal; adults fertile; hypersensitive to retinoid toxicity; increased retinyl ester in liver</td>
</tr>
<tr>
<td>Adh3</td>
<td>Oxidation of retinol to retinaldehyde</td>
<td>Postnatal growth deficiency; adults fertile but smaller litters, hypersensitive to retinoid deficiency</td>
</tr>
<tr>
<td>Adh4</td>
<td>Oxidation of retinol to retinaldehyde</td>
<td>Nonlethal; adults fertile; reduced postnatal survival during vitamin A deficiency</td>
</tr>
<tr>
<td>Rdh1</td>
<td>Oxidation of retinol to retinaldehyde</td>
<td>Nonlethal; adults fertile; increased weight and adiposity; increased retinyl esters</td>
</tr>
<tr>
<td>Rdh10</td>
<td>Oxidation of retinol to retinaldehyde</td>
<td>Lethal at E13.0; small forelimbs; craniofacial defects; lung and pancreas agenesis; kidney hypoplasia</td>
</tr>
<tr>
<td>Raldh1</td>
<td>Oxidation of retinaldehyde to RA</td>
<td>Nonlethal; adults fertile; perioptic mesenchyme defect when Raldh3 also null; protects against obesity in adults</td>
</tr>
<tr>
<td>Raldh2</td>
<td>Oxidation of retinaldehyde to RA</td>
<td>Lethal at E9.5; hindbrain and spinal cord defects; failure in embryonic turning due to somite defect; abnormal heart; lung and pancreas agenesis; posterior hindbrain absent; posterior foregut absent</td>
</tr>
<tr>
<td>Raldh3</td>
<td>Oxidation of retinaldehyde to RA</td>
<td>Lethal at birth; blockage of nasal passages; ventral retina defect; perioptic mesenchyme defect when Raldh1 is also null</td>
</tr>
<tr>
<td>Cyp26a1</td>
<td>Oxidation of RA to RA metabolite for degradation</td>
<td>Caudal region defects (truncated with spina bifida), axial skeleton, anterior hindbrain (abnormal patterning)</td>
</tr>
<tr>
<td>Cyp26b1</td>
<td>Oxidation of RA to RA metabolite for degradation</td>
<td>Maxillary-mandibular region and palate defects; defects in limbs and male gonads</td>
</tr>
</tbody>
</table>
Table 1.7 VAD-related defects observed in RAR compound mutants. *Taken from (91).

<table>
<thead>
<tr>
<th>RAR mutant</th>
<th>Defect</th>
<th>a1b2</th>
<th>ab2</th>
<th>alg</th>
<th>a1ga2&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>ag</th>
<th>b2g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eye</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coloboma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Retrolenticular membrane</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Others; unfused eyelids, abnormal corneal sac</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Respiratory tract</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung agenesis or hypoplasia</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Heart and aortic arches</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abnormal aortic arch pattern</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ventricular septum defect</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><strong>Kidney and ureter defects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Renal hypoplasia</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hydronephrosis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Ureter agenesis or ectopia</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Genital tract abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1.8 Non-VAD-related defects observed in RAR compound mutants.
Taken from (95).

<table>
<thead>
<tr>
<th>RAR mutant</th>
<th>Defect</th>
<th>a1b2</th>
<th>ab2</th>
<th>a1g</th>
<th>a1ga2+/-</th>
<th>ag</th>
<th>b2g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Craniofacial defects</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(Agenesis, dysplasia, and ectopias)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(Agenesis, aplasia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymus, thyroid, parathyroid hypoplasia or</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>ectopias</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Limb malformations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forelimb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hindlimb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Genital tract abnormalities</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absence of anal canal</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(Agenesis, dysplasia, and ectopias)
Table 1.9 RXRa acts synergistically with RARs (VAD-related defects observed in RAR/RXR and RAR/Rxra2° compound mutants). Adapted from (95).

<table>
<thead>
<tr>
<th>Defect</th>
<th>Rar(a,b,or g)/Rxra</th>
<th>Rar/Rar (a,b, or g)</th>
<th>Rxraaf-2/Rar(a,b, or g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eye defects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortening of the ventral retina</td>
<td>Rara/Rarab; Rara/Rarag</td>
<td>Rarab/Rarg</td>
<td>Rxraaf-2/Rar(a,or g)</td>
</tr>
<tr>
<td><strong>Respiratory tract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung agenesis or hypoplasia</td>
<td>Rara</td>
<td>Rara/Rarb</td>
<td>Rxraaf-2/Rar(a,or b)</td>
</tr>
<tr>
<td><strong>Heart and aortic arches</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal aortic arch pattern</td>
<td>Rara/Rarab; Rara/Rarag; Rara/Rarg</td>
<td>Rara/Rarb; Rara/Rarg</td>
<td>Rxraaf-2/Rar(a,or b)</td>
</tr>
<tr>
<td><strong>Kidney and ureter defects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal hypoplasia</td>
<td>Rara/Rara</td>
<td>Rara/Rarb</td>
<td>Rxraaf-2/Rar(a,or g)</td>
</tr>
<tr>
<td>Agenesis of mullerian ducts</td>
<td>Rara/Rara</td>
<td>Rara/Rarb</td>
<td>Rxraaf-2/Rara</td>
</tr>
</tbody>
</table>
Table 1.10 Examples of paracrine function of retinoic acid signaling.
Taken from (54,65).

<table>
<thead>
<tr>
<th>Retinoic Acid Source</th>
<th>Retinoic Acid Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somitic mesoderm (<em>Raldh2</em>)</td>
<td>Hindbrain neuroectoderm (anteroposterior patterning)</td>
</tr>
<tr>
<td>Somitic/lateral plate mesoderm (<em>Raldh2</em>)</td>
<td>Cardiac mesoderm (anteroposterior patterning)</td>
</tr>
<tr>
<td>Somitic mesoderm (<em>Raldh2</em>)</td>
<td>Sprinal cord neuroectoderm (motor neuron formation)</td>
</tr>
<tr>
<td>Lateral plate mesoderm (<em>Raldh2</em>)</td>
<td>Foregut endoderm (pancreas and lung induction)</td>
</tr>
<tr>
<td>Mesonephros (<em>Raldh2</em>)</td>
<td>Ureteric bud growth</td>
</tr>
<tr>
<td>Optical vesicle mesenchyme (<em>Raldh2</em>)</td>
<td>Neural retina</td>
</tr>
</tbody>
</table>
Figure 1.7 Retinoids regulate many phases of motor neuron differentiation. RA signaling inhibits Fgf signaling to regulate onset of neurogenesis, and their further specification into neuronal subtypes such as lateral motor columns (LMC) and lateral LMC neurons. * Taken from (112)
Retinoids regulate many phases of pancreatic progenitor differentiation. RA signaling is required for differentiation of pancreatic progenitors into proendocrine progenitors and β-cells.

* Taken from (113)
Figure 1.9 The urinary tract. The urinary tract consists of kidneys, ureters, bladder and urethra. Nephrons in the kidney filter blood to generate urine which is propelled down a muscular tube called the ureter and stored in the bladder until ready for excretion through the urethra. * Taken from (122)
Table 1.11 Comparison of developmental stages between human and mouse species.
Taken from http://embryology.med.unsw.edu.au/OtherEmb/CStages.htm

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Days</td>
<td>20</td>
<td>22</td>
<td>24</td>
<td>28</td>
<td>30</td>
<td>33</td>
<td>36</td>
<td>40</td>
<td>42</td>
<td>44</td>
<td>48</td>
<td>52</td>
<td>54</td>
<td>55</td>
<td>58</td>
</tr>
<tr>
<td>Mouse</td>
<td>Days</td>
<td>9</td>
<td>9.5</td>
<td>10</td>
<td>10.5</td>
<td>11</td>
<td>11.5</td>
<td>12</td>
<td>12.5</td>
<td>13</td>
<td>13.5</td>
<td>14</td>
<td>14.5</td>
<td>15</td>
<td>15.5</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 1.10 Ureteric Bud Theory by Mackie & Stephens. Diagram explaining the relationship between ureteral orifice zones in bladder and urethra and points of origin from wolffian duct. Point of UB outgrowth A, E, and F are normal. If the UB sprouts more cranially on the WD, it’s final position will be more caudal in the bladder. If the UB sprouts more caudally on the WD, it’s final position will be more cranial. * Taken from (139)
**Figure 1.11** Mackie-Stephens model for ureter maturation. A schematic representation of ureter maturation and its role in trigone formation. The CND, the portion of the WD that separates the ureter from the bladder, translocates to the bladder. The new ureter orifice is established as the CND expands and differentiates into the trigone.

* Taken from (110)
Figure 1.12. The CND undergoes apoptosis as a new ureteral orifice is established. (a-f) Hoxb7-Gfp urogenital tracts. (a,b) The ureter (ur) and wolffian duct (WD) (green) join the urogenital sinus through the CND. (c,d) The CND expands laterally against the urogenital sinus. (e,f) CND is flattened in morphology and CND cells regress. Gfp+ cells are not observed in bladder (bl) trigone (dashed triangle). (g-l) Images of Hoxb7-Gfp embryos stained with laminin. (g,h) CND cells merge with urogenital sinus epithelium. (i,j,k) Ureter is separating from WD. (l) Growth of the bladder separates the ureter from the WD to a position at the base of the bladder. * Taken from (129)
The ureter orifice undergoes apoptosis within the sinus ridge

Figure 1.13. Apoptosis of the ureteral orifice is the final step in ureter maturation. (a-f) Initially the ureter is plugged against the bladder epithelium post ureter maturation, as visualized by HoxB7-Gfp reporter strain. From E15.5-E16.5, the ureter orifice undergoes apoptosis as the final ureter-bladder connection is established.

* Taken from (129)
Figure 1.14. Ret-Gdnf signaling pathway. Binding of Gdnf to Gfrα1 co-receptor activates phosphorylation of key intracellular tyrosine residues on the Ret receptor. Binding of adaptor proteins to phosphorylated Y residues initiates downstream signaling cascades such as Ras/Raf, PI3K/AKT, and MAPK, which regulate apoptosis, cell proliferation, migration, and cell adhesion. * Taken from (156)
Table 1.12. Urinary tract defects observed in VAD, RAR compound, and HoxB7Cre;RaraDN mutants.

<table>
<thead>
<tr>
<th>Defect</th>
<th>VAD</th>
<th>RAR Total</th>
<th>HoxB7Cre;RaraDN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horseshoe kidneys</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Renal aplasia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Renal hypoplasia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydronephrosis (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Ureter abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agenesis (partial or total, uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ectopia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ureteral hydronephrosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Genito-urinary tract abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminal vesicle agenesis (males)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vaginal agenesis (females)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Defect in Mullerian duct regression in males</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Persistance of Mesonephric duct in females</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Agenesis of the urinary bladder</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Defects in epithelial differentiation and/or keratinization of the urogenital sinus</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Defect in cloacal partitioning</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1.15 Current model of urinary tract development. Ret and RA signaling are required at multiple steps of urinary tract development. ND insertion plays a primary critical role at E9. UB formation at E10.5 and CND maturation occurs between E10 and E13. RA and Ret-mediated apoptosis at E11-E13 separates the ureter from the WD. Bladder growth and expansion separates the ureter from the WD to a position at the base of the bladder.

* Adopted from (127)
Intrinsic and extrinsic apoptotic pathways. (a) Intrinsic pathway of apoptosis is mitochondrial-mediated and acts through effector caspases 3, 6, and 7. (b) The extrinsic pathway is receptor-mediated and is initiated when pro-apoptotic ligands bind to death receptors. Cleavage of caspase 3 transduces apoptotic signals.

* Taken from http://www.biooncology.com/research-education/apoptosis/pathways/intrinsic/*
Figure 1.17 Bladder embryogenesis. (a) The bladder arises from an endodermal sac called the cloaca. (b) The cloaca is partitioned by the urorectal septum into the hindgut dorsally and urogenital sinus ventrally. The urogenital sinus gives rise to the bladder cranially and urethra caudally.

* Taken from (178)
Figure 1.18 Anatomy of the bladder and the urothelium (a) The bladder is made up of an epithelial layer surrounded by the serosa, detrusor muscle, muscularis mucosa, and the lamina propria (from outside to inside). (b) The bladder epithelium or urothelium contains basal, intermediate, and umbrella cells that face the bladder lumen. * Taken from (GUDMAP.org, 164)
**Figure 1.19 Dominant-negative Retinoic Acid Receptor α (RARaDN).** (a) Human RARa is truncated at the C-terminus that encodes transactivation domain. (b) RARaDN can be inserted into the Rosa26 locus, thus it’s activatable in the presence of cre-recombinase. (c) The mutant receptor is still able to bind to its ligand RA, can still heterodimerize with RXR, and can also bind to RA-response elements, thus rendering it a constitutive dominant negative receptor.

* Adopted from (212)
Chapter II

Retinoic acid signals from the bladder are crucial for proper connections between the upper and lower urinary tract and for bladder development

I. Abstract

Urinary tract formation depends on a series of events in which the ureter moves from its initial sprout site on the nephric duct (ND) to its final insertion site in the bladder. Congenital abnormalities of the urinary tract affect approximately 1% of humans and are associated with mal-positioned ureters that are irregularly joined to the bladder. Impaired vitamin A signaling, either by deleting Rars or by maternal vitamin A deficiency in mice, leads to syndromic urinary tract abnormalities similar to those seen in humans, such as renal hypoplasia, hydronephrosis, and hydroureter. Previous studies in our lab suggest that RA-dependent bladder-derived signals might play a role in regulating these processes. To test this hypothesis, we employed cell-type specific Cre recombinase to inactivate RA signaling via a dominant negative retinoic acid receptor (RaraDN) in the bladder. The observation that inactivation of RA signaling in the bladder can lead to ectopic ureters suggests that common nephric duct (CND) maturation is dependent on bladder-derived signals. Additionally, we find that retinoids play a diverse role in the same tissue by regulating bladder development.
II. Introduction

The urinary tract, including the kidneys, ureters, bladder and urethra, is crucial for the removal of toxic substances from the blood and for urine storage and excretion (126). The upper (kidney and ureter) and lower (bladder and urethra) urinary tract compartments derive from different germ layers and differentiate independently, but must be connected during development to establish a patent urinary outflow tract (125,127,128,129).

Formation of the nephric duct (ND) occurs on E9.5, prior to kidney development, and generates the initial connection between the upper and lower urinary tract compartments (131,132,160). During this process, the NDs epithelialize, extend along the anterior-posterior axis just beneath the surface ectoderm and cloacal plate, and insert into the cloaca (159,166,167,218,219). Renal development is initiated on E10.5 when the ureteric bud (UB) forms at the caudal aspect of the ND. The distal UB will form the renal collecting duct system, while the proximal UB forms the ureters (125,143). At this stage, the cloaca partitions into the hindgut and the urogenital sinus, which is the primordium to the future bladder and urethra. The ND has also established a patent connection with the urogenital sinus during this time; however, ureters are connected indirectly via the common nephric duct (CND), the posterior-most portion of the ND. Mature ureteral connections with the bladder are established during a process called ureter maturation, which occurs between E11.5 and E13.5, a time when the ureter separates from the ND and fuses with the bladder epithelium to generate a new ureteral orifice (127,129,164,165,220). Our studies suggest that this process depends on a number of events. The CND cells, which are initially organized into a tube, become flattened and elongated through cellular remodeling as they merge with the bladder epithelium and
undergo apoptosis. Insertion of the CND into the bladder, and its subsequent apoptosis, brings the ureter into contact with the bladder epithelium, where it inserts, forming a mature connection (125,162). Growth of the bladder from E13.5 to E17.5 repositions the new ureteral opening at the base of the bladder (Fig.). The ureteral orifice, which is initially plugged, becomes patent at E15 via apoptosis, prior to the onset of renal function (127).

The observation that maternal vitamin A deficiency, inactivation of RA-synthesizing enzymes and inactivation of RA-receptors generate overlapping sets of urinary tract malformations indicates that RA is the active vitamin A-derived signal that regulates urinary tract development (13,28,40,53,54,78,79,80,81,83,84,85). RA is a potent regulator of transcription that acts by binding and activating Rars, transcription factors that belong to the nuclear receptor superfamily (20). Our studies suggest that a major function of RA during urinary tract development is to regulate Ret, while Ret acts downstream of RA, regulating cellular rearrangements, proliferation, and apoptosis (161,162,163). RA and Ret are important for multiple functions in the lower urinary tract, in ND cells for migration to the cloaca, during ND-cloacal fusion at E9.5, and for CND remodeling and apoptosis. All of these processes are necessary for establishing primary and secondary connections between the upper and lower urinary tract compartments (Fig. 1.15) (129,160).

Studies from our lab alternatively suggest that ND insertion and CND remodeling also depend on unknown extrinsic signals from the bladder, another tissue that is also formed through vitamin A-dependent pathways (127,129). To test the requirement for RA-signaling in ND insertion, CND remodeling, and bladder development in these
studies, we have selectively inactivated RA-receptor signaling in the cloaca and in the urogenital sinus by using a Shh$^{Cre}$ driver to induce expression of a floxed dominant-negative RA-receptor (Rara$^{DN}$) which has been inserted into the Rosa26 locus (Fig. 1.19) (163). We find that RA-receptor signaling plays a distinct and crucial role in generating signals that regulate ND insertion into the cloacal epithelium, and CND remodeling in trans, in part through Ret. Strikingly, RA-dependent signaling from the bladder is also required for ureteral apoptosis, an end-stage step in ureter maturation that establishes the final ureter-bladder connection. Additionally, we find that RA acts independently of Ret during bladder development, where it promotes outgrowth of the urogenital sinus. Together our studies present a novel role for RA-signaling in lower urinary tract development.
III. Materials and Methods

Mouse strains and genotyping All matings were done with Swiss-Webster mice (Taconic). Embryonic day 0.5 (E0.5) was considered to be noon of the day when a vaginal plug was detected. Littermates were used for all experiments in which wild type and mutant embryos were compared. *HoxB7-Gfp* mice were obtained from F. Constantini (Columbia University) (141). *Rare-hsp68-lacZ* mice were obtained from the Rossant Laboratory (University of Toronto) (71). *RaraDN* mice were generated by the Mendelsohn lab (Columbia University) (163). The *Shh<sup>Cre</sup>* and *Shh<sup>CreERT2</sup>* mouse lines were generated in the Tabin laboratory (Harvard University) (221). The *Gli1<sup>CreERT2</sup>* line was generated by the Joyner laboratory (NYU) (222). All mice lines used in this study are listed on Table 2.1.

Genotyping was done by PCR of the tail or yolk sac. Genotyping of *HoxB7-Gfp* mice were done using 5'-AGCGCGATCACATGGTCCTG-3' and 5'-ACGATCCTGAGACTTCCACACT-3'. *Rare-hsp68-lacZ* mice were genotyped using primers 5'-CGTCGTCCCCTCAAACTGGCAGATGC-3' and 5'-TTCGGCGCTCCACAGTTTCGGGTTTTC-3' generating a 570 bp product. *RaraDN* mice were genotyped using primers for mutant band 5'-ATGGGTGTACACGTGTGCACC-3' and 5'-CACCTTCTCAATGAGCTCC-3'. For the wild type allele, we used primers 5'-TGGGCTGGTGTCAAAGAACTG-3' and 5'-TGGTCGGTAGAAAGGCAGAG-3'. Mutant and wild type bands were 210 bp and 426 bp, respectively. PCR genotyping of the *Shh<sup>Cre</sup>* mice was performed using primers for Cre: 5'-TGATGAGGTTCGCAAGAACC-3' and 5'-CCATGAGTGAACGAGCGAGG-3', generating a 400 bp product. Genotyping of *Shh<sup>CreERT2</sup>* mice were done using primers 5'-
AGGTGGACCTGATCATGGAG-3’ and 5’-ATACCGGAGATCATGCAAGC-3’. For 
\textit{Gli1}^{\text{CreERT2}} \textsuperscript{+}, we used Cre-S 5’-CAATGCTGTTTACTGTTATG-3’ and Cre-AS 5’- 
CATTGCCCTGTTTACTAC-3’. All PCR protocols were performed using a DNA 
Thermal Cycler PTC-100 (BIO-RAD, Hercules, CA, USA) with 40 cyles of 94°C for 30 
seconds, 53.5°C for 30 seconds and 72°C for 40 seconds, except for \textit{RaraDN}, where we 
performed 45 cycles of 94°C for 30 seconds, 54.5°C for 30 seconds and 72°C for 40 
seconds.

\textbf{Generation of Raldh2}\textsuperscript{-/-} embryos \textit{Raldh2} mice were obtained from K. Neiderreither 
(Baylor College of Medicine) and \textit{Raldh2}\textsuperscript{-/-} embryos were generated following 
supplementation with RA (72). Briefly, we replaced standard mouse chow with RA- 
supplemented chow (150µg per mouse, 4.5mg per kg body weight) for 24 h on E7.5. On 
E8 and E9, the dose of RA in the chow was increased to 13.3mg per kg body weight. On 
E10, we replaced RA-supplemented chow with normal standard chow until the day of 
dissection. We prepared RA-supplemented chow as follows: we mixed 50g of powdered 
food diet in water (Rodent diet 30, Picolab #5053) with the appropriate amounts of a 
stock solution of all-trans RA (5mg/ml in ethanol). The experimental design used in this 
study was approved by the institutional animal care and use committee (IACUC) at 
Columbia University. To genotype \textit{Raldh2}\textsuperscript{-/-} embryos, primers RALDH2KO-S 
5’GCCTGACCTATTGCATCTCCG-3’ and RALDH2KO-AS 
5’GCCATGTAGTGTATTGACCGATTCC-3’ were used for PCR amplification of the 
mutant allele. PCR protocol was performed using a DNA Thermal Cycler PTC-100 
(BIO-RAD, Hercules, CA, USA) with 40 cyles of 94°C for 30 seconds, 53.5°C for 30 
seconds and 72°C for 40 seconds.
**Histology, immunohistochemistry, and non-radioactive in situ hybridization**

Embryos were dissected into ice cold PBS, transferred to 4% paraformaldehyde (PFA) and then fixed overnight at 4°C on a soft shaker. Following fixation, embryos were either transferred to 70% ethanol at 4°C for paraffin sections or washed in a successive sucrose gradient (15% and 30% sucrose) overnight at 4°C for cryo sections. Embryos were embedded in paraffin or OCT. Sagittal cryo sections (10 µm) for *in situ* hybridization were cut, dried, and processed directly or stored at -80°C. Sagittal paraffin sections (5 µm) were cut and dried overnight. Hematoxylin and Eosin (HE) staining was performed according to standard procedures (161).

We carried out *in situ* hybridization analysis with digoxigenin-labeled riboprobes essentially as described (161). cDNAs were linearized and riboprobes were generated as previously described (223). The cDNA probe encoding Ret was linearized with SacII, and T3 was used to produce a 3.3-kb riboprobe (144); Raldh2 was linearized with BamHI and T7 was used to produce a 2-kb antisense riboprobe (162); Shh was linearized with EcoRI and T7 was used to produce the probe; Ptc was linearized with BamHI and transcribed with T3; Pea3 was linearized with Apal and transcribed with SP6; Gata3 was linearized with EcoIII and transcribed with T7 polymerase to generate the antisense probe (236). Briefly, slides were washed in PBS/0.05% Tween-20 (PTW), then digested for 30 min with proteinase K (10 µg/ml). After two washes in PTW, slides were pre-hybridized at 68°C for 30 min (in 50% formaldehyde, 5xSSC, 5mM EDTA pH 8, 10 µg/ml yeast RNA, 0.05% Tween-20, 0.5% CHAPS and 0.1 mg/ml heparin). Hybridization with digoxigenin-labeled riboprobes was done at 68°C overnight, followed by 3 washes in hybridization solution at 68°C, 1 wash at 68°C in 1:5:4 TBST (0.125 M NaCl, 0.003 M
KCl, 0.05% Tween-20, 0.025 M Tris, pH7.5)/hybridization buffer/H2O and subsequently at RT with 1xTBST (3 times for 10 min). Slides were preincubated in 5% heat-inactivated goat serum (HIGS), bovine serum albumin (1 mg/ml) for 30 min, then overnight at 4°C with 1:5000 dilution of anti-digoxigenin antibody (Roche). The next day, slides were washed with TBST, then with NTMT (0.1 M NaCl, 0.1 M Tris pH9.5, 0.05 M MgCl2, 0.05% Tween-20) followed by substrate application. The staining reaction progressed anywhere between 15 min to 2 h, depending on the probe, and was stopped with TBST.

For immunohistochemistry (224), cryostat sections (10 µm) were washed in 1xTBST 3 times for 10 minutes each. They were then incubated in 10% horse serum blocking solution for 2 h. We applied antibodies in 1% horse serum overnight in the dark at 4°C as specified in Table 2.2a. The following day, we washed out the primary antibody solutions with 1xTBST 3 times for 10 minutes each and applied secondary antibodies as listed in Table 2.2b for 2 h at RT. Paraffin sections (5 µm) were deparaffinized using HistoClear and rehydrated through a series of ethanol and 1xPBS washes. The procedure for immunostaining paraffin sections is similar to cryostat sections from hereon. For vibratome sections (100-150 µm), tissue was fixed for 2 h in 4% PFA, washed in 1xPBS 3 times for 10 min, and then embedded in 3% agarose for sectioning. Sections were then permeabilized with 0.3% H2O2 in cold methanol for 20 min, washed in 1xPBS/0.1% Triton X-100 for 30 min then processed for immunostaining as cryo- and paraffin sections.

Detection of apoptotic and proliferating cells: To recognize apoptotic cells during ureter maturation, we fixed E11.5, E12.5, and E13.5 embryos in 4% PFA overnight. The
following day, embryos were washed in 1xPBS and embedded for cryostat or paraffin sections. The immunostaining protocol was followed essentially as described above. For apoptosis, we stained with rabbit antibody to activated Caspase-3, and for proliferation we stained with mouse antibody to phosphohistone H3 (Table 2.2). Images were generated from each sample at low magnification (10x) and apoptotic labeled-cells in the CND were counted. For proliferation, each urogenital sinus image was divided into two parts and then cells positive for phosphohistone H3 were counted. The Student’s t-test was used for statistical analysis.

**Organ culture** The procedure for organ culture was followed as previously described (163). *HoxB7-Gfp;ShhCre;RaraDN* mutant and control E9.5 embryos were dissected into ice-cold DMEM/F12 medium then placed on Transwell Clear filters (Costar) frontally and fixed with 4% PFA for 1 hr. Samples were then washed in 1xTBST and then cultured in serum-free medium (DMEM/F12) with the following additives: 5 µg per ml insulin, 5 µg per ml transferring, 5 ng per ml selenium (Sigma), with Pen/Strep/Glu (Sigma). Embryos were incubated at 37°C in 5% CO2 atmosphere for up to 24 h. We added all-trans retinoic acid (Sigma) and 9-cis-retinoic acid (Biomole) to the culture medium to a final concentration of 200 nM. All-trans retinol (Sigma) was used at a final concentration of 1 µm/mL.

**Imaging** *HoxB7-Gfp;ShhCre;RARαDN* mutant and control E9.5 embryos were placed on Transwell Clear filters with medium. Images were taken every 30 min for 4-8 h using an environmentally controlled live imaging system. We used Zeiss Axiovert 200M equipped with microscopy top stage system set at 37°C, 5% CO2 and Hamamatsu ORKA-ER
camera. For immunohistochemistry fluorescent images, we used Zeiss Axiovert 200M with Apotome.
III. Results

RA is required for outgrowth of the bladder from the urogenital sinus and for generating bladder-derived signals that control ureter insertion. Vitamin A deficiency results in defects in ND and ND-derived tissue that are related to loss of Ret expression, and results in bladder defects that have yet to be investigated. To begin to characterize the role of RA-signaling in the bladder, we analyzed Raldh2 mutants, a mouse line in which RA-synthesis is globally inhibited (66,68,72). Histological analysis at E18 revealed that mutant bladders were abnormally smaller than controls (Fig. 2.1a,b, \( n=4 \)). Despite their small size, mutant bladders retained normal radial patterning, such that concentric layers of submucosa and muscle encircle the epithelium (Fig. 2.1b.). However, epithelium outgrowth from the urogenital sinus that normally forms the bladder appeared to be stunted. The hindgut separated from the urethra, indicating that cloacal septation was not defective. Development of the urethra, urethral plate, and genital tubercle (GT) was also similar to wild type embryos (data not shown, \( n=4 \)), suggesting that the role of RA in bladder growth is specific and is not required for formation of other cloacal-derived organs. In wild type embryos, the epithelium extends from the anterior portion of the urogenital sinus at E11.5 to give rise to the future bladder. Analysis of Raldh2 mutants at this stage revealed little, if any, detectable differences in the size or morphology of the urogenital sinus and the surrounding mesenchyme (Fig. 2.1c,d, \( n=3 \)). These observations suggest that differentiation of the mesenchymal and epithelial bladder compartments occurs after E11.5 and via distinct pathways, where RA-signaling is required specifically in the urogenital sinus epithelium during bladder formation.
To determine how Raldh2 regulates bladder formation, we investigated its distribution during development. Analysis of the distribution of Raldh2 at E9, when NDs insert into the cloaca, revealed intense expression in the mesenchyme surrounding the cloaca and the NDs (Fig. 2.2a,b). This pattern of expression persisted until E11.5 (Fig. 2.2c). After E11.5, when bladder formation is taking place, Raldh2 expression became restricted to the serosa of the bladder and subepithelial stroma, a domain that is rich in expression of signaling molecules, including Wnt, Bmp4, and Fgf, all thought to be important for differentiation of the bladder and the urothelium (Fig. 2.2d.).

**RA signaling plays a cell-autonomous role in bladder development and ureter maturation**

Since RA is diffusible, RA-generated by Raldh2 in mesenchyme may function either cell-autonomously, acting locally on Rars to generate signals necessary for epithelial differentiation, or may activate RA-receptor-dependent transcription in epithelial cells that overlie the stroma. To address this, we used the Cre-lox recombination system to selectively block RA-mediated transcription in the epithelial and mesenchymal compartments. We used cell-type specific Cre lines to drive expression of the RaraDN allele, which is inserted into the Rosa26 locus following a floxed transcriptional and translational STOP sequence (Fig. 1.19). In cells expressing Cre recombinase, the floxed STOP sequence is removed and expression of the dormant RaraDN mutant receptor is activated. Using Rare-lacZ, a transgenic RA-reporter mouse line, we found no detectable RaraDN activity in cells lacking Cre recombinase, which indicates that expression of the construct is not leaky (data not shown). Previous studies have demonstrated that RaraDN effectively blocks RA-receptor signaling in a dose-
dependent manner (163). More importantly, expression of RaraDN mutant receptor appears to affect Rar but not Rxr-dependent signaling pathways. Our lab, along with others, have observed numerous abnormalities associated with RaraDN that are similar to those reported in VAD, RA-receptor knockouts and Raldh2 null embryos, but found none that could reflect disruption of non-RA signaling pathways such as PPAR, TR, and VDR (214,215,217).

We used Shh\textsuperscript{Cre} and Shh\textsuperscript{CreERT2} lines to drive expression of RaraDN\textsuperscript{floox/+} in the cloaca, the urogenital sinus, and the bladder epithelia, and Gli1\textsuperscript{CreERT2} to block RA-signaling in the urogenital sinus subepithelial mesenchyme (123). We first verified the specificity of the Shh\textsuperscript{Cre} and Gli1\textsuperscript{CreERT2} drivers by crossing these lines with a Rosa26RmTmG reporter mouse. In this model, cells expressing Cre will express Gfp, while cells not expressing Cre will be labeled with mCherry red (225,226,227). We found that Shh\textsuperscript{Cre} was active in the cloacal endoderm and neural cord as early as E9.5, and labeled 95% of Gfp\textsuperscript{+} cells in the bladder epithelium at E14 (Fig. 2.3a,b, n=3). Unlike Shh transcript expression, which is detected in the ureters at E14, Shh\textsuperscript{Cre} activity was not detected in the ureters until after birth, indicating that Shh\textsuperscript{Cre} driver is restricted to the bladder compartment (data not shown). In contrast, induction of urothelial cells at E11 using the Shh\textsuperscript{CreERT2} line labeled only 59% of Gfp\textsuperscript{+} cells in the bladder epithelium at E14. In agreement with previous findings, Gli1\textsuperscript{CreERT2} promoter was restricted to pericloacal-mesenchyme and labeled 67% of Gfp\textsuperscript{+} mesenchymal cells in a pattern complimentary to Shh (Fig. 2.3d n=3).

To determine whether there is a requirement for RA signaling in the mesenchyme/stromal compartment, we crossed the RaraDN\textsuperscript{floox/+} mouse with Gli1\textsuperscript{CreERT2}
mice to determine whether there were bladder abnormalities similar to those observed in Raldh2 mutants. Interestingly, wholmount analysis revealed that bladder development and ureter maturation were similar in controls and in Gli1CreERT2;RaraDNflox/+ mutants, suggesting that RA-signaling is unlikely to be important in the mesenchymal compartment for bladder development (Fig. 2.4a,b, n=3).

To investigate the requirement for RA-signaling in the epithelial compartment, we analyzed ShhCre controls and ShhCre;RaraDNflox/+ E18 embryos. Whole mount analysis revealed that in comparison to control embryos, mutant embryos had smaller sized bladders, and massive bilateral hydroureters, a phenotype observed previously in VAD embryos (Fig. 2.4c,d, n=5). These observations together suggest that RA generated in sub-epithelial stroma by Raldh2 may be normally important for bladder development and/or ureter development by activating transcription of RA-receptors in urogenital sinus epithelial cells.

**RA-signaling from the cloaca and urogenital sinus regulates ND insertion and ureter maturation.** The observation that inhibition of RA-signaling in the urogenital sinus results in dilated ureters suggests that RA-signaling from the bladder may be normally important for ureter maturation. To rule out the possibility that intrinsic ureteral abnormalities such as defective muscle formation is the cause of obstruction, we first stained E18 ShhCre;RaraDNflox/+ mutants and controls with SMαA, a marker of smooth muscle, and uroplakin, a marker for ureter epithelium. Despite their dilation, this analysis revealed abundant expression of SMαA and UP in ureters of ShhCre;RaraDNflox/+ mutants at levels comparable to controls (Fig. 2.5a,b, n=3). In addition, we analyzed ureter
development prior to the stages of urine formation, which alters ureteral morphology. We found little, if any, difference in the histology of the mutant versus wild type ureter at E15 (Fig. 2.5a,b inset, n=4). Taken together, the presence of smooth muscle, urothelial markers, and normal ureteral histology at stages prior to dilation suggests that hydronephrosis in Shh\textsuperscript{Cre};Rara\textsuperscript{DNGflax/+} mutants is not due to intrinsic ureteral abnormalities.

We next examined the ureter-bladder connection in mutants to determine whether there was obstruction. Brightfield images of vibratome sections of Shh\textsuperscript{Cre};Rara\textsuperscript{DNGflax/+} control and mutant E18 embryos revealed that ureters entered the bladder, but were obstructed by a thin layer of tissue and failed to open into the bladder lumen-an indication that ureter maturation had not occurred normally (Fig. 2.5c, n=3). This abnormality is virtually identical to ureteroceles, malformations that occur in humans and result in obstruction and hydronephrosis (133,134,136).

To identify the primary cause of ureteroceles in Shh\textsuperscript{Cre}; Rara\textsuperscript{DNGflax/+} mutants, we analyzed ureter maturation at earlier developmental stages. In E15.5 control embryos, ureters were fused with the bladder epithelium, as visualized with Pax2 and p63 nuclear staining, respectively. In mutants, however, Pax2-positive CND cells were still joined to the WD and lay alongside p63-positive urothelial cells suggesting that there was a defect in CND remodeling (Fig. 2.5d,e, n=4). Analysis of embryos at earlier stages revealed that the position of the ND was also abnormal. In addition to CND defects, at E11, in comparison to control NDs, the mutant NDs were joined to the urogenital sinus epithelium at an abnormally posterior position-at the level of the hindgut, suggesting that ND insertion at E9.5 may have also occurred abnormally (Fig. 2.5f,g, n=3).
To visualize ND insertion at E9.5 *in vivo*, we took advantage of *HoxB7-Gfp* mice, a transgenic line that expresses *Gfp* in epithelia of the mesoderm derivatives including the kidneys, ureters, and WDs (141). Analysis of whole mount wild type *HoxB7-Gfp* embryos revealed, as expected, that NDs had reached the cloacal epithelium at E9.5 (*Fig. 2.5h, n=3*). However in *ShhCre;RaraDN^floxb/+;HoxB7-Gfp* littermates, NDs extended close to the cloaca but failed to join (*Fig. 2.5i, n=3*). This phenotype was present in all mutants examined. Analysis of the cloaca did not reveal any overt malformations, suggesting that abnormal signaling between the ND and the cloaca may be a cause of ectopic ureters. In agreement with this finding, we have previously observed that NDs in *Raldh2-* and *Ret* mutants also fail to join the cloaca at E9.5, which was thought to be the underlying cause of ectopic ureters and hydronephrosis. Interestingly, a number of studies in amphibians and fish suggest that ND guidance depends on exogenous signals from the cloaca. Here our data suggests that the bladder-derived signals that depend on RA may play a role in ND migration and fusion with the cloaca.

**RA-signaling in the bladder is required continuously for ND insertion and CND remodeling.** It is unclear from this data whether ND insertion, which takes place at E9.5, is the primary cause of ureter abnormalities or whether RA signaling is also required independently at later stages of ureter maturation. To test this, we inhibited RA signaling in the bladder at E11.5, when CND maturation is well underway. Expression of *RaraDN* allele at E11.5 using *Shh^CreERT2* inducible model, which targets approximately 59% of all bladder epithelial cells at E11.5, does not interfere with CND maturation. However, inhibition of RA signaling at this stage prevents degeneration of the caudal ureter, which
is normally required for establishing a patent ureter-bladder connection (Fig. 2.6a,b, \( n=3, p=0.02 \)). This novel finding suggests that RA-dependent signaling, first from the cloaca, then from the urogenital sinus and bladder, is necessary for ND-cloacal fusion, CND maturation and distal ureter canalization. No defects are observed when we inhibit RA signaling at E14 (data not shown), when bladder outgrowth and ureter insertion are complete, suggesting that the entire process of ureter insertion takes place within a precise developmental time frame and is temporally regulated by RA-signaling.

**RA-signaling from the bladder epithelium regulates CND apoptosis and Ret expression.** Our previous studies have suggested that apoptosis of the CND, which generates a new ureteral orifice, depends on signals from the urogenital sinus (127,129,164). To determine whether defects in apoptosis cause the ureterocele in \( Shh^{Cre}\backslash RaraDN^{lox/+} \) mutants, we compared ureter maturation in mutants and controls. Analysis at E11.5 and E12.5, when CND maturation is taking place, reveals intense apoptosis in the CND of wild type embryos (Fig. 2.7a,c, \( n=3, p=0.0001 \)). In mutants, however, apoptosis was significantly reduced and CND cells had failed to merge with the urogenital sinus epithelium (Fig. 2.7b,c, \( n=3, p=0.0001 \)). This suggests that apoptosis not only depends on bladder-derived signals, but also may depend on integration of CND cells with bladder epithelial cells.

We next asked which signaling pathways, including \( Shh, Ret, Fgf \), and if transcription factors \( Gata2 \) and \( Gata3 \), were disrupted in our mutants. \( Shh \) and \( Fgf \) signaling are thought to play a role in nephric lineage specification (228). Within the lower urinary tract, \( Gata2 \) and \( Gata3 \) are expressed in the ND epithelia and the cloaca
and are proposed to play a role in urinary tract development; Gata2 null mutants present defects that resemble aspects of CAKUT (congenital anomalies of the kidney and urinary tract) such as hydronephrosis and renal hypoplasia, while loss of Gata3 results in premature ND cell differentiation and loss of Ret expression (160,166,229). Analysis by in situ hybridization of wild type and mutant Shh\textsuperscript{Cre};RaraDN\textsuperscript{flox/+} E14 embryos revealed no differences in expression patterns of Shh, Gata3 or Pea3, a downstream Fgf signaling target (Fig. 2.8a,b,c,d,e,f, n=3). However, Gata2 expression was downregulated in the mutant bladder epithelia, but was unaffected in the epithelia of the WD (Fig. 2.8g,h, n=3). Interestingly, Gata2 hypomorphic mutants suffer from megaureter and hydronephrosis that is thought to be due to defects in ureter maturation (235). While there was abundant Ret expression in wild type embryonic epithelial cells at the base of the WDs, the region that intercalates with CND cells and is thought to be important for their apoptosis, expression of Ret was undetectable in Shh\textsuperscript{Cre};RaraDN\textsuperscript{flox/+} mutants, suggesting that expression of Ret in the urogenital sinus epithelia may also be important for ureter maturation (Fig. 2.8i,j, n=3). The proto-oncogene Ret is expressed in a number of sites in the urinary tract, including ND epithelia, the UB, the UB tips in the kidney, the CND, and in epithelial cells at the base of the WD (160,162,163,164). Ret signaling mediates a number of functions, including migration, proliferation and apoptosis (143,156,230). Our previous studies have suggested that expression of Ret depends on RA signaling, and that inactivation of Ret causes a range of malformations similar to those seen in RA knockout mutants (146,160,162,163,164). Loss of Ret expression in the bladder epithelia is thus a potential cause of the impaired ureter maturation and distal ureter abnormalities seen in Shh\textsuperscript{Cre};RaraDN\textsuperscript{flox/+} mutants. Interestingly, no bladder defects are observed in Ret\textsuperscript{-/-}. 
mutants (data not shown), suggesting that the sole function of Ret in the dorsal bladder epithelial cells might be for facilitating CND cell migration, rearrangements, and apoptosis. In order to test the requirement for Ret signaling in the bladder for CND and ND insertion for future studies, we would need to selectively inactivate Ret signaling in Shh-expressing cells.

RA-signaling in the urogenital sinus epithelium temporally regulates bladder development. The finding that epithelial outgrowth from the urogenital sinus is stunted in Raldh2 mutants suggests that RA is also required for bladder development. In Shh\textsuperscript{Cre}:RaraDN\textsuperscript{flox/+} E18 mutants, in which RA-signaling is inhibited at E9, bladders are abnormally smaller in size compared to control littermates. To begin to understand bladder growth abnormalities in our mutants, we first asked whether there was a defect in proliferation. To assess this, urogenital tract sections between E11.5 and E13.5 from control and Shh\textsuperscript{Cre}:RaraDN\textsuperscript{flox/+} mutant embryos were stained with anti-phospho-histone H3, which labels all cells in mitosis. In comparison to controls, there was a significant reduction in the number of proliferating bladder epithelial cells in Shh\textsuperscript{Cre}:RaraDN\textsuperscript{flox/+} mutants at E11.5, while there was no difference at E12.5 or E13.5 (Fig. 2.9, n=3, p=0.001), suggesting that like ureter maturation, RA may also temporally regulate bladder growth. Similarly, there was a significant difference in proliferating mesenchymal cells at E11.5, while there was no difference at E12.5 and E13.5 (Fig. 2.9, n=3, p=0.03).

To further understand the cause of bladder hypoplasia in Shh\textsuperscript{Cre}:RaraDN\textsuperscript{flox/+} mutants, we stained E18 control and mutant vibratome sections with UP, a marker of the
differentiated urothelium, and SMαA. Our analysis revealed abundant expression of SMαA in Shh\textsuperscript{Cre};RaraDN\textsuperscript{flo} mutants, indicating that muscle had differentiated in the bladder of the mutants. However, in comparison to wild type embryos, UP expression was down-regulated in the bladder lumen of Shh\textsuperscript{Cre};RaraDN\textsuperscript{flo} mutants, suggesting that differentiation of the specialized epithelium was abnormal (Fig. 2.10, n=3). A similar but less severe defect in urothelial differentiation was also observed when we inhibited RA signaling at E11, but not at E14, suggesting that RA is required during a limited critical time frame for bladder development and differentiation. A more detailed investigation of the role of RA-signaling in urothelial differentiation is discussed in Chapter III.

We next attempted to determine which signaling pathways, including Shh, Fgf, Ret, and if transcription factors Gata2, and Gata3, were affected in the bladder of the Shh\textsuperscript{Cre};RaraDN\textsuperscript{flo} mutants. Within the developing bladder, Shh is expressed as early as E9 in the cloaca and continues to be expressed in the adult bladder epithelia (123). Furthermore, Shh null mutants display hypoplastic bladders suggesting that Shh signaling is normally important for bladder development (123). Studies suggest that Fgf signaling also plays a role in bladder development and is upregulated during urothelial cancers; fibroblast growth factor 7 (Fgf7) is secreted by stromal cells underlying the bladder epithelium and binds to Fgf receptor 2 (Fgfr2) in the epithelium. While Gata2 and Gata3 are expressed in the bladder epithelium, their role in bladder development is unknown.

As mentioned before, no difference in expression patterns for Shh, Ptc, Pea3 and Gata3 were observed (Fig. 2.8). However, Ret and Gata2 were downregulated in the bladder epithelium (Fig. 2.8). Interestingly, no bladder defects are observed in Ret\textsuperscript{−/−} mutants, suggesting that RA does not regulate bladder development through Ret.
III. Discussion

**RA-signaling from the bladder regulates many phases of ureter insertion.** Lower urinary tract obstruction is often accompanied by renal hypoplasia or dysplasia, and single or duplicated ureters that fail to join the bladder properly, either ending blindly in the WD or joining the bladder more posterior or lateral to the normal insertion site (133,136,138,139). In duplicated UB systems, ureter connections follow a general trend called the Weigert-Meyer rule: The upper-pole kidney is often obstructed due to its distal ureter connecting at an abnormally posterior site (137). The ureter associated with the lower pole kidney tends to join the bladder properly, and as a result is unobstructed. Mackie and Stephens incorporated this idea into the Ureteric Bud Theory and proposed that the caudal-most ND segment, the CND, is incorporated into the bladder where it differentiates into the trigone, a muscular region at the base of the bladder (138). Studies from our lab support this model and suggest that ND insertion and ureter maturation occurs in close proximity with the primitive bladder, where it is hypothesized to be regulated by bi-directional signaling between the ND or CND and the bladder, and apoptotic elimination of the CND (129,164). *Ret*, whose expression in the NDs, CND and renal collecting duct system depends on RA, is important both for ND insertion and CND maturation (129,162,163,164). Here our studies suggest that proper ND insertion and ureter maturation also depends on signaling from the bladder. More importantly, our studies also suggest that RA-dependent signaling from the urogenital sinus is continuous, initially to direct ND fusion with the cloaca, for PCD of the CND and distal ureter, and for outgrowth of the bladder. While ureter maturation is dependent on Ret signaling in
the urogenital sinus epithelia, bladder formation is Ret independent. A model for lower urinary tract development based on these findings is shown in Fig. 2.11.

**RA-dependent bladder derived signals regulate PCD of the CND and distal ureter.**

In Shh\(^{Cre}\); RaraDN\(^{flox/+}\) mutants, ND and CND cells sit alongside the bladder epithelium and either fail to undergo apoptosis, or apoptosis is delayed and later compensated by non-intrinsic apoptotic pathways such as necrosis. In our studies, we used activated anti-caspase 3 antibody to measure apoptosis, which does not discriminate between intrinsic and extrinsic apoptotic pathways (Fig. 1.16). While the intrinsic apoptotic pathway is mitochondrial-mediated and acts through effector caspase-9, the extrinsic pathway is receptor-mediated and functions through caspase-8. In embryos lacking receptor protein tyrosine phosphatase (RPTP), an inducer of cell death, apoptosis of the CND through the intrinsic pathway is found to be defective (165,220,231). In support of this data, recent unpublished studies from our lab (Ekatherina Batourina) also suggests that caspase-9, but not caspase-8, is necessary and sufficient for PCD of the CND; in embryos lacking caspase-9, apoptosis of the CND is severely delayed. The close phenotypic similarities between caspase-9 null mutants and Shh\(^{Cre}\); RaraDN\(^{flox/+}\) mutants suggests that RA-signaling in the bladder is responsible for executing the intrinsic apoptotic pathway. In order to test this hypothesis, we will need to stain Shh\(^{Cre}\); RaraDN\(^{flox/+}\) mutants with caspase-9 antibody. A similar requirement for the intrinsic apoptotic pathway is also observed in the developing kidneys; inhibition of caspase-3 and caspase-9, but not caspase-8, disrupts nephrogenesis (231). Interestingly, even in the absence of caspase-3 in mice, cell death is observed, highlighting the redundancy in apoptotic pathways and
possibly explaining the residual apoptosis observed in the NDs and CNDs of $Shh^{Cre;RaraDN^{lox+}}$ mutants.

**RA-dependent Ret and Gata2 expression in urogenital sinus epithelia may be important for ND migration towards the cloaca and for rearranging CND cells.** Up until now, studies in ND insertion and ureter maturation have been primarily focused on signaling within the ND or CND, and little attention has been paid to the signals emanating from the urogenital sinus. In these studies, inhibition of RA-signaling in the cloaca and urogenital sinus leads to a persistent CND that is linked to defects in ND insertion and downregulation of Ret expression in the bladder epithelia. An interesting explanation for this abnormality is that incorporation of the ND and CND epithelial cells into the bladder may be a function of bi-directional Ret-dependent cell movement from ND and CND cells and from the cloaca and the bladder. Recent work in Ret chimeras suggests that Ret-dependent cell movement is required for UB morphogenesis (230). Our previous data suggests that ND migration towards the cloaca is dependent on RA and Ret in the ND (160); NDs fail to reach the cloaca in $Raldh2^{−/−}$ and $Ret^{−/−}$ mutants. Here, Gdnf might act as a chemoattractant guiding the trajectory of ND cells toward the cloaca via a Ret-dependent mechanism. Gdnf has been shown to serve as a chemoattractant in culture and for migrating neural crest cells. Since Ret-expressing cells are also present in the dorsal urogenital sinus, the region where NDs fuse and CND maturation takes place, it is plausible to hypothesize that these cells may direct ND and CND cells through a reverse cell migration towards the cloaca and urogenital sinus. It will be interesting to see how Ret-expressing ND and CND cells fuse with Ret-expressing cloacal and urogenital sinus
cells; and if Ret-positive distal ND and CND cells are progressively incorporated into the basement membrane where they merge with Ret-positive bladder epithelial cells and undergo apoptosis. In order to test this hypothesis in our future studies, we will selectively remove Ret expression in cloacal and bladder epithelial cells by driving expression of Ret\textsuperscript{flox/flox} under the control of Shh\textsuperscript{Cre} driver.

Recent studies in Gata2 hypomorphic mutants suggest that Gata2 is necessary for establishing patent ureter-bladder connections (235). In the urinary tract, Gata2 is expressed in a number of sites, including the UB, the ureters, the WDs, and the urogenital sinus wall that is in direct contact with the WDs (235). Our studies reveals that Gata2 is down-regulated in the urogenital sinus epithelium, suggesting that it might play a role in maturation of the CND that is in close contact with the bladder. A closer analysis of Gata2 mutants will reveal its role in ureter maturation, and conditional inactivation of Gata2 in Shh-expressing cells will help us determine whether or not Gata2 is required in the bladder for ureter maturation.

What factors contribute to bladder development? The mature bladder is derived from Shh-responsive percloacal mesenchyme and Shh-positive cloacal endoderm. Studies by Shiroyanagi et al., (2007) suggests that reciprocal epithelial-mesenchymal interactions are crucial for growth and differentiation of the bladder, and that Shh is a necessary epithelial signal for bladder growth and smooth muscle differentiation (178,179,209,233,234). Shh-signaling is dependent on patched (Ptc) receptors in the subepithelial stroma. In the absence of Shh, Ptc represses the activity of smoothened (Smo); once Shh binds Ptc, the inhibitor effect is relieved, and initiates a signal
transduction cascade that activates transcription factors Glil, Glil2, and Glil3. Glil and Glil2 are then thought to induce expression of Shh target genes such as Bmp4, which directs smooth muscle differentiation. Our studies indicate that Shh and Ptc expression are not affected in ShhCre; RaraDNflax/+ mutants, which partially explains why there are no defects in smooth muscle formation. This data also implies that Shh-signaling does not play a role in epithelial differentiation and that the defects we observe are solely attributable to inhibition of RA-signaling. Interestingly, we have not observed bladder epithelial or smooth muscle defects in Shh+/− embryos (Chapter III), which is in contrast to what has been previously observed in the literature (123).

In ShhCre, RaraDNflax/+ mutants, there is a defect in outgrowth of the epithelium from the urogenital sinus and a defect in proliferation. Studies suggest that Gata2 is necessary for migration of neuronal precursor cells (232). The observation that Gata2 is downregulated in the bladder epithelia but not in the WD suggests that RA might regulate bladder proliferation through Gata2. Thus, it will be interesting to see if Gata2 is involved in migration of bladder epithelial cells. Recent data suggests that Gata2 hypomorphic mutants result in uropathies resembling human CAKUT such as hydronephrosis and defects in bladder morphogenesis (235). Thus, analysis of bladders from Gata2 hypomorphic embryos might reveal a functional role for Gata2 in bladder development through RA-Gata2 pathway. However, because there are Rare sites in the Gata2 promoter, it is also possible that the absence of Gata2 might simply reflect disruption of RA-signaling with no functional consequence.
RA signaling from the bladder is required for many stages of lower urinary tract development. These studies suggest that RA signaling from the bladder is required for multiple developmental processes in the lower urinary tract, including ND insertion, CND maturation, and bladder development (Fig. 2.11). While ND insertion and CND maturation may be Ret and Gata2-dependent, bladder growth from the urogenital sinus is Ret-independent. Importantly, inhibition of RA-signaling in the cloaca and bladder by expressing RaraDN recapitulates abnormalities observed in VAD and Rar compound mutants, such as ND, CND, and bladder defects (Table 2.3).
Table 2.1 Summary of mouse strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shh&lt;sup&gt;Cm&lt;/sup&gt;</td>
<td>Targeted knock-in mouse strain. (221)</td>
</tr>
<tr>
<td>Shh&lt;sup&gt;CmERT2&lt;/sup&gt;</td>
<td>Tamoxifen-inducible targeted knock-in mouse strain. (221)</td>
</tr>
<tr>
<td>Gli1&lt;sup&gt;CreERT2&lt;/sup&gt;</td>
<td>Tamoxifen-inducible targeted knock-in mouse strain. (222)</td>
</tr>
<tr>
<td>Rare-LacZ</td>
<td>Transgenic mouse strain in which 3 copies of RARE present in Rarb2 gene is fused to hsp promoter and hspLacZ. (71)</td>
</tr>
<tr>
<td>mT/mG</td>
<td>Double-fluorescent reporter that expresses membrane-targeted dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted GFP (mG) after excision. (225)</td>
</tr>
<tr>
<td>Rarat403 (RaraDN)</td>
<td>Transgenic mouse strain in which truncated human Rara receptor is inserted into the Rosa26 locus and is activatable in the presence of Cre-recombinase. (163)</td>
</tr>
<tr>
<td>HoxB7-Gfp</td>
<td>Transgenic mouse strain that selectively expresses Gfp in all ND derivatives (under the control of HoxB7 promoter). (141)</td>
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</table>
Table 2.2a. Primary antibodies used in this study.

<table>
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<th>Antigen</th>
<th>Supplier</th>
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<th>Dilution</th>
<th>Method</th>
</tr>
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<tr>
<td>p63</td>
<td>Santa Cruz Biotechnology</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>Paraffin</td>
</tr>
<tr>
<td>p63</td>
<td>Santa Cruz Biotechnology</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Cryosection</td>
</tr>
<tr>
<td>Cytokeratin 18</td>
<td>Abcam Ab59248</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Keratin 5</td>
<td>Covance Keratin 5 (AF 138): PRB-160P</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Keratin 10</td>
<td>Covance Keratin 10 PRB-159P</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Cryosection</td>
</tr>
<tr>
<td>Uroplakin Total</td>
<td>Gift from Dr. T. T. Sun at NYU Medical Center</td>
<td>Rabbit IgG</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Smooth Muscle Anti-Actin-Cy3</td>
<td>Sigma Life Science C6198</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>Paraffin, Cryosection</td>
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<tr>
<td>Beta-galactosidase</td>
<td>Biogenesis 4600-1409</td>
<td>Goat IgG</td>
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<td>Paraffin, Cryosection</td>
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<tr>
<td>BrdU</td>
<td>Abcam Ab6326</td>
<td>Rat IgG</td>
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<td>E-Cadherin</td>
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<td>Laminin</td>
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<td>P-Histone H3 (S10)</td>
<td>Cell Signaling 9701L</td>
<td>Rabbit IgG</td>
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<tr>
<td>P-Histone H3 (S10) 6G3</td>
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<td>Paraffin</td>
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<td>Invitrogen 716000</td>
<td>Rabbit IgG</td>
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<td>Paraffin</td>
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<tr>
<td>Activated Caspase 3</td>
<td>Promega G748A</td>
<td>Rabbit IgG</td>
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<td>Paraffin, Cryosection</td>
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Table 2.2b. Secondary antibodies used in this study.

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<th>Supplier</th>
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<td>Alexafluor 488 donkey anti-goat</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexafluor 488 donkey anti-rabbit</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-rabbit</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-mouse</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexafluor 488 goat anti-mouse</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-rat</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-goat</td>
<td>Invitrogen</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cy5-conjugated donkey anti-mouse</td>
<td>Jackson Immunoresearch</td>
<td>IgG (H+L)</td>
<td>1:500</td>
</tr>
<tr>
<td>Cy5-conjugated donkey anti-goat</td>
<td>Jackson Immunoresearch</td>
<td>IgG (H+L)</td>
<td>1:500</td>
</tr>
<tr>
<td>Cy5-conjugated donkey anti-rabbit</td>
<td>Jackson Immunoresearch</td>
<td>IgG (H+L)</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Figure 2.1 *Raldh2* is necessary for bladder development. (a,b) Epithelial outgrowth is stunted in *Raldh2* mutants (arrows). (c,d) Bladder defects are not observed at E11.5 (arrowheads) (c,d).

(bl, bladder; urth, urethra; hg, hindgut)
Figure 2.2 *Raldh2* is expressed in the ND, CND, and cloaca, urogenital sinus, and bladder. (a,b) Whole mount wild type E9 embryos were probed with *Raldh2* for *in situ* hybridization analysis. Frontal and saggital views of *Raldh2* expression. (c) At E12, *Raldh2* expression remains restricted to mesenchyme surrounding the urogenital sinus, ureters and WD. (d) At later stages, expression of *Raldh2* is restricted to subepithelial mesenchyme and mesenchyme surrounding the WD (arrows).

(nd, nephric duct; cl, cloaca; ugs, urogenital sinus; wd, wolffian duct)
Figure 2.3 Shh\textsuperscript{Cre}, Shh\textsuperscript{CreERT2} and Gli1\textsuperscript{CreERT2} reporter activity using mTmG reporter (a,b) Shh reporter activity in E9 and E14 embryos. Cre is active in cloaca at E9 and E14. (c) Shh\textsuperscript{CreERT2} reporter activity at E14, induced with tamoxifen at E11. (d) Gli1\textsuperscript{CreERT2} reporter activity is complimentary to Shh. (e) Percent of Gfp+ cells in reporter mice strains.

(cl, cloaca; nc, neural cord; bl, bladder)
Figure 2.4 RA-signaling in bladder epithelium but not mesenchyme is necessary for lower urinary tract development. (a,b,c) In comparison to wild type urinary tracts, Shh<sup>Cre</sup>;RaraDN<sup>floxed+/+</sup> mutants have dilated ureters and abnormally small bladder at E16. In contrast, no uropathies are observed in urogenital tracts from Gli1<sup>Cre</sup>;RaraDN<sup>floxed+/+</sup> embryos. Note patent ureter-bladder connections in wild type and Gli1<sup>Cre</sup>;RaraDN<sup>floxed+/+</sup> UGTs (arrowhead).

(ur, ureter; ut, uterus; bl, bladder; ki, kidney; ad, adrenal gland)
Figure 2.5 RA-dependent signaling from the cloaca regulates ureter maturation and ND insertion (a,b) Up and Sma expression in wild type and $Shh^{Cre}\text{;}RaraDN^{\text{floxed}}/+$ mutant ureters. (c) Ureters in $Shh^{Cre}\text{;}RaraDN^{\text{floxed}}/+$ mutants are obstructed by thin layer of tissue. (d,e,f,g) Analysis at E15 reveals a persistent CND. (f,g) Analysis at E11 reveals that the ND is inserted at an abnormally posterior position in comparison to wild type NDs. (h,i) At E9.5, NDs are a distant away from the cloaca in $Shh^{Cre}\text{;}RaraDN^{\text{floxed}}/+$ mutants.

(ur, ureter; wd, wolffian duct; bl, bladder; cnd, common nephric duct; nd, nephric duct; cl, cloaca)
Figure 2.6 RA-dependent bladder derived signals are also necessary for ureteral apoptosis. (a,b) H/E staining of E15 urinary tracts from wild type and Shh<sup>CreERT2;Rara<sup>DN<sub>flox/+</sub></sup></sup> embryos. (c) K5 p63 staining showing ureterocele in Shh<sup>CreERT2;Rara<sup>DN<sub>flox/+</sub></sup></sup> embryo. (d,e) Ureter in wild type embryos have joined the embryo and are separated from the WD. A piece of tissue separates ureters from the bladder in Shh<sup>CreERT2;Rara<sup>DN<sub>flox/+</sub></sup></sup> mutants (arrowhead).
Figure 2.7 RA-derived UGS signals are necessary to drive CND maturation. (a,b) Number of apoptotic cells in CND of Shh^{Cre};RaraDN^{lox/+} mutants is significantly lower in comparison to controls. (c) Percent of active caspase-3+ cells in wild type and mutant embryos.

(ur, ureter; ugs, urogenital sinus; wd, wolffian duct; cnd, common nephric duct; nd, nephric duct; cl, cloaca)
Figure 2.8 RA-signaling from the bladder regulates Gata2 and Ret expression. (a,b,c,d,e,f) Shh, Gata3, and Pea3 expression is not altered in Shh\textsuperscript{Cre};Rara\textsuperscript{DN}\textsuperscript{lox/lox} mutant bladders and WDs and ureters in comparison to controls. (g,h) Gata2 is down-regulated in Shh\textsuperscript{Cre};Rara\textsuperscript{DN}\textsuperscript{lox/lox} mutant bladders but is still expressed in the WD. (i,j) Ret expression is down-regulated in Shh\textsuperscript{Cre};Rara\textsuperscript{DN}\textsuperscript{lox/lox} mutant bladders but not in the WD.

(ur, ureter; wd, wolffian duct; bl, bladder)
Figure 2.9 RA temporally regulates bladder growth from the UGS. (a,b,c,d) Number of H2A+ proliferating cells is significantly less in Shh\textsuperscript{Cre;RaraDN\textsuperscript{flox+/+}} mutant ugs epithelia and mesenchyme at E11.5 than at E12.5 and E13.5.

**C** Percent of Proliferating Cells in UGS Epithelial Compartment

**D** Percent of Proliferating Cells in UGS Mesenchymal Compartment
Figure 2.10 Retinoids regulate urothelial differentiation. (a,b) Upk expression is down-regulated in Shh<sup>Cre;RaraDN<sub>flox/+</sub></sup> mutant bladder lumen (arrowheads) but not in the ureters. SMaA expression is similar to controls.

(ur, ureter; bl, bladder)
Figure 2.11 New model of urinary tract development. Ureter insertion and maturation depends on bidirectional signaling from the ND and ureters and from the cloaca. RA signaling first from the cloaca then from the urogenital sinus and bladder are required at multiple steps of urinary tract development. ND insertion plays a primary critical role at E9.5 and its insertion is dependent on RA-derived signals from the cloaca. RA and Ret-mediated apoptosis from the bladder at E11-E13 separates the ureter from the WD. Bladder growth and expansion is dependent on RA. * Modified from (127)
### Table 2.3. Urinary tract defects observed in VAD, RAR compound, HoxB7Cre;RaraDN, and ShhCre;RaraDN mutants.

<table>
<thead>
<tr>
<th>Defect</th>
<th>VAD</th>
<th>RAR Total</th>
<th>HoxB7Cre;RaraDN</th>
<th>ShhCre;RaraDN</th>
</tr>
</thead>
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<tr>
<td><strong>Kidney abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
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<td>Horseshoe kidneys</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<td>Renal aplasia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Renal hypoplasia (uni- or bilateral)</td>
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<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Hydronephrosis (uni- or bilateral)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Ureter abnormalities</strong></td>
<td></td>
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<tr>
<td>Agenesis (partial or total, uni- or bilateral)</td>
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<td>Defect in Mullerian duct regression in males</td>
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<td>Persistance of Mesonephric duct in females</td>
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<tr>
<td>Agenesis of the urinary bladder</td>
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<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Defects in epithelial differentiation and/or keratinization of the urogenital sinus</td>
<td>+</td>
<td>-</td>
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</table>
Chapter III

Retinoid-dependent regulation of urothelial progenitors is crucial for urothelial differentiation

I. Abstract

The urothelium is a stratified epithelium that provides a crucial barrier between the urinary tract and blood. It contains basal, intermediate, and superficial umbrella cells that are specialized for synthesis and trafficking of uroplakins, a family of secreted proteins that assemble into a protective crystalline plaque that lines the apical surface of the urothelium (183). Umbrella cells have an extremely long half-life (6 months to a 1 year) but are prone to damage by inflammation, disease, chemicals, or urinary tract infections (UTIs) (180,181,183,209,210,237). Recent studies suggest that the Shh-expressing population in the adult bladder contains progenitors that can repopulate the urothelium post-damage (209). However, little is known about the progenitors that are normally important for urothelial differentiation and the subpopulations that can give rise to bladder tumors, including papillary tumors, carcinoma in situ, and squamous carcinoma, which differ in cellularity (238,239,240). Here we take advantage of a dominant-negative retinoic acid (RA) receptor (RaraDN) mouse line that enables us to generate global inactivation of RA-signaling in cells expressing the Cre-recombinase (163,212). We find that inhibition of RA-dependent signaling in Shh-expressing progenitors blocks urothelial differentiation and regeneration. Furthermore, our studies reveal that in the absence of RA-signaling, Shh-progenitors differentiate into squamous epithelia instead of forming intermediate and umbrella cells. Using an indelible lineage analysis technique and marker analysis, we also find that umbrella cells are established early in development and that
K5+-basal cells are not progenitors of intermediate and umbrella cells. Interestingly, we observe a robust requirement for RA-signaling at stages prior to urothelial stratification, when inhibition of RA signaling leads to down-regulation of uroplakin and loss of the intermediate cell population, which are likely to be umbrella cell precursors. Analysis of urothelial regeneration in $Shh^{CreERTmTmG;RaraDN}$ mutants reveals a dramatic decrease in umbrella cell formation, suggesting that RA-signaling is also critical for urothelial regeneration after damage. Our findings suggest that, in the urothelium, RA temporally regulates urothelial differentiation by determining the fate of $Shh$-progenitors.
II. Introduction

The function of the bladder in mammals is to collect and store urine until it can be excreted through the urethra. The urothelium, the mucosa that extends the urinary tract from the renal pelvis to the proximal urethra, is a unique epithelial barrier that prevents water, ions, and nitrogenous wastes from passing between urine and blood (183). The urothelium contains basal, intermediate and superficial cells that are more commonly referred to as umbrella cells. Unlike basal or intermediate cells, umbrella cells have characteristic features including high-resistance tight-junctions and apical plaque units composed of uroplakins that create a water-resistant barrier along the urinary outflow tract. Umbrella cells are specialized for the synthesis and delivery of uroplakins and contain a specialized vesicle system (fusiform vesicles) that transport uroplakins to and from the apical surface of umbrella cells, enabling them to adjust their surface area in response to stretch (183,184,187,237). They are large, polyploid cells that cover as many as twenty intermediate cells in a filled bladder state.

The urothelium is slow cycling epithelia with a turnover rate of 6 months to a year; however it is susceptible to damage by chronic disease such as interstitial cystitis, urinary tract infections (UTIs) or cancer (237). Due to their unique properties, studies have shown that umbrella cells cannot be replaced by cell-grafting with epithelial cells from other organs, such as the gut (237,238,239); therefore, methods to grow urothelial cells in vitro and to regenerate the urothelium in vivo have been the focus of recent studies. Identifying progenitors and genetic pathways that are normally important for umbrella cell formation will be significant for regeneration studies, and may also pave the way for studies aimed at identifying cells that can give rise to urothelial tumors.
RA, the active derivative of vitamin A, is required for formation of most cells and tissues in the embryo and for maintenance of vision, fertility, and specialized epithelia in adults (54). Wolback and Howe originally reported the requirement for vitamin A in preventing keratinization of a number of epithelia, including: the esophagus, cervix, and the bladder (80,81,206). In animals bred on RA-deficient diet, the bladder epithelium is replaced with cornifying squamous epithelium that resembles skin, suggesting that RA may be normally important for steady state maintenance of the urothelium. Studies by T.T. Sun’s group suggest that in the absence of vitamin A, uroplakin expression is down-regulated and squamous markers are upregulated, suggesting that either the urothelium has undergone replacement by neighboring invading cells or undergone transdifferentiation (207). In vitro, RA can induce differentiation of endodermal ES cells into two populations of cells that express urothelial markers typical of intermediate and umbrella cells, suggesting that RA may also be important for formation of urothelial cells during development (208).

RA-dependent transcription is induced when RA binds to retinoic acid receptors (Rars), transcription factors that belong to the nuclear receptor (NR) superfamily. Rars bind to retinoic acid response elements (Rare) located in regulatory sequences of target genes with Rxrs, a second family of nuclear receptors (53). In the absence of RA, Rar/Rxr heterodimers are constitutively bound to DNA with co-repressor complexes. Binding of RA to the Rar partner releases corepressor complexes and recruits coactivator complexes (10). RA can also regulate transcription indirectly, by activating expression of primary transcription factors, or by interacting with factors that induce epigenetic changes such as chromatin modification (2).
Recent lineage analysis in adult mice reveals that the Shh-expressing urothelial progenitors can contribute to umbrella cell formation during regeneration (209). Here we report that RA-receptor-dependent signaling temporally regulates Shh-expressing urothelial progenitors, and is required for formation of intermediate and umbrella cells, and their regeneration after damage. Interestingly, in the absence of RA-signaling, Shh-progenitors undergo a fate change, down-regulating uroplakins and up-regulating squamous markers, suggesting that RA is normally important in Shh-progenitors for negatively suppressing squamous differentiation or positively regulating urothelial differentiation. We also find that the differentiation potential of the Shh-population changes with time, as does the sensitivity to RA-inhibition. Shh-progenitors generate large numbers of labeled daughters when TM induction is at E11.5, prior to stratification, and RA-dominant negative expression dramatically reduces umbrella cell and intermediate cell formation. Differentiation potential and sensitivity to RA-inhibition decreases after E14.5, as basal cells, the population that has been suggested to be an urothelial stem cell during development and in adults begins to form. Direct examination of the differentiation potential of basal cells using K5\textsuperscript{CreERT2} reporter, reveals that basal cells are unipotent and are able to generate basal cells but rarely, if ever, intermediate or umbrella cells-during development and after damage-induced regeneration. We conclude that the formation of umbrella cell precursors, which are established during early development, depends on RA-mediated signaling.
III. Materials and Methods

**Mouse strains and genotyping** All matings were done with Swiss-Webster mice (Taconic). Embryonic day 0.5 (E0.5) was considered to be noon of the day when a vaginal plug was detected. Littermates were used for all experiments in which wild type and mutant embryos were compared. *Rare-hsp68-lacZ* mice were generated by the Rossant Laboratory (University of Toronto) (71). *RaraDN* mice were generated by the Mendelsohn lab (Columbia University) (163). The *Shh*<sup>GfpCre</sup> and *Shh*<sup>CreERT2</sup> mouse lines were generated in the Tabin laboratory (Harvard University) (221). The *Gli1*<sup>CreERT2</sup> line was generated by the Joyner laboratory (NYU) (222). The *Krt5*<sup>CreERT2</sup> line was generated in the Metzger laboratory (Institut de Genetique et de Biologie Moleculaire et Cellulaire, College de France) (241). The *UP2*<sup>Cre</sup> line was generated by Wu Xue-Ru in T.T. Sun laboratory (NYU Medical Center) (242), and the *UP2*<sup>CreERT2</sup> line was generated in C.C. Cordon laboratory (Mount Sinai Medical Center). The *UP2*<sup>CFP</sup> line was generated by the Mendelsohn lab (Columbia University), and *Shh*<sup>LacZ</sup> line was generated by A. Kottmann and provided by T. M. Jessell (Columbia University) (243). The *T83LacZ* mouse line was generated in the Metzger laboratory (Institut de Genetique et de Biologie Moleculaire et Cellulaire, College de France) (241). All mice lines used in this study are listed on Table 3.1.

Genotyping was assessed by PCR of the tail or yolk sac. *Rare-hsp68-lacZ* mice were genotyped using primers 5’-CGTCGTCCTCCCTCAAAACTGGCAGATGC-3’ and 5’-TTCGGCGCTCCACAGTTTCGGGTTTTC-3’ generating a 570 bp product. *RaraDN* mice were genotyped using primers 5’-ATGGTGTCACGCAGTGCACC-3’ and 5’-CACCTTCTCAATGAGCTCC-3’. For the wild type allele, we used primers 5’-
TGGCTCGTGTCAGGAGACTG-3’ and 5’-TGGTCGGTAGAAAGGCAGAG-3’ to generate a 210 bp mutant and a 426 bp wild type band. PCR genotyping of Shh\textsuperscript{Cre} and \textit{UP2}\textsuperscript{Cre} mice was performed using primers for Cre: 5’-TGATGAGGTTCGCAAGAACC-3’ and 5’-CCATGAGTAGACGAACCTGG-3’, generating a 400 bp product. Genotyping of \textit{Shh}\textsuperscript{Cre\textsubscript{ERT2}} mice was done using primers 5’-AGGTGGACCTGATCATGGAG-3’ and 5’-ATACCGGAGATCATGCAAGC-3’. For \textit{Gli1}\textsuperscript{Cre\textsubscript{ERT2}} and \textit{UP2}\textsuperscript{Cre\textsubscript{ERT2}} mice, we used Cre-S 5’-CAATGCTGTTCCTGTTATG-3’ and Cre-AS 5’-CATTGCCCCTGTTCGCAAGC-3’. Genotyping of \textit{Shh}\textsuperscript{Cre\textsubscript{ERT2}} mice was done using primers 5’-AGGTGGACCTGATCATGGAG-3’ and 5’-ATACCGGAGATCATGCAAGC-3’. For \textit{Gli1}\textsuperscript{Cre\textsubscript{ERT2}} and \textit{UP2}\textsuperscript{Cre\textsubscript{ERT2}} mice, we used Cre-S 5’-CAATGCTGTTCCTGTTATG-3’ and Cre-AS 5’-CATTGCCCCTGTTCGCAAGC-3’. Genotyping of \textit{Krt5}\textsuperscript{Cre\textsubscript{ERT2}} mice was done using primers TK139 (sens) 5’ ATTTGCTGTCACTGACCGTC 3’ and TK141 (reverse): 5’ ATCAACGCCTGTTTTCGGA 3’ to generate a 350 bp product. Genotyping of \textit{ShhLacZ} mice was done using primers 5’. For \textit{RareLacZ}, we used primers 5’ CGTCGTGCCCTCAAGACTGCGATGC 3’ and 3’ TTTCCAGGGTCAAGGAAGG 5’. For genotyping \textit{T83LacZ} mice, we used primers 5’ GGAGTTTGTGACAGCAGG-3’ and 3’ CCTTCCAGGGTCAAGGAAGG 5’. For genotyping \textit{UP2Cfp} mice, we used primers 5’ CACTCCGAGCAGAATCAGCTACC 3’ and 3’ CGTGCTCTTGGAAGATGGT 5’.

All PCR protocols were performed using a DNA Thermal Cycler PTC-100 (BIO-RAD, Hercules, CA, USA) with 40 cyles of 94°C for 30 seconds, 53.5°C for 30 seconds and 72°C for 40 seconds, except for \textit{RaraDN}, for which we performed 45 cycles of 94°C for 30 seconds, 54.5°C for 30 seconds and 72°C for 40 seconds.
**Histology, immunohistochemistry, and non-radioactive in situ hybridization**

Embryos were dissected into ice cold PBS, transferred to 4% paraformaldehyde (PFA) and then fixed overnight at 4°C on a soft shaker. Following fixation, embryos were either transferred to 70% ethanol at 4°C for paraffin sections or washed in a successive sucrose gradient (15% and 30% sucrose) overnight at 4°C for cryosections. Embryos were embedded in paraffin or OCT. Sagittal cryosections (10 µm) were cut, dried, and processed directly or stored at -80°C. Sagittal paraffin sections (5 µm) were cut and dried overnight. Hematoxylin and Eosin (HE) staining was performed according to standard procedures (161).

For immunohistochemistry (224), cryostat sections (10 µm) were washed in 1xTBST three times for 10 minutes each. They were then incubated in 10% horse serum blocking solution for 2 hours. We applied antibodies in 1% horse serum overnight in the dark at 4°C as specified in **Table 3.2a**. The following day, we washed out the primary antibody solutions with 1xTBST three times for 10 minutes each and applied secondary antibodies as listed in **Table 3.2b** for 2 hours at RT. Paraffin sections (5 µm) were de-paraffinized using HistoClear and rehydrated through a series of ethanol and 1xPBS washes. The procedure for immunostaining paraffin sections is similar to cryostat sections from hereon. For vibratome sections (100-150 µm), tissue was fixed for 2 hours in 4% PFA, washed in 1xPBS three times for 10 min, and then embedded in 3% agarose for sectioning. Sections were then permeabilized with 0.3% H₂O₂ in cold methanol for 20 min, washed in 1xPBS/0.1% Triton X-100 for 30 min then processed for immunostaining as cryo- and paraffin sections.
**Imaging** Fluorescent images were collected using a Zeiss Axiovert 200M with Apotome microscope. All images were processed and collected identically, with focus on the urogenital sinus at early ages, and bladder at later embryonic stages.
IV. Results

RA is required for urothelial differentiation. Data from ES and RA-deficiency studies suggest that RA is likely to be important for urothelial differentiation and for its maintenance in adults (80,81,206,208). To determine whether retinoid signaling plays a cell-autonomous role in Shh-expressing cells, a population that in adults, contains progenitors that can regenerate the urothelium after damage, Shh<sup>Cre</sup> mice (221) were crossed with Rara<sup>DN</sup> mice, a line that harbors a dormant dominant-negative RA-receptor in R26R locus. In Cre-expressing cells, the floxed STOP sequence is excised and the RaraDN mutant receptor is expressed, generating a global blockade of RA-receptor signaling in cells expressing Cre. Studies from our lab and others have demonstrated that the RaraDN used in this study is specific and efficiently blocks RA-signaling in adult and embryonic tissues in a dose-dependent manner (163). Importantly, RaraDN generates a large number of phenotypes that are present in RA-deficiency models, strongly suggesting that the defects observed are due to retinoid-specific repression and not due to non-specific action from other nuclear receptor pathways (Table 3.3) (80,81,83,84,93,96,160,163,162).

Analysis of E18 vibratome sections of wild type and mutant UGT sections stained with UP, a marker of the urothelium, and SMaA, a marker of smooth muscle, reveals that smooth muscle had differentiated normally. However, while there was robust UP expression in the bladder lumen of wild type UGTs, there was little or no detectable UP expression in the lumen of Shh<sup>Cre</sup>;RaraDN<sup>fl</sup> mutants (n=0/3), suggesting that either RA is important for expression of UP, or that RA is required for differentiation of UP-expressing umbrella cells (Fig. 3.1a,b).
To address this, we stained bladders from E18 control and Shh$^{Cre}; Rara^{DN\text{floxed}+}$ mutants with markers of umbrella cells including UP, K20, and K18, and with markers for basal and intermediate cells including keratin-5 (K5), a protein present in basal cells of most epithelia such as the skin, and p63, a marker of epithelial cells (Fig. 3.2a,c,e). Analysis of wild type and mutant urothelia for basal cell markers revealed the presence of basal cells that were K5 and p63-positive (Fig. 3.2a,b $n=5/5$). On the other hand, we did not detect markers of intermediate or umbrella cells (Fig. 3.2c,d,e,f $n=5/5$), suggesting that RA signaling is necessary for intermediate and umbrella cell formation. A summary of urothelial populations between wild types and Shh$^{Cre}; Rara^{DN\text{floxed}+}$ mutants is listed in Table 3.4.

To directly confirm the absence of umbrella cells E18 Shh$^{Cre}; Rara^{DN\text{floxed}+}$ mutants, we also performed transmission and scanning electron microscopy in collaboration with T.T. Sun’s laboratory (NYU Medical Center). Unlike controls, mutant superficial cells lacked characteristic plaques on their apical surface and did not contain fusiform vesicles, another prominent feature of mature umbrella cells and consistent with loss of UP expression (Fig. 3.2g,h, $n=3/3$). Instead, the apical surface was replaced with microvilli that are normally not present on umbrella cells.

**The urothelium is composed of 4 distinct cell types, 3 of which express Shh.** The urothelium forms from endoderm, cells that initially express Shh and p63. Stratification occurs on E14.5-E15.5, prior to the onset of renal function. However, it is unclear when Shh-progenitors normally differentiate into basal, intermediate and umbrella cells. To begin to address this, we examined the composition of Shh-population over time. Since Shh is a secreted protein, we used Shh$^{\text{LacZ}}$ and Shh$^{\text{GfpCre}}$ knock-in mice to identify Shh-
expressing cells during development and in the adult bladder. \( Shh^{Gfp\text{Cre}} \) mice harbor a targeted mutation in which \( Gfp\text{Cre} \) cassette is inserted into the endogenous \( Shh \) locus resulting in the production of Gfp in cells that normally express \( Shh \) mRNA. We validated the specificity of these reporter alleles by also looking at expression of \( Shh \) during different developmental times points (see below, Fig. 3.4d,e,f,g) Because uroplakin is also secreted, we used an \( UP2^{Gfp} \) transgenic mouse, which label cells with active \( UP2 \) promoter with Cfp, to identify uroplakin-expressing cells. Surprisingly, we find that the Shh population contains 3 distinct cell types that change over time; urothelial-progenitors, basal cells and intermediate cells, whose relative numbers change over time. At E11.5, most urogenital sinus cells are marked by \( Shh^{Gfp} \) and p63, which we have defined as an undifferentiated urothelial (U-O) progenitor (Fig. 3.3a). At E12.5, 32% of urothelial cells \((n=650)\) display uroplakin (UP), Shh, and p63 expression, and are likely to be intermediate cells (Fig. 3.3e). We did not observe cells expressing basal cell markers, but did observe about 3% of cells that were not p63-positive \((UP^+/p63^-)\) that we call immature umbrella cells (Fig. 3.3e). At E13.5, however, the number of intermediate cells begins to increase and comprises 85% of total epithelial cells \((n=1802)\), while immature umbrella cells constitute about 6.13% of the epithelial population (Fig. 3.3f). At this stage, K5+-basal cells have not formed; however, a small percentage of U-O progenitor cells are still present \((8.5\%, n=1802 \text{ total } \# \text{ urothelial cells})\) (Fig. 3.3f). Stratification begins on E14.5 as the first few double-labeled K5+ and p63+-basal cells are detected \((0.39\%, n=1345 \text{ total } \# \text{ urothelial cells})\), while the majority of urothelial cells are either intermediate \((69.1\%, n=1345 \text{ total } \# \text{ urothelial cells})\) and immature umbrella cells \((29.44\%, n=1345 \text{ total } \# \text{ of urothelial cells})\) (Fig. 3.3g, and data not shown). \( Shh^{Gfp} \)
and p63 labeled cells are now observed in the basal and intermediate cells but are absent in the immature umbrella cells (Fig. 3.3b). By E16.5, basal cells have expanded considerably and make up 44% of the population (n=2243), while intermediate and immature umbrella cell populations make up 36.5% and 18% of the population (n=2243), respectively (Fig. 3.3h, and data not shown). By E18, K5+ basal cell population comprises 68.3% of the total urothelial population (n=1869) (Fig. 3.3i). At this stage, we begin to observe few mature umbrella cells that are morphologically distinguishable from intermediate and immature umbrella cells; they begin to develop into large oval multinucleated apical mature umbrella cells (Fig. 3.3i,j). The adult urothelium is fully differentiated and contains distinct urothelial cell types that are morphologically distinguishable (Fig. 3.3k,l). Analysis of bladder from adult ShhLacZ reporter mice reveals that the majority of epithelial cells are basal and Shh-positive (72.3%, n=1357), while Shh+-intermediate cells comprise 23% of the population (Fig. 3.3c,d). Large oval multinucleated Shh-negative umbrella cells, however, makeup only 4.4% of the population.

We quantified less than 1% of the remaining cells as U-O progenitor cells (Fig. 3.3k,l). These studies suggest that intermediate and umbrella cells are established early in development and are likely to form from undifferentiated ShhGFP+/p63+ U-O progenitor cells, which decrease in number over time (Fig. 3.3m). Additionally, because the number of intermediate cells decrease in time as umbrella cells form, this data suggests that umbrella cells form from intermediate cells (Fig. 3.3m). Given that intermediate and immature umbrella cells form before onset of basal cells, these studies also imply that basal cells are not likely progenitors of umbrella cells.
**Umbrella cell formation is temporally regulated during development.** The above results suggest that $Shh^{GFP+}/p63+$ U-O cells are the likely progenitors of umbrella cells during early development. To test this hypothesis directly, we used a tamoxifen (TM)-inducible $Shh^{CreERT}$ mouse strain in conjunction with a Cre-inducible bi-fluorescent $mTmG$ reporter to permanently mark $Shh$-expressing cells at discrete developmental time points. Prior to Cre-mediated recombination, cells express membrane-targeted Tomato ($mT$) and express membrane-targeted Gfp ($mG$) post-recombination (225). To trace the fate of $Shh$-descendents, we induced $Shh$-expression at E11.5, prior to urothelial stratification and when Shh+ p63+ U-O progenitors are present, and at E14.5, when basal cells begin to form and stratification begins. TM has been shown to induce nuclear translocation of CreER within 6 h of treatment and remains in the nucleus for another 36 h (244). To ensure that we identified the earliest cells that respond to tamoxifen, we designated the initial population of $Shh^{CreERT}:R26RmTmG$-marked cells within 24 h of TM treatment.

Induction with tamoxifen at E11.5 targets approximately 56% of all urogenital sinus cells at E12.5 ($n=1265$) and induction at E14.5 labels 21% of urothelial cells at E15.5 ($n=2061$) (**Fig. 3.4a,b,c**). The initial populations of $Shh^{CreERT}:R26RmTmG$-marked cells were also compared to $Shh$ mRNA expression at the time of TM treatment and were found to be similar. Analysis by in situ hybridization revealed that $Shh$ is expressed in all cloacal and urogenital sinus cells at E9.5, E11.5, and E12.5, before onset of bladder development (data not shown, and **Fig. 3.4d,e n=3**). At E14.5, its expression is restricted to basal and intermediate cells but excluded in immature umbrella cells (**Fig. 3.4f, n=3**). This $Shh$-expression pattern persists at E18 and in the adult bladder (**Fig. 3.4g, n=3**).
When embryos were exposed to TM at E11.5, when Shh is expressed in all urogenital sinus cells, at E18.5 the recombined mG allele labeled 85% of K5+/p63+ basal cells (n=3118 total # of Gfp+ cells) and 7.7% of UP+/p63- immature umbrella cells (n=3183 total # of Gfp+ cells) (Fig. 3.5a,d, and data not shown). In contrast, introduction of TM at E14.5 or at P17 with analysis at 2Y-when Shh expression is restricted to basal and intermediate cells-results in the labeling of mG-positive cells that contribute to only 2% (n=1429 total # of Gfp+ cells) and 0.7% (n=1890 total # of Gfp+ cells) of UP+/p63- umbrella cells, respectively (Fig. 3.5b,c,d), with no difference in contributions to the basal population (TM at E14.5, 92%, n=4693 total # of Gfp+ cells; TM at P17, 91%, n=2293 total # of Gfp+ cells) (Fig. 3.5b,c and data not shown). This data suggests that Shh-expressing urothelial progenitors generate the bulk of umbrella cells during early development, before onset of stratification. However, the observation that mG-labeled umbrella cells are detected after TM induction at E14.5 and in the adult demonstrates that the Shh-expressing U-O progenitor population persists during development and in the adult bladder and that new umbrella cells are continually formed, albeit at a considerably slower rate.

**RA temporally regulates umbrella cell formation.** The results above suggest that RA may be required for umbrella cell formation prior to stratification. To test the temporal requirement for RA in umbrella cell formation, we inhibited RA-signaling at E11.5, when the potential of Shh+/p63+ U-O progenitors to give rise to umbrella cells is highest, and at E14.5, when stratification begins and the potential of U-O progenitors to give rise to umbrella cells decreases. Expression of RaraDN at E11.5 under the control of ShhCreERT2, with analysis at E18.5, gives rise to a phenotype that is similar to that observed in
*Shh*<sup>C</sup>*Cre;RaraDN* mutants and is characterized by loss of intermediate and umbrella cells (Fig. 3.6a,b, *n*=5). However, basal cells were still present, suggesting that RA does not regulate or is not needed for basal cell formation (Fig. 3.6a,b, *n*=5). In contrast, there are no difference in urothelial populations between controls and *Shh*<sup>C<sub>ERT2</sub></sup>*Cre;RaraDN* mutants when RA-signaling is inhibited at E14.5 (Fig. 3.6c,f, *n*=3). The similarity in phenotypes between *Shh*<sup>C</sup>*Cre;RaraDN* and *Shh*<sup>C<sub>ERT2</sub></sup>*Cre;RaraDN* mutants suggests that there is a temporal requirement for RA in umbrella cell formation and that RA is likely to be important in Shh+ p63+ U-O progenitors before E14.5 for regulating intermediate and umbrella cell formation.

**RA-signaling is active before stratification and during regeneration.** Within the lower urinary tract, analysis of *Raldh2*, a RA-synthesizing enzyme, reveals that it is expressed in urogenital sinus epithelia and mesenchyme surrounding the urogenital sinus at E11.5, and in the bladder sub-epithelial stroma at E14.5 (Chapter II, www.gudmap.org).

Because Rars are widely expressed in the lower urinary tract (LUT), we sought to map the distribution of active RA-signaling within the urothelium through use of *Rare-lacZ* reporter. *Rare-lacZ* reporter mice harbor three copies of stably integrated RA-response element from *Rarb2* promoter fused to the lacZ gene. Promoter activity is activated in cells in which RA is available and RA-receptors are expressed. At E11.5, we observed strong lacZ activity in the urothelium and the surrounding mesenchyme (Fig. 3.7a, *n*=3). This pattern of expression persisted up until E14.5 (data not shown and Fig. 3.7b, *n*=3).

However, LacZ expression dramatically decreased at E14.5 onwards (Fig. 3.7c, *n*=3, and data not shown). In the mature adult urothelium, we were not able to find a single LacZ positive cell (Fig. 3.7d, *n*=3). The activity of RareLacZ observed in these studies is
consistent with a role for RA-signaling in Shh+ p63+ urothelial progenitors during development; LacZ expression is high when the potential of urothelial progenitors to form umbrella cells is high, but decreases in time as the potential of urothelial progenitors also decreases (Fig. 3.7h).

Under normal physiological conditions, the urothelium has a slow rate of turnover. However, when damaged, it undergoes rapid proliferation and restores lost urothelial cells (237). The observation that RA-signaling is necessary for intermediate and umbrella cell formation during development led us to ask whether it is also required during urothelial regeneration. To test this, we exposed RareLacZ reporter mice to one round of urothelial damage by IP injection of cyclophosphamide, an anticancer drug whose toxic byproducts are excreted into the urine and cause severe bladder inflammation and regeneration (245). We analyzed bladders at 24, 48, and 72 h time points. Interestingly, we observed an up-regulation in RA-reporter activity within 24 h of urothelial damage, and quantitated LacZ expression in 33% of basal and in 48% of umbrella cells 48 h post CPP damage (Fig. 3.7e,f,g, n=3358). These results suggest that RA may likely to be important for urothelial differentiation during regeneration.

**RA-signaling is required for umbrella cell formation during regeneration.** To directly test the requirement of RA-signaling for umbrella cell formation during regeneration, we exposed TM-induced Shh$^{CreERT2;RaraDN}$ mutants crossed with R26RmTmG mice to one round of IP injection of CPP and analyzed Shh$^{CreERT2}$ controls and Shh$^{CreERT2;RaraDN}$ mutant bladders 2 weeks after treatment. We also analyzed bladders from Shh$^{CreERT2;R26RmTmG}$ and Shh$^{CreERT2;R26RmTmG;RaraDN}$ animals that were given TM but were not subjected to CPP injection. Newly formed umbrella cells
were scored by the presence of Gfp and lack of p63. In comparison to 
Shh\textsuperscript{CreERT2};R26RmTmG controls (13.3%, \(n=3\)), there is a significant decrease in the number of new Gfp+/p63- umbrella cells in Shh\textsuperscript{CreERT2};R26RmTmG;RaraDN mutant bladders that were treated with CPP (1.8%, \(n=3\)) (Fig. 3.8b,c,d \(p=0.001\)). In contrast, there is no difference in the number of newly generated umbrella cells between Shh\textsuperscript{CreERT2};R26RmTmG and Shh\textsuperscript{CreERT2};R26RmTmG;RaraDN animals without CPP treatment (data not shown and Fig. 3.8a). This data suggests that Shh-expressing U-O progenitors are able to regenerate umbrella cells, and that RA is also required during urothelial regeneration in Shh-progenitors for restoration of umbrella cells.

**RA-signaling is likely to control the fate of Shh-progenitors.** Vitamin A deficiency or impaired RA signaling leads to squamous metaplasia in the urothelium (80,81,83,207). It has been unclear, however, whether metaplasia is due to replacement of the resident urothelium with exogenous squamous epithelial cells, or whether urothelial cells undergo a fate change in the absence of RA. To address this, induced expression of RaraDN in Shh-expressing cells at post-natal stage P2, when the bladder expands and grows considerably, and analyzed the urothelium 3 months later. Strikingly, our results revealed a phenotype that is similar to that reported in vitamin A deficiency. Compared to controls, Shh\textsuperscript{CreERT2};R26RmTmG;RaraDN mutants had foci of squamous-keratinized cells that had replaced the normal urothelium (Fig. 3.9a,b, \(n=3\)). Additionally, analysis of mutants on an R26RmTmG background revealed squamous foci containing Shh\textsuperscript{CreERT2} descendents that express keratin-1/10 (K1/K10), squamous markers that are absent in the urothelium of controls but is expressed in the skin and in keratinized epithelia. UP expression was not detected in these mutants (data not shown). These findings together
suggest that squamous epithelial cells in RA-deficiency may derive from \textit{Shh}-expressing progenitors that undergo abnormal differentiation, indicating that RA is likely to be required normally for regulating progenitor cell differentiation.

We were then interested in seeing if U-O progenitors also differentiate into non-urothelial cell types during development. To answer this, we stained wild type and \textit{Shh}^{\text{CreERT2};\text{R26R};\text{RaraDN}} mutant bladders that were induced with TM at E11.5 and at E14.5 with antibody to K1. Surprisingly, we find that inhibition of RA-signaling at E11.5, but not at E14.5 (data not shown), also gives rise to \textit{Gfp}+ cells that co-label with K1+ cells, suggesting that in the absence of RA-signaling, Shh+ U-O progenitors misdifferentiate into non-urothelial cell types (Fig. 3.9c,d,e,f,g,h, \textit{n}=3). However, it is not known whether this is a primary defect of RA-inhibition in K5+ cells, or secondary to defective umbrella cell differentiation.

**K5-basal cells are not progenitors of umbrella cells during development.** In most stratified epithelia, such as the skin and lung, K5+ basal cells are thought to be the progenitors of suprabasal cells (246,247,248). In the urothelium, however, the observation that intermediate and immature umbrella cell types are present before onset of K5+basal cells suggests that basal cells are not likely to be progenitors of UP2-expressing intermediate and umbrella cells. To test the differentiation potential of K5 cells directly, we performed a lineage analysis using \textit{K5}^{\text{CreERT2}} transgenic mouse and \textit{T83LacZ} reporter to trace the long-term contribution of K5-expressing cells to the urothelium after TM administration. In \textit{T83LacZ} reporter mice, the LacZ protein is fused to a nuclear localization signal (NLS) that allows the protein to translocate to the nuclear after \textit{Cre} recombination. To our surprise, introduction of TM at E14.5, when the first few
basal cells are detected, resulted in the generation of only 0.1% intermediate and umbrella cells at post-natal stage P31, while 99.3% of basal cells were formed, suggesting that K5+ basal cells are unipotent cells that rarely give rise to UP-expressing intermediate or umbrella cells (Fig. 3.10a, b, n=12 out of 4208 LacZ+ cells). As expected, we detected K5-expressing progenies in the suprabasal layers of the skin (Fig. 3.10c).
IV. Discussion

What is the urothelial stem cell? The multi-layered bladder epithelium consists of a luminal layer of specialized cells called umbrella cells, which overlie intermediate cells and basal cells. The urothelium has a slow rate of turnover under normal physiological conditions; however, in response to injury, the bladder epithelium is capable of undergoing rapid division, suggesting the existence of a stem cell/progenitor population that is capable of self-renewal and restoring all cell types, including umbrella cells. Our studies suggest that Shh+ p63+ urothelial progenitors are the likely progenitors of basal, intermediate and umbrella cells. By mapping the distribution of urothelial cell types within the Shh-expressing population, we find that undifferentiated Shh+ p63+ progenitors are present during early development, but that their numbers decrease over time, with less than 1% of them remaining in the mature adult urothelium. Using an UP2Cfp transgenic mouse model, we detected a small number of intermediate cells—which we believe are the precursors to mature umbrella cells—as early as E12.5. We confirmed the specificity of UP2Cfp mouse line, which is an indicator of UP2 promoter activity, by independently looking at expression of UP antibody at different developmental stages. We find that as umbrella cells are formed, the number of intermediate cells decrease over time, suggesting that umbrella cells are most likely to form from transit amplifying (TA) intermediate cells. Our preliminary work supports this model. By performing lineage analysis and BrDU analysis of Shh$^{CreERT2;R26RmTmG}$ cells in the adult bladder during regeneration, our data suggests that intermediate cells may fuse to form large multinucleated umbrella cells.
What is the contribution of \( Shh \)-expressing progenitors towards umbrella cell formation during development and during regeneration. Our study shows that the differential potential of \( Shh^+ \) U-O progenitors to form umbrella cells changes with time, and suggests that the bulk of umbrella cells are formed during early development. The observation that \( Shh \)-progenitors can give rise to umbrella cells before the appearance of basal cells further suggests that basal cells are the unlikely progenitors of umbrella cells, which is in contrast to previous findings (209,250, see below for a more detailed discussion). As the number of \( Shh^+ \) p63+ urothelial progenitors decreases with time, so does their capacity to give rise to umbrella cells. However, when challenged with chemical damage, \( Shh \)-expressing cells rapidly restore all urothelial populations, suggesting the existence of a stem cell population. Because \( Shh \) is expressed in both basal and intermediate cells in the adult urothelium, it is difficult to conclude from this experiment which cell type regenerates umbrella cells. One of our current experiments is to determine if UP2-expressing intermediate cells have the capacity to restore umbrella cells after urothelial damage. Using a TM-inducible \( UP2^{CreERT2} \) transgenic mouse line with a fluorescent reporter, we will be able to trace the progeny of Gfp+/-labeled cells. If we find more lineage-tagged intermediate and umbrella cells in comparison to controls without damage, it would support a model in which intermediate cells are a progenitor population that can renew the urothelium after damage.

RA-regulates the fate of \( Shh \)-progenitors. Our studies suggest RA is normally important in \( Shh \)-progenitors for either positively regulating urothelial differentiation or negatively suppressing squamous differentiation. Interestingly, as the differentiation potential of \( Shh \)-population changes with time, so does the sensitivity to RA inhibition.
Formation of intermediate and umbrella cells is dramatically reduced when expression of RA-dominant negative is induced at E11.5, prior to stratification and when Shh-progenitors generate large numbers of labeled daughter umbrella cells; however, differential potential and requirement for RA-dependent intermediate and umbrella cell formation decreases after E14.5. We propose a model in which RA-signaling is required during early development in Shh+ p63+ urothelial progenitors cells for formation of intermediate and umbrella cells. In the absence of RA-signaling, Shh-progenitors assume a non-urothelial fate, down-regulating uroplakins and up-regulating squamous markers (Fig. 3.11). Nonetheless, it is not clear at the moment whether RA also plays a role in K5-expressing basal cells to suppress squamous differentiation or if it is required in intermediate and umbrella cells for their formation and maintenance.

**K5+ basal cells are not progenitors of P2-intermediate or P3-umbrella cells during development.** Self-renewing epithelial tissues like the skin and intestine contain stem cells that reside in specialized niches (248,249). Since the bladder lacks crypts and ducts, the presumed niche for urothelial stem cells is along the basement membrane. Studies with $^3$H-thymidine labeling and 5-bromo-2’-deoxyuridine (BrdU) label retention assays to identify potential urothelial stem cells, have demonstrated that the long-term proliferative compartment of the urothelium is localized to its basal layer (250). In addition, recent studies by Shin et al., 2011 suggests that Shh-expressing basal cells are the multi-potent urothelial stem cells that are capable of self-renewal and differentiating into basal, intermediate, and UP+ umbrella cells *in vivo* and *in vitro* (209). Using a genetic fate mapping approach, we labeled K5-expressing cells during development and traced their long-term contribution. To our surprise, we find that the progeny of K5-
expressing cells rarely give rise to intermediate or umbrella cells. However, these results do not rule out the possibility that basal cells might act as a reserve SC population that can restore umbrella cells in the adult urothelium during damage. We are currently testing the requirement of K5+ cells for umbrella cell formation during regeneration by exposing TM-inducible K5<sup>CreERT2</sup>:R26RmTmG animals to CPP. If we do not observe any Gfp<sup>+</sup> cells in superficial cell layers, then we can conclude that K5+ cell are not progenitors of umbrella cells during regeneration.

**Model for urothelial differentiation.** Based on our data, we propose that Shh+/p63+ U-O progenitors are multipotent and can give rise to basal, intermediate, and umbrella cells. However, it’s not clear from this data whether umbrella cells form from intermediate cells in a linear fashion, or whether umbrella cells form independently from intermediate cells. It’s also not clear if U-O progenitor is responsible for giving rise to all three-cell types. While we find that RA is required in U-O cells for regulating formation of intermediate and umbrella cells, and suppressing squamous differentiation, it is presently not clear if RA is also required in basal cells for its formation and maintenance and in intermediate cells for formation of umbrella cells (**Fig. 3.11**).
Shh<sup>Cre</sup> | Targeted knock-in mouse strain. (221)
---|---
Shh<sup>CreERT2</sup> | Tamoxifen-inducible targeted knock-in mouse strain. (221)
Gli1<sup>CreERT2</sup> | Tamoxifen-inducible targeted knock-in mouse strain. (222)
K5<sup>CreERT2</sup> | Tamoxifen-inducible transgenic mouse line under the control of bovine keratin 5 promoter. (241)
UP2<sup>Cre</sup> | Transgenic mouse line under the control of bovine UPII promoter. (242)
UP2<sup>CreERT2</sup> | Tamoxifen-inducible transgenic mouse strain under the control of UPII promoter. (C.C. Cordon, not published)
Shh<sup>LacZ</sup> | Transgenic reporter mouse line used to study Shh expression. (243)
UP2<sup>Cfp</sup> | Transgenic reporter mouse line used to study UPII expression. (Mendelsohn lab, not published)
Rare-LacZ | Transgenic mouse strain in which 3 copies of RARE present in Rarb2 gene is fused to hsp promoter and hspLacZ. (71)
mT/mG | Double-fluorescent reporter that expresses membrane-targeted dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted GFP (mG) after excision. (225)
T83LacZ | Reporter mouse strain that expresses NLS-tagged LacZ reporter upon Cre-mediated excision. (241)
Rarat403 (RaraDN) | Transgenic mouse strain in which truncated human Rara receptor is inserted into the Rosa26 locus and is activatable in the presence of Cre-recombinase. (163)
Table 3.2a. Primary antibodies used in this study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Supplier</th>
<th>Ig Type</th>
<th>Dilution</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63</td>
<td>Santa Cruz Biotechnology p63 (4A4): sc-8431</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>Paraffin</td>
</tr>
<tr>
<td>p63</td>
<td>Santa Cruz Biotechnology p63 (H-137): sc-8343</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Cryosection</td>
</tr>
<tr>
<td>Cytokeratin 18 (K18)</td>
<td>Abcam Ab52948</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>Paraffin, cryosection</td>
</tr>
<tr>
<td>Keratin 5 (K5)</td>
<td>Covance Keratin 5 (AF 138): PRB-160P</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>Paraffin, cryosection</td>
</tr>
<tr>
<td>Keratin 10 (K10)</td>
<td>Covance Keratin 10 PRB-159P</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Cryosection</td>
</tr>
<tr>
<td>Keratin 20 (K20)</td>
<td>DAKO Clone K5 20.8</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>Paraffin</td>
</tr>
<tr>
<td>Uroplakin Total</td>
<td>Generous gift from Dr. T. T. Sun at NYU Medical Center</td>
<td>Rabbit IgG</td>
<td>1:1000</td>
<td>Paraffin, cryosection</td>
</tr>
<tr>
<td>Smooth Muscle Anti-Actin-Cy3</td>
<td>Sigma Life Science C6198</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Beta-galactosidase</td>
<td>Biogenesis 4600-1409</td>
<td>Goat IgG</td>
<td>1:100</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>BrdU</td>
<td>Abcam A06326</td>
<td>Rat IgG</td>
<td>1:100</td>
<td>Paraffin, cryosection</td>
</tr>
<tr>
<td>Click-it EdU Alexa Fluor Azide Kit</td>
<td>Invitrogen E10415</td>
<td></td>
<td></td>
<td>Paraffin</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>R&amp;D Systems AF748</td>
<td>Goat IgG</td>
<td>1:100</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Laminin</td>
<td>Sigma Life Science L9393</td>
<td>Rabbit IgG</td>
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<td>Paraffin, Cryosection</td>
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<tr>
<td>P-Histone H3 (S10)</td>
<td>Cell Signaling 9701L</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>P-Histone H3 (S10) 6G3</td>
<td>Cell Signaling 9703</td>
<td>Mouse IgG</td>
<td>1:100</td>
<td>Paraffin</td>
</tr>
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<td>Pax2</td>
<td>Invitrogen 716000</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Paraffin</td>
</tr>
<tr>
<td>Activated Caspase 3</td>
<td>Promega G748A</td>
<td>Rabbit IgG</td>
<td>1:100</td>
<td>Paraffin, Cryosection</td>
</tr>
</tbody>
</table>
Table 3.2b. Secondary antibodies used in this study.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Ig Type</th>
<th>Dilution</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexafluor 488 donkey anti-goat</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Alexafluor 488 donkey anti-rabbit</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-rabbit</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-mouse</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Alexafluor 488 goat anti-mouse</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-rat</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Alexafluor 594 donkey anti-goat</td>
<td>IgG (H+L)</td>
<td>1:1000</td>
<td>Paraffin, Cryosection</td>
</tr>
<tr>
<td>Cy5-conjugated donkey anti-mouse</td>
<td>Jackson Immunoresearch</td>
<td>IgG (H+L)</td>
<td>1:500</td>
</tr>
<tr>
<td>Cy5-conjugated donkey anti-goat</td>
<td>Jackson Immunoresearch</td>
<td>IgG (H+L)</td>
<td>1:500</td>
</tr>
<tr>
<td>Cy5-conjugated donkey anti-rabbit</td>
<td>Jackson Immunoresearch</td>
<td>IgG (H+L)</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Table 3.3. Urinary tract defects observed in VAD, RAR compound, HoxB7Cre;RaraDN, and ShhCre;RaraDN mutants.

<table>
<thead>
<tr>
<th>Defect</th>
<th>VAD</th>
<th>RAR Total</th>
<th>RaraDN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horseshoe kidneys</td>
<td>+</td>
<td>+</td>
<td>+ FoxD1Cre</td>
</tr>
<tr>
<td>Renal aplasia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+ HoxB7Cre;FoxD1Cre</td>
</tr>
<tr>
<td>Renal hypoplasia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+ HoxB7Cre</td>
</tr>
<tr>
<td>Hydronephrosis (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre/HoxB7Cre</td>
</tr>
<tr>
<td><strong>Ureter abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agenesis (partial or total, uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+ HoxB7Cre</td>
</tr>
<tr>
<td>Ectopia (uni- or bilateral)</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre/HoxB7Cre</td>
</tr>
<tr>
<td>Ureteral hydronephrosis</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre/HoxB7Cre</td>
</tr>
<tr>
<td><strong>Genito-urinary tract abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminal vesicle agenesis (males)</td>
<td>+</td>
<td>+</td>
<td>+ HoxB7Cre</td>
</tr>
<tr>
<td>Vaginal agenesis (females)</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre</td>
</tr>
<tr>
<td>Defect in Mullerian duct regression in males</td>
<td>+</td>
<td>+</td>
<td>+ HoxB7Cre</td>
</tr>
<tr>
<td>Persistance of Mesonephric duct in females</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre</td>
</tr>
<tr>
<td>Agenesis of the urinary bladder</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre</td>
</tr>
<tr>
<td>Defects in epithelial differentiation and/or keratinization of the urogenital sinus</td>
<td>+</td>
<td>+</td>
<td>+ ShhCre</td>
</tr>
<tr>
<td>Defect in cloacal partitioning</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 Retinoids regulate urothelial differentiation. (a,b) Upk expression is down-regulated in $Shh^{Cre};RaraDN^{\text{flo}x/+}$ mutant bladder lumen (arrowheads) but not in the ureters. SMaA expression is similar to controls.

(ur, ureter; bl, bladder)
Figure 3.2 RA-signaling in Shh-progenitors is required for umbrella and intermediate cell formation. (a,b) Shh\textsuperscript{Cre}\textsubscript{WT};RaraDN\textsuperscript{lox+/+} E18 mutants lack UP+ umbrella cells in comparison to controls. (c,d) K5+ p63+ basal cells are still present. (e,f) K20, another marker of umbrella cells is also absent in Shh\textsuperscript{Cre};RaraDN mutants. (g,h) SEM reveals the loss of umbrella cells but presence of microvilli, a characteristic of undifferentiated cells.
Table 3.4. Umbrella cells are absent in $Shh^{Cre};RaraDN^{flox/+}$ mutant E18 embryos.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>$Shh^{Cre};RaraDN^{flox/+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (K5+/p63+)</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Intermediate (K5-/p63+/UP+)</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Umbrella (K5-/p63-/UP+)</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>K20</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>K18</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>
Figure 3.3 Ontogeny of urothelial differentiation. (a) Most urogenital sinus cells at E11.5 express ShhLacZ and p63. (b) Shh expression coincides with p63 and is restricted to basal and intermediate cells at E14.5. (c,d) Shh is not expressed in umbrella cells in the adult. (e,f) UP2Cfp transgenic reveals presence of UP2-expressing cells. (g,h) K5 expression is seen in most basal cells by E16.5. (i,j) Umbrella cells are morphologically distinguishable by E18.5. (k,i) Marker expression in adult urothelia. (m) Developmental ontology.
Figure 3.4 *Shh*<sup>CreERT;R26RmTmG</sup> and *Shh* mRNA marks initial population of *Shh*-expressing cells. (a,b) Labeling of urothelial cells with *Shh*<sup>CreERT;R26RmTmG</sup> 20 h post-induction. (c) Percent of urothelial cells labeled at E12 and E15. (d,e) *Shh* is expressed in all urogenital sinus cells at early stages. (f,g) Expression of *Shh* is restricted to basal and intermediate cells and excluded from superficial layer E14.5 onwards.
Figure 3.5 Umbrella cell precursors are established early in development. (a) Labeling of urothelial cells with Shh-CreERT;R26RmTmG at E11.5 and analysis at E18.5. (b,c) Labeling of urothelial cells with Shh-CreERT;R26RmTmG at E14.5 and analysis at E18.5 and 2Y. (d) Percent of umbrella cells that are labeled decrease when induction is later developmental stages.
Figure 3.6 RA-signaling temporally regulates umbrella cell formation. (a) Labeling of urothelial cells with Shh\textsuperscript{CreERT}/R26RmTmG at E11.5 and analysis at E18.5. (b,c) Labeling of urothelial cells with Shh\textsuperscript{CreERT}/R26RmTmG at E14.5 and analysis at E18.5, and 2Y (d) Percent of umbrella cells that are labeled decrease when induction is later developmental stages.
Figure 3.7 RA-signaling is active during early development and during regeneration. (a,b,c) LacZ expression is high at E11.5 and this pattern of expression persists up until E14.5. (d) No LacZ expression is detected in the adult bladder. (e,f,g) LacZ expression is upregulated in umbrella and basal cells after damage.

D. Gandhi, A. Molotkov
Figure 3.8 RA-signaling is required in Shh-expressing U-O progenitors for umbrella cell formation. (a) Without CPP treatment, few umbrella cell are generated. (b,c) In the absence of RA-signaling, formation of umbrella cells is drastically reduced.

D. Gandhi, A. Molotkov
Figure 3.9 RA-signaling is required in Shh-expressing U-O progenitors for prevention of squamous differentiation. (a,b) Inhibition of RA-signaling at P2 leads to K1-expressing squamous cells. (d,e,f,g,h,i) Shh-descendants express K1 markers when RA-signaling is defective. 

D. Gandhi, A. Molotkov
Figure 3.10 K5+ basal cells are not progenitors of intermediate or umbrella cells. (a) \( \text{LacZ}^+ \) cells are not found in intermediate or umbrella cells. (b) Percentage of basal and intermediate cells formed when induced at E14.5 and analyzed at P31. (c) K5-labeled cells give rise to all layers in the skin.

A. Molotkov
Figure 3.11 Model for urothelial differentiation. In the absence of RA-signaling, Shh+ U-O progenitors misdifferentiate into non-urothelial K1-expressing cell types. RA is needed in U-O progenitors for intermediate and umbrella cell formation.
Chapter IV

Conclusions and Perspectives

Retinoids are essential for development and control many aspects of cell proliferation, differentiation, apoptosis, and epigenetic mechanisms (1,2,14). The active derivative of vitamin A, retinoic acid (RA), exerts its effects through receptor-mediated signaling. Previous studies from our lab have suggested that vitamin A-signaling, through Ret, plays multiple roles in the urinary tract and is required for kidney development and ureter maturation. By inducing expression of a floxed dominant-negative mutant Rar receptor (RaraDN) in the bladder, for the first time we show that bladder-derived RA-receptor signaling is required for establishing both mature ureter-bladder connections, and for bladder growth and differentiation of the bladder epithelia. The implications of these novel findings are discussed below.

I. Almost 1% of human infants are born with urogenital abnormalities that are often associated with abnormal connections between the upper and lower urinary tracts (129). The diagnosis and treatment of urinary tract birth defects depends on the type of malformation. The identification of animal models that resemble human urinary tract malformations has helped elucidate key physical and molecular events underlying urinary tract morphogenesis. Our lab, along with others, have shown that ureter maturation, the process by which ureters migrate from their initial integration site in the wolffian duct (WD) to their final insertion site in the bladder, depends on RA-mediated apoptosis of the common nephric duct (CND), the portion of the ureter that remains attached to the WD (127). In this study, we show that proper ureter connections with the bladder depends on bi-directional signaling first between the nephric duct (ND) and the cloaca, and then
between the CND and the bladder epithelium; defects in these processes can lead to abnormalities commonly observed in infants.

Previous studies have suggested that loss of Ret, Pax2/8, or Gata3, results in failure of ND to extend and fuse with the cloaca (165). In our lab, we showed that inhibition of RA-signaling in the ND results in a similar phenotype, in part through regulation of Ret (160). Here we show that proper ND insertion with the cloaca not only depends on signaling in the ND, but also relies on RA-dependent signaling cues from the cloaca. In the absence of RA-signaling in the cloaca, NDs fail to join the cloaca, resulting in a persistent CND, and subsequent hydroureter and hydronephrosis. Additionally we show that inhibition of RA-signaling in the bladder epithelia at later stages can lead to formation of an ureterocele, a common birth defect. Thus, our studies provide a new way of looking at lower urinary tract development and a novel model that can be used to link clinical conditions in infants and adults to distinct events during early development.

II. Bladder cancer is the fifth most common human disease and the second most frequent diagnosed genitourinary tumor after prostate cancer (240). It is a recurring disease that is associated with high medical costs due to the requirement for frequent bladder resections. The majority of bladder cancers are urothelial carcinomas. The majority of bladder neoplasms (75-85%) are low-grade superficial papillary tumors, while the remaining are high-grade carcinoma in situ (CIS) and precursors of invasive cancer (241). Superficial and invasive bladder cancers are associated with distinct genotypic and phenotypic patterns. For example, gain-of-function mutations in proto-oncogenes Ras, PI3K, or Fgfr3 occur in most papillary tumors. On the other hand, high-grade invasive carcinomas in situ (CIS) are linked to loss of mutations in tumor
suppressor proteins p53 along with deficiency in Pten or Rb (Fig. 4.1) (242). Recent studies by Cordon-Cardo and Abate-Shen labs revealed that inactivation of tumor suppressor genes p53 and Pten results in bladder tumors that resemble CIS with areas of squamous and sarcomatoid carcinoma (242). Despite these findings, little is known about the progenitors that are normally important for urothelial differentiation and the urothelial subtypes that contribute to tumorigenesis (238,239,240).

The urothelium is a stratified epithelium that lines the major portion of the lower urinary tract, from the renal pelvis to the urethra. It contains basal, intermediate, and superficial umbrella cells. The existing theory of urothelial differentiation is based on the linear differentiation pattern of the epidermis; K5+ basal cells are progenitors that give rise to intermediate cells, which in turn give rise to umbrella cells (241). Recent lineage studies suggest that Shh-expressing progenitors have the capacity to repopulate all cell-types of the urothelium after damage, suggesting the existence of a stem-cell population in the adult urothelium (209). However, it is unclear when and how Shh-progenitors differentiate into basal, intermediate, and umbrella cells during normal urothelial development. By utilizing a collection of reporter mouse lines, we identified in this study an urothelial progenitor population (termed U-O) that expresses Shh and p63. We show that Shh+/p63+ cells are present as early as E11.5, before the urogenital sinus matures into the future bladder. Using lineage tracing, we show that umbrella cell precursors are established by Shh-expressing progenitors early in development, before K5+ basal cells are detected. Furthermore, our data indicates that K5+ basal cells are not progenitors of intermediate or umbrella cells during development. Based on our observations, we propose the existence of an Shh+/p63+ U-O progenitor population that gives rise to two
distinct cell lineages during development and in the adult: basal cells that are K5+ p63+
but negative for UP, and intermediate and umbrella cells that are UP+ (Fig. 3.11). The
distinct characteristics of these populations could explain the phenotypic and genotypic
variations in bladder cancer. Based on our findings, it is possible that loss of tumor
suppressing proteins such as p53 and Pten in the U-O progenitor can give rise to CIS and
squamous cell carcinoma that express K5. On the other hand, over-expression of
oncogenes such as Ras in U-O progenitors may give rise to superficial papillary tumors
(Fig. 4.2).

The identification of an U-O Shh+/p63+ progenitor population also paves the way
for bladder regeneration for cancer patients who require bladder surgery. Current
methods for bladder reconstructive surgery include cell grafting from the gastrointestinal
tract (238,239). However, there are many complications associated with use of bowel
such as infection, and intestinal obstruction. Isolation of the Shh+/p63+ U-O progenitor
from human bladder samples and methods to grow them in vitro may prove to be useful
for bladder regeneration grafting procedures.
Figure 4.1 Molecular pathways of urothelial tumorigenesis. Bladder tumors are classified into two separate pathways with distinct histopathological patterns, molecular alterations, and clinical behavior.

Taken from (241).
Figure 4.1 Model for tumor formation. Inactivation of Ras or PI3K in umbrella cell progenitors leads to papillary tumors, while inactivation of p53 and Pten leads to carcinoma in situ (CIS).
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