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Retinoid-signaling in progenitors controls specification and regeneration of the urothelium

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Abstract

The urothelium is a stratified epithelium that prevents exchange of water and toxic substances between the urinary tract and blood. It is composed of Keratin-5-expressing-basal-cells (K5-BCs), intermediate cells and superficial cells specialized for synthesis and transport of uroplakins that assemble into the apical barrier. K5-BCs are considered to be a progenitor cell type in the urothelium and other stratified epithelia. Fate mapping studies however, reveal that P-cells, a transient population, are urothelial progenitors in the embryo, intermediate cells are superficial cell progenitors in the adult regenerating urothelium, and K5-BCs are a distinct lineage. Our studies indicate that retinoids, potent regulators of ES cells and other progenitors, are also required

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in P-cells and intermediate cells for their specification. These observations have important implications for tissue engineering and repair, and ultimately, may lead to treatments that prevent loss of the urothelial barrier, a major cause of voiding dysfunction and bladder pain syndrome.

INTRODUCTION

The urothelium is a stratified epithelium derived from endoderm (Wells and Melton, 1999) that extends from the renal pelvis to the proximal urethra that serves as a crucial barrier between the blood and urine. The mature urothelium consists of a layer of *Keratin-5* expressing basal cells (K5-BCs), intermediate cells (I-cells) and a luminal layer of superficial cells (S-cells). S-cells are a terminally differentiated and are specialized for synthesis and transport of uroplakins (Upks), a family of molecules that assemble into apical crystalline plaque that is water proof and damage resistant [reviewed in: (Khandelwal et al., 2009; Wu et al., 2009)]. Damage to the urothelial barrier can compromise bladder function, lead to inflammation, and expose sub-urothelial nerve fiber receptors to urinary toxins, a possible mechanism behind chronic bladder pain or interstitial cystitis (Wyndaele and De Wachter, 2003). Thus, identification of urothelial progenitors and the signaling pathways that regulate them will be important for designing strategies for tissue augmentation and regeneration.

The urothelium is distinguishable in the mouse embryo on E11.5 when the bladder begins to form at the anterior aspect of the urogenital sinus. It is thought to assemble in a linear sequence, beginning with K5-BC progenitors that produce I-cells and S-cells that populate upper layers (Shin et al., 2011). The adult urothelium is quiescent but can rapidly regenerate in response to acute damage such as urinary tract infection or exposure to drugs and toxins [reviewed in: (Khandelwal et al., 2009)]. The injury response begins with desquamation of the damaged urothelium, followed by a massive wave of proliferation that reconstitutes the urothelial barrier within 72h, observations that suggest the existence of a progenitor population. Fate mapping studies using a TM-inducible $Shh^{CreERT2}$;*mTmG* to indelibly label *Shh*+ cells support the existence of a population of *Shh*-expressing progenitors in the adult which are proposed to be K5-BCs (Shin et al., 2011). It remains unclear, however, whether these progenitors are also important for generating the urothelium during embryonic development.

Retinoic acid (RA) is a potent signaling molecule that regulates self-renewal and pluripotency and specification in ES cells and other progenitors, by inducing chromatin modifications in regulatory regions of RA-responsive genes (Kashyap et al., 2011). Retinoids are important in adults for vision and fertility, maintaining a wide variety of specialized epithelia (Wolbach and Howe, 1925), and are critical regulators of organogenesis. RA is synthesized from retinol, an inactive precursor that is taken up by cells and converted to retinoic acid in a two step process by retinol dehydrogenase-10 *(Rdh10)* and retinaldehyde dehydrogenase-2 *(Raldh2)*, enzymes that are selectively expressed in cells where active RA signaling is required (Duester, 2008; Niederreither and Dolle, 2008). Once available, RA regulates transcription by binding to and activating RA receptors (*Rars*), a family of eight transcription factors that are widely expressed in adults and embryos. *Rars*

control transcription by binding to RA response elements in promoter regions of target genes in association with *Rxrs*, a second family of nuclear receptors. In the absence of RA, Rar/Rxr heterodimers are frozen in an inactive conformation, however RA binding to the Rar/Rxr heterodimer induces a conformational change, converting the inactive complex to a transcriptionally active state (Samarut and Rochette-Egly, 2012)

The observations that RA regulates the adult steady state urothelium (Liang et al., 2005), together with recent studies showing that RA can induce ES cells to differentiate into urothelial cells (Mauney et al., 2010) suggest that retinoids may be important regulators of urothelial differentiation *in vivo*. To address this, we examined the requirement for RA-signaling in urothelial cells by expressing a dominant inhibitory form of *Retinoic acid receptor alpha (Rara), RaraT403* in urothelial progenitors. *RaraT403* lacks the ligand dependent activation domain that is critical for recruiting histone modifiers (Kashyap et al., 2011) and is thus a potent inhibitor of endogenous RA signaling in vivo and in vitro (Blumberg et al., 1997; Damm et al., 1993). *RaraDN* has been inserted into the *Rosa26* locus (Soriano, 1999) after a floxed STOP sequence to generate *[Gt(ROSA)26Sor)]* mice (hereafter called *RaraDN* mice). We showed previously that Cre-dependent expression of *RaraDN* generates a collection of defects that are virtually identical to those observed in RA-deficiency and in mutants lacking components of the RA-signaling pathway (Table S1) that increase the severity of phenotypes in a dose dependent manner (Chia et al., 2011; Rosselot et al., 2010). Importantly, defects induced by expression of *RaraDN* appear to be specific for *Rar*-signaling, since we have not observed abnormalities that could be linked to inhibition of transcription via other nuclear receptor family members (Table S1).

The *Shh*-expressing population in the adult urothelium contains progenitors that have long term regenerative capacity and which have been proposed to be K5-BCs (Shin et al., 2011). We show here that the *Shh*-expressing population in embryos contains K5-BCs as well as two additional cell types: P-cells, which are present in the embryonic urothelium but not in the adult, and I-cells, which are present in the embryonic and adult urothelium. Lineage studies using a $Krt5^{CreERT2}$ line to indelibly label K5-BCs and their daughters indicate that that K5-BCs are unlikely to be progenitors in the embryo or in adults. On the other hand, we find that P-cells, a transient urothelial cell type, are progenitors in the embryo and I-cells are progenitors in the adult regenerating urothelium, and we show that retinoids are required both in P-cells and I-cells for their specification. These observations could have important implications for tissue engineering and repair and may lead to treatments for patients with voiding dysfunctions and/or painful bladder syndrome that are associated with loss of the urothelial barrier function.

RESULTS

Shh-expressing cells are progenitors in the embryonic urothelium

The mature urothelium is composed of a layer of basal cells that are positive for Krt5 and P63 (K5-BCs), 1-2 layers of intermediate cells (I-cells) that express Upk and P63 but not Krt5 and a luminal layer of superficial cells (S-cells) that express Upk, but not Krt5 or P63 [(Fig. 1A,F); in this figure and in subsequent figures, yellow arrowheads designate S-cells, purple arrowheads designate I-cells and green arrowheads designate K5-BCs]. Recent fate

mapping studies using *Shh*$^{CreERT2+/-}$*;mTmG* mice to indelibly label *Shh*+ cells and their daughters, support the existence of a population of *Shh*-expressing progenitors with long term regenerative potential (Shin et al., 2011). Based on the co-localization of Shh and Krt5, a marker of K5-BCs, it was proposed that the urothelial progenitor is a K5-BC. An interesting question, however, is whether this progenitor population also participates in d*e novo* urothelial formation in the embryo.

Shh$^{CreERT2+/-}$ mice which harbor a TM inducible form of Cre (Harfe et al., 2004), were crossed with *Gt(ROSA)26Sor*$^{tdTomato,-EGFP}$ [(*Rosa26*-membrane-*Tomato*/membrane-*Gfp* reporter mice); hereafter called *mTmG* mice]. In TM treated *Shh*$^{CreERT2+/-}$*;mTmG* mice, membrane bound *Gfp* is expressed in cells that undergo *Cre*-mediated recombination and membrane bound *Tomato* is expressed constitutively from the *Rosa26* promoter in cells where recombination has not taken place (Muzumdar et al., 2007). We used this reporter line in fate mapping experiments to evaluate the potential of the *Shh*+ population in the developing urothelium. We first examined the specificity of Cre-dependent recombination by comparing the distribution of *Shh* mRNA with the *Gfp*-lineage tag in *Shh*^{CreERT2}*;mTmG* embryos. In situ hybridization analysis indicates that *Shh* mRNA is expressed in virtually all urothelial cells between E10 and E13, becoming restricted to the basal and intermediate layers by E14, when S-cells begin to form in the upper layer (Fig. S1A-C). To evaluate the initial distribution of Cre-recombination, we analyzed the urothelium in TM treated *Shh*^{CreERT2}*;mTmG* embryos after a short chase. TM was administered at E11, when the urothelium begins to form, and at E14, when the urothelium is stratified, and embryos were analyzed 24h later. In E12 *Shh*^{CreERT2}*;mTmG* embryos exposed to TM at E11, *Gfp*-labeling was throughout the developing urothelium in a pattern that overlaps well with endogenous *Shh* mRNA (Fig. 1B,G; Fig. S1; compare A and D). In E15 *Shh*^{CreERT2}*;mTmG* embryos exposed to TM on E14, *Gfp*-labeling was present in the intermediate and basal layers where *Shh*-mRNA is expressed, but was undetectable in the superficial cell layer, where *Shh* mRNA is down-regulated (Fig. S1B,E-I). Together these findings indicate that Cre-dependent recombination in *Shh*^{CreERT2}*;mTmG* embryos is restricted to *Shh*-expressing cells.

To evaluate whether *Shh*+ cells can generate superficial cell daughters, *Shh*^{CreERT2}*;mTmG* embryos were exposed to TM on E11 or E14, and analyzed at E18 when the urothelium is stratified and mature S-cells occupy the luminal layer. In E18 embryos exposed to TM at E11, *Gfp*-expression was present in 70% of urothelial cells including the superficial cell compartment where labeling was in 45% of the population (Fig. 1C,D,H,I,K). In E18 embryos exposed to TM at E14, 25% of urothelial cells were *Gfp* labeled including 6% of the superficial cell population (Fig. 1E,J,K). Since Cre-mediated recombination peaks between 6 and 48h after TM exposure (Hayashi and McMahon, 2002), these observations suggest that *Shh*-expressing progenitors are present in the urothelium between E11.5 and E16.

The urothelium stratifies in a unique manner

According to the current thinking, the urothelium stratifies in a similar fashion as does the skin, beginning with K5-BC progenitors that produce mature cell types that progressively

populate the upper layers. A surprising observation however, is that *Krt5*, an early marker of K5-BCs, is barely detectable prior to E15, a stage when S-cells and I-cells have already formed (Fig. S2A-C,G-I). We therefore examined the composition of the *Shh*+ population to determine the ontogeny of different cell types and to identify potential progenitor populations. Shh is secreted and Upk, the definitive marker of both I-cells and S-cells, is expressed on the apical surface making it difficult to distinguish individual positive cells (e.g. Fig. S2G-L). We therefore used $Shh^{Gfp/Cre}$ and Shh^{nLacZ} reporter mice (Harfe et al., 2004; Lewis et al., 2004) to define cell types present in the *Shh*+ population and we generated *Up2-Cfp* reporter mice that express *Cfp* driven by *Up2* regulator sequences to evaluate the distribution of *Upk*-expressing cells (Fig. S2M,N).

Marker analysis of $Shh^{Gfp/Cre}$ and $Shh\text{-}^{nlacZ}$ mice revealed at that the *Shh*-expressing population in the embryonic urothelium contains 4 cell types: an undifferentiated endodermal population (**Foxa2+** Upk− **P63+ Shh+** Krt5−), P-cells (**Foxa2+ Upk+ P63+ Shh+** Krt5−): which are a transient cell type abundant between E11 and E13 but undetectable at later stages (Fig. 2A-E); I-cells (Foxa2− **Upk+ P63+ Shh+** Krt5−): which are abundant in the basal and intermediate layers at E14, and in adults reside in the intermediate layer where they comprise 5% of the urothelial population (Fig. 2,H,I,K,O); and K5-BCs (Foxa2− Upk− **P63+ Shh+ Krt5+):** which are first detected between E14 and E15 and by E18 are the majority of cells in the urothelium (Fig. 2J,K,N,O). S-cells, which are negative for *Shh*-expression (Foxa2− **Upk+** P63− Shh− Krt5−), are first detectable in an immature mononucleated form at E14 (Fig. 2F) and by E18 are multinucleated, resembling their mature counterparts (Fig. 2G). Analysis of the distribution of *UP2-Cfp* activity confirmed these observations: *Up2-Cfp*+ P-cells co-expressing Foxa2 were detected between E11 and E13, while *Up2-Cfp* expression was restricted to intermediate and S-cells at later stages (Fig. 2L-M and not shown). At birth, 90% of cells in the urothelium are K5-BCs, which occupy the basal layer, 5% are I-cells and 5% are S-cells (Fig. 2P).

Fate mapping indicates that the K5-BCs are not urothelial progenitors in the embryonic or adult urothelium

The observations that (i) K5-BCs are undetectable in the urothelium between E11 and E14 when progenitor potential is high, (ii) K5-BCs form after S-cells, I-cells and P-cells, suggests that K5-BCs are unlikely to be urothelial progenitors during development. To directly address this question we performed fate-mapping studies using Cre-lox recombination to indelibly label K5-BCs and their daughters. *Tg(KRT5CreERT2)* mice (hereafter referred to as $K5^{CreERT2}$ mice) express a transgene containing *Krt5* regulatory sequences fused to the TM-inducible *Cre/ERT2* cassette that drives Cre-dependent recombination in *Krt5* expressing cells, including epidermis (Indra et al., 1999). Based on the distribution of Krt5 expression, K5-BCs appear between E14 and E15. We therefore exposed $Krt5^{CreERT2}$*;mTmG* embryos to TM on E14 and analyzed the distribution of *Gfp*-positive cells after 4 days, which in experiments with the $Shh^{CreERT2}$*;mTmG* line as a lineage marker, was sufficient time to label 6% of the superficial cell population (Fig. 1E,J,K). In E18 $Krt5^{CreERT2}$*;mTmG* embryos exposed to TM on E14, Cre-dependent recombination occurred in about 22% of urothelial cells, and labeling was confined almost exclusively to the K5-BC population (Fig. 3A,C). Similar findings were obtained in

experiments where TM was administered at E14 *in utero* and embryos were analyzed after either one month or three months; lineage-tagged cells were almost exclusively K5-BCs with an occasional *Gfp* labeled cell in the intermediate layer (Fig. 3B,D,K and not shown). These results suggest that K5-BCs are not urothelial progenitors during development.

K5-BCs have been proposed to be progenitors with long-term regenerative capacity in the adult urothelium based on fate mapping experiments using the *ShhCreERT2;mTmG* line (Shin et al., 2011) which in adults, drives Cre-dependent recombination in I-cells as well as in K5-BCs (Fig. 2J,K; Fig. S1F-I). To directly determine whether K5-BC have regenerative potential in the adult urothelium, we performed parallel fate mapping experiments using the $Krt5^{Cre/ERT2}$*;mTmG* and $Shh^{CreERT2}$*;mTmG* as lineage markers for K5-BCs and *Shh*-expressing cells respectively, after treatment with cyclophosphamide (CPP), which induces a rapid cycle of injury and repair (Farsund and Dahl, 1978). To assess the kinetics of regeneration in our experimental setting, CPP-treated and untreated controls were injected with EdU (5-ethynyl-2′-deoxyuridine) to label cells in S-phase, and proliferation was measured at 24h, 48h and 72h. Consistent with the low rate of turnover in the adult urothelium, few proliferating cells were present in controls that had not been treated with CPP (Fig. S3A), however in CPP-treated animals, proliferation increased after 24h, peaking at 48h when 33% of cells in the urothelium were Edu+ (Fig. S3B,C). To evaluate the kinetics of urothelial regeneration during this 3day period, we stained CPP treated adults and controls for expression of Upk, which labels both intermediate and S-cells. Analysis 24h and 48h after CPP treatment revealed a decreased thickness of the urothelium and down-regulation of Upk expression compared to untreated controls, indicating that extensive exfoliation had taken place (Fig. S3D-F). By 72h after CPP-treatment, the thickness of the urothelium and expression levels of Upk-distribution were similar to controls, indicating that the urothelium was reconstituted (Fig. S3D,G).

We next performed damage and regeneration experiments with the $Shh^{CreERT2}$*;mTmG* and $Krt5^{CreERT2}$*;mTmG* lines. Adult mice were given 3 doses of TM over a one-week period to activate Cre-dependent recombination and expression of the *Gfp*-lineage tag. CPP was administered 1 week after the last TM injection to induce a round of damage and repair. Analysis was performed 2 weeks after CPP exposure when regeneration is complete and the urothelium has returned to a quiescent state. Analysis of $Shh^{CreERT2}$*;mTmG* mice that did not receive CPP revealed a small number of lineage-tag expressing cells (Fig. 3E), however in CPP-treated $Shh^{CreERT2}$*;mTmG* mice, *Gfp*-expression was present in 70% of the cells in the urothelium, including 50% of the superficial cell population (Fig. 3H,L). These findings demonstrate that *Shh*-expressing cells can generate superficial cell daughters after CPP-induced injury. In parallel experiments with $K5^{CreERT2}$*;mTmG* mice as a lineage marker, 60% of the urothelium was *Gfp*-positive after Tm-induction, indicating that recombination was robust, however expression of the lineage tag was almost entirely restricted to the K5-BC population in both CPP treated and untreated adults (Fig. 3F,G,I,J,L). The observation that K5-BCs do not generate detectable numbers of S-cells during regeneration suggests that I-cells rather than K5-BCs must be the superficial progenitor.

P-cells are a newly discovered progenitor population in the developing urothelium

Our studies suggest that P-cells (**Foxa2+ P63+ Shh+ Upk+** Krt5−) are a transient population present in the embryonic urothelium between E11 and E13; a period when fate mapping indicates that progenitor potential is high (Fig. 1). To evaluate whether P-cells can produce other urothelial cell types, we used a TM-inducible *Foxa2*^{CreERT} line (Frank et al., 2007) in fate mapping experiments. We first examined the specificity of Cre-dependent recombination in the *Foxa2*^{CreERT}*;mTmG* line by exposing embryos to TM at E11 and analyzing the distribution of the *Gfp* lineage marker after a short, 24h, chase. *Gfp* expression was seen in a small number of P-cells that co-express Foxa2, P63 and Upk, while *Gfp* labeled cells were undetectable in the urothelium of embryos that were not exposed to TM (Fig. 4A,B and not shown), indicating that Cre-dependent recombination is TM-dependent and is initially confined to P-cells. Analysis of TM pulsed embryos after a longer, 7day chase, revealed expression of *Gfp* in 10-14% of cells in the intermediate and superficial populations (Fig. 4C-F). Although almost 90% of urothelial cells are K5-BCs at this stage, expression of the *Gfp*-lineage tag was rare or undetectable in the K5-BC population (Fig. 4C-F), suggesting that K5-BCs arise from a distinct progenitor cell type.

In parallel experiments, we traced the fate of P-cells using the *Upk3aGCE;mCherry* line (www.gudmap.org), in which Tm-inducible *mCherry* expression is detected by antibody staining (*mCherry* is shown in green in Fig. 4 and in subsequent figures). Analysis of E12 *Upk3aCCE;mCherry* embryos 24h after Tm exposure revealed *mCherry* labeling in a small number of P-cells co-expressing Foxa2, Upk and P63 (Fig. 4G,H), indicating that recombination at this stage is restricted to the P-cell compartment. Analysis after a 7-day chase period revealed expression of the *mCherry* lineage tag in about 33% of the superficial cell population and 10% of the intermediate cell population (Fig. 4I,J), but again, we did not observe expression in K5-BCs. These studies suggest that P-cells are intermediate cell and superficial cell progenitors. However, since intermediate and superficial cells form within 24h of one another (E13, and E14, respectively), it is unclear from our studies whether superficial cells are direct descendants of P-cells or whether they are derived from intermediate cells, which are P-cell daughters. Together, these studies suggest that P-cells are intermediate cell and superficial cell progenitors. However, since intermediate and S-cells form within 24h of one another (E13, and E14, respectively), it is unclear from our studies whether S-cells are direct descendants of P-cells or whether they are derived from I-cells, which are P-cell daughters.

I-cells are likely to be superficial cell progenitors in the regenerating adult urothelium

Fate mapping studies indicate that the *Shh*-expressing population contains urothelial progenitors in adults (Shin et al., 2011). We find that *Shh* is localized in K5-BCs, which fate mapping studies suggest are unlikely to be urothelial progenitors (Fig. 3), and in I-cells which have not been assessed for progenitor potential. We therefore used the *Upk3aGCE;mCherry* line in fate mapping studies to determine whether I-cells can generate superficial cells in the adult regenerating urothelium. *Upk3aGCE;mCherry* adults were treated first with TM, then 1 week later with CPP to induce a round of regeneration, and analysis was performed 2 weeks later to evaluatethe distribution of *mCherry* expressing cells.

Analysis of TM induced *Upk3aGCE;mCherry* control mice that had not received CPP, revealed *mCherry*-labeling in I-cells and S-cells, however few were Edu+ (Fig. 5A), consistent with the low rate of proliferation in the adult steady state urothelium (Jost, 1989). However, analysis of *Upk3aGCE;mCherry* adults after CPP treatment revealed Edu labeling in 45% of I-cells and in 67% of S-cells (Fig. 5E). CPP induces death and desquamation of the superficial layer (Fig. S3), thus the presence of Edu+ lineage tagged S-cells strongly suggests that they are intermediate cell daughters. To evaluate whether I-cells can self-renew and produce superficial cell daughters after serial CPP damage and regeneration, *Upk3aGCE;mCherry* mice were *Upk3aGCE;mCherry* adults were given TM 3 times over a one week period to activate Cre-dependent recombination, then CPP was administered 1 week, 3 weeks and 5 weeks after the last dose of TM. Edu was given 48h after the first CPP dose to label cells in S-phase. Analysis after 3 rounds of injury and repair revealed an increase in the percentage of lineage-labeled S-cells [(92% compared to 67% after 1 dose of CPP (Fig. 5B,C,D,F,G,H,I)]. The observation that the numbers of lineage marked S-cells increase after serial damage and regeneration suggests that I-cells are superficial cell progenitors, and that they can self-renew. Since the Edu concentration is reduced by half each time a cell divides, the presence of S-cells expressing high levels of Edu suggests that they are either derived directly from intermediate cell progenitors that divide slowly, or are derived from a transit amplifying intermediary cell type.

Retinoid signaling is selectively expressed in P-cells during development, and is up regulated in the regenerating urothelium

Retinoic acid (RA) can induce embryonic stem cells to differentiate into urothelial cells in culture (Mauney et al., 2010), suggesting that retinoids may be important for controlling urothelial specification in vivo. To begin to address this, we analyzed wild type embryos to assess the distribution of RA-responsive cells using the *RARE-LacZ* reporter line. *RARE-LacZ* mice harbor a transgene containing *LacZ* fused to an RA-response element which is expressed in cells where RA is available and RA-receptor signaling is active (Rossant et al., 1991). Analysis of *RARE-LacZ* expression during development revealed that *LacZ*-positive cells were most abundant between E11 and E14 and decreased to low levels at later stages (Fig. 6A-D). Analysis at of the distribution of *RARE-LacZ* activity at E12 revealed expression in P-cells (Fig. 6E), and by E14, *LacZ* expression was localized predominantly in intermediate and S-cells (Fig. 6K). These studies suggest that the RA-responsive cells are most abundant in the embryonic urothelium between E11 and E14, when urothelial progenitors are also present (Fig.1).

RA-deficiency in mammals results in squamous metaplasia in the adult urothelium (Liang et al., 2005; Wolbach and Howe, 1925), indicating that retinoids are normally required for maintenance of the adult steady state urothelium. Analysis of the adult *RARE-lacZ* mice revealed low numbers of RA-responsive cells (Fig. 6G), suggesting that low levels of RA-signaling are adequate to maintain superficial cell renewal in the steady state urothelium, which has a very slow rate of turnover. To evaluate whether RA-signaling increases in response to injury, *RARE;LacZ* mice were treated with CPP then analyzed during the first 3 days post-treatment, to determine the numbers and distribution of *LacZ*-expressing cells. This analysis revealed a dramatic increase in the *LacZ+* population, which followed similar

kinetics as proliferation (Fig. 6G-H, Fig. S3C). Marker analysis during this 72h period revealed that the majority of *LacZ+* cells were intermediate and S-cells (Fig. 6I,J), suggesting that RA-signaling may be important in in these populations for regeneration after damage. These findings suggest that low levels of RA-signaling maintain steady state urothelial cell renewal, while high levels of RA-signaling may be important for renewal of the superficial layer after injury.

RA-receptors are only active when bound to RA, which is synthesized from retinol (vitamin A) in a temporally and spatially restricted manner by RA-synthesizing enzymes. To identify the source of RA that regulates urothelial formation and regeneration, we performed in situ hybridization analysis to assess the distribution of *Aldh1a2* (hereafter-called *Raldh2*), an enzyme required for RA-synthesis (Niederreither et al., 1999). These experiments reveal that *Raldh2* expression in the bladder is restricted to the mesenchyme just below the urothelium where expression was highest between E12 and E13 (Fig. 6F). In adults, *Raldh2* expression persisted in in the sub-urothelial stroma (Fig. 6L), a domain important for regulating urothelial maintenance and regeneration via Wnt, Bmp and Shh signaling (Mysorekar et al., 2009; Shin et al., 2011). These observations suggest that RA synthesized in the stromal compartment may be important for regulating RA-receptor signaling in the embryonic and adult urothelium.

RA signaling is required for urothelial specification

While the above experiments demonstrated that RA signaling is active in P-cells at a stage in embryonic development when progenitor potential is high, it was still unclear what role, if any, RA signaling has in urothelial specification. To directly address this question, we used Cre-Lox recombination to express *RaraDN*, a dominant inhibitory RA-receptor, in the *Shh*-positive population in the embryo, which our studies indicate contains urothelial progenitors (Fig. 1). To do this, we used the $Shh^{Cre/+}$ line (Harfe et al., 2004) which drives Cre-dependent recombination in more than 90% of cells in the developing urothelium in combination with the *Rosa26 mTmG* reporter [(Muzumdar et al., 2007) ; Fig. S1J)]. *RaraDN* is a truncated form of *Rara* that has been inserted in the *Rosa26* locus downstream of a floxed stop cassette where it is activated by Cre-mediated recombination. Our previous studies demonstrated that expression of the *RaraDN* mutant receptor blocks transcription by endogenous RA-receptors and importantly, we found that defects in embryos expressing 2 copies of the *RaraDN* allele are more severe than those in embryos expressing one allele (Chia et al., 2011; Rosselot et al., 2010), indicating that *RaraDN* inhibits RA-signaling in a dose-dependent manner.

To evaluate whether *RaraDN* is an efficient suppressor of RA-signaling in the urothelium, we generated $Shh^{Cre/+}$; $RaraDN^{+/+}$ mutants expressing two copies of the *RaraDN*, and we examined whether expression of the *RaraDN* led to a reduction in expression of RA-responsive genes. Analysis of E18 $Shh^{Cre/+}$ controls and Shh^{Cre}; $RaraDN^{+/+}$ mutants revealed that *Ret* and *Rarb2*, two genes whose expression is RA-dependent, were down regulated in mutants compared to controls (Fig. S4A,B,D,E). These findings suggested that the *RaraDN* was efficiently inhibiting RA-signaling. For further confirmation, we compared the distribution of RA-responsive cells in mutants and controls using the *RARE-lacZ*

reporter line. Analysis of *Shh^{Cre};RARE-LacZ* controls at E11, revealed large numbers of *LacZ*+ cells (Fig. S4C), however in *Shh^{Cre}; RaraDN;RARE-LacZ* mutants, the number of *LacZ*-expressing cells was greatly reduced (Fig. S4C,F). The observation that *Rarb* and *Ret* are down-regulated in *Shh^{Cre}; RaraDN* mutants, together with the reduction in the numbers of RA-responsive cells in the mutants, suggests that *RaraDN* driven by *Shh^{Cre}* efficiently inhibits RA-signaling in urothelial cells.

In E18 *Shh$^{Cre/+}$* control embryos, the urothelium is fully stratified epithelium containing K5-BCs, one to two layers of I-cells and a layer of mature, multinucleated S-cells (Fig. 7A). However, in 5/7 *Shh^{Cre}; RaraDN$^{+/+}$* mutants examined, there was only a single layer of P63-expressing cells, few if any morphologically distinguishable S-cells and Upk a marker of intermediate and S-cells, was down-regulated (Fig. 7F). On the other hand, K5-BCs expressing Krt5 and P63 lined the basal layer in mutants, suggesting that formation of this population is retinoid-independent (Fig. 7B,G). TEM analysis of the urothelium in controls revealed the prominent apical plaque and fusiform vesicles that are unique features of S-cells (Fig. 7C, black and white arrowheads, mark apical plaque and vesicles, respectively) were absent from S-cells in mutants, which instead displayed microvilli (Fig. 7H, black arrowhead), structures not found on the surface of wild type S-cells. These studies suggest that RA-signaling is normally required for formation of intermediate and S-cells.

We next investigated which cell types normally mediate RA-signaling. To begin to examine the temporal requirement for RA-signaling, we used the TM inducible *Shh^{CreERT2};mTmG* line to express *RaraDN* at E11, when our studies indicate that P-cell progenitors are abundant. *Shh^{CreERT2};mTmG* controls and *Shh^{CreERT2};mTmG*; *RaraDN* mutants were exposed to TM at E11 and analyzed at E18. This analysis revealed a moderate reduction in overall numbers of intermediate and S-cells compared to the controls, and a dramatic reduction in the numbers of *Gfp*-labeled S-cells (Fig. 7K,L), supporting the suggestion that RA-signaling is normally required in urothelial progenitors for formation of S-cells. Since *RaraDN* inhibits RA-signaling in a dose-dependent manner (Blumberg et al., 1997; Damm et al., 1993; Rajaii et al., 2008), the reduced severity of the urothelial phenotype in *Shh^{CreERT2};mTmG*; *RaraDN* mutants compared to the constitutive *Shh^{Cre}; RaraDN$^{+/+}$* line is likely to be due to expression of one vs. two copies of *DN*, respectively.

Impaired superficial cell formation at E18 could indicate a role for retinoids for survival of urothelial progenitors or could indicate a role for RA in specification of urothelial progenitors. We did not detect intermediate or S-cells in *Shh$^{Cre+/-}$; RaraDN$^{+/+}$* mutants at any stage examined (Fig. 7D,I), suggesting that these cell types failed to form. Consistent with this, TUNEL analysis of mutants did not reveal increased apoptosis in the urothelium compared to controls (data not shown). P-cells, the first urothelial cell type, are transiently present in the urothelium between E11 and E13 (Fig. 4). They are distinguishable from endoderm by expression of Upk, and from other urothelial cell types by expression of *Foxa2*, which is down-regulated after E13 (Fig. 2). Immunostaining of E14 *Shh$^{Cre+/-}$* controls revealed undetectable expression of Foxa2, as expected, however E14 *Shh$^{Cre+/-}$; RaraDN$^{+/+}$* mutants contained large numbers of Foxa2 expressing cells which were also positive for Shh and P63, but lack expression of Upk expression or Krt18, Krt20 and other urothelial markers (Fig. 7J and not shown). The persistence of this population expressing

endodermal markers in *Shh*$^{Cre+/-}$*; RaraDN*$^{+/+}$ mutants and absence of intermediate and S-cells, suggests that retinoids may normally be important in endoderm for specification of P-cells.

To directly examine the requirement for RA-signaling in P-cells, we first attempted to use the *Foxa2*^{CreERT2}*;mTmG* line to express a single copy of *RaraDN*, which in *Shh*^{CreERT2}*;mTmG mice* resulted in reduction of the number of lineage tagged S-cells. We were, however, unable to obtain *Foxa2*^{CreERT2}*;mTmG; RaraDN* mutants, most likely due to embryonic lethality, since *Foxa2*^{CreERT2} labels cells in the heart and vasculature which are also regulated by RA-signaling (Li et al., 2012). We therefore used the *Upk3aGCE* line to express the *RaraDN*, which our studies indicate selectively labels P-cells after a TM pulse at E11 (Fig. 4G,H). E11 *Upk3aGCE;mCherry* controls and *Upk3aGCE;mCherry;RaraDN* mutants were exposed to TM and analyzed at E18 to determine the distribution of lineage tagged intermediate and S-cells. Analysis of controls revealed abundant *mCherry* labeled superficial and I-cells as expected (Fig. 7M), however the number of *mCherry*-labeled cells in mutants was greatly reduced (Fig. 7N). This phenotype was virtually identical to that obtained in with *Shh*^{CreERT2}*;mTmG;DN* mutants, which also express one allele *of RaraDN* (compare Fig. 7K and 7L). Together these results suggest that retinoids control urothelial formation by regulating P-cell specification.

RA signaling is required for urothelial regeneration

We found that RA-signaling is selectively up regulated in the urothelium following CPP-induced injury, suggesting that retinoids may control regeneration (Fig. 6). To address this further, we first used the *Shh*^{CreERT2} line, which drives expression in urothelial progenitors, to express the *RaraDN*. *Shh*^{CreERT2}*; RaraDN;mTmG* mutants and *Shh*^{CreERT2}*;mTmG* controls were treated with TM, and CPP was administered after 1 week to induce damage and repair. Controls and mutants were analyzed after 2 weeks to evaluate the distribution of lineage tagged-cells in the CPP-treated urothelium. In animals that had not received CPP, *Gfp*-labeled cells were predominantly in the basal and intermediate layers and a small number of lineage-tagged S-cells were detectable (Fig. 8A,C). Analysis of *Shh*^{CreERT2}*;mTmG* controls after CPP treatment revealed expression of the *Gfp*-lineage tag in 40% of the superficial cell population indicating that these were daughters of *Shh*+ progenitors (Fig. 8B). In *Shh*^{CreERT2}*;RaraDN;mTmG* mutants however, there were 10-fold fewer *Gfp*-labeled S-cells compared to controls, (Fig. 8D). These results indicate that, RA signaling is important in an *Shh*-expressing cell type for urothelial regeneration.

To evaluate the requirement for RA-signaling in I-cells, which our studies suggest are superficial cell progenitors, we performed CPP-induced injury using *Upk3aGCE;mCherry* as a lineage marker. *Upk3aGCE;mCherry* mice were treated with TM then 1 week later with CPP. Edu was administered 48h after CPP treatment to label proliferating cells. Analysis of *Upk3aGCE;mCherry* control mice 2 weeks after CPP treatment revealed extensive *mCherry* labeling in the intermediate and superficial cell populations (Fig. 8E,F). In *Upk3aGCE;*mCherry; *RaraDN* mutants however, the overall numbers of S-cells were reduced by about 40% compared to controls and the proportion of *mCherry*-labeled S-cells was also reduced by nearly 50% (Fig. 8G,H), suggesting that RA-signaling is normally

required in I-cells for regeneration of the adult urothelium. As expected, we did not observe defects in regeneration in *Krt5^{CreERT2};RaraDN* mutants (data not shown).

Previous studies suggest that K5-BCs, which are progenitors in skin and other stratified epithelia, are also progenitors in the adult urothelium (Shin et al., 2011). We show by fate mapping however, that K5-BCs are unlikely to be progenitors either in the embryo or adult regenerating urothelium. Our studies suggest that formation and regeneration of the urothelium depends on distinct progenitor populations: P-cells a transient cell type abundant during early stages of urothelial development, and I-cells that serve as progenitors in the adult regenerating urothelium. We show that retinoids, potent signaling molecules that regulate specification and self-renewal of ES cells and other progenitor cell types, are required in P-cells for their specification during development, and in I-cells for regeneration in response to injury. The identification of novel urothelial progenitors and the observation that urothelial formation and regeneration depends on retinoid signaling in these progenitors could have important implications for tissue engineering and repair. Ultimately, these findings may lead to treatments that prevent loss of the urothelial barrier associated with chronic injury, a major cause of voiding dysfunction and bladder pain syndrome in humans.

DISCUSSION

Recent studies indicate that the adult urothelium contains a population of *Shh*-expressing cells that have long term regenerative potential and these cells have been proposed to be K5-BCs (Kurzrock et al., 2008; Shin et al., 2011; Thangappan and Kurzrock, 2009). Our fate mapping studies, however, suggest that K5-BCs rarely if ever produce intermediate or S-cells and that the intermediate/superficial cell compartment arises from a separate lineage. We show that P-cells, are transient progenitors in the embryonic urothelium, and we show that I-cells are superficial cell progenitors in the regenerating adult urothelium (see model). Retinoids are potent transcriptional regulators that can induce embryonic stem cells to form urothelial cells in vitro (Mauney et al., 2010). Our studies demonstrate that impaired RA-signaling leads to loss of the intermediate and superficial cell populations during development due to failure in P-cell specification, and, and we find that RA-signaling is also important in I-cells in adults, for regeneration after injury. That K5-BCs are unlikely progenitors in the embryo or adult challenges the current thinking, and raises the possibility that other specialized epithelia may develop from novel progenitor populations.

K5-BC cells and the intermediate/superficial populations originate from different lineages

Lineage studies using *Krt5^{CreERT2};mTmG* to permanently label K5-BCs and their daughters indicate that K5-BCs may self-renew, but rarely if ever generate other urothelial cell types. On the other hand, we find that neither P-cells nor I-cells can generate K5-BC daughters in lineage studies, suggesting that the intermediate/superficial cell compartment and K5-BCs are derived from independent lineages. Recent fate mapping studies using a constitutive *P63^{Cre}* line *[DeltaNp63$^{(+/Cre)}$;ROSA26(EYFP)]* to label P63-expressing cells and their daughters, suggest that K5-BCs and the intermediate/superficial cell populations arise from a common progenitor (Pignon et al., 2013). However, since the Cre line is constitutive, it will likely label P63-expressing endodermal population that generates epithelia lining the

digestive, respiratory and genital and urinary tract epithelia. Krt5-expressing cells are present in the urethra at early stages of bladder urogenital sinus development, but are only detected in the bladder after I-cells and S-cells form. Whether urothelial K5-BCs derive from these urethral Krt5-expressing cells is an interesting possibility.

RA-dependent transcription regulates multiple steps of urothelial development and regeneration

The endoderm is patterned along the rostro-caudal axis to generate a number of organs, including the thyroid, thymus, lung, stomach, intestine, pancreas, and the bladder, and retinoids have been shown to be important in endoderm for establishing this regional patterning and cell type specification (Bayha et al., 2009). An example of the multiple functions of RA-signaling in organ formation is the pancreas, where RA acts at the stage of specification (Martin et al., 2005; Molotkov et al., 2005) and at later stages, is required for formation of insulin-producing beta cells (Dalgin et al., 2011; Stafford and Prince, 2002). In addition, RA can also induce stem cells in culture to differentiate into pancreatic cell types culture (Shim et al., 2007). Retinoids may act in a similar manner in the urothelium. Retinoids induce ES cells to differentiate into urothelial cell types (Mauney et al., 2010) and our studies suggest RA signaling controls specification of P-cell progenitors in the embryonic urothelium and I-cell progenitors during regeneration. It would not be surprising if RA is also an important regulator of I- cells in the steady state adult urothelium, a question that we will address in future studies.

Retinoids control pluripotency and specification of progenitors and stem cell populations (Soprano et al., 2007; Wang et al., 2011; Wichterle and Peljto, 2008). Recent studies suggest that this RA regulates the state change from pluripotency/self-renewal to differentiation via an epigenetic mechanism in which RA-binding to Rar/Rxr complexes in regulatory regions of target genes relieves polycomb repression by inducing a conformational change in the RA-receptors [reviewed in: (Gudas and Wagner, 2011)]. This RA-induced conformational change is mediated by the ligand dependent activating domain (AF2) which is deleted in the *RaraDN* mutant receptor, hence it would not be surprising if *RaraDN* expression in urothelial progenitors inhibited their ability to undergo a state change. It will be interesting to determine whether RA signaling acts by positively regulating sets of target genes in urothelial progenitors or by relieving repression. The identification of novel urothelial progenitors whose specification is regulated by retinoids, could have important implications for tissue engineering and repair, and ultimately, may lead to treatments that prevent loss of the urothelial barrier, a major cause of voiding dysfunction and bladder pain syndrome in humans.

EXPERIMENTAL PROCEDURES

Mice

Animals were housed in the animal facility of Irving Cancer Research Center, Columbia University; all animal works were approved by IACUC protocol. Littermates were used for all experiments in which wild type and mutant embryos were compared and n=3 animals were analyzed unless otherwise specified.

Chemical injury

For chemical injury, cyclophosphamide (CPP, Sigma Cat#: C7397) was dissolved in PBS (15 mg/ml) and given to mice at a dose of 150 mg /kg by IP injection.

Proliferation

Mice were injected IP with EdU at dose of 0.1 mg/20g. Proliferating cells were detected on frozen sagittal bladder sections according to the manufacturers protocol (Click-iT® EdU cell proliferation assay kit, Invitrogen Cat#: C-10419).

Histology, immunohistochemistry, and non-radioactive in situ hybridization

Tissues were fixed o/n with 4% PFA. Cryosections were 7μm and 14μm for immunostaining and in situ hybridization, respectively. In situ hybridization analysis with digoxigenin-labeled riboprobes was essentially as described elsewhere (Mendelsohn et al., 1999).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES CITED

Bayha E, Jorgensen MC, Serup P, Grapin-Botton A. Retinoic acid signalingorganizes endodermal organ specification along the entire antero-posterior axis. PLoS ONE. 2009; 4:e5845. [PubMed: 19516907]

Blumberg B, Bolado J Jr. Moreno TA, Kintner C, Evans RM, Papalopulu N. An essential role for retinoid signaling in anteroposterior neural patterning. Development. 1997; 124:373–379. [PubMed: 9053313]

Chia I, Grote D, Marcotte M, Batourina E, Mendelsohn C, Bouchard M. Nephric duct insertion is a crucial step in urinary tract maturation that is regulated by a Gata3-Raldh2-Ret molecular network in mice. Development. 2011; 138:2089–2097. [PubMed: 21521737]

Dalgin G, Ward AB, Hao LT, Beattie CE, Nechiporuk A, Prince VE. Zebrafish mnx1 controls cell fate choice in the developing endocrine pancreas. Development. 2011; 138:4597–4608. [PubMed: 21989909]

Damm K, Heyman RA, Umesono K, Evans RM. Functional inhibition of retinoic acid response by dominant negative retinoic acid receptor mutants. Proceedings of the National Academy of Sciences of the United States of America. 1993; 90:2989–2993. [PubMed: 8096643]

Duester G. Retinoic acid synthesis and signaling during early organogenesis. Cell. 2008; 134:921–931. [PubMed: 18805086]

Farsund T, Dahl E. Cell kinetics of mouse urinary bladder epithelium. III. A histologic and ultrastructural study of bladder epithelium during regeneration after a single dose of cyclophosphamide, with special reference to the mechanism by which polyploid cells are formed. Virchows Archiv B: Cell pathology. 1978; 26:215–223.

Frank DU, Elliott SA, Park EJ, Hammond J, Saijoh Y, Moon AM. System for inducible expression of cre-recombinase from the Foxa2 locus in endoderm, notochord, and floor plate. Developmental

dynamics : an official publication of the American Association of Anatomists. 2007; 236:1085–1092. [PubMed: 17304540]

Gudas LJ, Wagner JA. Retinoids regulate stem cell differentiation. Journal of cellular physiology. 2011; 226:322–330. [PubMed: 20836077]

Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell. 2004; 118:517–528. [PubMed: 15315763]

Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol. 2002; 244:305–318. [PubMed: 11944939]

Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P, Metzger D. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen- inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res. 1999; 27:4324–4327. [PubMed: 10536138]

Jost SP. Cell cycle of normal bladder urothelium in developing and adult mice. Virchows Arch B Cell Pathol Incl Mol Pathol. 1989; 57:27–36. [PubMed: 2567547]

Kashyap V, Gudas LJ, Brenet F, Funk P, Viale A, Scandura JM. Epigenomic reorganization of the clustered Hox genes in embryonic stem cells induced by retinoic acid. J Biol Chem. 2011; 286:3250–3260. [PubMed: 21087926]

Khandelwal P, Abraham SN, Apodaca G. Cell Biology and Physiology of the Uroepithelium. Am J Physiol Renal Physiol. 2009

Kurzrock EA, Lieu DK, Degraffenried LA, Chan CW, Isseroff RR. Label-retaining cells of the bladder: candidate urothelial stem cells. Am J Physiol Renal Physiol. 2008; 294:F1415–1421. [PubMed: 18367656]

Lewis PM, Gritli-Linde A, Smeyne R, Kottmann A, McMahon AP. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. Dev Biol. 2004; 270:393–410. [PubMed: 15183722]

Li P, Pashmforoush M, Sucov HM. Mesodermal retinoic acid signaling regulates endothelial cell coalescence in caudal pharyngeal arch artery vasculogenesis. Dev Biol. 2012; 361:116–124. [PubMed: 22040871]

Liang FX, Bosland MC, Huang H, Romih R, Baptiste S, Deng FM, Wu XR, Shapiro E, Sun TT. Cellular basis of urothelial squamous metaplasia: roles of lineage heterogeneity and cell replacement. J Cell Biol. 2005; 171:835–844. [PubMed: 16330712]

Martin M, Gallego-Llamas J, Ribes V, Kedinger M, Niederreither K, Chambon P, Dolle P, Gradwohl G. Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. Dev Biol. 2005; 284:399–411. [PubMed: 16026781]

Mauney JR, Ramachandran A, Yu RN, Daley GQ, Adam RM, Estrada CR. All-trans retinoic acid directs urothelial specification of murine embryonic stem cells via GATA4/6 signaling mechanisms. PloS one. 2010; 5:e11513. [PubMed: 20644631]

Mendelsohn C, Batourina E, Fung S, Gilbert T, Dodd J. Stromal cells mediate retinoid-dependent functions essential for renal development. Development. 1999; 126:1139–1148. [PubMed: 10021334]

Molotkov A, Molotkova N, Duester G. Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn. 2005; 232:950–957. [PubMed: 15739227]

Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis. 2007; 45:593–605. [PubMed: 17868096]

Mysorekar IU, Isaacson-Schmid M, Walker JN, Mills JC, Hultgren SJ. Bone morphogenetic protein 4 signaling regulates epithelial renewal in the urinary tract in response to uropathogenic infection. Cell Host Microbe. 2009; 5:463–475. [PubMed: 19454350]

Niederreither K, Dolle P. Retinoic acid in development: towards an integrated view. Nat Rev Genet. 2008; 9:541–553. [PubMed: 18542081]

Niederreither K, Subbarayan V, Dolle P, Chambon P. Embryonic retinoic acid synthesis is essential for early mouse post- implantation development [see comments]. Nat Genet. 1999; 21:444–448. [PubMed: 10192400]

Pignon JC, Grisanzio C, Geng Y, Song J, Shivdasani RA, Signoretti S. p63-expressing cells are the stem cells of developing prostate, bladder, and colorectal epithelia. Proc Natl Acad Sci U S A. 2013; 110:8105–8110. [PubMed: 23620512]

Rajaii F, Bitzer ZT, Xu Q, Sockanathan S. Expression of the dominant negative retinoid receptor, RAR403, alters telencephalic progenitor proliferation, survival, and cell fate specification. Dev Biol. 2008; 316:371–382. [PubMed: 18329011]

Rossant J, Zirngibl R, Cado D, Shago M, Giguere V. Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev. 1991; 5:1333–1344. [PubMed: 1907940]

Rosselot C, Spraggon L, Chia I, Batourina E, Riccio P, Lu B, Niederreither K, Dolle P, Duester G, Chambon P, et al. Non-cell-autonomous retinoid signaling is crucial for renal development. Development. 2010; 137:283–292. [PubMed: 20040494]

Samarut E, Rochette-Egly C. Nuclear retinoic acid receptors: conductors of the retinoic acid symphony during development. Mol Cell Endocrinol. 2012; 348:348–360. [PubMed: 21504779]

Shim JH, Kim SE, Woo DH, Kim SK, Oh CH, McKay R, Kim JH. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia. 2007; 50:1228–1238. [PubMed: 17457565]

Shin K, Lee J, Guo N, Kim J, Lim A, Qu L, Mysorekar IU, Beachy PA. Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. Nature. 2011; 472:110–114. [PubMed: 21389986]

Soprano DR, Teets BW, Soprano KJ. Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. Vitamins and hormones. 2007; 75:69–95. [PubMed: 17368312]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–71. [PubMed: 9916792]

Stafford D, Prince VE. Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr Biol. 2002; 12:1215–1220. [PubMed: 12176331]

Thangappan R, Kurzrock EA. Three clonal types of urothelium with different capacities for replication. Cell Prolif. 2009; 42:770–779. [PubMed: 19765021]

Wang W, Yang J, Liu H, Lu D, Chen X, Zenonos Z, Campos LS, Rad R, Guo G, Zhang S, et al. Rapid and efficient reprogramming of somatic cells to induced pluripotent stem cells by retinoic acid receptor gamma and liver receptor homolog 1. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:18283–18288. [PubMed: 21990348]

Wells JM, Melton DA. Vertebrate endoderm development. Annual review of cell and developmental biology. 1999; 15:393–410.

Wichterle H, Peljto M. Differentiation of mouse embryonic stem cells to spinal motor neurons. Current protocols in stem cell biology Chapter. 2008; 1 Unit 1H 1 1-1H 1 9.

Wolbach SB, Howe PR. Tissue changes following deprivation of fat-soluble A vitamin. J Exp Med. 1925; 42:753–777. [PubMed: 19869087]

Wu XR, Kong XP, Pellicer A, Kreibich G, Sun TT. Uroplakins in urothelial biology, function, and disease. Kidney Int. 2009; 75:1153–1165. [PubMed: 19340092]

Wyndaele JJ, De Wachter S. The basics behind bladder pain: a review of data on lower urinary tract sensations. Int J Urol. 2003; 10(Suppl):S49–55. [PubMed: 14641415]

Highlights

- The urothelium regenerates, but can be damaged by chronic exposure to toxins or UTI
- We identify progenitors critical for urothelial formation and regeneration
- Development and repair utilize two separate populations of progenitors
- RA is critical in urothelial progenitors for specification and regeneration

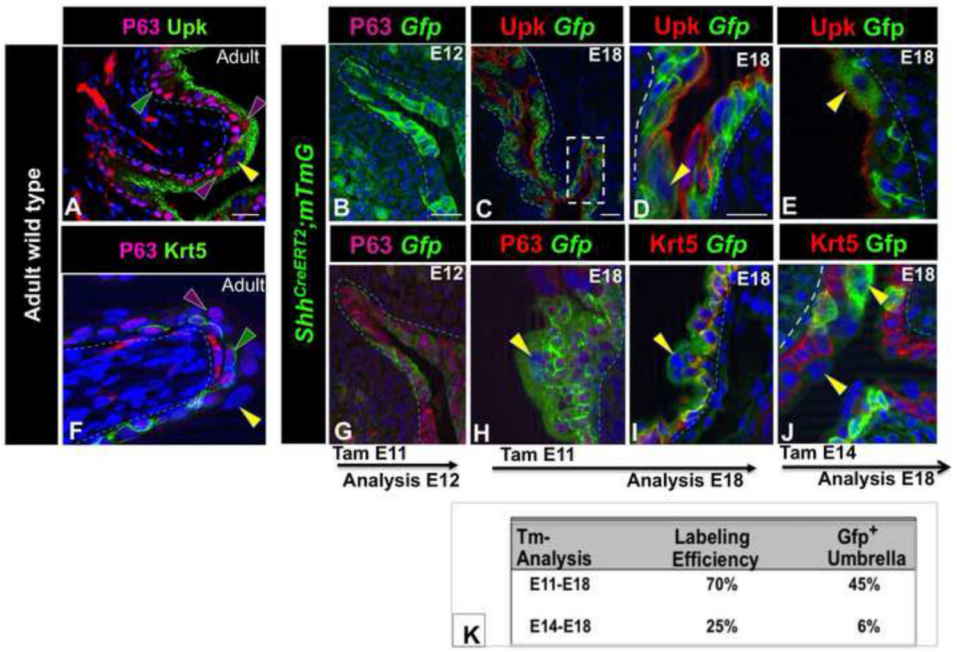

Figure 1. *Shh*-expressing cells are progenitors in the developing urothelium

A. A section from an adult urothelium stained with Uroplakin (Upk; green) and P63 (pink). B. A section from an E12 *Shh*^{CreERT2}*;mTmG* embryo treated with TM at E11. C. Upk-expression (red) in an E18 *Shh*^{CreERT2}*;mTmG* embryo exposed to TM on E11. D. Higher magnification of C. E. Upk expression (red) in a section from an E18 *Shh*^{CreERT2}*;mTmG* embryo exposed to TM on E14. F. A section from an adult urothelium stained with Krt5 (green) and P63 (pink). G. P63 expression in the urothelium from an E12 *Shh*^{CreERT2}*;mTmG* embryo treated with TM on E11. H. P63-expression (pink) in an E18 *Shh*^{CreERT2}*;mTmG* embryo exposed to TM on E11. I. Krt5-expression (red) in an E18 *Shh*^{CreERT2}*;mTmG* embryo exposed to TM on E11. J. Krt5-expression (red) in an E18 *Shh*^{CreERT2}*;mTmG* embryo exposed to TM on E14. K. A table showing the labeling efficiency after TM treatment at E11 vs. E14, and the percentage of S-cells in E18 *Shh*^{CreERT2}*;mTmG* embryos expressing the *Gfp* lineage tag 7 days and 4 days after TM treatment, respectively. Yellow arrowheads: S-cells, Green arrowheads, K5-BCs, purple arrowheads; I-cells. *mTmG* Gfp-positive cells are green. **Magnifications:** A,B,C,G,F 20X ; D,E, H,I,J 40X . All scale bars are 50μm. (See Also Figure S1).

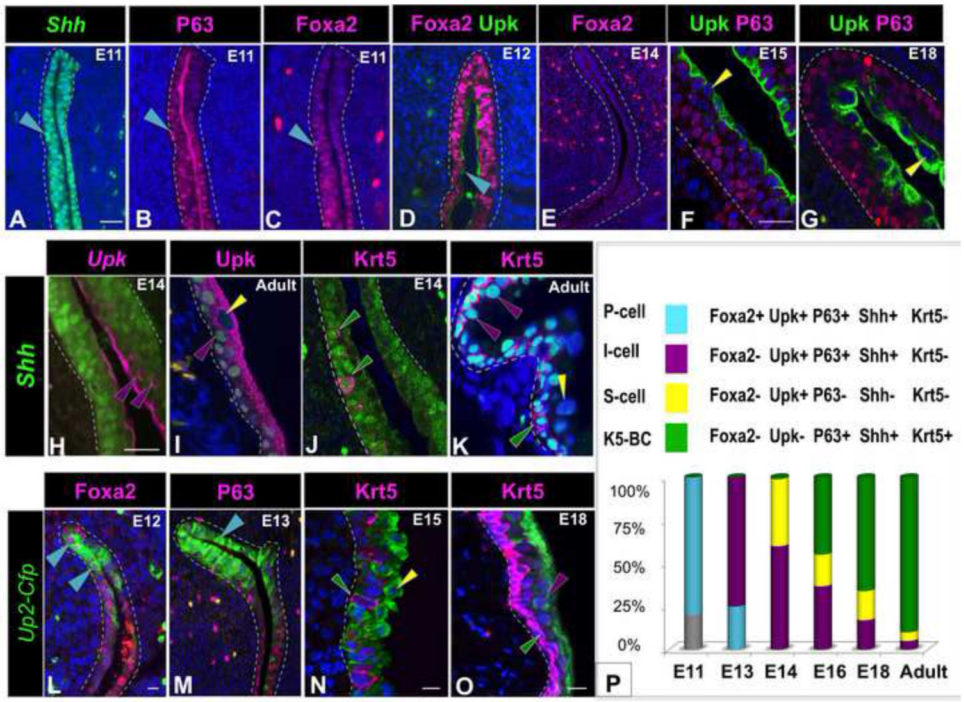

Figure 2. The *Shh*-population contains multiple cell types

A. A section from an E11 $Shh^{Gfp/Cre}$ embryo immunostained for expression of *Gfp* (green nuclear staining). B. A serial section from the same embryo as in (A) stained for expression of P63 (pink). C. A serial section from the same embryo as in (A) stained for expression of Foxa2. D. A section from an E12 embryo showing P-cells expressing Foxa2 (pink) and Upk (green) expression. E. A section from an E14 embryo stained with Foxa2 antibody (pink) which is undetectable. F. A section from an E14 embryo stained for expression of Upk (green) and P63 (pink). G. A section from the urothelium of an E18 embryo stained for expression of P63 (pink) and Upk (green). H. A section from an E14 $Shh^{Gfp/Cre}$ embryo (*Gfp* is green nuclear staining) stained for expression of Upk (pink). I. A section from an adult Shh^{nlacZ} embryo (*nlacZ* is green nuclear staining) stained with Upk antibody (pink). J. A section from an E14 $Shh^{Gfp/Cre}$ embryo (*Gfp* is green nuclear staining) stained for expression of Krt5 (pink). K. A section from an adult Shh^{nlacZ} embryo (*nlacZ* is green nuclear staining) stained for expression Krt5 (pink). L. A section from an E12 *Up2-Cf*p embryo (*Cfp* detected with anti-*Gfp* antibody is shown in green) stained for expression of Foxa2 (pink). M. A section from a E13 *Up2-Cfp* embryo stained for expression of P63 (pink). *Cfp* detected with anti-*Gfp* antibody is shown in green. N. A section from an E15 *Up2-Cfp* embryo stained for expression of Krt5 (*Cfp* detected with anti-*Gfp* antibody is shown in green). O. A section from an E18 *Up2-Cfp* embryo stained with Krt5 antibody (pink). *Cfp* detected with anti-*Gfp* antibody is shown in green. P. A schematic showing the color code for different urothelial cell types and the relative proportions in the embryonic and adult urothelium. In this and subsequent figures: S-cells are marked with yellow arrowheads, I-cells with purple arrowheads, K5-BCs with green arrowheads, and P-cells, with blue-green arrowheads. **Magnifications:** A-D; L,M 20X; E 10X; F,G 40X; H-K 40X ;N ,O x40. Scale bars: 50μm. (See also Figure S2).

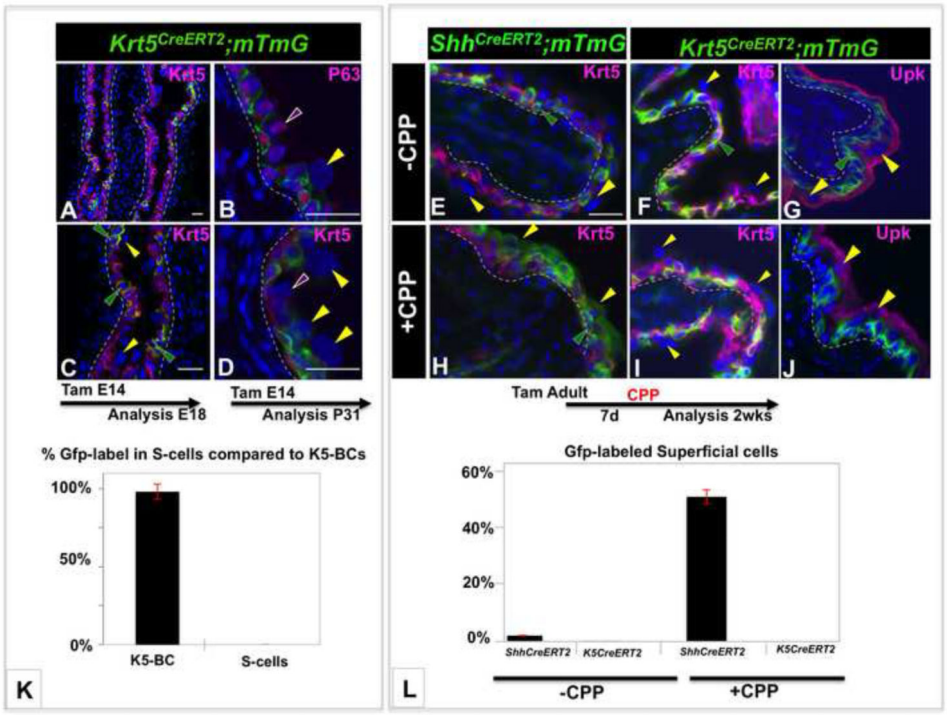

Figure 3. K5-BCs are unlikely to be urothelial progenitors

A-D. Lineage studies in the embryonic urothelium using the *Krt5^{CreERT2};mTmG* line to follow the fate of K5-BCs. A. section from a *Krt5^{CreERT2};mTmG* E18 embryo exposed to TM at E14 stained for expression of Krt5 (pink). Cells expressing the *Gfp*-lineage marker detected with Gfp antibody are green. B. A section from a *Krt5^{CreERT2};mTmG* embryo exposed to TM at E14 and analyzed 1 month later stained for expression of P63 (pink). Cells expressing the *Gfp*-lineage marker detected with Gfp antibody are green C. A higher magnification of the section in (A). D. A section from a *Krt5^{CreERT2};mTmG* embryo exposed to TM at E14 and analyzed after 1 month stained for expression of Krt5 (pink). Cells expressing the *Gfp*-lineage marker detected with Gfp antibody are green. E. A section from a TM treated adult *Shh^{CreERT2};mTmG* mouse that did not receive CPP, stained for expression of Krt5 (pink). Cells expressing the *Gfp*-lineage marker detected with Gfp antibody are green. F-G. Sections from adult TM treated *Krt5^{CreERT2};mTmG* mice that did not receive CPP, stained for expression of Krt5 (pink in F) and Upk (pink in G). H. A section from a TM treated adult *Shh^{CreERT2};mTmG* mouse analyzed 2 weeks after CPP administration stained for expression of Krt5 (pink). I-J. Sections from a TM treated adult *Krt5^{CreERT2};mTmG* mouse stained for expression of Krt5 (pink in I) or Upk (pink in J) 2 weeks after CPP treatment. Cells expressing the *Gfp*-lineage marker detected with Gfp antibody are green K. A graph showing the distribution of lineage tagged cells in the K5-BC and superficial compartments in *Krt5^{CreERT2};mTmG* mice exposed to TM on 14, *in utero* and analyzed at E18 or P31. L A graph showing a comparison of lineage tracing studies in *Krt5^{CreERT2};mTmG* and *Shh^{CreERT2};mTmG* mice with and without CPP treatment. S-cells are marked with yellow arrowheads, I-cells with purple arrowheads, K5-BCs with green arrowheads. For quantification, a minimum of three independent experiments were

performed, and the average ± SEM was plotted. **Magnifications:** A 20X ; C 40X; B,D 40X, E-J 30X. Scale bars: 50μm. (See also Figure S3).

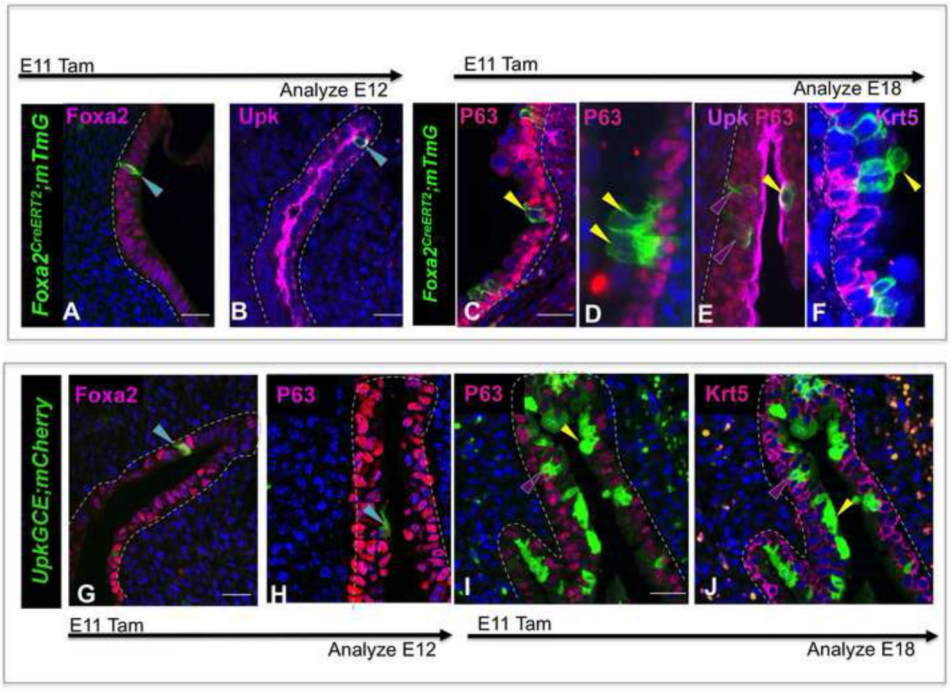

Figure 4. P-cells are a transient progenitor population in the embryonic urothelium

A-F. Lineage tracing with the *Foxa2^{CreERT2};mTmG line.* A. Expression of the Gfp lineage tag (detected with antibody, green) in P-cells stained for expression of Foxa2 cells (pink) in an E12 embryo, 24h after TM exposure on E11. B. Expression of the Gfp lineage tag (detected with antibody, green) in P-cells expressing Upk (pink) in an E12 embryo 24h after TM exposure on E11. C. A section from an E18 *Foxa2^{CreERT2};mTmG* embryo exposed to TM on E11 stained for expression of P63 (pink). D. A section from an E18 *Foxa2^{CreERT2};mTmG* embryo exposed to Tam at E11 stained for expression of Upk (pink) and P63 (red). E A section from an E18 *Foxa2^{CreERT2};mTmG* embryo exposed to Tam on E11stained for expression of Krt5 (pink). F. A section from an E18 *Foxa2^{CreERT2};mTmG* embryo exposed to Tam at E11, stained with Krt5 (pink) showing a cluster of lineage-marked cells (detected with Gfp antibody, green). G. Expression of the *mCherry* lineage tag (green, detected with an antibody directed against Rfp) in P cells in an E12 *Upk3aGCE;mCherry* embryo exposed to Tamoxifen on E11 and stained for expression of Foxa2 (pink). H. Expression of the *mCherry* lineage tag (green, detected with an antibody directed against Rfp) in P cells in an E12 *Upk3aGCE;mCherry* embryo exposed to Tamoxifen on E11 and stained for expression of P63 (pink). I. Expression of the *mCherry* lineage tag (green, detected with an antibody directed against Rfp) in intermediate and superficial cells in an E18 *Upk3aGCE;mCherry* embryo exposed to Tamoxifen on E11 and stained for expression of P63 (pink). J. Expression of the *mCherry* lineage tag (green, detected with an antibody directed against Rfp) in intermediate and superficial cells in an E18 *Upk3aGCE;mCherry* embryo exposed to Tamoxifen on E11 and stained for expression of Krt5 (pink). **Magnifications**: A,B, G 20X ; H 40X;C,D 20X;E,20X and F,2X. Scale bars: 50μm.

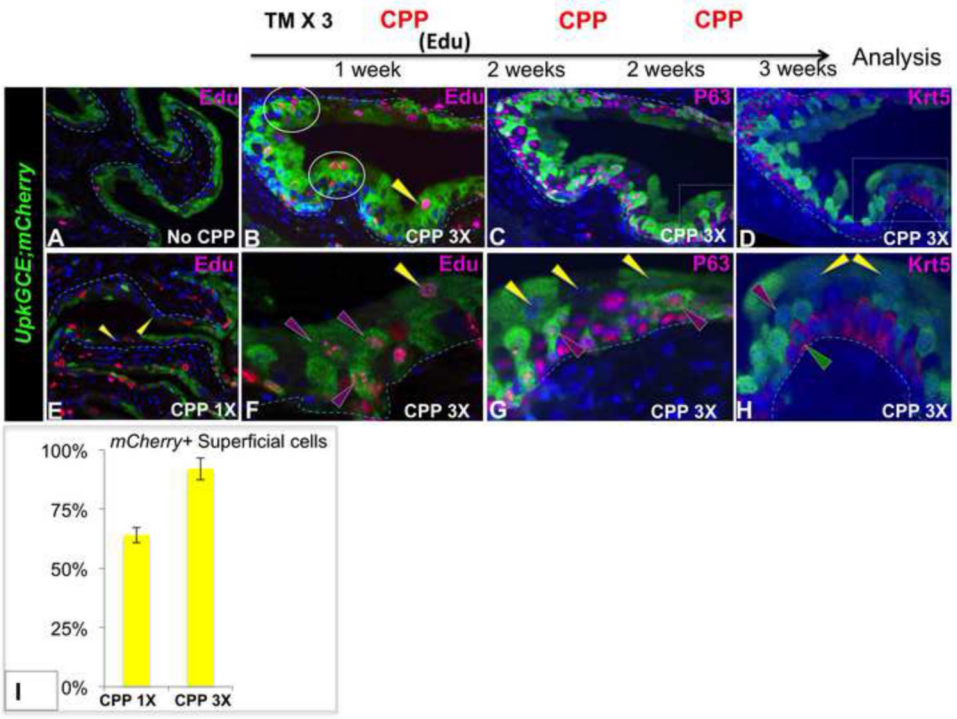

Figure 5. I-cells are a superficial progenitor population in adults

A. A section showing the urothelium of a *Upk3aGCE;mCherry* adult that did not receive CPP *mCherry* detected with an antibody directed against Rfp is shown in green and Edu-expressing cells are pink. B-H Sections from an *Upk3aGCE;mCherry* adult after 3 rounds of CPP-induced damage and repair. (B): Stained with *Edu and mCherry* (C) Stained with P63 and *mCherry* (D) Stained with Krt5 and *mCherry*. E. A section of an *Upk3aGCE;mCherry* adult after 1 round of CPP-induced damage and repair, showing the distribution of *mCherry* expression (green) and Edu (pink). F,G,H, Higher magnification of B,C,D, respectively. For quantification, a minimum of three independent experiments were performed, and the average ± SEM was plotted. **Magnifications**: A,E 20X; B,C,D 20X and 2x zoomed; F,G,H 40X and 2X zoomed. Scale bars 50uM.

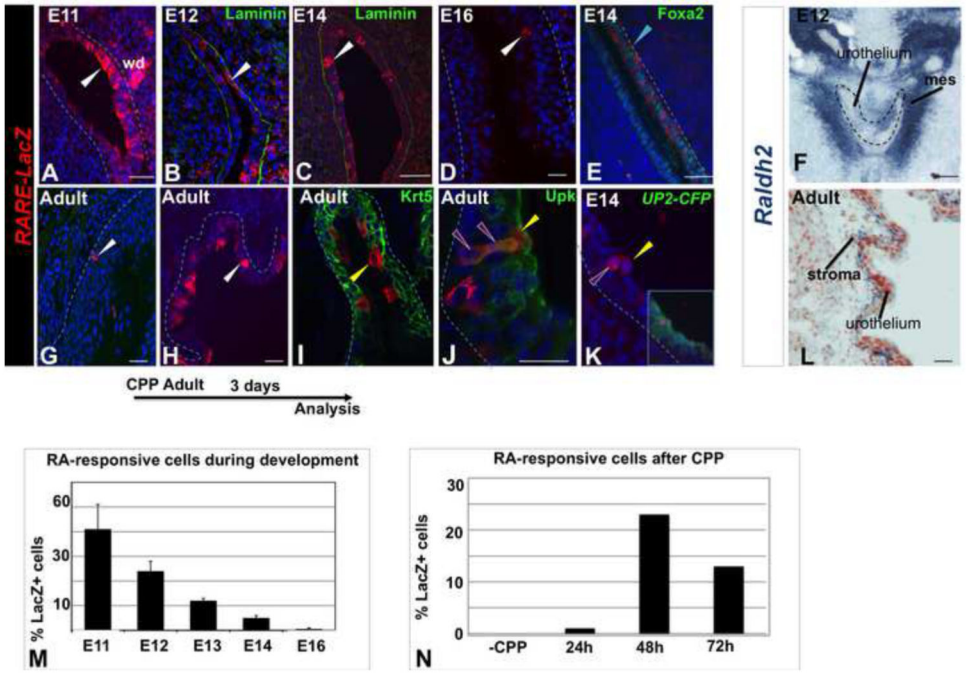

Figure 6. RA-signaling is selectively up-regulated in the embryonic urothelium and in the adult urothelium during regeneration

A. A section showing the urothelium in an E11 *RARE-lacZ* reporter embryo showing *LacZ* expression (red) detected with antibody staining. B. A section showing the distribution of RA-responsive cells in the urothelium of an E12 *RARE-lacZ* reporter (red). C. A section from an E14 *RARE-lacZ* reporter embryo stained for *lacZ* expression (red). D. A section from an E12 *RARE-lacZ* reporter embryo stained for *lacZ* expression (red) and Foxa2 (green). E. A section from an E14 *Up2-Cfp;RARE-lacZ* reporter embryo stained for *lacZ* expression (red) and *Up2-Cfp* (green). F. In situ hybridization showing expression of *Raldh2* in sub-urothelial mesenchyme in an E12 embryo. G. A section from the urothelium of adult *RARE-lacZ* reporter mouse stained for *lacZ* expression (red). H. G. A section from the urothelium of adult *RARE-lacZ* reporter mouse 48h after administration of CPP stained for *lacZ* expression (red) and Krt5 (green). I. A section from the urothelium of adult *RARE-lacZ* reporter mouse 48h after administration of CPP, stained for *lacZ* expression (red) and Upk (green). K. Same section as in (E) showing only the red channel. L. In situ hybridization showing expression of *Raldh2* in sub-urothelial stroma in a wild type adult mouse. For quantification, a minimum of three independent experiments were performed, and the average ± SEM was plotted.**Magnifications:** A –C,F 20X; D,E. Scale bars: 50μm

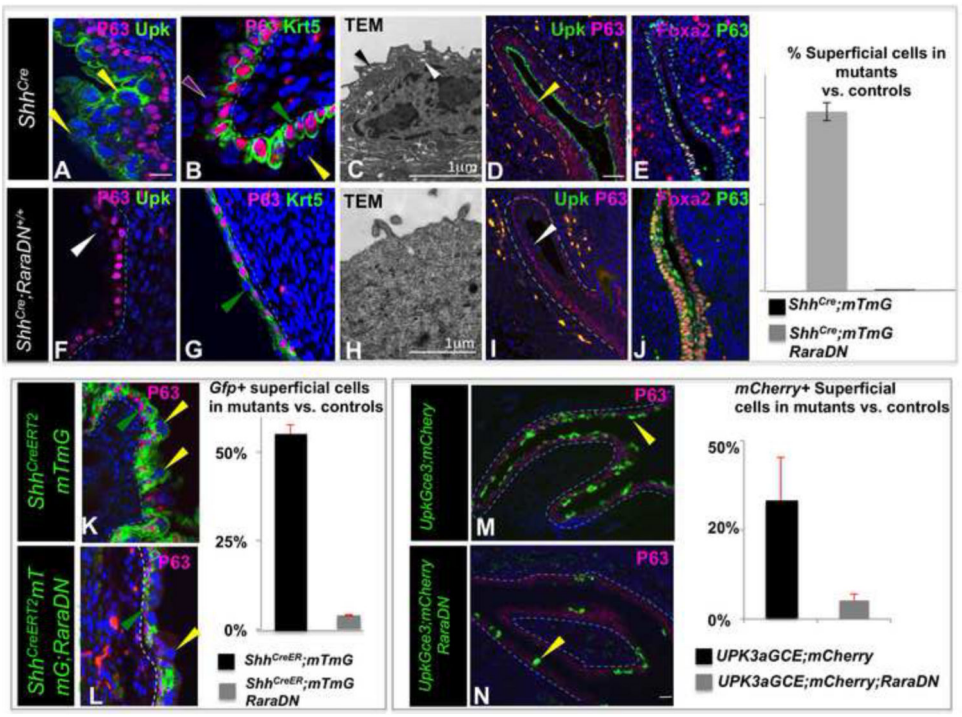

Figure 7. Retinoids are required for urothelial formation

A. P63 (pink) and Upk (green) staining in an E18 control *Shh*$^{Cre/+}$ embryo. B. Krt5 (green) and P63 (pink) staining in the urothelium of an E18 control *Shh*$^{Cre/+}$ embryo. C. Transmission electron microscopy showing the apical surface of an *Shh*$^{Cre/+}$ embryo. D. A section from an E14 *Shh*$^{Cre/+}$ control embryo stained with Upk (green) and P63 (pink). E. A section from an E14 *Shh*$^{Cre/+}$ embryo stained with Foxa2 (pink) and P63 (green). F. P63 (pink) and Upk (green) staining in a section from an E18 *Shh*$^{Cre/+}$; *RaraDN* mutant embryo. G. Krt5(green) and P63 (pink) staining in the urothelium of an E18 *Shh*$^{Cre/+}$*; RaraDN* mutant embryo. H. Transmission electron microscopy showing the apical surface of an E18 *Shh*$^{Cre/+}$*; RaraDN* mutant urothelial cell. I. A section from an E14 *Shh*$^{Cre/+}$*;RaraDN* mutant embryo stained with Upk (green) and P63 (pink). J. A section from an E14 *Shh*$^{Cre/+}$*;RaraDN* mutant embryo stained with Foxa2 (pink) and P63 (green). K. P63 (pink) staining in an E18 control *Shh*^{CreERT2}*;mTmG* embryo exposed to TM on E11 (the *Gfp* lineage-tag is green). L. P63 (pink) staining in an E18 *Shh*^{CreERT2}*;mTmG;RaraDN* mutant embryo exposed to TM on E11 (the *Gfp* lineage-tag is green). M. P63 (pink) staining in an E18 *Upk3aGCE;mCherry* control embryo exposed to TM on E11 (*mCherry* is shown in green). N. P63 (pink) staining in an E18 *Upk3aGCE;mCherry;RaraDN* mutant embryo exposed to TM on E11 (*mCherry* is shown in green). For quantification, a minimum of three independent experiments were performed, and the average ± SEM was plotted. Magnifications: A,B,D,E,F,G,I,J 20X; C,H 31,000X; KL, 40X; M,N 20X. Scale bars: 50μm. (See also S4).

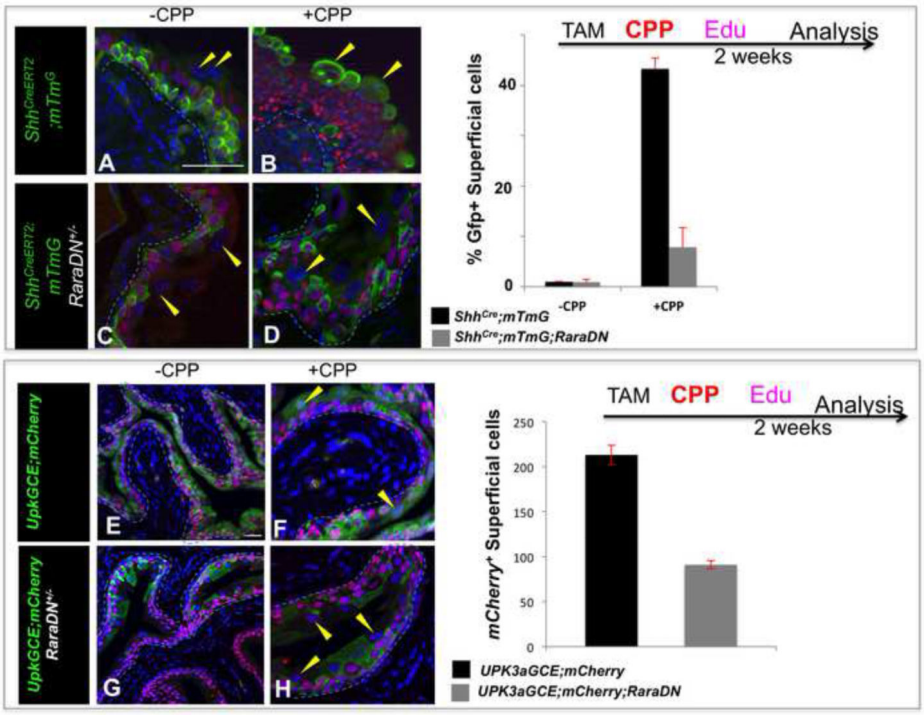

Figure 8. Retinoids are required for urothelial regeneration

A. P63 expression in a control *Shh*^{CreERT2}*;mTmG* adult that has not received CPP. B. P63 expression (pink) in a *Shh*^{CreERT2}*;mTmG* adult analyzed 2 weeks after CPP treatment. C. P63 expression in a mutant *Shh*^{CreERT2}*;mTmG; RaraDN* adult that has not received CPP. D. P63 (pink) expression in a CPP-treated *Shh*^{CreERT2}*;mTmG;RaraDN* mutant adult analyzed 2 weeks after CPP treatment. E. P63 expression (pink) in a control *Upk3aGCE;mCherry* adult that has not received CPP (*mCherry* is shown in green). F. P63 (pink) expression in a CPP-treated *Upk3aGCE;mCherry* adult analyzed 2 weeks after CPP treatment. *mCherry* is shown in green. G. P63 (pink) expression in a *Upk3aGCE;mCherry;RaraDN* mutant adult that did not receive CPP. *mCherry* is shown in green. H. P63 (pink) expression in a *Upk3aGCE;mCherry RaraDN* mutant 2 weeks after CPP treatment. *mCherry* is shown in green. For quantification a minimum of three independent experiments were performed, and the average ± SEM was plotted. **Magnifications:** A-H 20X. Scale bars 50μm.