Higher-Order Kidney Organogenesis from Pluripotent Stem Cells

Graphical Abstract

Highlights

- Functional and molecular profiling reveals insights into in vivo UB maturation
- Induction of UB from PSCs and nephron progenitors requires distinct cues
- Induced UB and MM generate kidney organoids with higher-order architecture
- Induced UBs are useful tools to dissect roles of genes in human kidney development

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In Brief
Taguchi and Nishinakamura define signals that specifically induce ureteric bud and nephron progenitor lineages from PSCs. Co-culturing these progenitors can generate organoids that mimic organotypic architecture of the embryonic kidney with nephrons interconnected by branched epithelium, showing that higher-order organogenesis can be recapitulated in PSC-derived organoids.

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Higher-Order Kidney Organogenesis from Pluripotent Stem Cells

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SUMMARY

Organogenesis generates higher-order structures containing functional subunits, connective components, and progenitor niches. Despite recent advances in organoid-based modeling of tissue development, recapitulating these complex configurations from pluripotent stem cells (PSCs) has remained challenging. In this study, we report assembly of kidney organoids that recapitulate embryonic branching morphogenesis. By studying the distinct origins and developmental processes of the ureteric bud, which contains epithelial kidney progenitors that undergo branching morphogenesis and thereby plays a central role in orchestrating organ geometry, and neighboring mesenchymal nephron progenitors, we established a protocol for differential induction of each lineage from mouse and human PSCs. Importantly, reassembled organoids developed the inherent architectures of the embryonic kidney, including the peripheral progenitor niche and internally differentiated nephrons that were interconnected by a ramified ureteric epithelium. This selective induction and reassembly strategy will be a powerful approach to recapitulate organotypic architecture in PSC-derived organoids.

INTRODUCTION

Recent progress in biology has enabled the induction of various types of functional organ subunits from pluripotent stem cells (PSCs). In particular, strategies employing the cellular “self-organization” phenomenon have enabled successful generation of three-dimensional (3D) “organoids” in a dish (Lancaster and Knoblich, 2014; Sasai, 2013). However, most of the currently available organoids lack module-module connections and a progenitor niche, namely, the “higher-order structure” of the embryonic organ essential for development of the systemic organ anatomy and functions. Thus, we focused on innate branching morphogenesis by epithelial tissue, which plays a critical role in orchestrating organ geometries (Ochoa-Espinosa and Affolter, 2012).

A rudiment of the kidney, the embryonic metanephros, develops by mutual interaction of the metanephric mesenchyme (MM; including nephron progenitors [NPs] and stromal progenitors [SPs]) and the ureteric bud (UB) (Costantini and Kopan, 2010). The UB undergoes branching morphogenesis to form urine-collecting systems, and the tips of the UB signal to maintain undifferentiated NPs and induce differentiation of a subset of NPs. In this process, a transient Wnt signal from the UB induces mesenchymal-to-epithelial transition (MET) of NPs, and each epithelialized nephron then attaches to the UB tips for connection to the collecting duct. In turn, the undifferentiated NPs produce Gdnf to maintain UB tip proliferation, and the surrounding cortical SPs support ureteric branching by maintaining Ret receptor tyrosine kinase expression in the UB tips. This triad interaction enables concomitant NP maintenance and differentiation, thereby producing millions of nephrons with systemic connections. Hence, the roles of the UB, including dichotomous branch formation, NP maintenance, and NP differentiation, are essential for organ-scale kidney morphogenesis.

Recently, several groups have reported induction of the renal lineage from PSCs. We and another group demonstrated selective induction of the NP lineage (Taguchi et al., 2014; Morizane et al., 2015). Other groups have shown the derivation of a UB-like population by selective (Xia et al., 2013) or simultaneous (Takasato et al., 2015) induction with NP and SP populations. Most protocols that aimed to include the UB lineage resulted in epithelial nephron-like structure formation to a certain extent (Taguchi et al., 2014; Morizane et al., 2015; Takasato et al., 2015). However, the induced UB-like cells did not show branching morphogenesis and the UB induction/maintenance capacity was not proved, and therefore the inter-nephron connection by the collecting ducts was lacking (Xia et al., 2013; Takasato et al., 2015). These findings suggest that the currently available UB induction protocols are not sufficient to induce a functional UB, which could be partly due to the lack of precise knowledge about the differentiation signals for the early-stage UB lineage (Costantini and Kopan, 2010).

Previously, we identified spatiotemporally distinct origins of the UB and the MM (NP+SP) (Taguchi et al., 2014; Taguchi and Nishinakamura, 2015). The UB differentiates from the T+ immature mesoderm at embryonic day (E) 7.5. Subsequently, at E8.5, the immature mesoderm becomes the Osr1+/Lhx1+/Pax2+/T- anterior intermediate mesoderm (AIM). The anteriorly located committed UB lineage precursors extend and migrate caudally to form an elongated epithelial tube, the Wolffian
Figure 1. Wolffian Duct Acquires a Branching Capacity by Developmental Maturation

(A) Previously proposed model for kidney lineage specification.

(B) Schematic of the dissociation/reaggregation assay.

(C) Schematic of the WD developmental process. The parts of the WD used for the reconstruction assays or microarray analyses are outlined by the dashed lines. a, anterior part of WD; p, posterior part of WD.

(D) Fluorescence images of reconstructed kidney tissue at the indicated stages of WD or UB development. WD, Wolffian duct; WDa, anterior Wolffian duct; WDp, posterior Wolffian duct.

(E) Fluorescence images of reconstructed kidney tissue at the indicated stages of WD or UB development. WD, Wolffian duct; WDa, anterior Wolffian duct; WDp, posterior Wolffian duct.

(F) Trends of expression levels for UB lineage markers, Tip markers, and Maturation markers during WD development.

(legend continued on next page)
duct (WD). The precursor of the NP is maintained in the caudal T^+ immature state longer than the UB lineage, at least up to E8.5, and then differentiates into the Osr1^+ /Wt1^+ /Hox11^+ posterior intermediate mesoderm (PIM) at E9.5 (Figure 1A). These results suggested that the period in the T^+ state determines the anteroposterior positioning within the intermediate mesoderm, the precursor domain of the urogenital system. Indeed, we succeeded in selective induction of metanephric NPs from PSCs (Taguchi et al., 2014), by recapitulating the prolonged exposure to Wnt signals essential to induce and maintain the T^+ nascent mesoderm (Wilson et al., 2009). Consistent with our findings, a recent report showed preferential induction of an AIM-like population by shortening the exposure period to Wnt signaling (Takasato et al., 2015). However, the signal that distinguishes the AIM and PIM besides the Wnt exposure period has remained unclear. Furthermore, it remains to be elucidated how the AIM undergoes MET to differentiate into the epithelialized WD and finally mature to form the UB that possesses the branching capacity.

Thus, we addressed the WD developmental process using in vivo analysis and in vitro directed differentiation systems, to identify the key signals that are sufficient to induce the UB from PSCs. To develop our multistep protocol, we used the reverse induction approach established in our previous NP lineage induction study (Taguchi et al., 2014). In brief, we first focused on the latest maturation stage of UB development (from E9.5 WD to E11.5 UB) and then focused on an earlier stage (from E8.75 WD progenitor to E9.5 WD) using sorted embryonic precursors. Finally, the differentiation factors from the pluripotent state to the E8.75 WD progenitor stage were examined by employing mouse embryonic stem cells (mESCs).

### RESULTS

**WD Acquires a Robust Branching Capacity by Developmental Maturation**

To evaluate the functional maturation process of the early-stage WD, we employed the Hoxb7-GFP transgenic mouse line (Srinivas et al., 1999) and established a kidney reconstruction assay by modifying previously reported methods (Auerbach and Grobstein, 1958; Ganeva et al., 2011; Grobstein, 1953). E11.5 MMs, including NPs and SPs, were reaggregated with a WD or UB from E9.5, E10.5, and E11.5 stage embryos (Figures 1B, 1C, and S1A). The UB or WD from the E11.5 embryo showed robust branch formation, whereas the WD from E10.5 and E9.5 embryos showed less branch formation (Figures 1D and 1E). Intriguingly, the branch numbers did not differ significantly between the WD at the caudal and rostral parts of the E10.5 or E11.5 embryos (Figure 1E). These results suggest that the functional MM, but not the in situ neighboring mesonephric mesenchyme, induces branching morphogenesis to the WD. Furthermore, the branching capacity, which is retained regardless of the anteroposterior position in the WD, is acquired during developmental progression.

To identify markers that can monitor these maturation processes, we performed gene expression array analyses for each stage of the UB, WD, and progenitors from E8.75 to E11.5 (Figures 1C, 1F, and S1A). Non-biased clustering and similar entity analyses of representative UB marker genes identified several groups showing different gene expression kinetics (data not shown; Figure S1C). One group containing many key transcription factors with roles in early WD development (Pax2, Lhx1, Emx2, Sim1, and Gata3) was enriched in the UB lineage and already expressed in the E8.75 WD progenitor. These factors were maintained regardless of the developmental stage or anteroposterior position (Figures 1F and S1C). Another group was enriched with ureteric tip marker genes (En2, Wnt11, and Ret) that showed higher expression in the leading tip region of the WD or UB. Meanwhile, the expression of the other set of genes (E-cadherin, Calb1, Wnt9b, and Hnf1b) increased with progression of development and could be useful for monitoring maturation.

**Retinoic Acid, Wnt, and FGF/GDNF Signaling Induce Maturation of WD Progenitors into UB-like Cells**

We established a protocol to generate the UB by a reverse induction approach. The first step was to identify factors that induce maturation of the E9.5 WD into E11.5 UB-like cells. Our microarray analysis data identified accumulated expression of retinoic acid (RA) synthetic enzyme (Raldh3), Wnt co-receptor (Lgr5), and FGF receptor/target genes in the WD throughout early development (Figure S2A). Thus, we sorted WD cells from E9.5 embryos and reaggregated the cells in the presence of combinations of these factors. The combination of RA, Wnt agonist CHIR99021 (CHIR), and Fgf9 synergistically maintained lineage markers (Pax2 and Emx2) and a tip type marker (Ret) and induced mature WD/UB markers (Hnf1b, Wnt9b, and Calb1) (Figure 2A). However, the aggregates did not maintain Wnt11 expression and lacked the morphology of bud formation. Therefore, we employed Gdnf, a well-known inducer of Wnt11 and UB attractant (Majumdar et al., 2003; Pichel et al., 1996; Sainio et al., 1997). As expected, the optimized condition with Gdnf successfully induced Wnt11 expression and bud-like structure formation (Figure 2A; data not shown). These results support previous genetic loss-of-function studies showing the requirement for each growth factor signal (Zhao et al., 2004; Marose et al., 2008; Mendelsohn et al., 1994).

We then focused on maturation cues at an earlier stage of the WD precursor from E8.75 to E9.5, given that Hoxb7-GFP^+ committed WD precursors were first clearly detectable at E8.75 in the anterior body trunk of the embryo (12 somite stage) (Figure S1A). Similar to the E9.5–11.5 stage induction, sorted Hoxb7-GFP^+ precursors maintained Emx2 and Ret expression in the presence of RA, Wnt agonist, and Fgf9. Conversely, the Wnt agonist and Fgf9 at these concentrations (3 μM and 100 ng/mL, respectively) were not effective for the expression of certain maturation marker genes (E-cadherin, Calb1, and Hnf1b) at this stage (Figure 2B). Thus, we concluded that the combination of RA and low concentrations of Wnt agonist (1 μM) and
Fgf9 (5 ng/mL) is optimal for induction. At this step, we could maintain the expression of Wnt11 by Fgf9 without Gdnf. These results suggest the existence of somewhat different modes of gene regulatory circuits between the early and late developmental stages of the WD maturation process.

Finally, we sequentially applied the combined 3-day induction protocol to sorted E8.75 WD progenitors (Figure 2C). During the culture, the progenitors showed similar gene expression kinetics to those in vivo. At day 3 of induction, we observed the formation of a UB-like structure and quantitatively comparable gene expression levels to the embryonic UB in E11.5 embryos (Figures 2D and 2E).

**Induction of the WD Progenitor from mESCs**

Next, we investigated the factors that induce the E8.75 WD progenitor-like population from mESCs. First, to enable isolation and quantitative evaluation of the efficiency of WD progenitor induction, we searched for cell-surface molecules that are specifically expressed in committed WD progenitors at E8.75. The in vivo microarray analysis data identified the combination of Cxcr4 and Kit as efficiently sortable markers (Figures 2F, S2B, and 3A). Most WD progenitors at E8.75 were highly positive for Cxcr4 and Kit (Figure 2F; 94.6%), and the labeled population formed an isolated cluster in the entire E8.75 embryo proper (Figure S2C).

For induction of WD progenitors from mESCs, we first considered the in vivo anteroposterior patterning process within the intermediate mesoderm. Our previous lineage segregation model of the UB and NP indicated that the UB lineage differentiates earlier than the NP lineage from the T* immature mesoderm state that is maintained by strong Wnt signaling (Taguchi et al., 2014). Thus, we first tentatively shortened the incubation period with a high concentration of Wnt agonist to 1.5 day for the UB in contrast to 2.5 days for NP induction (Figure 3A; step 2).

Based on the minimally conserved gene expression profile between the AIM (Osr1*, Lhx1*, and Pax2*) and the PIM (Osr1*, Wt1*, and Hox11*), we hypothesized that there would be partly different signaling for the AIM differentiation step compared with that for the PIM differentiation step (Figure 3A; step 3). We identified RA as a common inducer for both the AIM and PIM (Figures 3B and S4A) (Taguchi et al., 2014). Although the endogenous FGF signal was sufficient for PIM induction, the high concentration of Fgf9 further enhanced AIM marker expression. In contrast to the PIM, addition of Activin A (Activin) was inhibitory for the induction of AIM markers. Intriguingly, suppression of the Smad2/3 pathway by SB431542 enhanced AIM marker induction, suggesting a principal role of Activin/Tgfb signaling for AIM versus PIM fate determination. These results may agree with a previous report showing the involvement of these signals in anteroposterior body patterning (Green et al., 2011; McPherron et al., 1999). Conversely, either addition or inhibition of Bmp4 (Bmp) suppressed AIM induction, indicating the requirement for an optimal level of Bmp signaling for AIM specification.

We then explored factors that specify the differentiation of the AIM to Cxcr4+/Kit+ WD progenitors (Figure 3A; step 4). At this step, we found a synergistic effect of RA, Wnt, and Fgf9. In particular, removal of the Wnt agonist dramatically reduced the Cxcr4+/Kit+ population induction, suggesting a crucial role for committed WD progenitor induction (Figure 3C).

To further fine-tune and understand the UB lineage differentiation process, we re-examined the optimal timing for efficient lineage differentiation of the nascent mesoderm into the AIM by changing the Wnt incubation period (Figure 3A; step 2). Surprisingly, the permissive time window for AIM induction was quite restricted at around day 4.5 (36 hr of Wnt treatment), and the efficiency was dramatically reduced at day 4 or 5 (Figure 3D). This observation may reflect the very narrow anteroposterior AIM domain (pronephric anlagen) in vivo, which initially appears within the 2-somite width (8th to 10th somite level) in the intermediate mesoderm at E8.5 (Bouchard et al., 2002; Grote et al., 2006).

Previous reports showing early-stage mesoderm patterning prompted us to further investigate earlier fate specification signals within the epiblast and primitive streak/nascent mesoderm (Attila et al., 2012; James and Schultheiss, 2003) (Figure 3A; steps 1 and 2). Indeed, we observed concentration-dependent patterning by Activin/Bmp signaling from day 2 to day 3 (step 1) and day 3 to day 4.5 (step 2) of the differentiation. During the mesoderm formation/patterning step (step 1), the UB had a preference for a higher concentration of Activin compared with the NP (Figures 3F and S3). Combination analysis of these two steps showed a reciprocal pattern in the optimal concentration range for the UB or NP (Figure S3). These results suggest that the cell-fate patterning of the UB and NP starts before and during the formation of the immature mesoderm. By employing these optimizations, we obtained an average 35.6% Cxcr4+/Kit+ population at day 6.25 of mESC differentiation (Figure 3G). We analyzed the gene expression kinetics from immature mESCs to day 6.25-induced committed WD progenitors (iWD; corresponding to E8.75 WD progenitors) (Figures 3A and S3). Notably, the induced spheroids showed quantitatively comparable gene expression levels to E8.75 WD progenitors and were devoid of PIM and metanephric NP marker expression (Figure 3H; Hoxd11, Wt1, and Six2), indicating successful selective induction of the UB lineage.
Induced UB Reconstructs the Higher-Order Structure of the Embryonic Kidney

We applied the WD maturation factors to the mESC-derived iWD. To visualize the branching morphogenesis in the following experiments, we established mESCs from Hoxb7-GFP transgenic mice. Minimal modifications in the initial induction steps successfully induced Hoxb7-GFP+/Cxcr4+/Kit+ iWD at day 6.25 of differentiation (Figure S4A). The sorted GFP+/Cxcr4+/Kit+ population was reaggregated and cultured in the WD maturation condition established with the E8.75 WD (Figures 2C and 4A). The aggregates formed UB-like structures at day 9.25 of induction and expressed UB markers that were comparable to the E11.5 UB (Figures 4B and 4C). To establish a single exit and confirm the branching capacity, we manually isolated a single bud from a spheroid and reaggregated it with an isolated E11.5 MM (including NPs and SPs) (Figure 4B). In the presence of the MM, the induced UB (iUB) underwent dichotomous branching up to the 6th–7th generations (1 generation/day) (Figure 4D). The final tip number from the single iUB grew to 141 ± 12 (n = 6), which was comparable to that induced by the E11.5 embryo-derived UB (Figure 4E). We also assessed the branching capacity of the iUB in a cell-free branching culture condition established by modifying a previously reported method (Rosines et al., 2007). We again observed bifurcation up to the 6th generation from the single iUB (Figure S4B).

Whole-mount staining of the reconstituted organoid at day 7 identified the Six2+ NPs maintained on each iUB tip in the periphery of the organoid, which was reminiscent of the nephrogenic zone of embryonic kidneys (Figures 4F and S4C). Conversely, on the inner side of the organoid, we observed differentiated nephrons composed of E-cadherin+ distal tubule segments, LTL+ proximal tubule segments, and Nephrin+ glomerular structures, thereby confirming the nephron induction capacity of the iUB (Figure 4G; Movie S1). Importantly, the distal end of each nephron was connected to the ureteric tips, giving that interconnection of nephrons is essential for urine drainage (Figure 4H). The typical UB tip marker Sox9 was expressed in the periphery (Reginensi et al., 2011), while cytokeratin 8 (CK8) showed stronger expression in the medullary region of the organoid, similar to the embryonic kidney, indicating proper tip-stalk patterning (Figure 4I). The ubiquitous expression of Gata3 and Calb1 in the entire ureteric epithelium further confirmed the embryonic lineage-specific feature of this ramified epithelium (Figure 4J). Collectively, the mESC-derived iUB fulfilled the functional criteria of the UB, including a branching morphogenic capacity, NP maintenance capacity, and nephron differentiation capacity. We then addressed the functional advantage of reconstructing a kidney organoid with the iUB and MM over the conventional UB substitution method that utilizes embryonic spinal cord for differentiating the MM to epithelialized nephrons (Grobstein, 1953, Taguchi et al., 2014). We combined manually isolated E11.5 MM with embryonic spinal cord or iUB. Organoids cultured for 7 days were transplanted and harvested after 15 days (Figure 4K) as previously described (Sharmin et al., 2016). The reconstructed organoid developed greater than 10 times more nephrons compared with the conventional method (Figure 4L), indicating that integration of the iUB, which maintains the nephrogenic progenitor niche, can enhance the final nephron endowment.

Reconstruction of a Kidney Organoid by mESC-Derived iUB, INPs, and Embryonic SPs

As we previously established a condition for NP induction from the mESCs (iNP), we replaced the embryonic NPs with iNPs. For this purpose, we first tested the individual requirements of the MM-composing population, namely, NPs and SPs. We previously showed that most NPs at E11.5 reside in the Itgα8+/Pdgfra+ fraction, while SPs express Pdgfra (Taguchi et al., 2014). Thus, we sorted a pure NP fraction from iNP spheroids and an SP fraction from E11.5 kidneys and reaggregated them with the iUB. The SPs did not support UB branching in the absence of INPs. Meanwhile, the INPs induced fewer and aberrant branch formation without the SP population (Figures S4D and S4E). A magnified image identified atypical UB tips with a multangular shape rather than the typical bifurcation or trifurcation. In addition, as shown in Figure S4-E, each site of the iUB tip contained a thickened layer of iNPs, being reminiscent of a previously reported knockout mouse phenotype lacking the functional stromal population (Das et al., 2013; Hatini et al., 1996; Ohmori et al., 2013). These findings indicate the requirement of both SP and NP populations for proper organization of ureteric branching morphogenesis. To confirm the selective contribution of each population, we employed a Pdgfra+ SP population from mouse embryos ubiquitously expressing tdTomato. The results showed a restricted contribution of the embryonic cells to the stromal region, but not to the epithelial structure of the organoid (Figures S4G and S4H).

Thus, we reconstructed a kidney organoid with INPs, an iUB, and the Pdgfra+ SP population sorted from E11.5 embryonic kidneys. Similar to the reconstruction with the iUB and whole embryonic MM, the reassembled kidney tissue showed robust branching with NP niches and differentiated nephron components (Figures 5A–5D). The 3D reconstructed image showed each segment contiguously forming nephron structures, including glomeruli, proximal tubules, distal tubules, and ureteric epithelium (Figure 5C). Staining of NKCC2 further confirmed the development of the loop of Henle located in the medullary part of the organoid.
the organoid (Figure 5E). Of note, the reconstructed organoid did not contain vascular endothelial cells (Figure S4F), because they were eliminated from the Pdgfra \(^+\) SP fraction. These findings suggest that endothelial cells are not primarily required for the initial branching morphogenesis of the kidney.

Finally, to examine the developmental maturity of the organoid, we carried out cDNA microarray analyses from embryonic mouse kidneys and reconstructed organoids (iUB+iNP+SP). We selected the serially upregulated (1,604) or downregulated (1,754) genes and performed hierarchical clustering analyses. In both probe sets, the organoids cultured for 7 days were most closely located to the E11.5 kidney (Figures 5F and S5A). Accordingly, individual examinations of mature nephric tubule/collecting duct markers mostly revealed intermediate expression levels between E13.5 and E15.5 (Figures 5G and S5B). In previous reports, artificial triggers of nephron differentiation with exogenous Wnt agonist treatment resulted in immediate exhaustion of the progenitors (Morizane et al., 2015; Takasato et al., 2015). In contrast, in our reconstructed organoid, most of the UB progenitor genes (UB-tip genes) and NP genes were maintained (Figure 5G), thereby confirming the proper maintenance of the nephrogenic niche. Collectively, these data indicate the functionality of both mESC-derived iPNS and iUB that can interact to form the higher-order structure of the kidney organoid.

### Induction of the UB from Human-Induced PSCs

We applied our UB induction protocol for mESCs to human-induced PSCs (hiPSCs). We first induced the nascent mesoderm by Activin followed by a high concentration of Wnt agonist (Figure 6A; steps 1 and 2). We then applied AIM induction factors and committed WD induction factors by combining RA, Fgf9, and Tgfβ inhibitor or RA, Wnt agonist, and Fgf9, respectively (steps 3 and 4). In contrast to mESC differentiation, addition of Bmp inhibitor LDN193189 (LDN) further enhanced both of these differentiation steps. In particular, administration of LDN at the AIM induction step (step 3) largely enhanced AIM marker gene expression and CXCR4\(^+\)/KIT\(^+\) WD progenitor induction, as confirmed by quantification at day 4.5 and day 6.25, respectively (Figures 6B and 6C).

We examined the differences in the optimal time windows for induction of the AIM or PIM from the nascent mesoderm. We changed the incubation period of the Wnt agonist and applied the UB or metanephric NP lineage-specific factors for the following differentiation. The induction efficiency of CXCR4\(^+\)/KIT\(^+\) WD progenitors or the ITGAb\(^+\)/PDGFR\(A\) NP fraction was examined at day 6.25 or day 12, respectively. Similar to mESCs, the human UB lineage required a rigid time window for AIM induction, which was maximized at day 2.5 (1.5 days of CHIR treatment). The induction efficiency of the NP lineage peaked by day 7 of administration (6 days of CHIR treatment) of the PIM induction factors (Figure 6D), confirming our previous results (Taguchi et al., 2014). Importantly, compared with the permissive time window of the CHIR for NP induction (5–7 days), the UB lineage was not induced beyond the optimal time window (1.5 days). Thus, the UB lineage was not induced in the time frame for NP differentiation.

We then optimized the Activin/Bmp concentration at the earliest phase of differentiation. At the epiblast patterning stage (Figure 6A; step 1), the UB showed a preference for a higher Activin signal (Figures 6E and S6), consistent with the mESC results. Moreover, addition of a low concentration of Bmp at this step further enhanced the WD progenitor induction, peaking with application of the combination of 10 ng/mL Activin and 1 ng/mL Bmp. In contrast, NP induction was enhanced at a lower concentration of Activin in the absence of Bmp, which was maximized by 1 ng/mL Activin (Figures 6F and S6). During the following mesoderm induction/patterning step by CHIR treatment (differentiation days 1–2.5 in the UB and days 1–7 in the NP), we examined the effect of co-administered Bmp. The UB progenitor was most efficiently induced by addition of 1 ng/mL Bmp, whereas NPs were most efficiently induced in the absence of Bmp ligand or antagonist (Figures 6G and S6). Taken together, these data strongly suggest mutually independent early lineage specification processes between the UB and metanephric NP lineage.

The final optimized condition induced approximately 51.2% CXCR4\(^+\)/KIT\(^+\) WD progenitors at day 6.25 of induction (Figure 6H). Kinetics analysis from day 0 to day 6.25 showed efficient induction of a set of WD markers (Figure S6I).

We sorted the CXCR4\(^+\)/KIT\(^+\) WD progenitor fraction at day 6.25 and reaggregated it in the WD maturation culture condition. For human WD progenitor differentiation, continuous...
administration of Fgf1 and LDN further enhanced mature WD marker gene expression in addition to mouse WD maturation cocktails (Figure 6J). The optimized culture condition induced HNF1b, E-cadherin, and CALB1, and multiple bud formation at day 6 of culture (day 12 of induction) (Figure 6K; data not shown). Importantly, the iUB showed a branching capacity in the gel culture setting. In this culture condition, we observed the first branching at around day 7 of culture and the second generation of bifurcation at the end of week 2 (Figure 6L), being much slower than mouse UB branching but comparable to that reported in vivo human development (de Bakker et al., 2016). The branched UB organoid showed stronger expression of SOX9 in the tip region and CK8 in the stalk region of the ureteric epithelium, showing tip-stalk patterning in the structure (Figure 6M). Magnified imaging of the tip region identified the typical ampulla or dichotomous bifurcation stained for PAX2 and E-cadherin, providing evidence for reconstruction of human UB branching morphogenesis in vitro (Figure 6N).

**PAX2 Is Cell-Autonomously Required for Induction and Maturation of the Epithelialized UB from Human PSCs**

Because our selective induction method for the NP and UB allows analysis of the lineage-specific roles of developmental genes, we examined the cell-autonomous role of the PAX2 gene in human NP and UB lineages.

Interestingly, deletion of PAX2 from the iNP did not result in a gross abnormality, at least in terms of NP induction and MET toward nephrons (Kaku et al., 2017). In contrast, we observed a defective phenotype in iUB differentiation. At day 6.25 of differentiation, we observed a slight decrease in the efficiency of CXCR4+KIT+ iWD induction between the two independent control and knockout clones (Figures 7A and 7B). Nevertheless, significant percentages of CXCR4+KIT+ iWD were obtained from both the control and knockout clones, which showed generally comparable WD marker gene expression profiles, except for PAX2 (Figure 7C). Thus, we further cultured the sorted iWD population in the WD maturation condition (Figure 6A).

At day 8.5 of induction (maturation culture day 2), we did not observe a gross morphological difference between the control and knockout clones (Figure 7D). At day 10.5, the control clones showed active cellular protrusion formation reminiscent of the migrating WD tip morphology (Soofi et al., 2012). The knockout clones showed less protrusion and gradual decreases in expression of previously reported PAX2 target genes including LHX1, GATA3, and RET (Figures 7E and S7A) (Bouchard et al., 2002; Grote et al., 2006; Torres et al., 1995). At day 12.5 of culture (maturation culture day 6), the control spheroid developed a massive UB-like structure, whereas the knockout clones showed a rough surface without clear bud formation (Figure 7D). Indeed, the expression level of E-cadherin in the knockout clones was much lower compared with that in the control clones (Figure 7E). The continuous expression of N-cadherin further suggested failure of proper MET in the knockout clones (Figure S7A), consistent with a previously observed phenotype in mice (Soofi et al., 2012). When we analyzed the day 12.5 control spheroid by whole-mount immunostaining, we observed ubiquitous expression of PAX2 in the nuclei and clearly accumulated E-cadherin signaling in the extracellular membrane of the basal region of the iUB. In contrast, the knockout clones showed weak cytoplasmic expression of E-cadherin without membranous localization (Figures 7F and S7B). Subsequent in-gel culture further confirmed the branching capacity in the control clones, but not the knockout clones (Figure 7D).

Collectively, these results indicate that the loss of PAX2 in iUB differentiation is initially permissive for the induction of committed WD progenitors but fails to induce MET during the following maturation stage. These observations are consistent with the in vivo knockout phenotype in mice, thereby emphasizing the authenticity of our human UB induction method.

**DISCUSSION**

The two major precursors of the kidney, the UB and metanephric NPs, share several transcription factors during the developmental process. Such molecular redundancy and regional proximity have hindered our understanding of their developmental relationship, especially in early embryogenesis. However, genetic knockout of NP lineage-specific genes allows normal development of the WD (Kreidberg et al., 1993) or vice versa (Grote et al., 2006) until about E10.5 when the mutual interaction starts. Similarly, mechanical obstruction of WD elongation in the chick embryo does not hamper activation of mesenchymal gene expression (Gruenwald, 1937; Waddington, 1938; Soueid-Baumgarten et al., 2014). In addition, our previous lineage-tracing study identified spatiotemporally distinct origins of the NP and UB (Taguchi et al., 2014). All these data strongly suggest the necessity of independent specification cues for each lineage induction.

Indeed, during the epiblast/mesoderm patterning stages examined in the present study (corresponding to steps 1 and 2 in Figures 3A and 6A), slight differences in Activin/Bmp signaling largely affected the future differentiation competency of NP and UB progenitors. These findings raise the possibility that, from the early epiblast and nascent mesoderm (primitive streak) stages, the fates of the NP and UB lineages are already separate
have shown, the exposure time to the Wnt signal in the nascent necessary in the presence of a functional UB. As we and they CHIR treatment for nephron differentiation, which would not be insufficiently branching ureteric tree or the progenitor niche, suggest-edly the cocktail of WD maturation factors, including RA, Wnt, and FGF/GDNF, overlaps with the late stage UB branching/propagation condition after E11.5 (Rosines et al., 2007; Yuri et al., 2017), suggesting the existence of a somewhat stereotypic envi-ronment around the WD and UB tip throughout the early to late developmental process. In conclusion, we identified mutually distinct inductive signals between the NP and UB lineages in every step of differentiation, including epiblast patterning, meso-derm patterning, intermediate mesoderm specification, and maturation (Figure 7G).

In contrast to our results, Takasato et al. (2015) recently showed “simultaneous” induction of multiple kidney compo-nents by a single protocol. Although they claimed induction of the ureteric epithelium (UE; a UB derivative), NPs, SPs, and even endothelial cells, their protocol did not induce the dichoto-mously branching ureteric tree or the progenitor niche, suggest-ing that the induced populations using this method are not fully functional. For example, the organoid requires exogenous CHIR treatment for nephron differentiation, which would not be necessary in the presence of a functional UB. As we and they have shown, the exposure time to the Wnt signal in the nascent mesoderm state largely determines the anteroposterior position within the intermediate mesoderm. The limited functionality of the induced UE-like cells could be partly attributable to their strategy for inducing the UB and NPs by a single protocol in which the cells receive an intermediate Wnt treatment period between the AIM and PIM induction conditions that are devoid of lineage-specific maturation conditions.

In our organoid reconstruction method, reassembly of single iUB, INPs, and SPs recapitulated the physiological interaction and enabled branching morphogenesis from the single trunk. This is a prerequisite configuration for the drainage system. However, the specification signals that induce the SP lineage remain to be elucidated. In addition, to further achieve functional ureter development that connects to the bladder, it may be necessary to consider the heterogeneity of the stromal cells that promote ureter stalk elongation/maturation processes (Bohnenpoll and Kispert, 2014).

For human UB lineage induction, we evaluated the feasibility by the expression of multiple marker genes and branch forma-tion using the in-gel organoid culture method. We also combined the human iNP and iUB, but primary human embryonic SPs were unavailable. Our preliminary attempts showed some elongation and bifurcation of the iUB up to day 7 of culture, but further branching morphogenesis was not observed (Figure S7C). This may indicate that either or both of the human iUB and iNP are not completely programmed to undergo the physiological inter-actions. Alternatively, the stromal cells may play a substantial role for human UB branching. It is also possible that the kidney reconstruction condition is still suboptimal for human tissue. However, it is currently difficult to experimentally validate these possibilities because of the lack of a human SP induction method and the limited access to primary human fetal kidney tissues, which would serve as a reliable reference to examine the species difference (O’Brien et al., 2016). Nevertheless, the in-gel epithelial-organoid culture system was useful to demonstrate the cell-autonomous requirement of the PAX2 gene in human iUB development, in contrast to its dispensable role in epithelializa-tion of the iNP (Kaku et al., 2017). Thus, our selective induction
strategy will also be effective for lineage-specific analyses during human development.

Regardless of the recent progress in somatic tissue induction from PSCs, there have been few strategies to reconstitute higher-order organ structures. Here, we have shown the efficiency of integration of the branching epithelium into an organoid as an organizer of tissue geometry. In addition to this morphogenic force, the branching epithelium serves the progenitor niche essential for expansion of the organ size. To further develop organ-scale tissue reconstruction and functional maturation, the expandability of our reassembly strategy will be beneficial. For this purpose, differentially induced functional blood vessels and a blood circulation system could be integrated. Thus, our selective induction and reassembly method, which recapitulates the in vivo developmental context, will be a globally applicable strategy to reconstruct the higher-order structure of an embryonic organ, thereby enabling systemic organ functions from PSCs in the future.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, one table, and one movie and can be found with this article online at https://doi.org/10.1016/j.stem.2017.10.011.

**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the Lead Contact, Atsuhiro Taguchi (ataguchi777@gmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Hoxb7-GFP mice (Srinivas et al., 1999) and Wt1-GFP mice (Zhou et al., 2008) were purchased from Jackson Laboratory, and maintained on an inbred (C57BL/6J; CLEA Japan Inc.) and outbred background (Jcl:ICR; CLEA Japan Inc.). Mafb-GFP mice (Moriguchi et al., 2006) were kindly provided by Dr. Satoru Takahashi (University of Tsukuba) and maintained on an outbred background (Jcl:ICR; CLEA Japan Inc.). For the transplantation experiments, immunodeficient 8–10-week-male mice (NOD/ShiJic-scidJcl) were purchased from CLEA Japan Inc. Mice were housed in a specific pathogen-free animal facility in plastic cages on a 12-h/12-h light/dark cycle, and fed an irradiated CE-2 diet (CLEA Japan Inc.). All animal experiments were performed in accordance with institutional guidelines and approved by the licensing committee of Kumamoto University (A29–040).

Except for the transplantation experiments, both male and female mice were used. The developmental stages of the mice used for the respective experiments are described in the relevant sections of the Method Details. For analyses conducted during embryonic stages, noon of the day when vaginal plugs of mated females were identified was considered to be E0.5.

mESC line generation and maintenance

The mESC line (Osr1-GFP) was described previously (Taguchi et al., 2014) and maintained on mitotically inactivated murine embryonic fibroblasts (MEFs) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 15% FBS, 1% (vol/vol) nonessential amino acids (Life Technologies), 1% (vol/vol) sodium pyruvate (Life Technologies), 0.1 mM 2-mercaptoethanol (Nacalai Tesque), and 1,000 U/ml leukemia inhibitory factor (Millipore).

The Hoxb7-GFP line was generated as described below. Fertilized 8-cell stage eggs were obtained by crossing ovarian hyperstimulated female 129/sv mice with male Hoxb7-GFP C57BL/6J mice. The harvested embryos were cultured for 24 h in M2 medium (ARK Resource). Embryos grown to the blastocyst stage were transferred to mESC maintenance medium on 0.1% gelatin-coated plastic dishes. After 6 days, the propagated cells were passaged to mESC maintenance medium on mitotically inactivated MEFs. The established mESCs were further propagated, and an early passage clone exhibiting a robust differentiation capacity to the nephron progenitor lineage according to a previously established protocol (Taguchi et al., 2014) was used for differentiation experiments (clone B6-5, female). The established mESC line (Hoxb7-GFP) was maintained on mitotically inactivated MEFs. The formulation of Hoxb7-GFP mESC maintenance medium is as follows. Glasgow’s minimal essential medium (Life Technologies) supplemented with 14% knockout serum replacement (Life Technologies), 1% FBS (Japan Bioserum), 1% (vol/vol) nonessential amino acids, 1% (vol/vol) sodium pyruvate (Life Technologies), 0.1 mM 2-mercaptoethanol, 1,000 U/ml leukemia inhibitory factor, 1.5 μM CHIR, and 0.5 μM PD0325901.

All mESCs were cultured in a humidified atmosphere of 5% CO_{2} at 37°C. Cells were passaged every other day.
hiPSC line and maintenance
The 201B7 hiPSC line from a healthy female donor (Takahashi et al., 2007) was maintained on iMatrix-511 (Nippi) in StemFit AK03N medium (AJINOMOTO). The cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C, and passaged every 6 days by treatment with Accutase (ESGRO). The cell culture medium was changed on days 2, 4, and 5. The PAX2 knockout hiPSC line was generated from the 201B7 hiPSC line (Kaku et al., 2017) and maintained as described above.

METHOD DETAILS

Experimental design
Each experiment was performed in triplicate at least, unless stated otherwise. No specific methods were used for randomization. The investigators were not blinded to the identity of the samples. No statistical methods were utilized to determine the sample size. All animal experiments were designed to use the minimum number of animals possible. Embryos that did not fulfill the staging criteria were excluded from the analysis. The experiments investigating the role of the PAX2 gene in human iUB differentiation were performed using two independent knockout lines and replicated three times for each line.

Kidney reconstitution assay
The metanephros was manually dissected from E11.5 ICR mouse embryos. WDs from E9.5 to E11.5 embryos were manually isolated using sharpened tungsten needles and forceps. Intact UBs were isolated by incubating the kidneys in DMEM/10% FBS containing 1 mg/ml type XI collagenase (Sigma) for 4 min at 37°C. UBs were manually isolated from the MM using 30G needles (Dentronics). The harvested MM was washed in PBS once and dissociated by incubation in 0.05% trypsin/EDTA at 37°C for 5 min. Dissociated cells were resuspended at 75,000 cells/150 μl mouse kidney reconstitution medium and seeded into a 96-well low cell-binding U-bottom plate (Thermo). The cells were pelleted by centrifugation (210 x g, 3 min). An isolated UB, WD, or iUB was placed onto the deposited sheet-like MM cells. The MM cells spontaneously aggregated and encapsulated the UB, finally forming a spheroid after 24 h of culture. The reaggregated spheroids were transferred to a 24-well transwell insert (Corning) containing 50 μl of 50% Matrigel in DMEM/F12 with 10% FBS and penicillin/streptomycin. The transwell was placed in DMEM/F12 with 10% FBS and penicillin/streptomycin and cultured for 7 days. The branched tip numbers were counted at day 7 of organ culture, when each organoid had ceased branching. The composition of the serum-free kidney reconstitution medium was as follows: DMEM/F12 (Invitrogen) supplemented with 1% (vol/vol) ITS (insulin-transferrin-selenium), 0.1 μM dexamethasone, 10 mM nicotinamide, 5 mM HEPES, 5% (vol/vol) KSR (KnockOut Serum Replacement), 2 mM L-glutamine, 50 μM β-mercaptoethanol, and 0.5% (vol/vol) penicillin/streptomycin.

Kidney reconstitution from the differentially sorted progenitor fraction
For reconstitution of a kidney organoid from mESC-derived NPs and embryonic SPs, each fraction was sorted by FACS. The mESC-derived NP embryoid bodies (EBs) were dissociated by incubation in 0.25% trypsin/EDTA at 37°C for 6 min and then harvested by staining and sorting of the Itga8+/Pdgfra- fraction. The manually dissected E11.5 mouse embryonic MM was dissociated by incubation in 0.05% trypsin/EDTA at 37°C for 5 min, and the Pdgfra+ stromal cell fraction was sorted. After sorting, 25,000 Itga8+/Pdgfra- NP cells and 50,000 Pdgfra+ stromal cells were mixed and seeded into a 96-well low cell-binding U-bottom plate. The isolated iUB was placed onto the deposited sheet-like cells. Aggregated spheroids were transferred to a transwell insert containing 50 μl of 50% Matrigel in DMEM/F12 with 10% FBS and penicillin/streptomycin on the next day (24 h later).

Kidney reconstitution from the differentially induced human iNPs and iUB
To visualize the branching morphogenesis in this experiment, we utilized hiPSCs transfected with a CAG-GFP construct for iUB induction. For hiPSC-derived iNP and iUB reconstruction, the respective differentiations were slightly modified to enhance the maturation of each tissue (please refer to the sections for human iNP and iUB induction methods). The day 13 NP EBs were dissociated by incubation in Accumax at 37°C for 6 min. After removing the supernatant by centrifugation, cell washing buffer (1 x Hank’s balanced salt solution [HBSS] containing 20% FBS, 50 ng/ml DNAsel, and 0.035% NaHCO₃) was applied and the cells were dissociated into single cells by gentle pipetting. The dissociated single EBs of cells were resuspended in hiPSC differentiation medium containing 10 μM Y27632 (Wako) and 5 ng/ml human Fgf9 (R&D Systems), and seeded into a 96-well low cell-binding V-bottom plate. The day 13 iUB was manually cut into four parts and placed in a well containing the iNP cell suspension. After 48 h of culture, spontaneously aggregated spheroids were transferred to a transwell insert in DMEM/F12 with 10% FBS and penicillin/streptomycin.

Sorted embryonic cell culture
We sorted WD progenitors (Hoxb7-GFP+/Flk1- fraction) by flow cytometry, as we noticed that the Hoxb7-GFP transgenic line leaked GFP fluorescence into part of the vascular endothelial population (Figure S1B). For embryonic tissue cultures at E9.5, posterior regions were harvested from the forelimbs of 22–26 somite stage embryos. The harvested tissues were dissociated into single cells by incubation in DMEM/10% FBS containing 1 mg/ml type XI collagenase for 6 min at 37°C and then in 0.25% trypsin with 50 ng/ml DNAsel for 6 min at 37°C. After blocking in normal mouse serum (Thermo Scientific), cell surface marker (Flk1) staining was carried out in 1 x HBSS containing 1% BSA and 0.035% NaHCO₃. FACS-sorted Hoxb7-GFP+/Flk1- cells were centrifuged and resuspended in mESC differentiation medium (Taguchi et al., 2014), and seeded at about 1,600 cells/well in V-bottom 96-well low cell-binding plates (Sumitomo Bakelite; Cat# MS-9096V). After centrifugation (210 x g, 4 min), the supernatant was replaced with the Step 6
differentiation medium including 10 μM Y27632 (Wako), 0.1 μM RA (Sigma), 3 μM CHIR (Axon), 5 ng/ml human Fgf9 (R&D Systems), 1 ng/ml human Gdnf (R&D Systems), and 10% growth factor-reduced Matrigel (BD). After 24 h, the aggregated spheroids were transferred to the Step 7 medium including 10 μM Y27632, 0.1 μM RA, 3 μM CHIR, 2 ng/ml human Gdnf, and 10% growth factor-reduced Matrigel.

For embryonic tissue cultures at E8.75, regions caudal to the heart primordia of 12–15 somite stage embryos were harvested, and Hoxb7-GFP+/Flk1- cells were sorted by FACS. The sorted cells were aggregated at about 1,200 cellsaggregate in V-bottom 96-well low cell-binding plates. For the first 24 h, the Step 5 differentiation medium including 10 μM Y27632, 0.1 μM RA, 3 μM CHIR, 5 ng/ml human Fgf9, and 10% growth factor-reduced Matrigel was applied. The subsequent differentiation was performed by transferring the spheroid to Step 6 and 7 media for 24 h each.

UB lineage (WD progenitor) induction from mESCs
ESC differentiation was carried out in serum-free medium as follows. ESCs were dissociated with Accutase (ESGRO) and cultured in serum-free mESC differentiation medium (Taguchi et al., 2014). Harvested cells were aggregated at 1,000 cellsaggregate in 96-well U-bottom low cell-binding plates to form EBs. After 48 h (on day 2), the EBs were dissociated with Accutase and reaggregated in serum-free differentiation medium with 10 ng/ml human Activin A (R&D Systems) (Step 1). After 24 h, the medium was switched to medium containing 0.3 ng/ml human Bmp4 (R&D Systems) and 10 μM CHIR (Step 2). After 36 h (on day 4.5), the medium was changed to Step 3 medium containing 0.1 μM RA, 100 ng/ml human Fgf9, and 10 μM SB431542 (Wako). On day 5.5, the medium was changed to Step 4 medium containing 0.1 μM RA, 100 ng/ml human Fgf9, and 5 μM CHIR. The mESC differentiation medium consisted of 75% Iscove’s modified Dulbecco’s medium and 25% Ham’s F12 medium with 0.5 × N2 (Thermo Fisher Scientific), 0.5 × B27 without retinoic acid (Thermo Fisher Scientific), 0.5 × penicillin/streptomycin, 0.05% BSA (Sigma), 2 mM L-glutamine (Life Technologies), 0.5 mM ascorbic acid (Sigma), and 4.5 × 10-4 M 1-thioglycerol (Sigma).

For Hoxb7-GFP ESC line differentiation factors, please see Figure S4A.

mESC-derived committed WD progenitor maturation culture
On day 6.25, induced spheroids were harvested and dissociated by incubation in 0.25% trypsin/EDTA for 6 min at 37°C. After blocking in normal mouse serum, cell surface marker (Cxcr4/Kit) staining was carried out in 1 × HBSS containing 1% BSA and 0.035% NaHCO3. A total of 3,000 FACS-sorted Hoxb7-GFP+/Cxcr4+/Kit+ cells were aggregated in V-bottom 96-well low cell-binding plates. After 48 h (on day 2), the EBs were dissociated with Accutase and reaggregated in medium containing 0.3 ng/ml human Bmp4 (R&D Systems) and 10 μM CHIR (Step 2). After 36 h (on day 4.5), the medium was changed to Step 3 medium containing 0.1 μM RA, 100 ng/ml human Fgf9, and 10 μM SB431542 (Wako). On day 5.5, the medium was changed to Step 4 medium containing 0.1 μM RA, 100 ng/ml human Fgf9, and 5 μM CHIR. The mESC differentiation medium consisted of 75% Iscove’s modified Dulbecco’s medium and 25% Ham’s F12 medium with 0.5 × N2 (Thermo Fisher Scientific), 0.5 × B27 without retinoic acid (Thermo Fisher Scientific), 0.5 × penicillin/streptomycin, 0.05% BSA (Sigma), 2 mM L-glutamine (Life Technologies), 0.5 mM ascorbic acid (Sigma), and 4.5 × 10-4 M 1-thioglycerol (Sigma).

For Hoxb7-GFP ESC line differentiation factors, please see Figure S4A.

mESC-derived single UB branching culture
On day 9.25, mESC-derived iUBs were manually isolated using sharpened tungsten needles. The isolated UBs were embedded into 24-well transwell inserts (Corning) and mounted in 150 μl of branching medium. The medium consisted of DMEM/F12 with 50% Matrigel, 10% FBS, 0.1 μM RA, 100 ng/ml human Rspondin1 (R&D Systems), 2 ng/ml human Gdnf, and 100 ng/ml mouse Fgf1. The transwell inserts were cultured on 500 μl of branching medium without Matrigel.

NP lineage induction from mESCs
The previously reported NP lineage induction protocol for mESCs (Taguchi et al., 2014) was applied with minimal modification. Before initiation of differentiation, mESCs were passaged once onto feeder cell-free, gelatin-coated dishes in DMEM (Invitrogen) supplemented with 15% FBS, 1% (vol/vol) nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1 × penicillin/streptomycin (Life Technologies), 1,000 U/ml leukemia inhibitory factor, 3 μM CHIR (Axon), and 1 μM PD0325901 (Wako). EBs were formed as aggregates of 1,000 cells in 96-well U-bottom low cell-binding plates. After 48 h (on day 2), the EBs were dissociated with Accutase and reaggregated in serum-free differentiation medium with 1 ng/ml human Activin A. After 24 h, the medium was switched to medium containing 10 μM CHIR. After 36 h (on day 4.5), the medium was replaced with medium containing 10 μM Y27632 and 10 μM CHIR. On day 5.5, the medium was changed to medium containing 10 ng/ml human Activin A, 3 ng/ml Bmp4, 3 μM CHIR, 0.1 μM RA, and 10 μM Y27632. On day 6.5, the medium was changed to medium containing 1 μM CHIR, 5 ng/ml human Fgf9, and 10 μM Y27632.

See also Figure S4A.

UB lineage induction from hiPSCs
Cells were reaggregated in the hiPSC differentiation media at 10,000 cellsaggregate in V-bottom 96-well low cell-binding plates to form EBs. After 48 h (on day 2), the EBs were dissociated with Accutase and reaggregated in medium containing 10 μM Y27632, 10 ng/ml human Activin A, and 1 ng/ml human Bmp4. After 24 h (on day 1), the spheres were transferred to U-bottom 96-well low cell-binding plates containing Step 2 medium including 10 μM CHIR and 1 ng/ml human Bmp4. After 36 h (on day 2.5), the medium was changed to Step 3 medium containing 0.1 μM RA, 100 ng/ml human Fgf9, 100 nM LDN193189 (Wako), and 100 μM SB431542. On day 4.5, the medium was changed to Step 4 medium containing 0.1 μM RA, 5 μM CHIR, 100 ng/ml human Fgf9, and 30 nM LDN193189. The serum-free hiPSC differentiation medium consisted of DMEM/F12 (Invitrogen) supplemented with 2% (vol/vol) B27 (without retinoic acid), 2 mM L-glutamine, 1% (vol/vol) ITS, 1% (vol/vol) nonessential amino acids, 90 μM β-mercaptoethanol, and 0.5% (vol/vol) penicillin/streptomycin (Taguchi et al., 2014).
hiPSC-derived committed WD progenitor maturation culture
On day 6.25, induced spheroids were harvested and dissociated by incubation in 0.25% trypsin/EDTA for 6 min at 37°C. After blocking in normal mouse serum, cell surface marker (CXCR4/KIT) staining was carried out in 1 × HBSS containing 1% BSA and 0.035% NaHCO3. FACs-sorted CXCR4+/KIT+ cells (5,000) were seeded in V-bottom 96-well low cell-binding plates and pelleted by centrifugation (210 × g, 4 min). The supernatant was replaced with Step 5 medium containing 10 μM Y27632, 0.1 μM RA, 1 μM CHIR, 5 ng/ml human Fgf9, 100 ng/ml human Fgf1 (R&D Systems), 10 nM LDN193189, and 10% growth factor-reduced Matrigel. On day 8.5, the spheroids were transferred to wells containing Step 6 medium including 10 μM Y27632, 0.1 μM RA, 3 μM CHIR, 5 ng/ml human Fgf9, 1 ng/ml human Gdnf, 100 ng/ml human Fgf1, 10 nM LDN193189, and 10% growth factor-reduced Matrigel. For human iNP and iUB reconstruction experiments in particular, the final step was continued for 3 days.

hiPSC-derived UB branching culture
On day 12.5, hiPSC-derived induced UB spheroids were embedded in 24-well transwell inserts and mounted in 150 μl of branching medium. The medium consisted of DMEM/F12 containing 50% Matrigel, 10% FBS, 0.1 μM RA, 100 ng/ml human Rspodin1, 2 ng/ml human Gdnf, 100 ng/ml human Fgf1, 30 ng/ml human Fgf7 (R&D Systems), and 10 nM LDN193189. The transwell inserts were cultured on 500 μl of branching medium without Matrigel.

NP lineage induction from hiPSCs
The previously reported protocol (Taguchi et al., 2014) was modified. Cells were reaggregated at 10,000 cells/aggregate in V-bottom 96-well low-cell-binding plates to form EBs in the presence of 10 μM Y27632 and 1 ng/ml human Activin A. For the human iNP and iUB reconstruction, 20 ng/ml human bFGF was added in addition to these factors. After 24 h (on day 1), the spheres were transferred to U-bottom 96-well low cell-binding plates containing mesoderm-inducing medium with 10 μM CHIR and 10 μM Y27632. Subsequently, half of the culture medium was refreshed with new medium every other day (on days 3 and 5). On day 7, the medium was changed to ABC3R medium containing 10 ng/ml human Activin A, 3 ng/ml human Bmp4, 3 μM CHIR, 0.1 μM RA, and 10 μM Y27632. On day 9, the medium was changed to C1F medium containing 1 μM CHIR, 5 ng/ml human Fgf9, and 10 μM Y27632. For human iNP and iUB reconstruction experiments, the ABC3R medium culture was continued for 3 days, and on day 10 the culture condition was switched to modified C1F medium containing 1 μM CHIR, 5 ng/ml human Fgf9, 30 nM LDN193189, 10 μM BMS493 (TOCRIS), and 10 μM Y27632 for 3 days. All data shown are representative examples of at least three independent experiments unless indicated otherwise.

Transplantation of reconstructed organoids
To examine the final nephron number, we employed Mafb-GFP knock-in mice that specifically express fluorescence in their glomeruli (Moriguchi et al., 2006). Manually isolated 3 MMs from Mafb-GFP knock-in mouse embryos at E11.5 was reaggregated with mouse ESC-derived iUB or combined with E11.5 embryonic spinal cord and cultured for 7 days using the organ culture settings described above. The 7-day cultured organoids were harvested and the transplantation procedure was performed as previously reported (Sharmin et al., 2016). Briefly, the host kidney capsule was incised at approximately 2 mm from the caudal end of the kidneys and rods were carefully inserted into the lateral side with forceps. The inserted rods were arranged to create a V-shaped free space and briefly cauterized with the capsule membrane by electric cautery to prevent them shifting from their initial positions. Finally, an organoid was inserted through the incised window by a 20-gauge plastic indwelling needle connected by a P-200 Gilson pipette. Immunodeficient 8–10-week-old male NOD/SCID mice were used as the host animal and anesthetized with normal saline containing 0.75 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol.

Whole-mount immunohistochemistry
Organoids were fixed in 4% paraformaldehyde in PBS for 60 min, washed with 0.1% Triton X-100 in PBS three times, and blocked in PBS containing 10% goat serum (Nippon Bio-Test Laboratories), 1% Triton X-100, and 2% dry skim milk for 1 h twice. The tissues were incubated overnight with primary antibodies on a rocking shaker at 4°C, washed with 1% Triton X-100 in PBS for 1 h three times, and incubated with secondary antibodies conjugated with Alexa Fluor 488, 568, 594, 633, or 647. After immunostaining, the tissues were incubated overnight with primary antibodies on a rocking shaker at 4°C, washed with 1% Triton X-100 in PBS for 1 h three times, and incubated with secondary antibodies conjugated with Alexa Fluor 488, 568, 594, 633, or 647 for 1 h at room temperature. The sections were incubated for 3 h with primary antibodies at room temperature, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488, 568, 594, 633, or 647 for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Whole-mount images were captured with an upright Axioscope system (Zeiss) or by confocal microscopy (FV1000-MPE; Olympus). Images were reconstructed by software (Imaris; Bitplane or LAS X; Leica).

Section immunohistochemistry
Samples were fixed with 4% paraformaldehyde in PBS for 60 min, washed with PBS, dehydrated with 10%, 20%, and 30% sucrose in PBS, embedded in optimal cutting temperature compound (Tissue Tek), and cryosectioned at 10-μm thicknesses. For fluorescence immunohistochemistry, the sections were washed with PBS three times and blocked by incubation with 3% BSA in PBS for 1 h at room temperature. The sections were incubated for 3 h with primary antibodies at room temperature, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488, 568, 594, or 633, or 647 for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Whole-mount images were captured with an upright Axioscope system (Zeiss) or by confocal microscopy (FV1000-MPE; Olympus). Images were reconstructed by software (Imaris; Bitplane or LAS X; Leica).
counterstained with 4,6-diamidino-2-phenylindole (Roche). Fluorescence images were captured by confocal microscopy (TSC SP8; Leica). Detailed antibody information is provided in the Key Resources Table.

**RNA extraction, reverse transcription, and quantitative RT-PCR**

Harvested spheroids or cells were homogenized, and total RNA was isolated using an RNeasy Plus Micro Kit (Qiagen). The RNA was reverse-transcribed with random primers and Superscript III (Invitrogen). Quantitative PCR was carried out using a Real-Time PCR System (Takara Bio) and Thunderbird SYBR qPCR Mix (Toyobo). Relative mRNA expression levels were analyzed by the ΔΔCT method and normalized to β-actin gene expression. Detailed primer information is provided in the Key Resources Table.

**Flow cytometric analysis**

Induced cell aggregates from embryonic tissues or mESCs/hiPSCs were dissociated and blocked with normal mouse serum for 10 min on ice. Cell surface marker staining was carried out in 1× HBSS containing 1% BSA and 0.035% NaHCO3 for 15 min on ice. Stained cells were analyzed using a FACSCanto II (BD). For cell sorting, stained cells were sorted using a FACSSORP Aria (BD). Data analyses were performed with FlowJo software (Treestar). Detailed antibody information is provided in the Key Resources Table.

**Microarray analysis**

For analysis of early-stage WD/UB development, we performed gene expression array analyses for each stage of the UB, WD, and progenitors from E8.75 to E11.5 utilizing Hoxb7-GFP transgenic mice. To determine UB lineage-specific markers, we also used Wt1-GFP knock-in mice (Zhou et al., 2008) and compared UB precursors with the sorted Wt1+ intermediate mesoderm of E9.5 embryos, the posterior part of which gives rise to the NP (Figure 1A).

For analysis of late-stage kidney/kidney organoid development, wild-type ICR mouse embryonic kidneys at the E11.5, E13.5, E15.5, E17.5, and P0 stages were compared. The extrarenal ureters were removed from the kidneys at all stages. From the E13.5 stage, the adrenal glands and kidney capsules were manually removed from the kidneys. The reconstructed mESC-derived organoids at Day 0 (24 h after U/B+NP+SP reaggregation; before starting organ culture) and Day 7 (after 7 days of organ culture) were analyzed in triplicate specimens.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Counting of UB tips or glomeruli in reconstructed tissues was performed in at least three organoids, unless stated otherwise. The quantification data, including qRT-PCR and flow cytometric data, were presented as mean ± SEM. Student’s t-test was applied for statistical analysis of differences between two groups. Differences with values of p < 0.05 were considered statistically significant.

**Microarray analysis**

Microarray analyses for the early-stage WD/UB maturation process were performed using an Agilent SurePrint G3 mouse gene expression (8×60K) microarray. The data were normalized by GeneSpring GX software (Agilent). The normalized gene lists were further limited by the 20.0th–100.0th percentile expression. Extraction of groups with different gene expression kinetics was performed by the analysis command “Find Similar Entities,” utilizing the Euclidean method.

For analysis of late-stage kidney/kidney organoid development, microarray analyses were performed using an Agilent SurePrint G3 Mouse Gene Expression v2 Microarray. The data were normalized by the GeneSpring GX software. The normalized gene lists were further limited by the 75th percentile expression. Genes that continuously showed higher signal values compared with earlier embryonic stages (P0 > E17.5 > E15.5 > E13.5 > E11.5) were extracted as “serially upregulated genes” (1604 entities). Genes that continuously showed lower signal values compared with earlier embryonic stages (P0 < E17.5 < E15.5 < E13.5 < E11.5) were extracted as “serially downregulated genes” (1754 entities). In both probe sets, the data were analyzed by unsupervised hierarchical clustering with the Manhattan distance and complete linkage method using GeneSpring GX software.

The gene expression kinetics of mature nephric tubule, nephron progenitor, and ureteric tip markers in embryonic kidneys/reconstructed organoids were analyzed using the microarray analysis data. The relative expression levels against the normalized median value for each probe were presented. Data for 7-day cultured organoids were averaged from triplicate samples.

**DATA AND SOFTWARE AVAILABILITY**

The accession numbers for the microarray data for the early-stage WD/UB development and late-stage kidney/kidney organoid analyses reported in this paper are NCBI GEO: GSE96524 and GEO: GSE102773, respectively.
Supplemental Information

Higher-Order Kidney Organogenesis
from Pluripotent Stem Cells

Atsuhiro Taguchi and Ryuichi Nishinakamura
Figure S1. In vivo analyses of the early WD development process (related to Figure 1).

(A) GFP expression in Hoxb7-GFP transgenic mouse embryos at the indicated stages. Scale bars, E8.5, E8.75, E10.5, E11.5: 200 µm; E9.5: 500 µm.

(B) Immunostaining of E9.5 Hoxb7-GFP embryos (left three panels). As we noticed that the Hoxb7-GFP transgenic line leaked GFP fluorescence into part of the vascular endothelial population, we sorted WD progenitors (Hoxb7-GFP+ /Flk1- fraction) by flow cytometry. Right panel: analysis by flow cytometry. Arrows: WD; arrowheads: Flk1+ endothelial cells. Scale bars: 100 µm.

(C) Examples of marker genes grouped by similarity to the indicated genes. WD: Hoxb7-GFP+ Wolffian duct; WD-A: anterior Wolffian duct; WD-P: posterior Wolffian duct; IM: Wt1-GFP+ intermediate mesoderm at E9.5; IM-A: Wt1-GFP+ IM of anterior trunk region (domain of the future mesonephros); IM-P: Wt1-GFP+ IM of posterior region (posterior intermediate mesoderm; precursor of MM).

Similarity Gene Symbol Gene Name
1.000 Sim1 single-minded homolog 1 (Drosophila)
0.695 Thap2a transcription factor AP-2, alpha
0.658 Pacd3 platelet/endothelial cell adhesion molecule 3
0.628 Lh1 LIM homeobox protein 1
0.612 Emx2 empty spiracles homeobox 2

Cut-off range [0.6,1.0], Entry genes:105

Cut-off range [0.7,1.0], Entry genes:266
Figure S2. Screening of WD specific signals and cell surface markers (related to Figure 2).

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(A and B) Comparison of UB and MM progenitor marker gene expressions in sorted early-stage precursors. Relative expression levels against the normalized median value for each probe are shown as log2 fold changes. WD: Hoxb7-GFP+ Wolffian duct; WD-A: anterior Wolffian duct; WD-P: posterior Wolffian duct; IM: Wt1-GFP+ intermediate mesoderm at E9.5; IM-A: Wt1-GFP+ IM of anterior trunk region (domain of the future mesonephros); IM-P: Wt1-GFP+ IM of posterior region (posterior intermediate mesoderm; precursor of MM).

(A) Gene lists related to signaling pathways.
(B) Gene lists related to cell surface molecules.
(C) FACS analysis of Hoxb7-GFP/Flk1 and Cxcr4/Kit expression in the E8.75 embryo proper. The magenta dots show the Hoxb7-GFP+/Flk1- committed WD progenitor fraction. The green dots show all the other populations from the viable single cell fraction.
Figure S3. Induction efficiency of UB and NP lineages by Step 1/2 modulation (related to Figure 3).

The upper table and graph show the induction efficiency of Cxcr4+/Kit+ committed WD progenitors from mouse ESCs at day 6.25. The lower table and graph show the induction efficiency of Itga8+/Pdgfra- metanephric nephron progenitors from mouse ESCs at day 8.5. The applied concentrations of Step 1 Activin (A) and Step 2 Bmp4 (B) and CHIR99021 (C) are shown as follows: Activin and Bmp4: ng/ml; CHIR: µM.
Figure S4. mESC-derived UBs possess branching capacity (related to Figures 4 and 5).

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(A) UB and nephron progenitor induction protocols for the Osr1-GFP and Hoxb7-GFP mESC lines. A: Activin (ng/ml); B: Bmp4 (ng/ml); C: CHIR99021 (µM); F: Fgf9 (ng/ml); R: retinoic acid (µM); Y: Y27632 (µM); G: Gdnf (ng/ml); SB: SB431542 (µM).

(B) Upper panels: Bright-field images of induced UB branching in 50% Matrigel culture. Lower panels: Fluorescence images of the upper panels. Orange arrows and numbers show the generation of the indicated bifurcations. Scale bars, 200 µm.

(C) Reconstructed organoid from the induced UB and embryonic MM. The peripheral nephrogenic zone is shown. Note that the Six2⁺ nephron progenitors surround the CK8⁺ ureteric tips to form the capping mesenchyme. The right panel shows a magnified image of the enclosed area in the left panel. Scale bars, 100 µm.

(D) Reconstructed organoids from the indicated progenitor populations. Scale bars, 100 µm.

(E) Reconstructed organoid from the iUB plus iNPs. The right two panels show magnified images of the ureteric tip region. Scale bars, 100 µm.

(F) Immunostaining of sectioned organoids. Left panel: E14.5 embryonic kidney. Right panel: Reconstructed organoid. Note that the embryonic kidney, but not the organoid, contains CD31⁺ vascular endothelial cells. Scale bars, 200 µm.

(G, H) Reconstructed organoids from Hoxb7-GFP ESC-derived iNPs, iUB, and E11.5 tdTomato mouse embryonic kidney SPs. (G) Low-magnification images of the organoids are shown. Scale bars, 100 µm. (H) Immunostaining of sectioned organoids. The right panel shows a magnified image of the enclosed area in the left panel. Note that the Six2⁺ capping mesenchyme and E-Cadherin⁺ epithelial components (including developing nephron and ureteric epithelium) are negative for tdTomato signals. Scale bars, 50 µm (left panel), 20 µm (right two panels).
Figure S5 Reconstructed organoid resembles the E15.5 kidney in global gene expression profile (related to Figure 5).

(A) Comparison of kidney developmental gene expression profiles among mouse embryonic kidneys at various developmental stages (from E11.5 to P0), 7-day cultured E11.5 kidney, and reconstructed kidney organoids (day 0 and day 7). Genes downregulated with developmental progression were selected to undergo hierarchical clustering analysis. ORG1, 2, 3: triplicate samples of reconstructed organoids. Day 0 represents the iUB+iNP+SP reaggregated tissue before starting the organ culture.

(B) Gene expression kinetics of mature nephric tubule (proximal tubule) and collecting duct markers examined by microarray analysis data. Relative expression levels against the normalized median value for each probe are shown as log2 fold changes.
Figure S6. Induction efficiency of UB and NP lineage by Step 1/2 modulation (related to Figure 6).

The upper table and graph show the induction efficiency of CXCR4+/KIT+ committed WD progenitor from hiPSCs at day 6.25. The lower table and graph show the induction efficiency of ITGA8+/PDGFRA- metanephric nephron progenitor from hiPSCs at day 12. The applied concentrations of Step 1 Activin (A) and Step 2 Bmp4 (B) and CHIR99021 (C) are shown as follows: Activin and Bmp4: ng/ml; CHIR: μM.
Figure S7. PAX2 is cell-autonomously required for human iUB development (related to Figure 7).

(A) Temporal kinetics of marker gene expressions in two independent control and knockout clones. Relative expression levels of the transcripts to β-actin expression are presented (n=3).

(B) Immunostaining of a day 12.5 UB stage spheroid. High-magnification images are shown. Note that E-CADHERIN protein shows membranous accumulation in the control UB, but not in the knockout clone-derived UB. Scale bars, 10 µm.

(C) hiPSC-derived iNP and iUB were reconstructed and cultured in the organ culture settings. Fluorescence images of the encapsulated iUB tissues are shown in the lower panels. Arrowheads indicate the budding sites of the iUB. Scale bars, 200 µm.

(A) Temporal kinetics of marker gene expressions in two independent control and knockout clones. Relative expression levels of the transcripts to β-actin expression are presented (n=3).

(B) Immunostaining of a day 12.5 UB stage spheroid. High-magnification images are shown. Note that E-CADHERIN protein shows membranous accumulation in the control UB, but not in the knockout clone-derived UB. Scale bars, 10 µm.

(C) hiPSC-derived iNP and iUB were reconstructed and cultured in the organ culture settings. Fluorescence images of the encapsulated iUB tissues are shown in the lower panels. Arrowheads indicate the budding sites of the iUB. Scale bars, 200 µm.
Table S1. Related to Figures 1 to 7. Primer sequences.

Movie S1. Related to Figure 3. Z-stack imaging of reconstructed kidney organoid at day 7 of culture.
Table S1. (Related to Figures 1 to 7)

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