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In Vitro Propagation and Branching Morphogenesis from Single Ureteric Bud Cells

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SUMMARY

A method to maintain and rebuild ureteric bud (UB)-like structures from UB cells in vitro could provide a useful tool for kidney regeneration. We aimed in our present study to establish a serum-free culture system that enables the expansion of UB progenitor cells, i.e., UB tip cells, and reconstruction of UB-like structures. We found that fibroblast growth factors or retinoic acid (RA) was sufficient for the survival of UB cells in serum-free condition, while the proliferation and maintenance of UB tip cells required glial cell-derived neurotrophic factor together with signaling from either WNT-β-catenin pathway or RA. The activation of WNT-β-catenin signaling in UB cells by endogenous WNT proteins required R-spondins. Together with Rho kinase inhibitor, our culture system facilitated the expansion of UB tip cells to form UB-like structures from dispersed single cells. The UB-like structures thus formed retained the original UB characteristics and integrated into the native embryonic kidneys.

INTRODUCTION

Kidney development begins with outgrowth of the ureteric bud (UB) at the caudal end of the nephric duct into the surrounding metanephric mesenchyme (MM). After invasion into MM, the UB forms tip and stalk regions and starts branching morphogenesis to form the collecting system (Dressler, 2009; Costantini and Kopan, 2010; Nagalakshmi and Yu, 2015). UB tip cells have higher proliferation rates and act as progenitor cells to produce UB stalks, and further differentiate into collecting ducts (Costantini and Kopan, 2010; Michael and Davies, 2004; Shakya et al., 2005). Through canonical WNT9B signaling, UB cells play two opposing roles for MM cells, i.e., maintaining stemness of MM cells while inducing their differentiation to form the remaining nephron structures (Carroll et al., 2005; Karner et al., 2011). In turn, MM cells release glial cell-derived neurotrophic factor (GDNF), which regulates UB tip cell proliferation and branching morphogenesis through RET tyrosine kinase receptor and GFRA1 co-receptor (Vega et al., 1996; Takahashi, 2001).

The expression of RET tyrosine kinase receptor in UB tip cells is regulated by multiple signaling pathways. For example, WNT-β-catenin activity that first appears in UB and then persists in UB tip cells during kidney development was shown to be required for maintaining UB tip identity from studies using UB-specific β-catenin knockout models (Iglesias et al., 2007; Bridgewater et al., 2008; Marose et al., 2008). Retinoic acid (RA) derived from the stromal cells is another signaling pathway that maintains *Ret* expression in UB tip cells, as the expression of the dominant-negative form of RA receptor was found to inhibit both *Ret* expression and UB branching (Batourina et al., 2001; Rosselot et al., 2010). Another important signaling pathway for *Ret* expression and UB branching is fibroblast growth factor (FGF). FGF7 and FGF10 are expressed in stromal and MM cells, and loss of FRS2A/FGFRR2 receptor in UB cells was found to cause a reduction in *Ret* expression with fewer UB tips (Qiao et al., 1999b; Michos et al., 2010; Bates, 2011).

The in vitro UB culture system has been widely used to investigate the regulation of UB branching morphogenesis. These studies have unraveled important roles played by multiple growth factors, including endodermal growth factor, hepatocyte growth factor, epidermal growth factor (EGF)-EGF receptor, FGF, vascular endothelial growth factor A (VEGF-A)-VEGF receptor 2, and transforming growth factor β superfamily members (Perantoni et al., 1991; Santos and Nigam, 1993; Sakurai and Nigam, 1997; Qiao et al., 1999a, 2001; Bush et al., 2004; Woolf et al., 1995; Ishibe et al., 2009; Marlier et al., 2009), as well as soluble factors, such as pleiotrophin (Sakurai et al., 2001) and heregulin (Sakurai et al., 2005), and extracellular matrix, such as type I and IV collagen and Matrigel (Perantoni et al., 1991; Rosines et al., 2007). Serum was also found to be required to promote UB branching morphogenesis in vitro (Takayama et al., 2014). However, the use of culture media containing serum and/or conditioned medium from an MM cell line (BSN-CM) in these studies made it difficult to examine the effect of a specific signaling pathway.

We aimed in our present study to establish an MM- and serum-free culture system that enables the propagation of UB cells with defined factors, and to test the possibility of reconstructing UB structures from single UB cells maintained under this culture condition in vitro. We found that the combination of GDNF, FGF, WNT-β-catenin

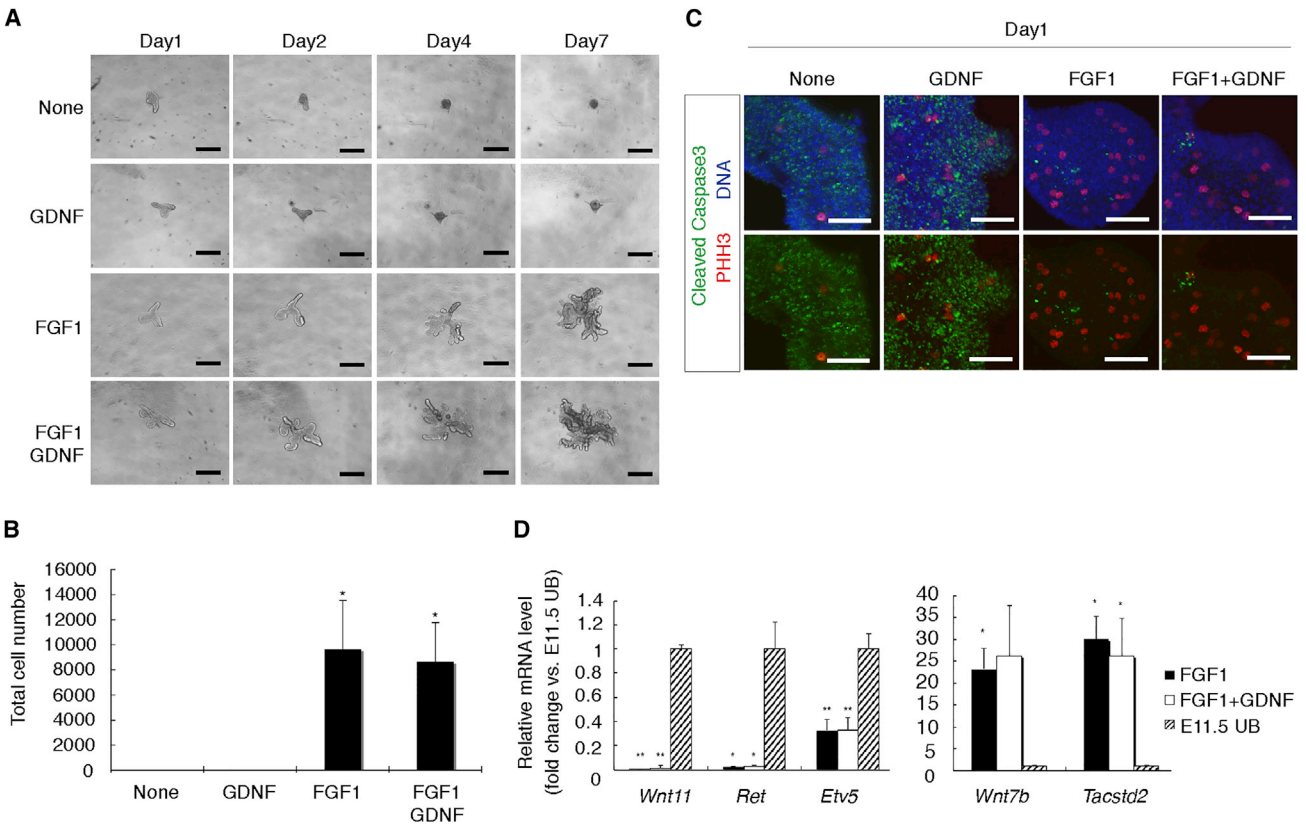


Figure 1. FGF Signaling Is Required for UB Survival

(A) Morphology of representative UBs in culture. UBs did not survive after 4 days in serum-free culture. Addition of GDNF alone was without effect. Addition of FGF1, with or without GDNF, supported UB survival and proliferation. Scale bars, 500 μm.

(B) The number of UB cells on day 7 increased only in the presence of FGF1. No additional effect with the addition of GDNF (n = 3 independent replicates; *p < 0.05 versus none).

(C) Immunostaining of cultured UB for apoptosis marker, cleaved caspase 3 (green), proliferation marker, PHH3 (red), and DNA (blue). Extensive apoptotic cells were detected in samples without or with GDNF treatment alone. Treatment with FGF1, with or without GDNF, reduced apoptotic cells and increased proliferating cells. Scale bars, 50 μm.

(D) qRT-PCR results showed significantly lower mRNA expression levels for UB tip marker genes (*Wnt11*, *Ret*, and *Etv5*) and higher expression levels for UB stalk marker genes (*Wnt7b* and *Tacstd2*) in UB treated with FGF1, with or without GDNF on day 7. Data were normalized by *Gapdh* expression levels and presented as fold changes from E11.5 UB cells on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus E11.5 UB).

signaling, and RA, together with Rho-associated kinase (ROCK) inhibitor, enabled the expansion of dispersed single UB cells to reconstruct UB-like structures that retained the in vivo characteristics of the original UB.

RESULTS

FGF Signaling Is Required for UB Cell Survival

As shown in Figure 1, UB isolated from embryonic day 11.5 (E11.5) kidneys did not survive in MM- and serum-free culture medium. Addition of GDNF alone was without effect. Under these conditions, UB cells showed extensive cleaved caspase-3 signals, detected as early as day 1 (Figure 1C), and eventually died by day 4 (Figure 1A). In contrast, addition of FGF1 allowed UB cells to survive and proliferate (Figures 1A and 1B). This was accompanied by a decrease in cleaved caspase-3 signals and an increase in $PHH3^{+}$ cells on day 1 (Figure 1C). No additional effect on UB cell proliferation was noted when GDNF was added on top of FGF1 (Figures 1B and 1C). However, treatment with FGF1, alone or in combination with GDNF, could not sustain the mRNA expression levels of UB tip markers, such as *Wnt11*, *Ret*, and *Etv5*, while the expression of UB stalk markers, such as *Wnt7b* and *Tacstd2*, increased significantly at day 7 compared with E11.5 UB (Figure 1D). We further examined the effects of FGF7 and FGF10, as they have also been

implicated in kidney development (Qiao et al., 1999b; Michos et al., 2010). As shown in Figure S1, we found that while both FGF7 and FGF10 allowed UB cell survival, FGF7 was more effective than FGF10 in promoting UB cell proliferation, and no additional effect of GDNF was detected. However, compared with FGF1 and FGF10, FGF7 was more effective in sustaining the expression of UB tip markers *Wnt11* and *Etv5*. For an unknown reason, FGF7 could not sustain the expression of *Ret* even when combined with GDNF. These results therefore suggest the involvement of an additional FGF-independent pathway(s) in the maintenance of *Ret* expression.

WNT-β-Catenin Signaling Potentiates the Proliferation of UB Cells

Since GDNF-RET signaling is a key regulator for UB tip cell proliferation (Michael and Davies, 2004; Pepicelli et al., 1997), and WNT-β-catenin signaling has been shown to be important in maintaining UB tip cell identity (Bridgewater et al., 2008; Marose et al., 2008), we next examined the effect of WNT-β-catenin signaling by first testing the effect of a selective GSK3β inhibitor, CHIR99021 (Polychronopoulos et al., 2004). As shown in Figure 2A, UB isolated from E11.5 kidneys did not survive when CHIR99021, alone or together with GDNF, was added. When combined with FGF1, CHIR99021 potentiated the stimulatory effect of FGF1 on UB cell proliferation (Figures 2A and 2B). When GDNF was added on top of FGF1 and CHIR99021, it further boosted UB cell proliferation and formed extensive branching structures (Figures 2A and 2B). Parallel to these results, addition of CHIR99021 to FGF1 and GDNF further reduced cleaved caspase-3 signals and increased $PHH3^+$ cells (Figure 2C). qRT-PCR results showed that the combination of CHIR99021 with FGF1 and GDNF increased mRNA expression levels of UB tip markers, including *Wnt11*, *Ret*, and *Etv5*, while the expression of UB stalk markers, such as *Wnt7b* and *Tacstd2*, was reduced (Figure 2D). These results suggest that canonical WNT-β-catenin signaling, when combined with FGF1 and GDNF, contributes to the proliferation and enrichment of UB tip cells.

R-Spondin Mediates the Effect of WNT-β-Catenin Signaling in UB Cells

Despite these CHIR99021 results, we could not reproduce a similar positive effect by adding WNT9B or WNT11 directly (Figures S2A and S2B), both of which are known to be expressed in UB cells that activate WNT-β-catenin signaling (Karner et al., 2011; Friedman et al., 2009). Since R-spondin (RSPO) proteins are known to serve as agonists that mediate the activation of WNT-β-catenin signaling (de Lau et al., 2012), and UB branching abnormality was reported in an RSPO receptor, LGR4, knockout model (Mohri et al., 2011), it is conceivable that RSPO proteins may be required for the activation of WNT-β-catenin signaling in UB cells. The recent reports of the involvement of LRP6, a low-density lipoprotein receptor-related protein 6, for *Ret* induction by canonical WNT signaling (Wang et al., 2016; Matsumoto et al., 2014) are also consistent with such notion. To test this possibility, we first examined the expression of *Rspo1–4* and their receptors, *Lgr4–6* (Carmon et al., 2011; de Lau et al., 2011; Wang et al., 2013), in manually dissected MM and UB from mouse E11.5 kidneys by qRT-PCR. As shown in Figure 3A, we found that both *Rspo1* and *Rspo3* were expressed in MM, while *Rspo1–4* were not detected in UB. On the other hand, *Lgr4* was found to be expressed in both MM and UB, while *Lgr5* was detected mainly in UB and *Lgr6* was barely detectable in both tissues (Figure 3A). These results thus suggest that R-spondin ligand-receptor relationship is conserved between MM and UB. We therefore tested the effects of RSPO1 and RSPO3 on UB proliferation and branching morphogenesis. Similar to CHIR99021, we found that addition of RSPO1, alone or together with GDNF, was without effect on UB cells (Figures 3B and 3C). When combined with FGF1, RSPO1 enhanced UB cell proliferation (Figures 3B and 3C). With the combination of RSPO1 with GDNF and FGF1, we observed a significant UB cell proliferation with extensive branching morphogenesis (Figures 3B and 3C). This was associated with reduced cleaved caspase-3 signals and an increase in $PHH3^+$ cells (Figure 3D). qRT-PCR results showed an increase in the expression of UB tip markers, *Wnt11*, *Ret*, and *Etv5*, with decreased expression in UB stalk markers, *Wnt7b* and *Tacstd2* (Figure 3E). Immunostaining for ETV5 also showed an increased population of tip cells with an increased expression level of ETV5 at the tips of the branching structure when GDNF and FGF1 were combined with RSPO1 (Figure 3F). A similar effect was observed with RSPO3 (Figure S3). In support of the fact that the effects of RSPO1 were mediated through activation of WNT-β-catenin signaling, we found that both XAV939, a specific WNT-β-catenin signaling inhibitor (Huang et al., 2009), and IWP2, a WNT secretion inhibitor (Chen et al., 2009), abolished the effects of RSPO1 on UB cell proliferation, branching morphogenesis, and the expression of UB tip markers (Figures 3G–3I). These results therefore indicate that RSPO proteins act as agonists mediating the activation of Wnt-β-catenin signaling by endogenous WNT proteins in UB cells, and constitute an additional important player for the maintenance of UB tip cell identity.

Retinoic Acid Promotes the Proliferation and Enrichment of UB Tip Cells

Since RA is known as another signaling pathway regulating *Ret* expression and plays a crucial role in kidney

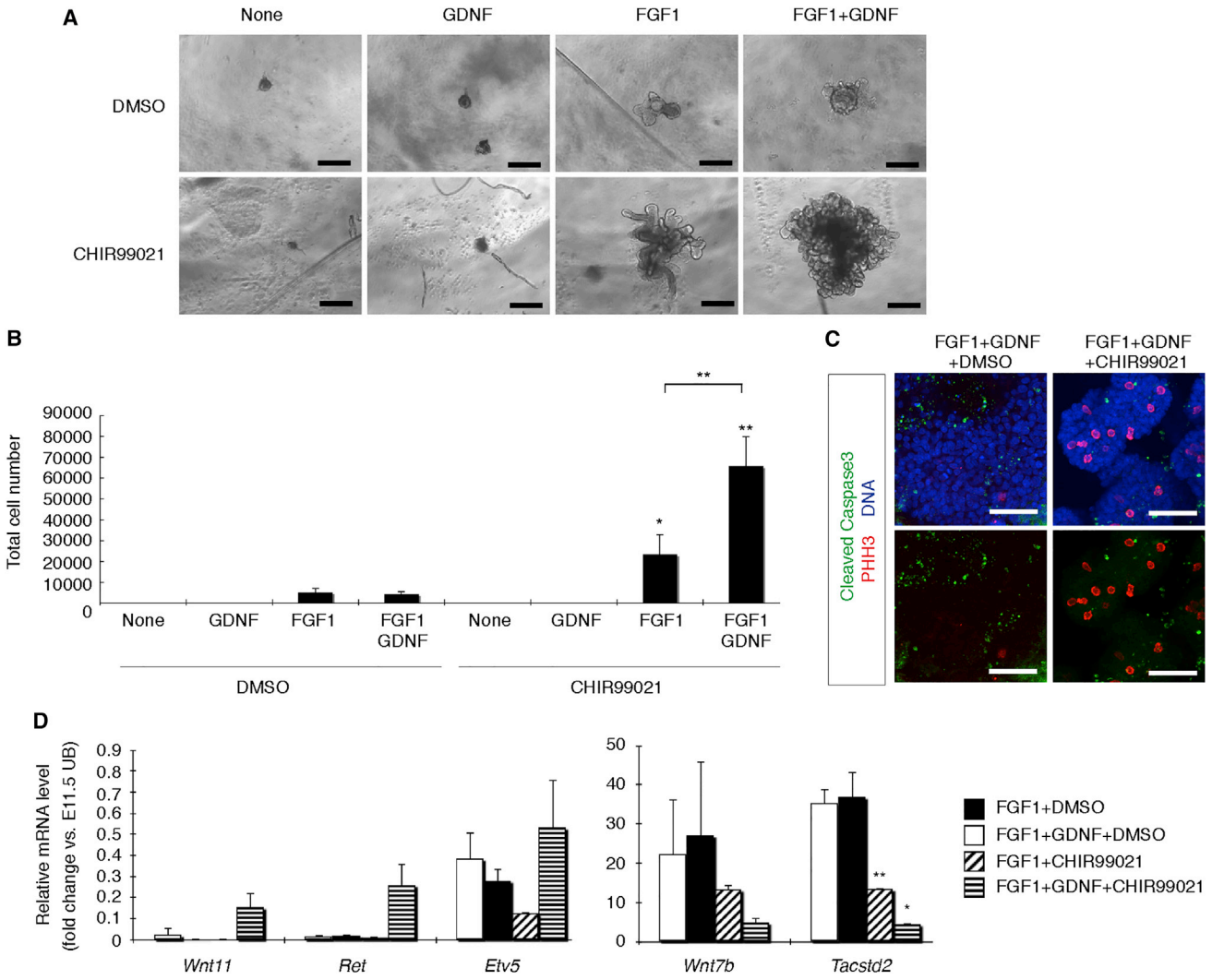


Figure 2. CHIR99021, a GSK3β Inhibitor, Promoted UB Proliferation When Combined with FGF1 and GDNF

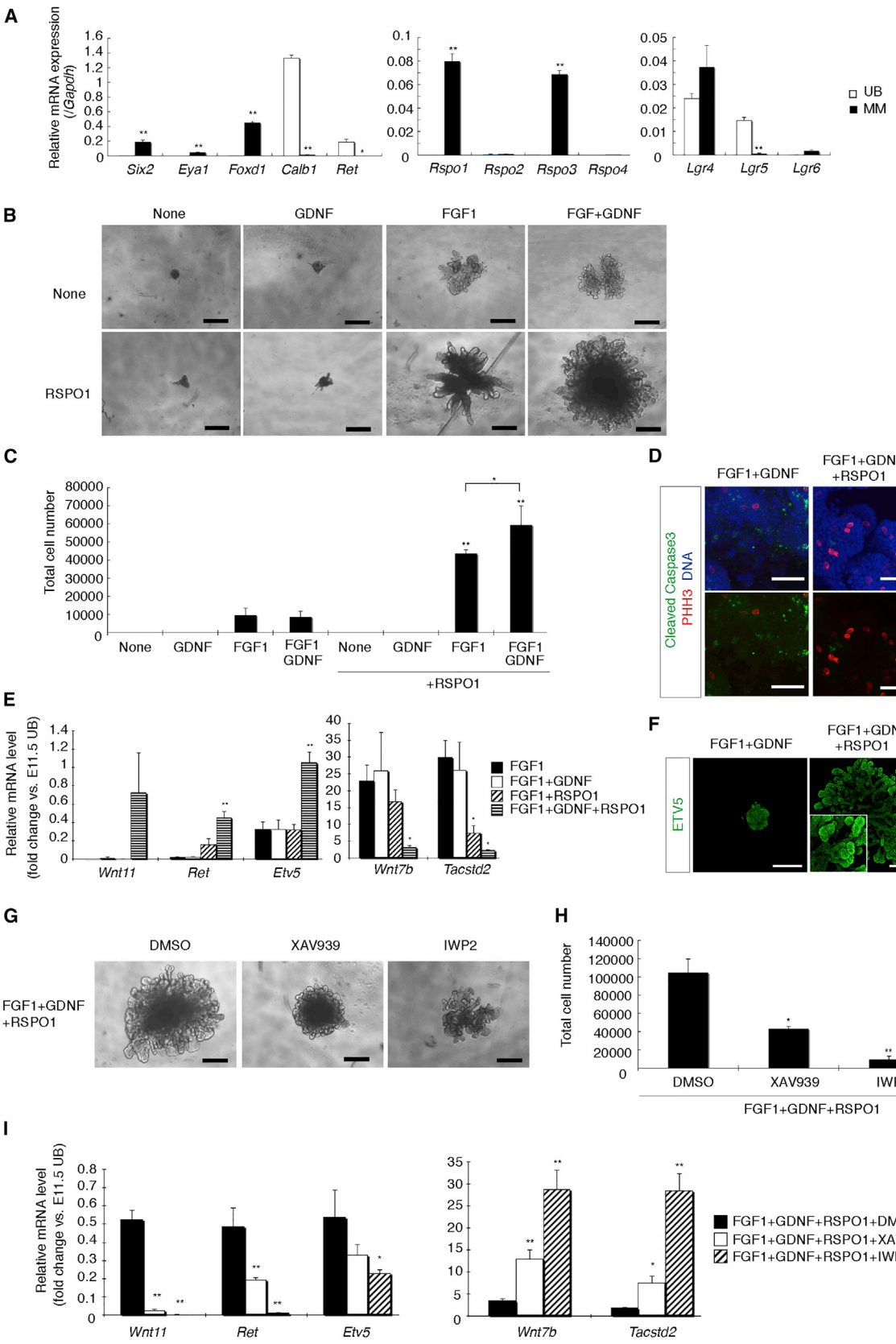
(A) Morphology of representative UBs on day 7 in culture. No effect was found with the addition of CHIR99021, with or without GDNF. When combined with FGF1, CHIR99021 potentiated the stimulatory effect of FGF1 on UB cell proliferation. Extensive UB proliferation and branching were observed when UB was treated with GDNF, FGF1, and CHIR99021. Scale bars, 500 μm.

(B) The number of UB cells on day 7 increased significantly when UB was treated with FGF1 and CHIR99021. Combination of CHIR99021 with GDNF and FGF1 further boosted the proliferation of UB cells (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus corresponding DMSO samples).

(C) Immunostaining of cultured UB on day 7 for apoptosis marker, cleaved caspase 3 (green), proliferation marker, PHH3 (red), and DNA (blue). Fewer apoptotic and more proliferating cells were detected in UB treated with GDNF, FGF1, and CHIR99021. Scale bars, 50 μm.

(D) qRT-PCR results showed higher mRNA expression levels on day 7 for UB tip marker genes (*Wnt11*, *Ret*, and *Etv5*) and lower expression levels for UB stalk marker genes (*Wnt7b* and *Tacstd2*) in UB treated with FGF1, GDNF, and CHIR99021. Data were normalized by *Gapdh* expression levels and presented as fold changes from E11.5 UB cells on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus corresponding DMSO samples).

development (Batourina et al., 2001; Rosselot et al., 2010), we tested its effect in our UB culture system. We found that RA alone allowed UB cells to survive and expand, albeit only to a limited extent (Figures 4A and 4B). However, when combined with GDNF, with or without FGF1, RA stimulated UB cell proliferation significantly (Figures 4A and 4B). This was associated with a decrease in cleaved caspase-3 signals and an increase in PHH3^{+} cells (Figure 4C). qRT-PCR results showed an increased expression in UB tip markers, *Wnt11*, *Ret*, and *Etv5*, with a decreased expression in UB stalk markers, *Wnt7b* and *Tacstd2* (Figure 4D). Immunostaining for ETV5 again showed an increase in



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tip cell population with increased level of ETV5 expression at the tips of the extensive branching structure (Figure 4E). These results thus indicate that RA plays two different roles for UB cells in culture: one is for the proliferation and maintenance of UB tip cell identity through GDNF-RET signaling, and the other is for the survival of UB cells independent of GDNF-RET signaling.

Plasticity of UB Tip and Stalk Cells

While UB tip cells are considered to be the progenitor cells that give rise to stalk cells, previous studies have demonstrated the possibility of transition in the reverse direction with stalk cells becoming tip cells (Sweeney et al., 2008). We tested this phenomenon in our culture system and examined the effects of WNT-β-catenin and RA signaling systems. Tip and stalk regions were manually separated from E11.5 UB, and each structure was cultured with FGF1 and GDNF, together with either RSPO1 (Figures 5B and 5C) or RA (Figures 5D and 5E). Although visual inspection alone could not confirm complete separation between tip and stalk regions, the effective separation of these two structures was confirmed by the absence or lower expression of markers of the opposite structure by qRT-PCR (Figure 5A). After 7 days in culture, we found that both UB tips and stalks proliferated and formed extensive branching with tip-like structures (Figures 5B and 5D). qRT-PCR results also revealed an increase in UB tip markers with a decrease in UB stalk markers in cultures from UB stalk, and vice versa (Figures 5B and 5D). Immunostaining for ETV5 confirmed the presence of tip cells that express ETV5 at the tips from both groups of samples (Figures 5B and 5D). These results demonstrate that the differentiation between UB tip and stalk cells are interchangeable under our in vitro culture condition with defined factors.

Propagation and Reconstruction of UB-like Structure from Single UB Cells

Based on these findings with isolated E11.5 UB, we tested the possibility of expanding dispersed UB cells and reconstructing UB-like structures from single UB cells using our culture system. Dispersed UB cells were isolated from E11.5 Hoxb7-Venus^{+} mouse kidneys by fluorescence activated cell sorting (FACS) and seeded at approximately 100 cells per well. After 7 days in culture, we found that no colony formed in most of the combinations of factors, except for only few colonies (up to two per well) in combinations containing RA (Figures 6A and 6B). These results therefore show that dispersed single UB cells could not survive under the same conditions under which isolated E11.5 UB could. To investigate possible anoikis and apoptosis associated with isolated single cells, we tested the effect of Y-27632, a selective ROCK inhibitor known to inhibit apoptosis of human embryonic stem cells in single-cell culture (Watanabe et al., 2007). As shown in Figures 6A and 6B, we found that addition of Y-27632 allowed dispersed single UB cells to survive and develop colonies

Figure 3. R-Spondin1 Promoted UB Tip Proliferation via WNT-β-Catenin Signaling

(A) UB was manually separated from MM (MM + stroma) from E11.5 kidneys. The separation was confirmed by undetectable levels of MM markers, *Six2* and *Eya1*, and stroma marker, *Foxd1*, in UB, and undetectable levels of UB markers, *Calb1* and *Ret*, in MM. *Rspo1* and *Rspo3* were detected in MM. The receptor *Lgr4* was detected in both MM and UB, while receptor *Lgr5* was detected in UB only. All mRNAs were normalized with *Gapdh* expression level (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus UB).

(B) Morphology of representative UBs on day 7 in culture. No effect was found with the addition of RSPO1, with or without GDNF. When combined with FGF1, RSPO1 potentiated the stimulatory effect of FGF1 on UB cell proliferation. Extensive UB proliferation with branching occurred when treated with GDNF, FGF1, and RSPO1. Scale bars, 500 μm.

(C) UB cell number on day 7 increased significantly when treated with RSPO1 with FGF1. Combination of RSPO1 with GDNF and FGF1 further boosted the proliferation of UB cells (n = 3 independent replicates; *p < 0.05 versus FGF1 + RSPO1 samples and **p < 0.01 versus corresponding samples without RSPO1).

(D) Immunostaining of cultured UB on day 7 for apoptosis marker, cleaved caspase 3 (green), proliferation marker, PHH3 (red), and DNA (blue). Fewer apoptotic and more proliferating cells were detected in UB treated with GDNF, FGF1, and RSPO1. Scale bars, 50 μm.

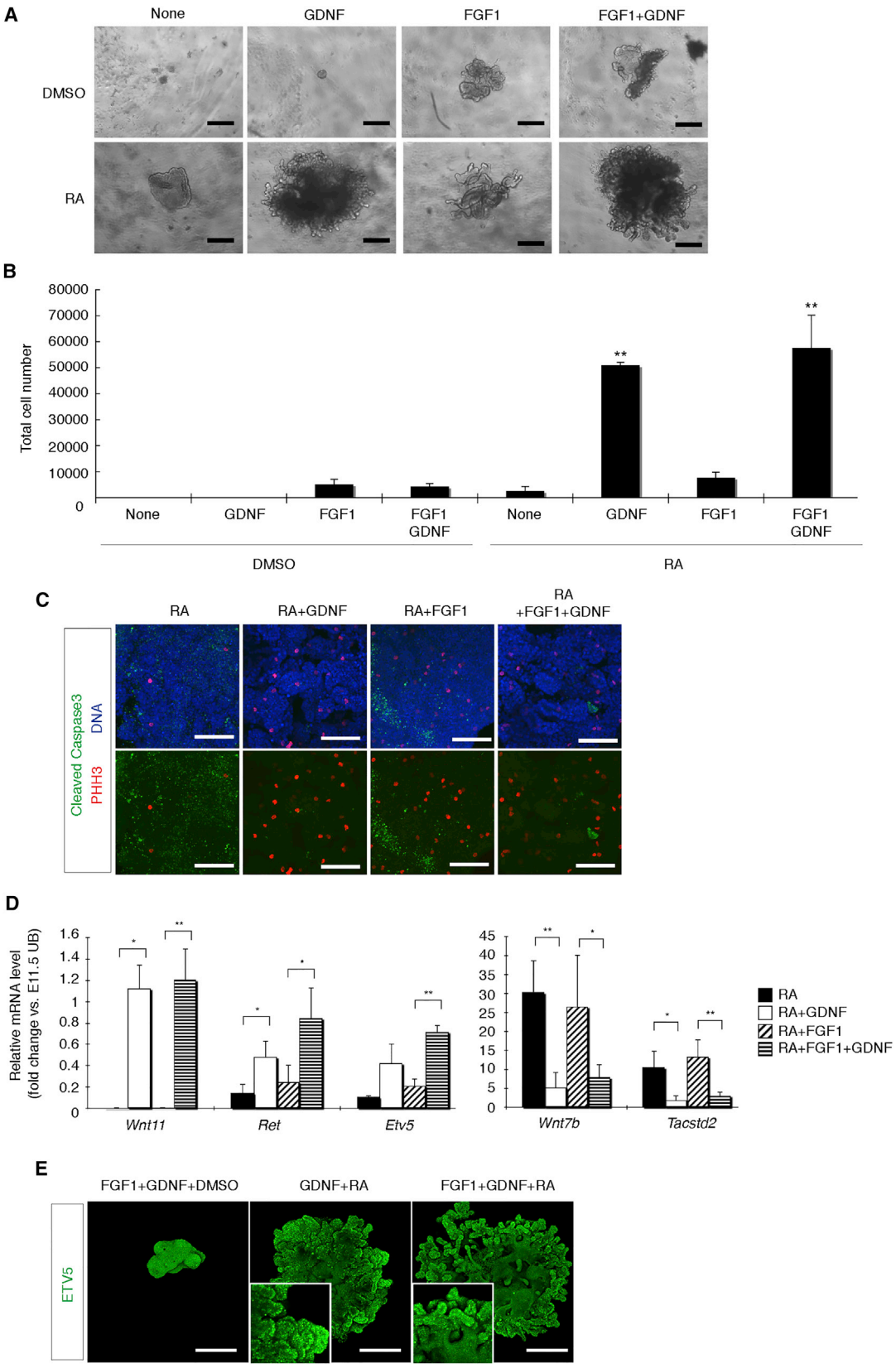
(E) qRT-PCR results showed higher mRNA expression levels for UB tip marker genes (*Wnt11*, *Ret*, and *Etv5*) and lower expression levels for UB stalk marker genes (*Wnt7b* and *Tacstd2*) in UB treated with GDNF, FGF1, and RSPO1 on day 7. Data were normalized by *Gapdh* expression levels and presented as fold changes from E11.5 UB cells on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus corresponding samples without RSPO1).

(F) Immunostaining of cultured UB on day 7 for UB tip marker, ETV5 (green). The stained nuclei of ETV5 mainly are positive in the tips of the structure with RSPO1. Scale bars, 500 μm.

(G) Morphology of representative UBs on day 7 showing the inhibitory effect of XAV939 or IWP2 on the stimulation of UB proliferation and branching by GDNF, FGF1, and RSPO1. Scale bars, 500 μm.

(H) The increase in UB cell number on day 7 by FGF1 + GDNF + RSPO1 was significantly reduced by XAV939 or IWP2 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus FGF1 + GDNF + RSPO1 samples).

(I) qRT-PCR results showed significantly reduced mRNA expression levels for UB tip marker genes and significantly higher expression levels for UB stalk marker genes in UB treated with XAV939 or IWP2 on day 7. Data were normalized by *Gapdh* expression levels and presented as fold changes from E11.5 UB cells on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus FGF1 + GDNF + RSPO1 samples).



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under most of the conditions tested. We also found that FGF signaling is essential for colony formation (data not shown). The number of colonies formed reached maximum when Y-27632 was combined with FGF1, GDNF, RA, and CHIR99021 (Figure 6A). After 9 days in our MM- and serum-free culture condition, we were able to demonstrate the development of UB-like structures from colonies that developed from single UB cells (Figure 6C). In particular, when treated with combinations that contained RA, we detected the initial formation of a cyst-like structure with central lumen as early as day 6, followed by extensive branching morphogenesis up to day 9 (Figure 6C). These results suggest that formation of these structures required RA, as they were undetected when cultured without RA (Figure 6C), and addition of CHIR99021 to the combination of GDNF, FGF1, and RA appeared to have an additive effect to further boost cell proliferation (Figure 6D) and promote branching morphogenesis (Figure 6C). qRT-PCR results showed that addition of RA to other growth factors was associated with increased expression in UB tip markers and decreased expression in UB stalk markers (Figure 6E). The relative enrichment in tip cells under such culture conditions may correlate with the shorter stalk segments in the resulting branching structure, compared with E13.5 embryonic kidneys (Figure 6C). However, we did notice the lengthening of stalks with an increase in stalk markers on qRT-PCR after longer incubation for up to 14 days (Figure S4). We also noted an elevated mRNA expression level of differentiated collecting duct marker, *Aqp2*, with the addition of RA to GDNF and FGF1, which was suppressed by CHIR99021 (Figure 6E).

In separate studies we tried the same combination of factors, i.e., GDNF, FGF1, RA, CHIR99021, and Y-27632, for 2D culture in either non-coated plates or plates coated with a thin or thick (1.5-mm) layer of Matrigel (Figure S5). We found that while both non-coated and thin-coated plates were able to sustain the proliferation of UB cells to some extent, the expression levels of UB tip cell markers were not maintained. It was only when the culture plates were coated with a thick layer of Matrigel, hence under a culture condition similar to that of 3D culture, that we were able to observe the formation of a branching structure, the proliferation of UB cells, and the maintenance of UB tip cell markers (Figure S5). Thus, the extracellular matrix used in 3D culture represents another important component required for the maintenance of UB tip cells under our culture conditions.

To further examine whether the UB cells contained in these reconstructed UB-like structures retained their original characteristics to maintain SIX2$^+$ nephron progenitor cells, we dissociated UB cells from these UB-like structures and reaggregated them with MM cells isolated from E11.5 kidneys, according to the conventional reaggregation assay procedure (Unbekandt and Davies, 2010; Ganeva et al., 2011; Yuri et al., 2015). We found that, similar to UB cells freshly isolated from E11.5 kidneys, when UB cells derived from the reconstructed UB-like structures were reaggregated with E11.5 MM cells and cultured for another 7 days, they were able to form branching structures and maintain SIX2$^+$ nephron progenitor cells to further differentiate to form LTL$^+$ and PODXL1$^+$ structures, markers for proximal tubular cells and podocytes, respectively (Figure 7A). In separate experiments, we also tested the function of the intact reconstructed UB-like structure, i.e., without dissociation into single cells, by replacing the UB from a metanephros with the reconstructed UB-like structure. We found that, similar to the original UB, the reconstructed UB-like structure was able to maintain SIX2$^+$ nephron progenitor cells, induce the differentiation of nephron progenitor cells to form LTL$^+$ and PODXL1$^+$ structures, and form branching structures that connect to distal tubules (Figures 7B and S6).

DISCUSSION

In this study we showed the feasibility of maintaining UB growth in vitro in an MM- and serum-free culture medium containing defined growth factors. This simplified culture

Figure 4. Retinoic Acid Supported UB Survival and Promoted UB Tip Proliferation When Combined with GDNF

(A) Morphology of representative UBs on day 7 in culture. Treatment with the combination of RA and GDNF, with or without FGF1, led to extensive UB proliferation with branching. Scale bars, 500 μm.

(B) The number of UB cells on day 7 increased significantly when treated with the combination of RA and GDNF, with or without FGF1 (n = 3 independent replicates; **p < 0.01 versus corresponding samples without RA).

(C) Immunostaining of cultured UB on day 7 for apoptosis marker, cleaved caspase 3 (green), proliferation marker, PHH3 (red), and DNA (blue). Fewer apoptotic and more proliferating cells were detected in UB treated with the combination of RA and GDNF, with or without FGF1. Scale bars, 100 μm.

(D) qRT-PCR results showed significantly higher mRNA expression levels on day 7 for UB tip marker genes (*Wnt11*, *Ret*, and *Etv5*) and lower expression levels for UB stalk marker genes (*Wnt7b* and *Tacstd2*) in UB treated with the combination of RA and GDNF, with or without FGF1. Data were normalized by *Gapdh* expression levels and presented as fold changes from E11.5 UB cells on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus corresponding samples without GDNF).

(E) Immunostaining of cultured UB on day 7 for UB tip marker, ETV5 (green). The stained nuclei of ETV5 are positive mainly in the tips of the branching structure in samples treated with RA. Scale bars, 500 μm.

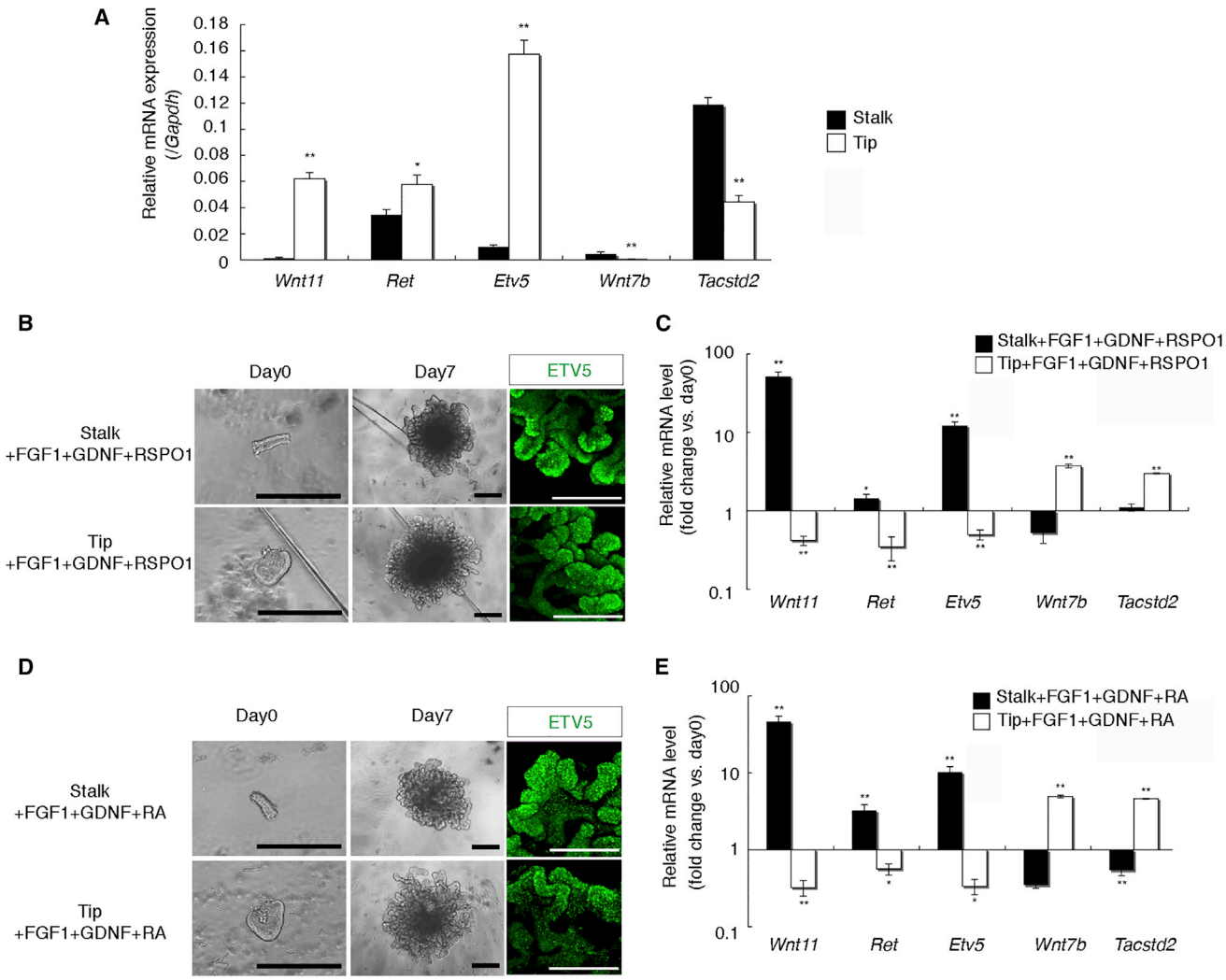


Figure 5. UB Tip and Stalk Conversion Occurred When Treated with the Combination of FGF1, GDNF, and RSPO1 or FGF1, GDNF, and RA

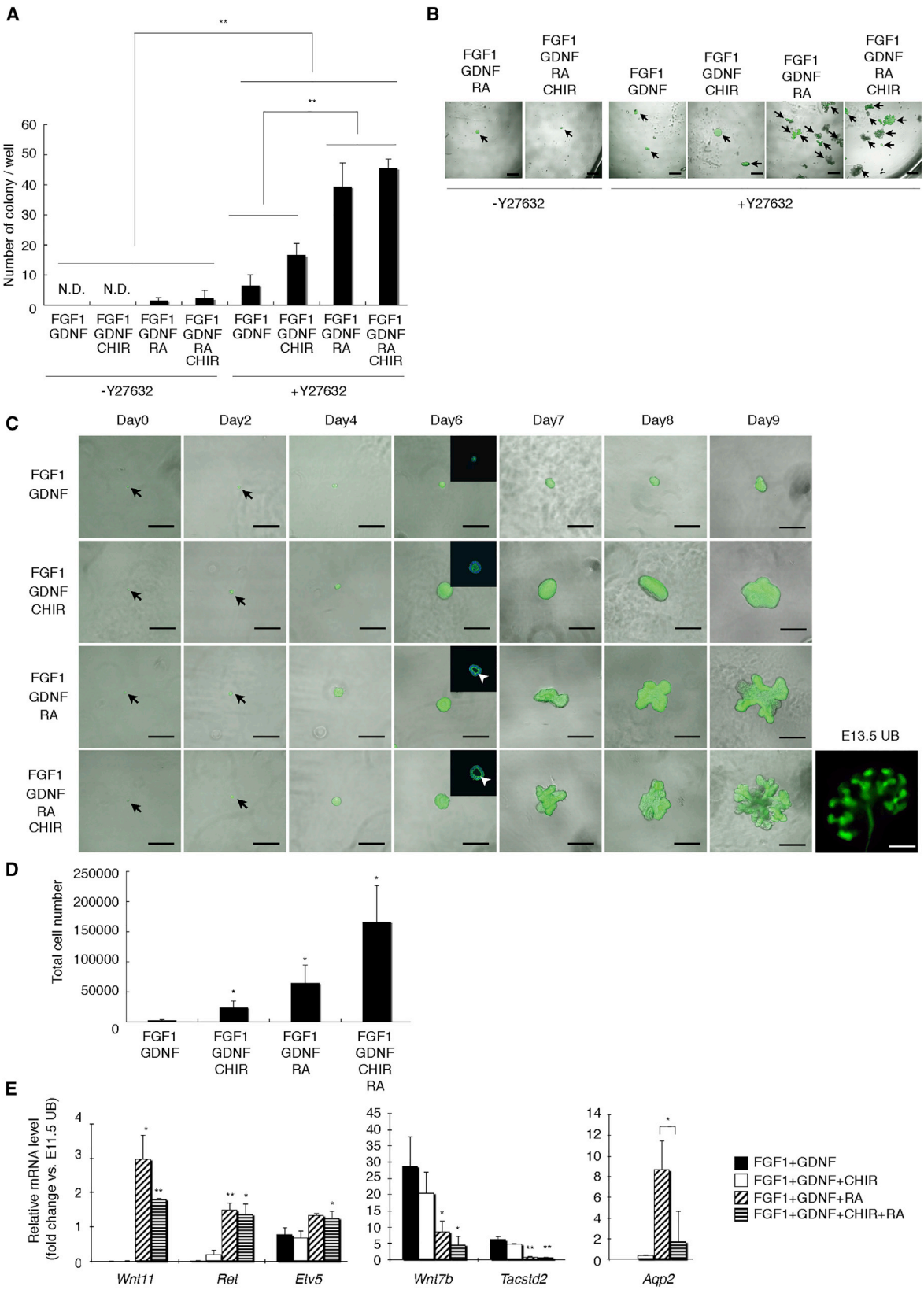
(A) qRT-PCR results showed the effective separation of UB tip and stalk tissues from E11.5 UB. The mRNA expression levels of UB tip markers, such as *Wnt11*, *Ret*, and *Etv5*, were significantly higher in the dissected UB tip tissues, while those of UB stalk markers, such as *Wnt7b* and *Tacstd2*, were significantly higher in the dissected UB stalk tissues. All mRNAs were normalized with *Gapdh* expression level (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus stalk).

(B) Morphology of a representative UB stalk and tip on day 0 and day 7 in culture. Treatment with the combination of FGF1, GDNF, and RSPO1 led to extensive proliferation with branching from both UB stalk and tip. Immunostaining revealed the expression of tip marker ETV5 at the tips of the branching structures developed from both UB stalk and tip. Scale bars, 500 μm.

(C) qRT-PCR results showed a significant increase in the expression of UB tip markers and decrease in expression levels for UB stalk markers from UB stalk tissues, and a significant decrease in expression levels for UB tip markers with significant increase in expression of UB stalk markers from UB tip tissues, after 7 days in culture with the combination of FGF1, GDNF, and RSPO1. Data were normalized by *Gapdh* expression levels and presented as fold changes from respective UB stalk or tip tissues on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus corresponding samples at day 0).

(D) Morphology of a representative UB stalk and tip on day 0 and day 7 in culture. Treatment with the combination of FGF1, GDNF, and RA led to extensive proliferation with branching from both UB stalk and tip. Immunostaining revealed the expression of tip marker ETV5 at the tips of the branching structures developed from both UB stalk and tip. Scale bars, 500 μm.

(E) qRT-PCR results showed a significant increase in the expression of UB tip markers and decrease in the expression of UB stalk markers from UB stalk tissues, and vice versa from UB tip tissues, after 7 days in culture with the combination of FGF1, GDNF, and RA. Data were normalized by *Gapdh* expression levels and presented as fold changes from respective UB stalk or tip tissues on day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus corresponding samples at day 0).



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condition allowed us to analyze the specific effect of different signaling pathways. With isolated E11.5 UB, we found that UB cells could not survive in serum-free medium with GDNF alone but required FGF or RA signaling, which worked independently to prevent apoptosis in UB cells and allow them to proliferate. In addition, we found that the maintenance of UB tip cell identity required RET induction by either activation of WNT-β-catenin signaling or RA. Our results therefore demonstrate that there are three critical steps involved in the propagation and maintenance of UB tip cells, i.e., cell survival, proliferation, and RET induction/activation (Figure 7C). The two minimal conditions that we have identified in our MM- and serum-free culture condition are therefore the combination of GDNF, FGF1 with WNT-β-catenin signaling, and the combination of GDNF with RA. However, since RA is known to promote the differentiation of UB cells to collecting duct cells (Takayama et al., 2014) and WNT-β-catenin is known to inhibit such a process (Marose et al., 2008), as we have also found in our present study (Figure 6E), WNT-β-catenin signaling is required to maximize the effect of GDNF and RA in maintaining the proliferation of UB cells. Together with the fact that FGF signaling promotes colony formation from single UB cells, we thus conclude that the combination of GDNF, FGF, RA, and CHIR99021, together with Y-27632, represents the optimum condition to maximize the expansion of dispersed UB cells while maintaining tip cell identity in our serum-free culture condition. Under this condition, FGF1 and FGF7 are equally effective (Figures S1F–S1H). However, we found that this culture condition could not sustain the proliferation of dispersed UB cells after the second round of dissociation from the reconstituted UB structures. Further improvement in our culture system is therefore required to perpetuate the propagation of dispersed UB cells in culture.

In recent years, we have witnessed remarkable advancement in kidney regeneration using the techniques of stem cell biology and tissue engineering. Aided by our knowledge of kidney development, many studies have reported protocols to effectively direct the differentiation of embryonic stem cells and induced pluripotent stem cells toward renal lineage progenitor cells, such as MM and UB cells (Kobayashi et al., 2005; Bruce et al., 2007; Vigneau et al., 2007; Morizane et al., 2009; Ren et al., 2010; Nishikawa et al., 2012; Xia et al., 2013, 2014; Taguchi et al., 2014; Takasato et al., 2014; Vanslambrouck and Little, 2015). Experimental procedures have also been developed to reconstruct kidney organoids by forming aggregates with dispersed MM and UB cells to recapitulate in vivo kidney development and form nephron structures (Unbekandt and Davies, 2010; Ganeva et al., 2011; Takasato et al., 2014, 2015; Xinaris et al., 2012). Together with these advancements, there is an increasing need to develop culture systems that can maintain and expand kidney progenitor cells in vitro by using defined factors in a serum-free condition. Examples of such culture systems have recently been reported for in vitro maintenance and propagation of MM cells (Yuri et al., 2015; Tanigawa et al., 2015; Brown et al., 2015). The MM- and serum-free culture system that we describe herein represents a culture system suitable for the maintenance and expansion of dispersed UB cells in vitro. While the applicability of our culture condition to human UB cells remains to be tested, the ability of single UB cells to reconstruct UB-like structures that retain in vivo characteristics of the original UB resembles the previously reported generation of intestinal and hepatic organoids from single $Lgr5^+$ stem cells (Sato et al., 2009; Barker et al., 2010; Huch et al., 2013a, 2013b, 2015), and could serve as a useful tool for kidney regeneration.

Figure 6. Proliferation and Reconstruction of UB-like Structure from Dispersed Single UB Cells

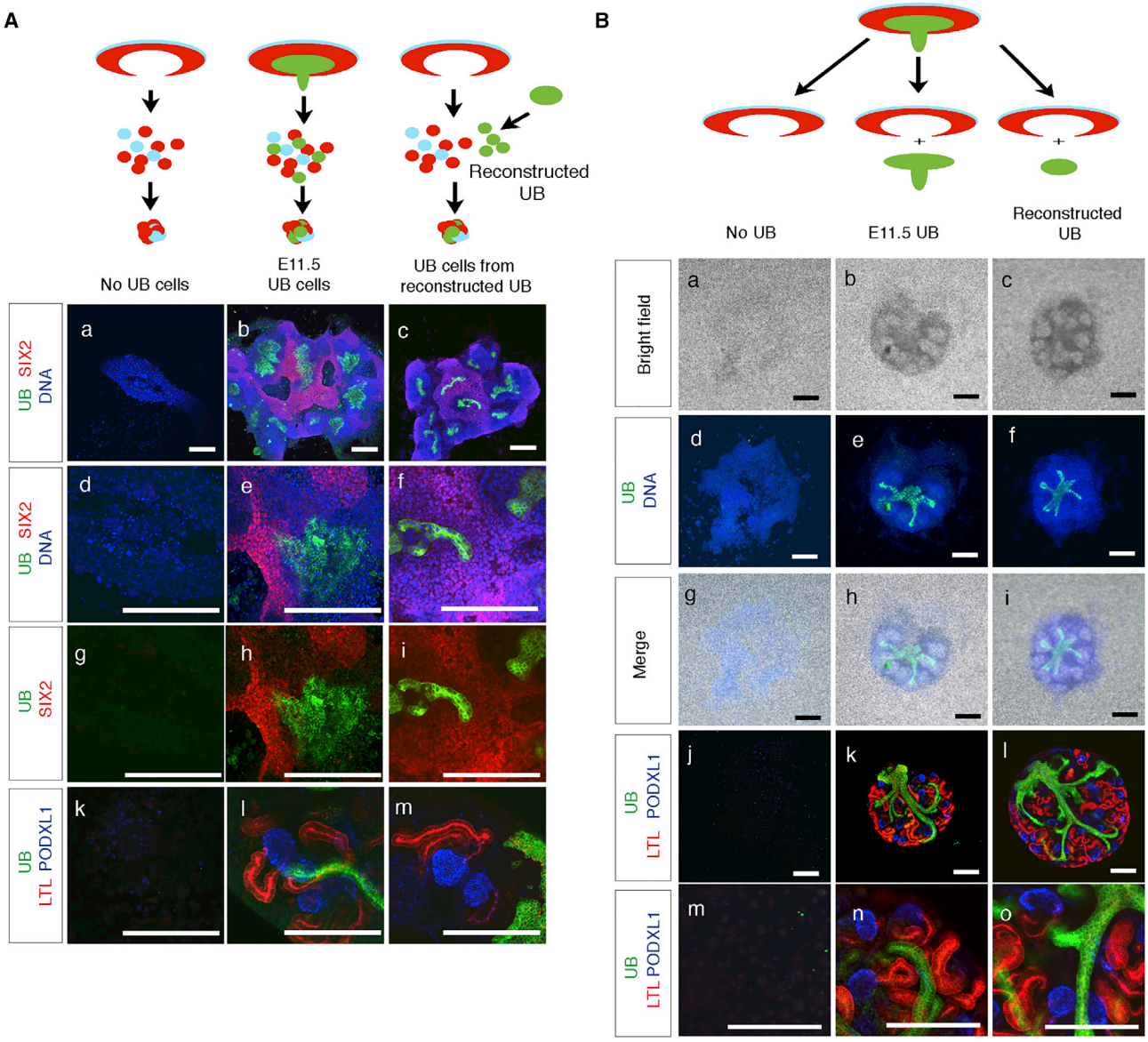
(A) The number of colonies formed from 100 single UB cells after 7 days in culture in serum-free medium containing various combinations of growth factors. The ROCK inhibitor, Y27632, is required for the survival and proliferation of UB cells to form colonies (n = 3 independent replicates; **p < 0.01 versus corresponding samples. N.D., not determined).

(B) Morphology of the colonies (arrows) formed from single UB cells on day 9 under various culture conditions. Scale bars, 500 μm.

(C) Morphology of representative colonies formed from single UB cells (arrows) under different culture conditions from day 0 to day 9. Lumen formation on day 6 with subsequent branching morphogenesis on day 9 was noted with samples treated with the combinations of GDNF, FGF1, and RA, with or without CHIR99021 (n = 10). The picture of an isolated E13.5 metanephros is shown for comparison. Scale bars, 200 μm.

(D) The number of UB cells increased the most with the combination of GDNF, FGF1, CHIR99021, and RA on day 8 (n = 3 independent replicates; *p < 0.05 versus GDNF + FGF1 samples).

(E) qRT-PCR results showed significantly higher mRNA expression levels on day 8 for UB tip marker genes (*Wnt11*, *Ret*, and *Etv5*) and significantly lower expression levels for UB stalk marker genes (*Wnt7b* and *Tacstd2*) in UB cells treated with the combination of GDNF, FGF1, and RA, with or without CHIR99021. We detected the expression of collecting duct marker, *Aqp2*, on day 8 after treatment with GDNF, FGF1, and RA, which was suppressed by CHIR99021. Data were normalized by *Gapdh* expression levels and presented as fold changes from E11.5 UB at day 0 (n = 3 independent replicates; *p < 0.05 and **p < 0.01 versus FGF1 + GDNF samples, and *p < 0.05 versus GDNF + FGF1 + RA samples for *Aqp2*).



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EXPERIMENTAL PROCEDURES

Dissection of Embryonic Kidney Cells and Cell Culture

Mouse embryonic kidneys were dissected free-hand from embryos at E11.5, using fine needles under a dissecting microscope (Olympus) in DMEM with 10% fetal bovine serum (Sigma). Embryonic kidneys were incubated in collagenase (Sigma) at 37°C for 10 min and UB was surgically separated with fine needles. The isolated UB was collected and placed on ice-cold AdDMEM/F12 (Invitrogen) to wash out the remaining collagenase. Matrigel was added into 96-well ultra-low attachment round-bottomed dishes (Corning) and UB were embedded into growth factor-reduced Matrigel (Corning) and cultured at 37°C and 5% CO_2 for 30 min to solidify the Matrigel. After solidification, AdDMEM/F12 containing 1× N2 supplement (Invitrogen), 1× B27 minus vitamin A supplement (Invitrogen), and 1 mM nicotinamide (Sigma) were added with indicated growth factors. For single-cell culture, Y27632 was added during the first 4 days and cell sorting was performed with Hoxb7-Venus$^+$ kidney at E11.5 by FACS (Jazz, BD Science). To ensure obtainment of single UB cells, we gated cells by forward scatter, side scatter, and pulse-width parameters. In all experiments where FACS was used to separate cells, the purity was close to 100% in positive fractions. Reaggregation assay was performed as previously described (Yuri et al., 2015). Chemicals and growth factors, such as DMSO (Sigma), 0.5 μM retinoic acid + 0.5 μM 9-*cis*-RA (Sigma), 250 ng/mL FGF1 (Peprotech), 250 ng/mL FGF7 (Peprotech), 250 ng/mL FGF10 (Peprotech), 125 ng/mL GDNF (Peprotech), 500 ng/mL RSPO1 (Peprotech or R&D Systems), 500 ng/mL RSPO3 (Peprotech or R&D), 500 ng/mL WNT9B (R&D), 500 ng/mL WNT11 (R&D) as well as inhibitors, such as GSK3β inhibitor, 1 μM CHIR99021 (Cellagen Technology), WNT inhibitor, 5 μM IWP2 (Stemgent), β-catenin inhibitor, 5 μM XAV939 (Cellagen Technology), and ROCK inhibitor, 10 μM Y27632 (Abcam Biochemicals), were added as indicated.

Ethics Statement

This study was carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. The protocol was approved by the Institutional Animal Care and Use Committee of VA Greater Los Angeles Healthcare System (Permit Number 01002-09).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.011.

AUTHOR CONTRIBUTIONS

S.Y. designed and conducted most of the experiments. M.N., N.Y., and O.D.J. performed experiments. N.Y. conceived and coordinated the project. S.Y. and N.Y. prepared the manuscript.

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Figure 7. Reconstructed UB-like Structures Retained the Original UB Characteristics

(A) UB cells were dissociated from UB-like structure that had developed from purified Hoxb7-Venus$^+$ single UB cells after treatment with GDNF, FGF1, CHIR99021, and RA for 7 days, and were reaggregated with freshly isolated MM cells from E11.5 kidneys. Aggregates formed with freshly isolated E11.5 MM cells, without or with freshly isolated E11.5 UB cells, were used as negative and positive controls, respectively. After 7 days in culture with Y27632, aggregates were immunostained for nephron progenitor cell marker, SIX2 (red), UB cell marker, DBA (green), and DNA with DRAQ5 (blue). UB cells dissociated from single UB cell-derived UB-like structure were positive for Hoxb7-Venus (green). Similar to positive control samples (b, e, h), nuclear positive SIX2 MM cells were maintained in aggregates produced from UB cells that were dissociated from the UB-like structures (c, f, l). In negative control samples, most of the SIX2$^+$ MM cells were lost (a, d, g). After 7 days in aggregate culture, where Y27632 was removed after day 1, aggregates were immunostained for proximal tubule marker, LTL (red) and podocyte marker, PODXL1 (blue). In negative control samples, no epithelial structure was detected (k). Similar to positive control (l), LTL- and PODXL1-positive structures were detected in the aggregate, where MM cells were combined with UB cells dissociated from reconstructed UB-like structure (m). Scale bars, 200 μm.

(B) An intact UB-like structure reconstructed from single UB cells was combined with an E11.5 metanephros where UB was removed, and further cultured for 4 days (a–i) or 7 days (j–o). As positive controls, a similar E11.5 metanephros without UB was recombined with the original fresh E11.5 UB. The E11.5 metanephros without UB was used as negative control. Bright-field images show the representative recombined tissue. Branching UB structure (DBA; green) and DNA (DRAQ5; blue) were observed under fluorescent microscope. Merged images show the development of branching structures from reconstructed UB-like structure similar to the positive control. Immunostaining for UB marker, Pan-cytokeratin (green), proximal tubule marker, LTL (red), and podocyte marker, PODXL1 (blue) shows the development of these differentiated tissues in both positive controls and samples recombined with the reconstructed UB-like structure. Scale bars, 200 μm.

(C) Schematic of different signaling pathways for the in vitro propagation and maintenance of UB cells in culture. FGFs and RA signaling act for the survival and proliferation of UB cells, and β-catenin signaling and RA act for the induction of RET. GDNF derived from MM cells acts to activate RET and promotes UB tip cell proliferation. The activation of β-catenin signaling by endogenous WNT proteins is mediated through RSPO1 and RSPO3 secreted from MM/stroma cells.

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