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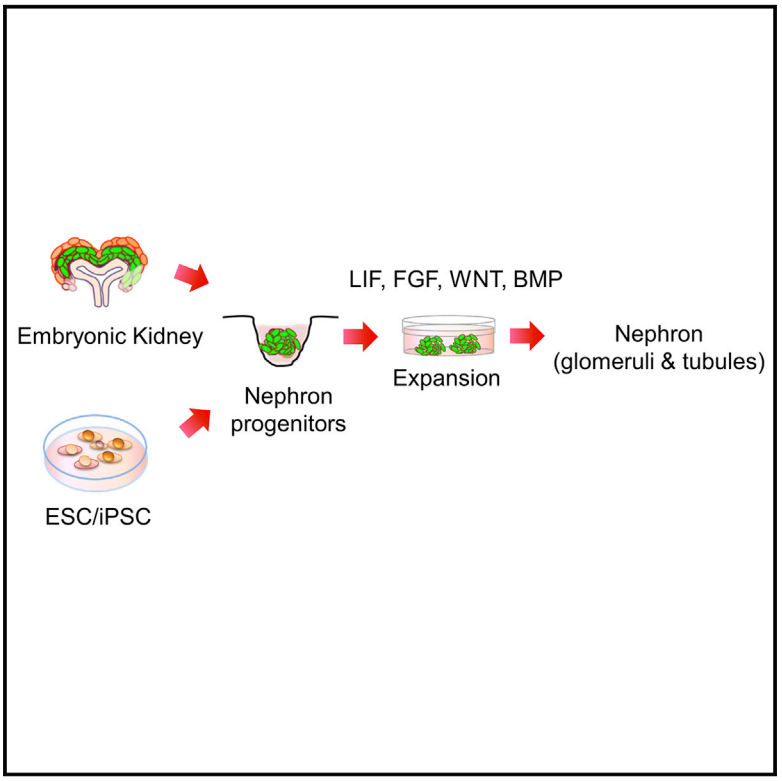
Selective In Vitro Propagation of Nephron Progenitors Derived from Embryos and Pluripotent Stem Cells

Graphical Abstract



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In Brief

Nephron progenitors are maintained during development but disappear around birth. Tanigawa et al. develop a method for selectively expanding nephron progenitors isolated from mouse embryos. The protocol is also applicable to nephron progenitors derived from mouse and human PSCs. Under these conditions, nephron progenitors retain nephron-forming potential.

Highlights

- Purified SIX2+ murine nephron progenitors are selectively expanded in vitro
- Expanded SIX2+ cells reconstitute 3D glomeruli and renal tubules
- Mouse ESC-derived nephron progenitors are expanded with nephron-forming potential
- Human iPSC-derived nephron progenitors are propagated and form nephrons

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Selective In Vitro Propagation of Nephron Progenitors Derived from Embryos and Pluripotent Stem Cells

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SUMMARY

Nephron progenitors in the embryonic kidney propagate while generating differentiated nephrons. However, in mice, the progenitors terminally differentiate shortly after birth. Here, we report a method for selectively expanding nephron progenitors in vitro in an undifferentiated state. Combinatorial and concentration-dependent stimulation with LIF, FGF2/9, BMP7, and a WNT agonist is critical for expansion. The purified progenitors proliferated beyond the physiological limits observed in vivo, both for cell numbers and lifespan. Neonatal progenitors were maintained for a week, while progenitors from embryonic day 11.5 expanded 1,800-fold for nearly 20 days and still reconstituted 3D nephrons containing glomeruli and renal tubules. Furthermore, progenitors generated from mouse embryonic stem cells and human induced pluripotent cells could be expanded with retained nephron-forming potential. Thus, we have established in vitro conditions for promoting the propagation of nephron progenitors, which will be essential for dissecting the mechanisms of kidney organogenesis and for regenerative medicine.

INTRODUCTION

A mammalian kidney contains a large number of nephrons (approximately one million in humans), which are functional units consisting of glomeruli and renal tubules. The kidney is formed by reciprocally inductive interactions, starting at embryonic day 10.5 (E10.5) to E11.5 in mice, between two precursor lineages the metanephric mesenchyme (MM) and the ureteric bud. The former contains nephron progenitors that express the transcription factor SIX2 (Kobayashi et al., 2008; Self et al., 2006), and these cells give rise to nephron epithelia in response to the canonical wingless-type mouse mammary tumor virus integration site family member (WNT) signal evoked by ureteric bud-derived *Wnt9b* (Carroll et al., 2005) and subsequent non-canonical signals including the $Ca2^{+}$-dependent pathway (Tanigawa et al., 2011). *Six2* opposes the *Wnt*-mediated differentiation signal, thereby maintaining nephron progenitors in the undifferentiated state (Park et al., 2012). Thus, the balance between propagation and differentiation of nephron progenitors is important for kidney organogenesis. Many other transcription factors, including *Sall1* (Kanda et al., 2014; Osafune et al., 2006), *Pax2* (Ranghini and Dressler, 2015), *Wt1* (Kann et al., 2015; Motamedi et al., 2014), and *Osr1* (Xu et al., 2014), are also involved in the maintenance of nephron progenitors, and their cooperative network is being elucidated. Nephron progenitors begin to express CITED1, which marks the most undifferentiated population, at E13.5, a few days after their initial appearance. Therefore, the CITED1+/SIX2+ fraction represents naive nephron progenitors from E13.5.

Nephron progenitors cease propagation and are terminally differentiated within a few days after birth in mice (Hartman et al., 2007; Short et al., 2014) and at 34 weeks of gestation in humans (Tank et al., 2012). In mice, a burst of differentiation occurs after birth, which leads to the formation of multiple nephrons per ureteric bud tip with altered distal-proximal patterning (Rumballe et al., 2011). Thus, no nephron formation occurs in the adult kidney, which may underlie the irreversible nature of diseased kidneys. Considering that nephron progenitors are formed at E10.5–11.5 in mice, they are maintained only for 10 days in vivo. Recently, Chen et al. reported that even the CITED1+ population changed its character during development and heterogeneity increased in older (postnatal day 0 [P0]) populations (Chen et al., 2015). Their data suggest that there are no age-resistant stem cells and that the majority of the cap mesenchymal cells intrinsically age in vivo. However, older cells were rejuvenated when transplanted in a younger niche, and cell-cell contacts are required for rejuvenation. Therefore, the niche environment may, at least partly, override the intrinsic aging of nephron progenitors. Thus, releasing them from their limited expansion will contribute greatly to the understanding of kidney development. It will also be beneficial for emerging regenerative medicine, because several groups, including ours, established the protocols to generate nephron progenitors from mouse embryonic stem cells (ESCs) and/or human induced pluripotent stem (iPSCs) (Taguchi et al., 2014; Takasato et al., 2014, 2015; Morizane et al., 2015).

Several extracellular signals have been implicated in the maintenance of nephron progenitors. While *Wnt9b* from the ureteric bud induces differentiation of nephron progenitors (Carroll et al., 2005), it is also required for maintaining the expression of progenitor-related genes in vivo (Karner et al., 2011). Mice deficient for *Fgf9* and *Fgf20* exhibit depletion of nephron progenitors (Barak et al., 2012), suggesting that fibroblast growth factor (FGF) ligands are promising maintenance candidates. In addition, *Fgf20* is partially responsible for the rejuvenation of older progenitors when they are transplanted into the younger niche (Chen et al., 2015). Indeed, addition of FGF ligands, including FGF9, sustains the survival of nephron progenitors in vitro, but they lose the potential to form nephrons within 2 days (Barak et al., 2012; Brown et al., 2011). Deletion of *Bmp7* in vivo leads to apoptosis of MM (Dudley et al., 1995), suggesting that *Bmp7* is required for the maintenance of nephron progenitors. However, addition of bone morphogenetic protein 7 (BMP7) in culture primes the progenitors for differentiation induced by WNT signals (Brown et al., 2013). Recently, Brown et al. found that inhibition of the SMAD pathway downstream of BMP4/7, accompanied by WNT and FGF activation, led to the in vitro expansion of CITED1+ nephron progenitors isolated from E13.5-P0 mouse kidneys, as well as ESC-derived human progenitors (Brown et al., 2015). However, the expanded cells formed renal tubules, but not glomeruli, the filtration apparatus of the nephron. In addition, their protocol was not applicable to mouse progenitors as early as E11.5. Because the progenitors induced from ESCs and iPSCs are likely to represent those at equivalent stages, a protocol to expand the early-stage progenitors will be useful for regenerative medicine.

We have independently established a method to propagate rat MM cells using a combination of leukemia inhibitory factor (LIF) and Y27632, in addition to FGF2 and transforming growth factor α (TGF-α) (Tanigawa et al., 2015). The rat MM cells could be passaged for 17 days, and they still retained the competence to form glomeruli and renal tubules. Although LIF is known to induce differentiation of rat MM in vitro (Barasch et al., 1999; Plisov et al., 2001), we found that the action of LIF on nephron progenitors was concentration dependent. A high concentration (50 ng/ml) evokes differentiation with activation of phospholipase C (PLC), whereas such activation is minimal at lower concentrations (1–10 ng/ml). The lower concentrations of LIF also maintain nuclear localization of SIX2 and YAP, both of which are critical for progenitor expansion. Concomitant treatment with Y27632, a Rho kinase inhibitor, attenuates LIF-induced c-Jun N-terminal kinase (JNK) activation, thereby enabling expansion. As a starting material, however, we used a heterogeneous population, i.e., the entire rat MM, which contained not only the nephron progenitors but also stromal cells. To initiate cultures from purified nephron progenitors and accurately measure the percentage of remaining progenitors during culture, we here use a mouse strain expressing GFP under the *Six2* promoter (*Six2-GFP* mouse) (Kobayashi et al., 2008) and present a robust protocol for expanding mouse nephron progenitors from as early as E11.5 kidneys. The expanded cells readily formed both glomeruli and renal tubules, and the same protocol was applicable to nephron progenitors generated from mouse ESCs and human iPS cells, derived using a protocol that we reported previously (Taguchi et al., 2014).

RESULTS

Partial Propagation of Nephron Progenitors from the MM

To precisely measure the percentage of nephron progenitors upon culture, we isolated the whole MM from *Six2-GFP* kidneys at E11.5 (Figure 1A) and cultured the cell clusters, without dispersal into single cells, on fibronectin-coated plates in the presence of LIF and Y27632 (LY) (together with FGF2 and TGF-α as used for the rat MM). While GFP+ cells comprised 23.9% of the freshly isolated MM, the percentage decreased to 5.0% after 7 days of culture (Figure 1B). In the absence of LIF and Y27632, only 0.1% of cells were positive for GFP, suggesting that LY exerted some effects on the progenitors, but not enough for their maintenance. Expression levels of *Six2* and *Pax2* at day 7 were also lower compared with levels in the freshly isolated MM (Figure 1C). Because Notch activation causes premature progenitor differentiation (Fujimura et al., 2010; Boyle et al., 2011), we tested the Notch inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) in addition to LY. We also compared fibronectin with the E8 fragment of laminin (iMatrix) as the coating matrix (Miyazaki et al., 2012) and found that a combination of iMatrix and DAPT was effective in maintaining the levels of *Six2* and, more prominently, *Pax2* (Figure 1C). In parallel, we tested the WNT agonist CHIR99021 (CHIR). While limited numbers of Six2-GFP+ cells were maintained in the presence of LIF alone (1–10 ng/ml), CHIR increased the number of GFP+ cells at culture day 7 (Figure S1A), and a combination of LIF and CHIR resulted in a massive expansion of GFP+ cells (Figure S1B). Thus, we tested the combination of LIF, Y27632, CHIR, and DAPT (CDLY) on iMatrix-coated plates and found that 26.3% of cells were positive after 7 days of culture (Figures 1D and 1E). We further added BMP7, which resulted in robust cell proliferation and GFP+ cells increased to 40.6% (Figures 1D and 1E). Expression levels of nephron progenitor markers, including *Six2*, *Pax2*, *Sall1*, Wt*1*, and *Osr1*, under these conditions (CDLY+BMP7: CDBLY) were comparable to those of the freshly isolated MM (Figure 1F). Therefore, these conditions may be suitable for propagation of nephron progenitors, although we started the culture from the whole MM, which contains a mixed cell population.

Selective In Vitro Propagation of Purified SIX2+ Nephron Progenitors from Embryonic and Neonatal Kidneys

Next, we applied the described culture conditions (CDBLY) to the purified Six2-GFP+ progenitors (Figure 2A). Because of the limited number of cells in E11.5 kidneys, we first used E15.5 and neonatal (P0) kidneys, and sorted the GFP+ cells, which comprised 21.1% and 8.1% of the total cells, respectively (Figure 2B). The sorted cells were aggregated for 2 days to form spheres, plated onto iMatrix-coated plates, and cultured for 5 more days in the conditions as described. As shown in Figures 2B and 2C, the total cell numbers increased (2.4-fold for E15.5, and 1.7-fold for P0) and the GFP+ ratio remained very high (94.3% for E15.5, 90.9% for P0). Expression levels of nephron progenitor markers at day 7 were comparable to those of the freshly isolated nephron progenitors

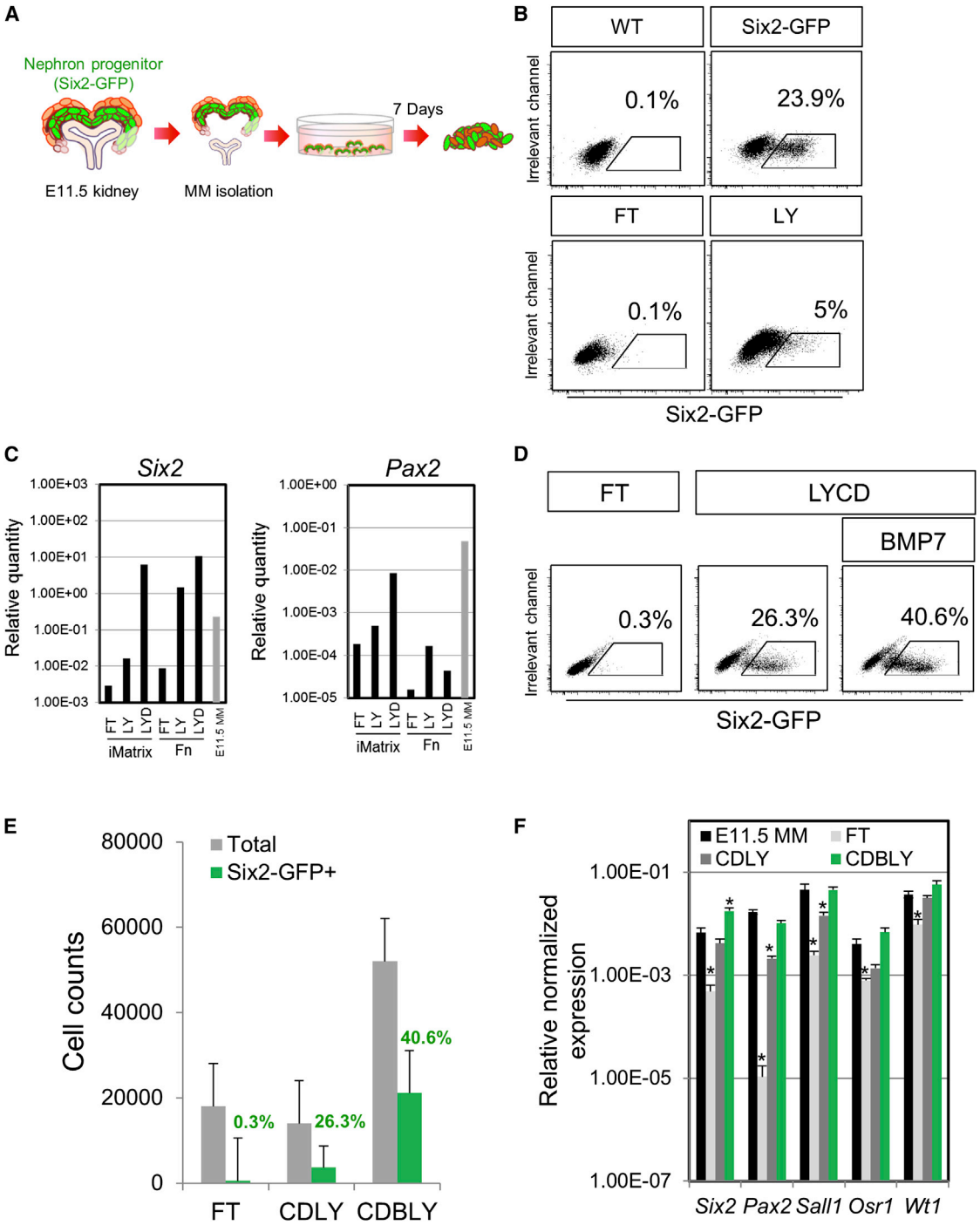

Figure 1. Partial Propagation of Nephron Progenitors from the Metanephric Mesenchyme

(A) Schematic of mouse MM culture.

(B) FACS analysis of wild-type and *Six2-GFP* E11.5 kidneys (top). Bottom: MM from *Six2-GFP* kidneys was cultured for 7 days in basal medium containing FGF2 and TGF-α (FT) or FT plus LIF (5 ng/ml) and Y27632 (10 μM) (LY).

(C) qPCR analysis of nephron progenitor markers. MM was cultured on iMatrix- or fibronectin-coated plates for 7 days. Freshly isolated MM at E11.5 was used as a positive control. LYD, LY plus DAPT.

(D) Percentages of Six2-GFP+ cells at culture day 7. Isolated MM was cultured on iMatrix-coated plates and analyzed by FACS.

(E) The numbers of GFP+ cells at day 7, calculated from the total cell numbers and the percentages described in (D).

(F) qPCR analysis of nephron progenitor markers at day 7. *p < 0.01. CDLY: CHIR, DAPT, LIF, and Y27632; CDBLY: CDLY plus BMP7.

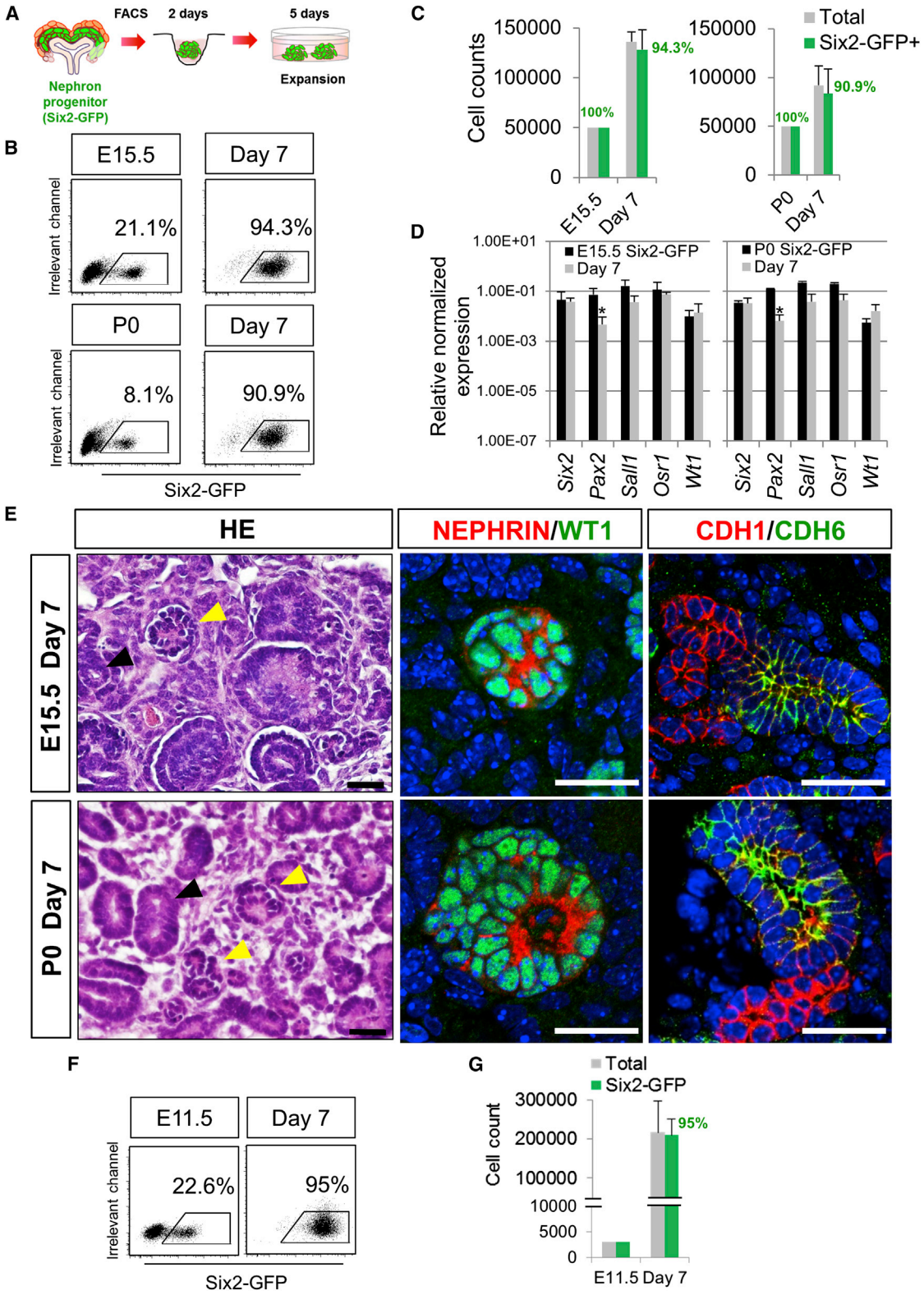

Figure 2. Selective In Vitro Propagation of Purified Nephron Progenitors from Embryonic and Neonatal Kidneys

(A) Sorted Six2-GFP+ progenitors (green) were aggregated for 2 days and cultured on the plate for an additional 5 days.

(B) FACS analysis of cultured *Six2-GFP* cells isolated from E15.5 or neonatal (P0) kidney. Left: results of freshly isolated kidneys.

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(Figure 2D). When these cultured cells were combined with embryonic spinal cord, a potent WNT-expressing inducer of the mesenchymal-to-epithelial transition (Taguchi et al., 2014; Tanigawa et al., 2011), tubulogenesis was observed within 3 days, and numerous glomeruli and renal tubules were detected 5 days after induction (Figure 2E). The spherical glomeruli were positive for the podocyte-specific markers WT1 and NEPHRIN. The induced renal tubules were regionalized into CADHERIN6+ proximal and CADHERIN1+ distal domains. Thus, our culture conditions enabled propagation of purified nephron progenitors while maintaining their competence to form three-dimensional nephron structures. It is noteworthy that the progenitors from the newborn mice were maintained for 7 days, considering that mouse nephron progenitors cease to proliferate and terminally differentiate within 2–3 days after birth. Therefore, our culture conditions maintained the propagation of the progenitors beyond their normal in vivo lifespan.

However, the increase in cell numbers was not dramatic (~2-fold; see Figure 2C). In addition, we initially optimized the culture conditions using the whole E11.5 MM (Figure 1). Therefore, we next purified Six2-GFP+ progenitors at E11.5. When the sorted cells were aggregated and cultured on the iMatrix-coated plates for 7 days, we observed a significant increase in cell numbers (30-fold), and more than 95% remained positive for GFP (Figures 2F and 2G). These data suggest that our culture condition was applicable to the E11.5 progenitors and that they may have more proliferative capacity compared with those at later developmental stages. Thus, our method sharply contrasts with the protocol by Brown et al., which does not support the propagation of E11.5 nephron progenitors (Brown et al., 2015).

Combinatorial and Concentration-Dependent Stimulation with LIF, FGF2/9, BMP7, and a WNT Agonist Is Critical for the SIX2+ Nephron Progenitor Expansion

Having determined fairly optimized conditions, we re-examined the requirement of each reagent for the expansion of SIX2+ nephron progenitors from E11.5 kidneys. In all the experiments described thus far, the medium (CDBLY) contained FGF2, based on our rat MM cultures (Tanigawa et al., 2015). We noticed that FGF signaling was critical in our culture conditions, because cells failed to proliferate in the absence of FGFs (Figure 3A). *Fgf9* is required for the maintenance of mouse nephron progenitors in vivo (Barak et al., 2012); therefore, we compared the effects of FGF2 and FGF9 in culture. Both FGF2 and FGF9 maintained the Six2-GFP+ cells at a very high percentage (>95%), and a significant increase in cell numbers was observed (30-fold for FGF2 and 60-fold for FGF9 at 50 ng/ml). Expression levels of nephron progenitor markers in both conditions were comparable to those of freshly isolated Six2-GFP+ cells at E11.5 (Figure 3B). Glomeruli and renal tubules were formed in both conditions, when combined with spinal cord (Figure S2A). The differences in proliferation between FGF2 and FGF9 may have reflected variable preparation of each reagent; however, we used FGF9 for subsequent experiments.

Next, we tested various concentrations of CHIR and found that SIX2+ cell numbers were increased in a concentration-dependent manner up to 1 μM (Figure 3C). However, 2.5 μM CHIR treatment failed to maintain the progenitors and led to expansion of GFP-negative cells, as well as upregulation of differentiation-associated markers, including *Lef1* and *Cadherin1* (Figure 3D). These data suggest that an optimal strength of WNT signal is required for progenitor propagation and that excessive signal induces differentiation accompanied with proliferation. We also found high concentrations of BMP7 (over 25 ng/ml) reduced the percentage of GFP+ progenitors, while lower concentrations (5 ng/ml) were optimal for selective expansion (Figure 3E). Expression of *Lef1*, the indicator of WNT activity, was not increased even at high concentrations of BMP7 (Figure S2B), which is consistent with a previous report showing that BMP7 only primes the progenitors for WNT signal-mediated differentiation (Brown et al., 2013). We further addressed the roles of LIF and BMP7 in our culture conditions (Figure 3F). When LIF was excluded from the culture, the percentage of GFP+ cells decreased to 84%. In the absence of BMP7, total cell numbers decreased, but the GFP+ percentage remained high. When LIF and BMP7 were excluded, the percentage of GFP+ cells was reduced dramatically, while cell numbers were higher than in the absence of BMP7 alone. We speculate that LIF may inhibit differentiation but also inhibit proliferation of both progenitors and differentiated cells, whereas BMP7 may enhance progenitor proliferation to counteract the negative effects of LIF on the progenitors, thereby enabling expansion of nephron progenitors. Although additional mechanistic studies are needed, our data indicate that both LIF and BMP7 are required for optimal progenitor propagation.

SIX2+ Nephron Progenitors from E11.5 Passaged for 19 Days Reconstitute 3D Glomeruli and Renal Tubules

Next, we tried to expand the E11.5 progenitors beyond a week. Dissociation into single cells at day 8 hindered further propagation of the cells. However, when the cells were dissociated into small clumps and divided into three plates, they continued to grow and could be passaged every 3–4 days until day 19 (Figure 4A). Cell numbers increased, and 97% were still Six2-GFP+ at day 19 (Figures 4B and 4C). In the absence of LIF, the percentage of GFP+ cells was decreased by day 8 and was 73% at day 19 (Figures 4C and 4D). In contrast, total cell

(C) The numbers of GFP+ cells at culture day 7.
(D) qPCR analysis of nephron progenitor markers. Freshly isolated GFP+ cells from E15.5 or P0 were used as positive controls. *$p < 0.01$.
(E) Reconstitution of three-dimensional nephron structures by cells expanded from E15.5 or P0 progenitors. Left: H&E staining; middle: immunostaining of NEPHRIN (red) and WT1 (green); right: immunostaining of CADHERIN1; CDH1 (red) and CADHERIN6; CDH6 (green). Yellow arrowheads, glomeruli; black arrowheads, renal tubules. Scale bars, 20 μm.
(F) FACS analysis at day 7 of cultured *Six2-GFP* cells isolated from E11.5 kidney. Left: freshly isolated kidney cells.
(G) The numbers of total and GFP+ cells at culture day 7.

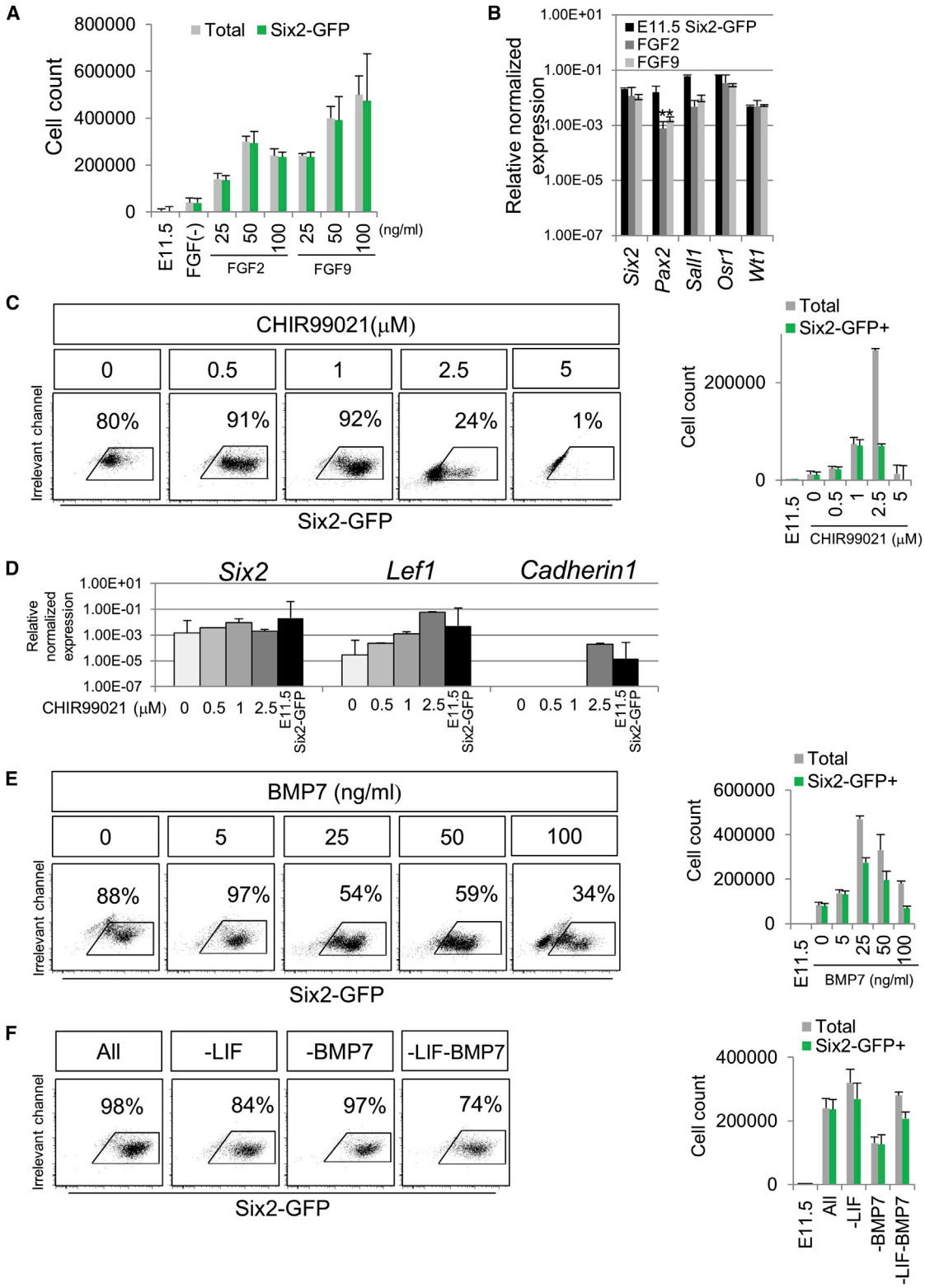

Figure 3. Combinatorial and Concentration-Dependent Stimulation with LIF, FGF2/9, BMP7, and a WNT Agonist Is Critical for Nephron Progenitor Expansion

(A) Effects of FGF2 and FGF9 on 7-day progenitor expansion from E11.5 *Six2-GFP* progenitors.

(B) qPCR analysis of nephron progenitor markers at day 7. Freshly isolated Six2-GFP+ cells at E11.5 were used as a positive control. *$p < 0.01$.

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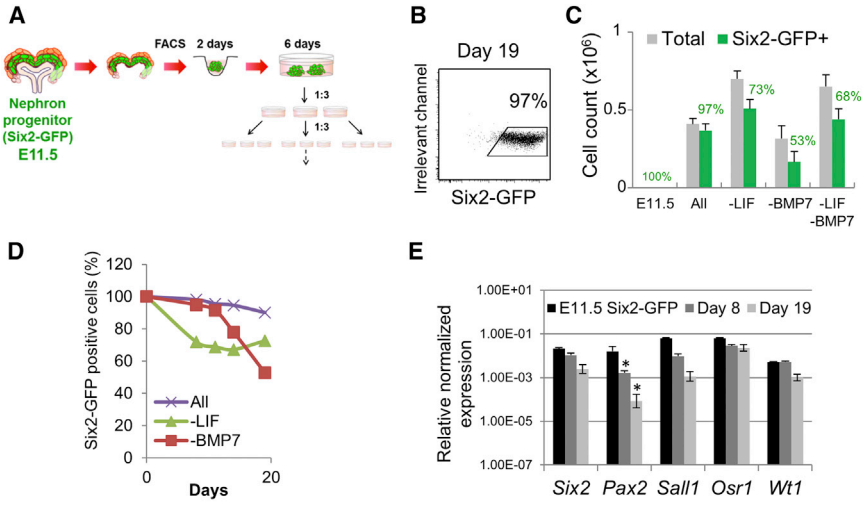

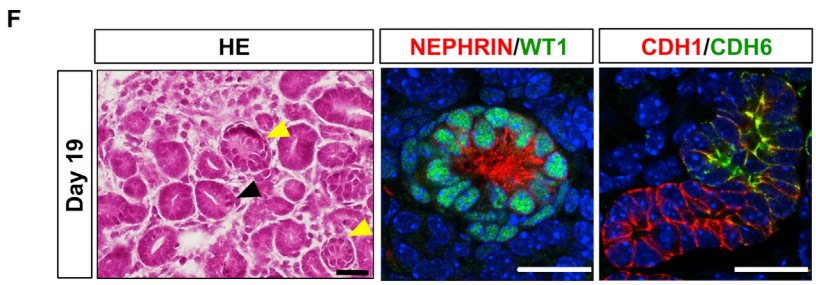

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Culture days	Day 8	Day19
Start cell number of culture	3000	3000
Number of passages attempted	0	3
Doubling times per day	1.05	2.27
Total cell number	222,912 ± 3,782	6,018,624 ± 102,114
Six2-GFP (%)	96.9 ± 1.2	93.4 ± 2.3
Six2-GFP+ cells	216,000 ± 2,592	5,621,395 ± 2,349
Fold increase	72 ± 11	1873 ± 1

Figure 4. E11.5 SIX2+ Nephron Progenitors Passaged for 19 Days Reconstitute Three-Dimensional Nephron Structures

(A) Sorted E11.5 Six2-GFP+ cells (green) were aggregated for 2 days and cultured on plates for an additional 6 days (day 8). Cells were then passaged at 1:3 dilutions until day 19.

(B) Ninety-seven percent of cultured cells were GFP+ in the optimized conditions at day 19.

(C) The numbers of total and GFP+ cells at day 19 in the optimized conditions (all) or in the absence of the indicated factors.

(D) Percentages of GFP+ cells during the culture.

(E) qPCR analysis of nephron progenitor markers at culture day 8 and 19. *$p < 0.01$.

(F) Reconstitution of nephron structures by cells cultured for 19 days. Yellow arrowheads, glomeruli; black arrowheads, renal tubules. Scale bars, 20 μm.

(G) Details of nephron progenitor expansion in vitro.

numbers were increased when compared with conditions including LIF. Interestingly, in the absence of BMP7, the percentage of GFP+ cells was retained at a high level until day 11 and then rapidly declined to 53% at day 19 (Figures 4C and 4D). Thus, BMP7 was essential, especially for long-term maintenance of GFP+ cells. Elimination of LIF and BMP7 from the culture failed to maintain SIX2+ cells (68%) despite the increase in the total cell number. Therefore, both LIF and BMP7 are required for propagation of nephron progenitors. Indeed, in these optimized conditions (CDBLY with FGF9), most of the nephron progenitor markers were retained at day 19 (Figure 4E). Immunostaining also showed that the percentages of cells positive for SIX2, PAX2, and WT1 at day 19 remained more than 85% (Figures S3A and S3B). Furthermore, the cells cultured for 19 days readily formed three-dimensional nephron structures when combined with spinal cord (Figure 4F). The actual cell numbers at the beginning and end of culture, the average doubling time, and fold increase are presented in Figure 4G, which shows that the E11.5 SIX2+ cells were expanded 72-fold at day 8 and 1,800-fold at day 19. We also calculated the progenitor numbers in vivo (Figure S3C) and found that progenitors in one kidney increased 18-fold within 9 days (from E11.5 to P0). Therefore, we have succeeded in expanding the nephron progenitors in vitro, while maintaining their differentiation potential, far beyond the physiological limits observed in vivo, both for cell numbers and lifespan.

Nephron Progenitors Retain CITED1 but Do Not Exhibit a Complete Self-Renewal In Vitro

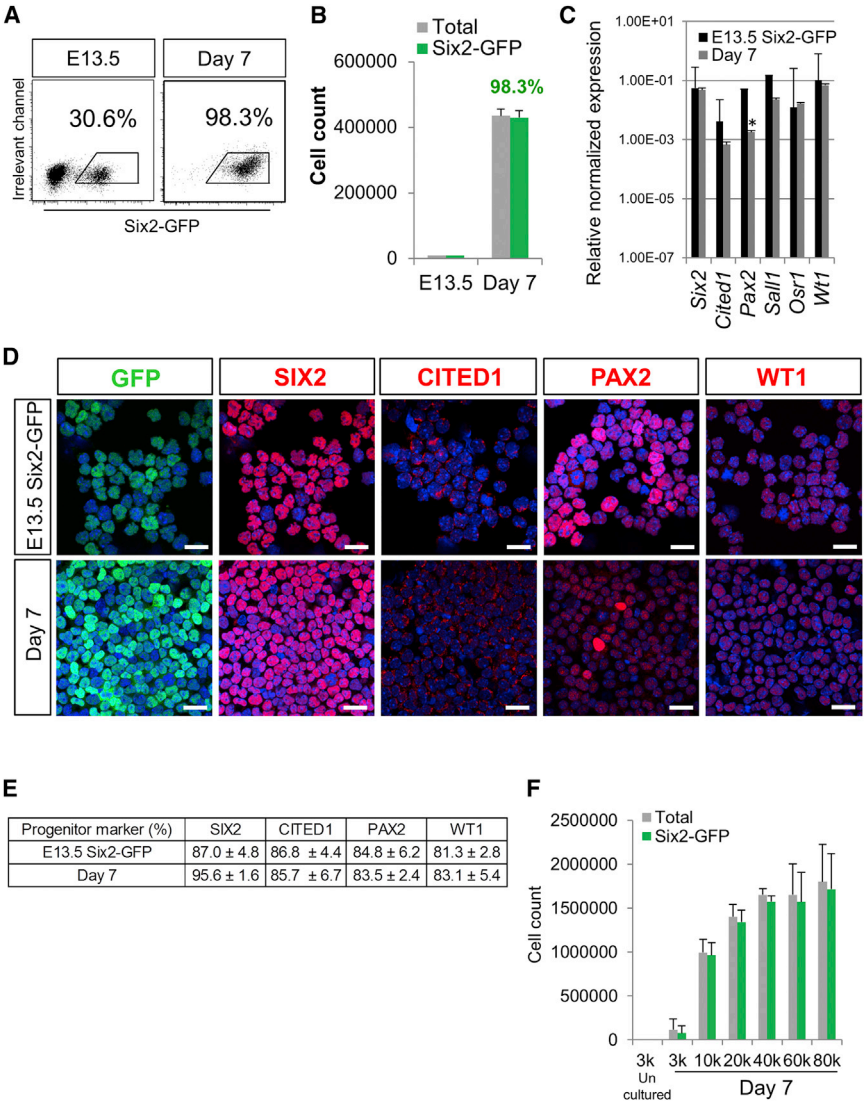
Despite retention of nephron-forming potential, our protocol does not support complete self-renewal of the progenitors. Expression levels of some nephron progenitor markers gradually decreased during long-term culture (Figure 4E). We performed microarray analysis of SIX2+ cells at E11.5 (starting material) and P0 (experiencing 8 days in vivo) and SIX2+ cells cultured for 8 days or 19 days. Unbiased principal component analysis (PCA) showed that the expanded cells in vitro were not identical to those in vivo but that the cells cultured for 8 days were aligned

(C) Optimal concentration of CHIR for expansion of nephron progenitors. Purified Six2-GFP+ cells were cultured for 7 days in the FGF9-based optimized (CDBLY) conditions with indicated concentrations of CHIR. The numbers of total and GFP+ cells are shown in the right panel.

(D) qPCR analysis of the cells cultured with the indicated concentrations of CHIR at day 7.

(E) Optimal concentration of BMP7 for expansion of nephron progenitors. Purified Six2-GFP+ cells were cultured for 7 days with indicated concentrations of BMP7. Right: the numbers of total and GFP+ cells.

(F) Requirement of LIF and BMP7 for progenitor expansion. E11.5 Six2-GFP+ cells were cultured in the FGF9-based optimized conditions (all) or in the absence of the indicated factors. Right: the numbers of total and GFP+ cells.



Progenitor marker (%)	SIX2	CITED1	PAX2	WT1
E13.5 Six2-GFP	87.0 ± 4.8	86.8 ± 4.4	84.8 ± 6.2	81.3 ± 2.8
Day 7	95.6 ± 1.6	85.7 ± 6.7	83.5 ± 2.4	83.1 ± 5.4

Figure 5. Nephron Progenitors Retain CITED1 but Do Not Completely Self-Renew In Vitro

(A) FACS analysis of cultured Six2-GFP+ cells isolated from E13.5. Left: the result of freshly isolated kidneys.

(B) Expansion of E13.5 Six2-GFP+ progenitors.

(C) qPCR analysis of nephron progenitor markers at culture day 7. *$p < 0.01$.

(D) Immunostaining at day 7 as compared with E13.5 progenitors freshly isolated for simultaneous staining. Scale bars, 20 μm.

(E) The percentages of cells expressing progenitor markers, as assessed by immunostaining.

(F) Cell-density-dependent expansion of nephron progenitors. The indicated numbers of cells are plated in a well of a 96-well plate.

on the similar coordinates of the primary PC axis (PC1) to P0 in vivo (Figure S4A). Thus, the character of cells in vitro may be changing gradually in a different manner from those in vivo. We next examined representative genes related to progenitor maintenance and differentiation (Figure S4B). Nephron progenitors in vivo at E11.5 and P0 exhibited similar but distinct expression profiles: some progenitor genes were moderately reduced while differentiation-related genes were increased at P0, which is consistent with the recent report of transcriptional changes in progenitors in vivo (Chen et al., 2015). Expression of aging-related genes reported by Chen et al. also showed similar changes in P0 progenitors in our analysis. In vitro, some of the progenitor markers were retained at day 8. Changes in differentiation- and aging-related genes at day 8 in vitro were not as marked as those at P0. However, at day 19 of culture, expression levels of aging-related genes were comparable to those at P0 in vivo, and expression levels of progenitor genes were lower than those at P0. Nevertheless, the expanded cells were able to form nephrons, which indicates that nephron-forming potential is tolerant of a relatively broad range of gene expression.

Next, we examined whether the expanded progenitors exited from the CITED1+/SIX2+ state. Because CITED1 is detected in nephron progenitors only from E13.5, we sorted Six2-GFP+ progenitors from E13.5 embryos and examined the expression of CITED1 upon culture. As expected, 98.3% of the cells remained GFP+ at day 7 and expanded 41.5-fold (Figures 5A and 5B). Expression of *Cited1*, as well as *Six2*, *Osr1*, and Wt*1*, were retained, while *Pax2* were decreased (Figure 5C), which is consistent with the data from previous experiments (Figures 2D and 4E and S4B). Immunostaining showed that the percentages of the cells positive for CITED1, SIX2, and WT1 remained high (Figures 5D and 5E). In contrast, a few cells expressed PAX2 strongly, although most of the remaining cells expressed PAX2 weakly (Figures 5D and 5E). Therefore, these data are likely to be explained not by the simple transition from CITED1+/SIX2+ state to CITED1−/SIX2+ state but by a global shift to the in-vitro-specific state. Nonetheless, the expanded cells formed glomeruli and renal tubules upon co-culture with spinal cord (Figure S5A).

Brown et al. (2015) reported nearly 100% retention of CITED1+ progenitors, despite isolating the starting materials by negative selection. Thus, we tested the culture conditions reported by Brown et al. on positively sorted E13.5 Six2-GFP+ progenitors and compared these conditions with ours. However, at least in our hands, only 20.8% of the cells were GFP+ at day 7, and expansion of SIX2+ cells was only 6.4-fold (Figure S6A). Expression of most progenitor marker genes, including *Cited1*, was significantly decreased (Figure S6B). We then combined the cultured cells with spinal cord, because CHIR, which was used

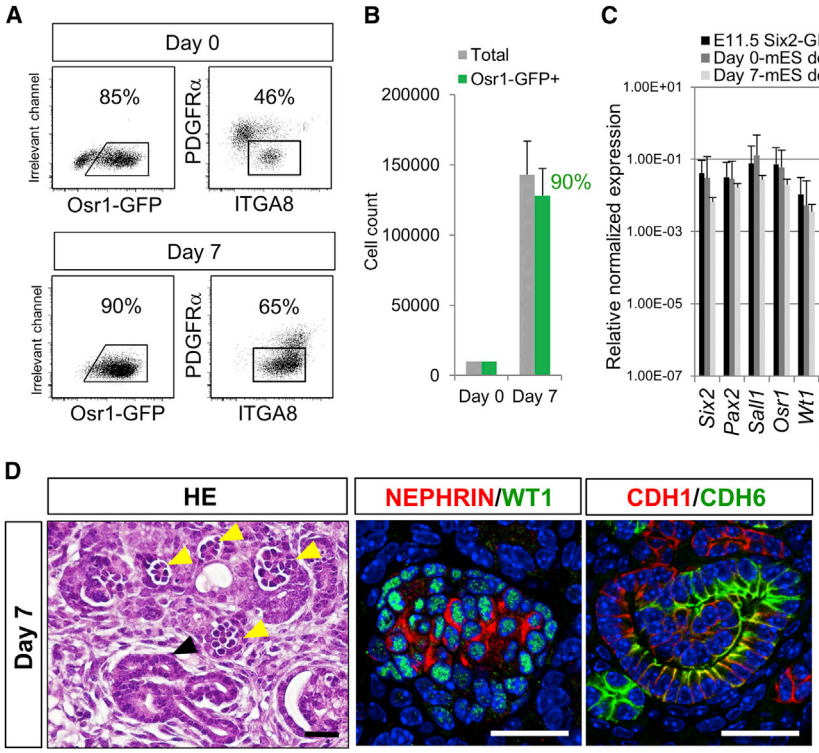


Figure 6. Nephron Progenitors Generated from Mouse ES Cells Can Be Expanded with Nephron-Forming Potential

(A) FACS analysis of nephron progenitors induced from mouse *Osr1-GFP* ESCs, before and after expansion. The Osr1-GFP+/INTEGRIN α8+/PDGFR α− fraction derived from ESCs (top) was cultured for 7 days (bottom).

(B) The numbers of total and Osr1-GFP+ cells at culture day 7.

(C) qPCR analysis of nephron progenitor marker genes expressed in the Osr1-GFP+/INTEGRIN α8+/PDGFR α− cells at culture day 0 and 7. Freshly isolated Six2-GFP+ cells at E11.5 were used as a positive control.

(D) Reconstitution of nephron structures from nephron progenitors derived from *Osr1-GFP* ESCs and expanded for 7 days. Yellow arrowheads, glomeruli; black arrowheads, renal tubules. Scale bars, 20 μm.

by Brown et al., is reported to skew differentiation away from glomeruli toward the distal nephron (Lindström et al., 2015). However, no glomeruli were formed, and a few tubules were observed (Figure S6C).

Progenitor Expansion Depends on Cell Density

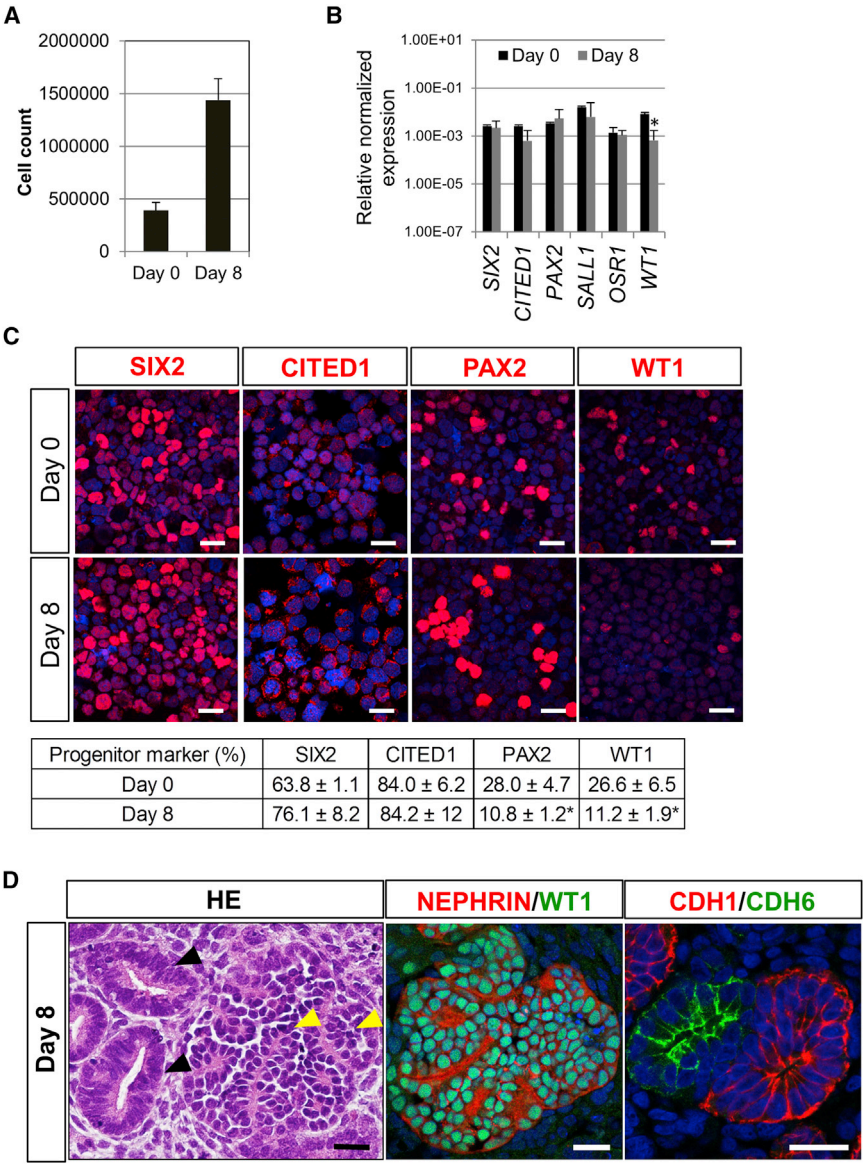
We cultured the cells as aggregates, because we were unable to propagate progenitors efficiently from a single cell. By using the singly dissociated cells from Six2-GFP kidneys at E13.5, we found that cells plated at a low density (3,000 cells/96-well) in our protocol resulted in poor propagation of GFP+ cells (Figures 5F and S5B). Increasing the initial cell density improved the expansion of GFP+ progenitors, although an aberrant Six2-GFP^{low} population appeared. Marker genes were retained, but *Pax2* did not reach the starting levels irrespective of the cell density (Figure S5C). Further passage was unsuccessful upon single-cell dissociation. These results suggest the importance of cell-cell community effects for the maintenance of nephron progenitors, which is consistent with the finding reported by Chen et al. (2015) and explains the aggregation-dependent improvement in progenitor expansion in our protocol.

Nephron Progenitors Generated from Mouse ES Cells Can Be Expanded with Nephron-Forming Potential

We previously reported the generation of nephron progenitors from mouse ESCs (Taguchi et al., 2014). Therefore, we addressed whether our expansion protocol could be applied to ESC-derived nephron progenitors. Because Six2-GFP ESCs with a differentiation potential to kidney lineages were not available, we used Osr1-GFP ESCs, which can successfully differentiate into nephron progenitors and subsequently into three-dimensional nephrons. The aggregates induced from mouse ESCs presumably correspond to nephron progenitors at E10.5–11.5. *Osr1* is expressed not only in nephron progenitors but also in the stroma at this stage and is restricted to nephron progenitors at later stages of development. Thus, we sorted the Osr1-GFP+/INTEGRIN α8+/PDGFR α− population, which represents the nephron progenitor fraction as described previously (Taguchi et al., 2014), and cultured it for 7 days (Figures 6A and 6B). The cells proliferated 15-fold, and 90.3% of them remained positive for Osr1-GFP, although the percentage of the INTEGRIN α8+/PDGFR α− fraction decreased to 65.2%. Expression levels of the nephron progenitor markers in the Osr1-GFP+ cells at day 7 were comparable to those of the SIX2+ cells in vivo at E11.5 (Figure 6C). Finally, these cultured cells formed three-dimensional nephron structures, including glomeruli and renal tubules, when stimulated by spinal cord (Figure 6D). Therefore, nephron progenitors generated from mouse ESCs can be expanded with sustained nephron-forming potential, demonstrating the robustness of our culture conditions.

Nephron Progenitors Derived from Human iPSCs Can Be Propagated and Form Glomeruli

We previously reported the successful induction of human nephron progenitors from iPSCs and showed that the resultant progenitor spheres readily form glomeruli and renal tubules upon culture with spinal cord (Taguchi et al., 2014). Therefore, these human iPSC-derived progenitors were subjected to our expansion protocol. Because there is no method available at present to purify human nephron progenitors, the spheres containing progenitors were dissociated into clumps and cultured for 8 days. We observed significant cell proliferation (Figure 7A), and expression of progenitor marker genes was maintained, except for *WT1* (Figure 7B). Immunostaining also showed maintenance of the



Progenitor marker (%)	SIX2	CITED1	PAX2	WT1
Day 0	63.8 ± 1.1	84.0 ± 6.2	28.0 ± 4.7	26.6 ± 6.5
Day 8	76.1 ± 8.2	84.2 ± 12	10.8 ± 1.2*	11.2 ± 1.9*

Figure 7. Nephron Progenitors Derived from Human iPSCs Can Be Propagated and Form Glomeruli

(A) The total cell numbers at day 8 of culture from human nephron progenitors.

(B) qPCR analysis of nephron progenitor marker genes at day 0 and 8. *$p < 0.01$.

(C) Immunostaining of progenitor markers at day 8 of culture, as compared with progenitors freshly prepared for simultaneous staining.

(D) Reconstitution of human nephron structures by cells cultured for 8 days. Yellow arrowheads, glomeruli; black arrowheads, renal tubules. Scale bars, 20 μm.

marker proteins, including SIX2 and CITED1, although the percentages of cells expressing PAX2 or WT1 were decreased to some extent (Figure 7C). When the expanded cells were aggregated and co-cultivated with spinal cord, they formed many glomeruli and renal tubules in all of the samples tested (eight out of eight aggregates) (Figure 7D). Although serially passaged cells failed to form nephron structures, our culture conditions preserved the human nephron progenitors while maintaining their competence to form both glomeruli and renal tubules.

Brown et al. reported a short-term expansion of the human nephron progenitors, which showed tubulogenesis, but not glomerulogenesis (Brown et al., 2015). There are several possible explanations for this. First, they generated the progenitors by a different method, which possesses minimal glomerulus-forming potential relative to ours. Second, they treated the progenitors with CHIR, which is reported to skew the differentiation away from glomeruli toward the distal nephron (Lindström et al., 2015). Finally, the progenitors expanded in their protocol may have lost the potential to form glomeruli. Therefore, we generated the human nephron progenitors by our method (Taguchi et al., 2014), cultured them as clumps in the conditions devised by Brown et al., and combined them with spinal cord. Although cells proliferated and marker genes were maintained (Figures S6D and S6E), seven out of eight aggregates showed limited tubulogenesis without glomerular formation (Figure S6F). Only a few glomeruli were detected in the remaining aggregate. We also tried single-cell culture, but no progenitor expansion occurred in either of the two protocols. Thus, our culture condition efficiently expands human nephron progenitors while retaining their competence to form both glomeruli and renal tubules.

DISCUSSION

The balance between propagation and differentiation of nephron progenitors is important for kidney organogenesis. However, shortly after birth, nephron progenitors cease propagation and terminally differentiate (Hartman et al., 2007). Here, we achieved the propagation of nephron progenitors in vitro beyond the physiological limits in vivo, both for cell numbers and lifespan, by modulating the extracellular cues. Murine progenitors harvested at E13.5, E15.5, and even P0 were maintained for 7–8 days. While the protocol by Brown et al. was applicable only to progenitors at or after E13.5 (Brown et al., 2015), our protocol allowed progenitors from E11.5 to be passaged for 19 days (1,800-fold expansion) with the retention of a potential to generate three-dimensional nephron structures: glomeruli and renal tubules. Considering that the progenitors induced from ESCs and iPSCs are likely to represent those at earlier stages, a protocol to expand the early-stage progenitors will be useful. Indeed, nephron progenitors generated from mouse ESCs and human

iPSCs were also expandable to form both glomeruli and tubules. The retention of glomerulus-forming potential is one of the features of our protocol that has not been achieved by other groups, suggesting a more efficient maintenance of the competence of the progenitors.

Combinations and concentrations of signaling factors are critical for the propagation of nephron progenitors in vitro. One of the major differences between our protocol and Brown et al.'s is the addition of LIF. We recently reported that LIF action on rat nephron progenitors was dependent on its concentration (Tanigawa et al., 2015). While high concentrations of LIF stimulated differentiation, low concentrations of LIF maintained rat nephron progenitors. Our present data suggest that the important role of LIF is conserved in mice. This may be somewhat puzzling, considering the minimal kidney phenotypes observed in mice deficient for *Lif* or its receptor, *gp130* (Barasch et al., 1999). One possibility is that redundant pathways compensate for the absence of LIF signaling in vivo. Alternatively, LIF signaling may be required only for artificial expansion of progenitors in vitro, as in the case of mouse ESCs. LIF is required for in vitro expansion of ESCs, whereas mice deficient for *Lif* or *gp130* do not show defects of the inner cell mass (Stewart et al., 1992; Yoshida et al., 1996), from which ESCs are derived. Because LIF plus BMP4 or a combination of LIF, CHIR, and MEK inhibitor (LIF+2i) sustains ESCs (Ying et al., 2003; Ying et al., 2008), the well-defined self-renewal mechanisms of ESCs will give insight into those of nephron progenitors.

LIF alone was unable to maintain the mouse progenitors, and addition of FGF9/CHIR/BMP7 clearly enhanced propagation of nephron progenitors. The absolute requirement of FGF in our culture conditions is consistent with the report that FGF9, in combination of FGF20, is essential for maintenance of nephron progenitors in vivo (Barak et al., 2012). *Wnt9b* is usually associated with differentiation of nephron progenitors (Carroll et al., 2005), while it is also essential for the maintenance of nephron progenitor markers in vivo (Karner et al., 2011); thus, *Wnt* has dual effects on the progenitors. Indeed, we found that higher concentrations of CHIR induced differentiation of progenitors, whereas lower concentrations were optimal for maintenance. Likewise, BMP7 concentration was critical for progenitor expansion. Brown et al. reported a requirement for LDN193189, which inhibits the SMAD pathway downstream of BMP4/7, because BMP7-mediated SMAD activation induces differentiation of nephron progenitors (Blank et al., 2009; Brown et al., 2013). Instead, we used lower concentrations of BMP7, and indeed SMAD1/5/8 phosphorylation was considerably lower at 5 ng/ml than 50 ng/ml BMP7 (Figure S2C). It is noteworthy that activation of JNK/p38, which is crucial for nephron progenitor maintenance (Blank et al., 2009; Brown et al., 2013; Muthukrishnan et al., 2015), was more prominent upon LIF stimulation than with BMP7 treatment. Therefore, our protocol does not simply elicit less SMAD activation but uses the maintenance program of the nephron progenitors more efficiently. Our protocol also contains DAPT, the Notch pathway inhibitor, owing to the previous findings that Notch activation causes premature progenitor differentiation (Fujimura et al., 2010; Boyle et al., 2011). Therefore, the combination of all these signals, as well as their optimal concentrations, is critical for progenitor expansion, and the decision between propagation and differentiation is based on the delicate balance between the multiple signaling pathways. The mechanism underlying concentration-dependent crosstalk will be an interesting topic for future studies.

Despite our advances, we have not yet achieved a condition for the complete long-term "self-renewal" of nephron progenitors. In extended cultures, the cell growth slowed and lost their competence to differentiate into nephrons. For example, E11.5 cells cultured for 30 days, or ESC-derived cells cultured for 2 weeks, failed to form glomeruli. The reduced expression levels of nephron progenitor markers at longer time points, as well as the microarray data, also indicate that our cultured cells gradually change their character. Recent data by Chen et al. (2015) suggest that there are no age-resistant "self-renewing" stem cells and that even the CITED1+ progenitors intrinsically age in vivo. However, our data, as well as those of Brown et al. (2015) suggest that the environment can override intrinsic aging of nephron progenitors and extend their lifespan to some extent. It is noteworthy that our culture conditions do not simply inhibit the in vivo aging processes. The cultured cells rather followed a distinct path from that observed in vivo but still retained nephron-forming potential. Therefore, nephron-forming competence is retained in cells in vitro and in vivo with a broader spectrum of gene expression patterns than expected.

Despite the limitations described above, our culture system will serve as a baseline for dissecting the maintenance mechanism of nephron progenitors. Nephron progenitors from mutant mice or gene deletion using CRISPR-Cas9 technology (Drost et al., 2015) will greatly advance our understanding of kidney development. The large numbers of cells amplified by our method will enable application of proteogenomic approaches, which are difficult to perform with conventional developmental nephrology approaches. In addition, our culture conditions are applicable to human nephron progenitors. If human nephron progenitors can be expanded for a longer term, they can be used for kidney organogenesis in vitro, which will contribute to regenerative medicine.

EXPERIMENTAL PROCEDURES

Animals

The *Six2GFPCre* transgenic mouse (Kobayashi et al., 2008), generated by bacterial artificial chromosome-based technology, was kindly provided by Andrew McMahon (University of Southern California). All animal procedures were performed following the guidelines of the Animal Care and Use Committee of Kumamoto University (#A27-018).

Expansion of Nephron Progenitors from the Embryonic Kidney

Six2-GFP+ cells were sorted using a FACSAria SORP (BD Biosciences) and seeded in U-bottom low-cell-binding 96-well plates (Thermo) at 3,000–10,000 cells per well. After 48 hr, cell aggregates were transferred onto the plates coated with iMatrix (Nippi: 0.5 $\mu g/cm^2$), and cultured for an additional 5 days (day 7). The basal medium was serum-free DMEM/F12 (Life Technologies) supplemented with tri-iodothyronine, hydrocortisone, insulin, transferrin, selenium, 10 ng/ml TGF-α (Peprotech), and 50 ng/ml FGF2 (Peprotech)/FGF9 (R&D Systems) (Perantoni et al., 1991; Tanigawa et al., 2011). The optimal conditions (CDBLY) required further addition of 1 μM CHIR99021 (Wako), 2.5 μM DAPT (Merck Millipore), 5 ng/ml BMP7 (R&D Systems), 5 ng/ml mouse LIF (Millipore), and 10 μM Y27632 (Wako).

At day 8, cultured cells were dissociated into small clumps using Dissociation solution (Reprocell) and divided into three plates. Medium was changed

every 24 hr for 3–4 days until the next passage. The numbers of nephron progenitors were calculated using the percentage of Six2-GFP+ cells identified by fluorescence-activated cell sorting (FACS) analysis.

Isolation of Nephron Progenitors Generated from Mouse ES Cells

Osr1-GFP mouse ESCs were used for induction of nephron progenitors (Taguchi et al., 2014). ESCs were passaged onto gelatin-coated plates for 2 days to remove feeders. Cells were then harvested using Accutase (Merck Millipore), aggregated, and differentiated according to the five-step protocol as described elsewhere (Taguchi et al., 2014). The induced spheres were harvested at day 8.5 and dissociated using 0.25% trypsin/EDTA. The cells were blocked with normal mouse serum and stained with anti-INTEGRIN α8 (R&D Systems) and anti-PDGFR α (Cell Signaling Technology) antibodies in FACS buffer. Osr1-GFP+/INTEGRIN α8+/PDGFR α− fractions were sorted on a FACSAria SORP (BD) and cultured in CDBLY-supplemented media.

Culture of Nephron Progenitors Derived from Human iPSCs

Human nephron progenitors were induced from iPSCs (201B7) (Takahashi and Yamanaka, 2006), based on the protocol that we previously established (Taguchi et al., 2014). Progenitor spheres were dissociated into small clumps using Dissociation solution (Reprocell), plated onto the iMatrix-coated plate, and cultured in CDBLY-supplemented media containing 5 ng/ml human LIF (Millipore). At day 8 of culture, the cells were dissociated into clumps with Dissociation solution, and aggregated for 48hr in the U-bottom low-cell-binding 96 well plates (Thermo Scientific) (200,000 cell equivalents per well) in CDBLY-supplemented media, followed by spinal cord recombination.

Statistical Analysis

Data were evaluated using the Student's t test. Statistical differences were considered significant if $p < 0.01$. All experiments were performed independently at least three times. Results are presented as the mean ± SEM.

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.celrep.2016.03.076.

AUTHOR CONTRIBUTIONS

S.T. performed the experiments and wrote the paper. A.T. contributed to FACS analyses and ESC and iPSC induction. N.S. and A.O.P. helped design the experiments. R.N. designed the experiments and wrote the paper.

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