Single adult kidney stem/progenitor cells reconstitute 3-dimensional nephron structures *in vitro.*

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Author contribution summary

Shinji Kitamura: Conception, design, financial support, collection, assembly of data, manuscript writing and final approval of manuscript

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Abstract

The kidneys are formed during development from two distinct primordial tissues, the metanephric mesenchyme and the ureteric bud. The metanephric mesenchyme develops into the kidney nephron, the minimal functional unit of the kidney. A nephron consists of several segments and regulates water, electrolyte and acid-base homeostasis in addition to secreting certain hormones. It has been predicted that the kidney will be among the last organs successfully regenerated *in vitro* due to its complex structure and multiple functions.

Here, we show that adult kidney stem/progenitor cells (KS cells), derived from the S3 segment of adult rat kidney nephrons, can reconstitute a 3-dimensional kidney-like structure *in vitro*. Kidney-like structures were formed when a cluster of KS cells was suspended in an extracellular matrix gel and cultured in the presence of several growth factors. Morphological analyses revealed that these kidney-like structures contained every substructure of the kidney, including glomeruli, proximal tubules, the loop of Henle, distal tubules and collecting ducts, but no vasculature. Our results demonstrate that a cluster of tissue stem/progenitor cells has the ability to reconstitute the minimum unit of its organ of origin by differentiating into specialised cells in the correct location. This process differs from embryonic kidney development, which requires the mutual induction of two different populations of progenitors, metanephric mesenchymal cells and ureteric bud cells.

Introduction

In the embryo, the kidney develops from two primordial cell types, metanephric mesenchymal cells and ureteric bud cells. These cells differentiate into more than a dozen distinct mature cell types in the kidney. The minimum unit of the kidney, the nephron, is composed of well-defined segments: the glomerulus, the proximal tubule (S1, PCT, S2 and S3), the loop of Henle, the distal tubule and the collecting duct (Fig. 1a) [1]. Because of this complicated structure, it is believed that the kidney will be among the last organs successfully regenerated *in vitro*.

Despite the challenge, a few attempts have been reported to aim (re)construction of the kidney. For example, GDNF-overexpressing human mesenchymal stem cells differentiate into nephron structure when they are injected near the ureteric bud of rat embryo [2]. This report "borrowed" the in vivo kidney organogenesis machinery. Recently, acellular kidney extracellular matrixes have been shown to serve as scaffolds for the functional new kidney [3], suggesting the importance of the three-dimensional structure of the kidney. Another group has tried to develop three-dimensional branching tubular structures from the embryonic ureteric bud or its primordial tissue, Wolffian duct, and let the structures induce freshly isolated metanephric mesenchyme cells to form nephron [4]. Their "neo-kidneys" attract host blood vessels toward them when they are transplanted into the kidney capsule.

In addition to these tissue engineering approaches, some groups have attempted to differentiate pluripotent stem cells into the kidney [5, 6]. A major problem of using such pluripotent stem cells is that undifferentiated cells may form ectopic tumors. In this regard, tissue stem cells are thought to be ideal source for kidney regeneration. Recently, Taguchi et al [7] has succeeded in pinpointing nephron progenitors in mouse embryo and has established an efficient induction method for ES cells to become nephron progenitors. Since Six2-positive embryonic nephron progenitor cells are reported be absent in the adult kidney [8], several other candidate cells for adult tissue stem cells have been proposed [9-11].

We previously established KS cells from adult kidney in an attempt to find a cell that has very high proliferating capacity [12] and demonstrated that these cells enhance the process of recovery from acute renal injury in rats [13]. KS cells in monolayer culture appears epithelial, rapidly proliferate and are shown to express stem cell markers such as Sca-1, c-Kit, Nestin and Mushashi-1 together with renal lineage marker such as Pax-2, WT-1. They seem to spontaneously differentiate into various renal tubular epithelial cells in 2D culture as they become positive for AQP1, AQP2, or THP and form

numerous tubular structures *in vivo* after injection into the muscles of immunodeficient mice [12]. Based on the expression pattern of a marker protein, KS cells are similar to metanephric mesenchymal cells, a population in which embryonic kidney tissue stem cells have been reported to exist. Because KS cells resemble embryonic kidney tissue stem cells and form numerous tubular structures *in vivo* after injection into the muscles of immunodeficient mice [12], we hypothesized that these cells may have the potential to undergo further differentiation into kidney like structures.

Materials and Methods

Establishment of rat kidney stem/progenitor cells (KS cells).

KS cells were obtained by microdissection from adult SD rat kidneys as previously described [12]. To harvest the KS cells, we isolated single nephron from SD rat (Clea Japan Inc, Tokyo, Japan) with microdissection methods. We microdissected each segments from nephron, such as glomeruli, proximal convoluted tubule (S1/ PCT), proximal straight tubule (S2, S3). Medullary thick ascending limb of Henle`s loop and collecting duct. The each segments were transferred into wells of 96-well plate that coated with type IV collagen(BD Biosciences, USA). The segments we cultured in a 1:1 mixture of culture supernatant (DMEM containing 10% FCS) from mouse mesenchymal cells (MCSs) provided from Dr. Sugaya T (CMIC Company, Limited, Tokyo, Japan) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium, supplemented with 10% FCS, 5 μg/ml insulin, 2.75 μg/ml transferrin, 3.35 ng/ml sodium selenious acid (GIBCO, USA), 50 nM hydrocortisone (Sigma, USA), 25 ng/ml hepatocyte growth factor (Sigma, USA) and 2.5 mM nicotinamide (Sigma, USA)) for 7days at 37℃ and 5% CO2/100% humidity. Outgrowing cells were harvested. We examined the morphology, the capacity of cell proliferation and gene expression described previously [12]. Then we harvested the KS cells. The KS cells were maintained on type IV collagen (BD Biosciences, USA) and in same previous culture condition, as undifferentiated culture condition. KS cells were differentiated in previous culture condition without MCSs, as differentiated culture condition described previously [12]. KS cells could be harvested from an adult rat kidney multiple times. We used the harvested other KS cells with characteristics similar to those of KS 56 cells.

Establishment of rat metanephric mesenchyme (MM) cells and ureteric bud (UB) cells.

Metanephric mesenchyme (MM) and ureteric bud (UB) tissues were dissected from the embryonic kidneys of timed pregnant SD rats (Crea JAPAN) at day 13 (e13) of gestation with fine forceps. Cells were cultured on type IV collagen (BD Biosciences, USA) and maintained in a 1:1 mixture of culture supernatant (DMEM containing 10% FCS) from mouse mesenchymal cells (MCS) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium, supplemented with 10% FCS, 5 μg/ml insulin, 2.75 μg/ml transferrin, 3.35 ng/ml sodium selenious acid (GIBCO, USA), 50 nM hydrocortisone (Sigma, USA), 25 ng/ml hepatocyte growth factor (Sigma, USA) and 2.5 mM nicotinamide (Sigma,

USA) at 37℃ and 5% CO2/100% humidity. Outgrowing cells were observed after 7 days. We harvested the outgrowing cells and divided them to obtain single cells using the limiting dilution method. Finally, we harvested a monoclonal MM cell line and a UB cell line. We used cells after 4-8 passages for this study.

Three-dimensional culture.

The KS cell sheets were incubated with trypsin and harvested. We generated a cell cluster from a KS cell sheet using the hanging drop method. The cell suspensions were incubated at 50 μl/well using the hanging drop method. One well contained from 6.25 X 10^3 to 200 X 10^3 KS cells. We cultured the cell clusters for 6-8 hour using the same condition employed for the hanging drop method. After making KS cell clusters, we placed the cell clusters into a half Matrigel (BD Biosciences, DMEM/F12=1:1). The half Matrigel was situated on top of Transwell filters (Costar, USA). Each cluster was cultured in DMEM/F12 supplemented with 10% FCS, 250 ng/ml GDNF (R&D systems, USA), 250 ng/ml b-FGF (Calbiochem, USA) and 250 ng/ml HGF (Sigma, USA) for 3-7 days until it displayed budding. After budding, we added 500 ng/ml EGF (R&D systems, USA) and 250 ng/ml BMP-7 (R&D Systems, USA) to the samples and incubated them at 37℃ and 5% CO2/100% humidity for 2 weeks. The kidney like structures were observed reproducibly as described previously.

Electron microscopy.

For electron microscopic analysis, pieces of specimens were cut into small blocks and immersed in 0.1 M cacodylate buffer containing 2.5 % glutaraldehyde (pH 7.2) for 2 hours, dehydrated through ascending grades of ethanol and finally embedded in Epon 812. Thin sections were stained with uranyl acetate and citrate and examined using a transmission electron microscope (H-700, Hitachi, Japan).

Immunostaining.

Cells were cultured on 16-well chamber slides (Nunc) coated with type IV collagen (BD Biosciences, USA). And frozen tissue sections of kidney-like structures, 17-day-old embryonic rat kidneys and adult rat kidneys were subjected to immunofluorescence staining using primary antibodies raised against Nephrin (Progen), Thy-1 (Santa Cruz), vWF (Chemicon), CD31 (Ab cam), aquaporin-1 (Chemicon), Na-K-Cl cotransporter 2 (NKCC2) (Lifespan Biosciences), Tamm-Horsfall glycoprotein (AbD), aquaporin-2 (Chemicon), vimentin (Sigma), Six2 (proteintech), CD133 (Santa Cruz) and PDX-1

(Santa Cruz);, secondary antibodies conjugated with FITC or rhodamine (Chemicon or Invitrogen, Molecular Probes); and DAPI (Roche) as previously described (12). For immunohistochemistry, the samples were fixed with acetone for approximately 5 min at 4°C. Images were recorded using a confocal fluorescence microscope (ZEISS Confocal Laser Scanning Microscope Model LSM510), FSX-100 (Olympus, Japan) or BIOZERO BZ-8000 fluorescent microscope (Keyence, Japan).

PCR.

To release the kidney-like structures from the half Matrigel ECMs, we used a cell recovery solution (BD Biosciences, USA). We washed the kidney-like structures and the half Matrigel twice with Ca⁻Mg⁻ PBS. We then added cell recovery solution on top of the half Matrigel ECMs, allowed it to sit for 10-20 min, removed the kidney-like structures using fine forceps under a microscope, and placed the kidney-like structure in tubes. Total RNA was extracted using the RNase plus Mini Kit (QIAGEN). The total RNA was reverse transcribed into cDNA. As RT-PCR, amplified using the GeneAmp RNA PCR core kit (Roche) with specific primer pairs for each gene. The reactions were subjected to agarose gel electrophoresis and visualised as previously described [12]. The primers are listed in Table 1.

Establishment of single KS cell colony from KS cells.

The KS cells were separated to single KS cell by using limiting dilution methods. The single KS cell was cultured on type IV collagen (BD Biosciences, USA) and were maintained in a 1:1 mixture of culture supernatant (DMEM containing 10% FCS) from mouse mesenchymal cells (MCSs) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium, supplemented with 10% FCS, 5 μg/ml insulin, 2.75 μg/ml transferrin, 3.35 ng/ml sodium selenious acid (GIBCO, USA), 50 nM hydrocortisone (Sigma, USA), 25 ng/ml hepatocyte growth factor (Sigma, USA) and 2.5 mM nicotinamide (Sigma, USA)) at 37℃ and 5% CO2/100% humidity as previous described [12].

Statistical Analysis.

The number of kidney like structure formation from single KS cells data were compared

between group using Student t-tests. Significance was defined as P < 0.05.

Results

Character of Kidney stem / progenitor cells.

KS cells express stem cell markers such as Sca-1, c-Kit, Nestin and Mushashi-1 together with renal lineage marker such as Pax-2, WT-1. In addition, KS cells expressed Six2 [8] (Fig.1b), CD133 [10, 14] (Fig.1c), and Vimentin [10] (Fig.1d), which are reported as kidney stem cell markers that were expressed in embryonic kidney (Fig. 1e-g) [8, 10 and 14]. The Musahi-1 positive cells decreased when we cultured the KS cells under differentiated culture condition (supplementary Fig. S1a, b). In addition, aquaporin-1 altered the expression and distribution when we cultured KS cells under differentiated culture condition (supplementary Fig. S1c, d). Based on these findings, KS cells may be very close to adult kidney-specific tissue stem cells. Thus, in this study, we explore their potential to self-organize and reconstruct the kidney.

Reconstituting kidney-like structure from kidney stem/progenitor cell cluster.

The KS cells grew like cobbled stone in 2-dimensional condition (Fig.2a). The KS cells did not show any signs of morphogenesis in the presence of any growth factors when tested under 2-dimensional culture conditions (Fig. 2b). Therefore, we generated cell clusters from the KS cells using the hanging drop method (Fig. 2c). The cell clusters (Fig. 2d) were transferred into 3-dimensional extracellular matrix (ECM) gels and cultured in the presence of multiple growth factors. These growth factors were chosen because they reported to be critical in certain stages of kidney development [15-19]. Different growth factor combinations have been tested (Fig. 2e-o) and a combination of GDNF, b-FGF, HGF, EGF and BMP-7 consistently induce the most kidney-like structure (Fig. 2m), defined as a central large cyst (pelvis-like structure) with multiple tubular projections (collecting duct/kidney tubule-like structure), with ball-like termini (glomerulus-like structure) after 3-4 weeks of culture. The efficiency of inducing such structures was more than 70%. As long as sufficient numbers of KS cells were put into the cluster (describe later), the kidney-like structures developed 9-10 times out of 12 attempts.

This morphogenesis seemed unique to KS cells as either embryonic kidney derived UB cell cluster or metanephric mesenchyme cell cluster did not undergo such a process (compare Fig. supplementary Fig. S2a,b, and c). Moreover, another tissue stem cell type, a pancreatic and duodenal homeobox-1 (PDX-1)-positive cell line (Fig.

supplementary Fig. S2d, e) that has been reported to be a pancreas progenitor cell [20], did not reconstitute the renal structure under the same culture conditions (Fig. supplementary Fig. S2f). However, the PDX-1 positive cell cluster reconstituted pancreatic like formation in another culture condition (Fig. supplementary Fig. S2g). These results suggested that each tissue stem cell has a unique ability to differentiate into its organ of origin.

We also noticed that three-dimensional structure of KS cells depended on the size of the initial KS cell cluster (Fig. 3): 1) when less than 6.25×10^3 cells were used for the cluster, only cyctic structures developed 2) long, distinct tubular structures became apparent as the initial cell number was increased up to 50×10^3 cells 3) kidney-like structures developed when the initial cell number exceeded 100×10^3 cells (Fig. 3).

Morphological analysis of kidney-like structure.

To characterize the kidney-like structures further, we examined each substructure at the cellular level. Ball-like structures at the tips of numerous tubules contained many cells (Fig. 4a). We examined each substructure at the cellular level. Round, glomerulus-like structures were observed at the tips of numerous tubules after 4 weeks of culture (Fig. 4b). The glomerular configurations consisted of lumens covered by Bowman's capsule-like structures (Fig. 4c). They were distinct from the cysts and looked similar to the kidney glomeruli by electron microscopy (EM) analysis (compare Fig. 4d, e with Fig. 4f, g). The tubules that connected directly to the glomerulus-like structures were slightly thicker than those in the centre of the tubular structures (Fig. 4h). These tubules were composed of cubic-columnar epithelial cells with many villi on the lumen side (Fig. 4i). This structure was similar to that of the kidney proximal tubules *in vivo*, which also have the brush border (compare Fig. 4j with Fig. 4k, l). Following the proximal tubule-like structures, another distinct tubular structure was observed, in which the lumen was surrounded by ciliated (Fig. 4m), flatter epithelial cells resembling the structure of the more distal kidney tubules. Thus, the kidney-like structure turned out to contain at least 3 distinct segments, similar to kidney glomerulus, proximal tubules and distal tubules.

Molecular marker analysis of kidney-like structure.

An immunohistochemical analysis revealed that Nephrin [21] (a podocyte marker, Fig. 5a) and Thy-1 [22] (a mesangial cell marker, Fig. 5a) were expressed in the

glomerulus-like structures, and Nephrin-positive cells and Thy-1-positive cells were observed at distinct sites in the glomerulus-like structures (Fig. 5a). The Nephrin and Thy-1 protein distribution of glomerular structure in kidney-like structure is very close to that of embryonic day 17 kidney glomeruli (compare Fig. 5a with Fig. 5f, k). Although we found foot process-like protrusion in the EM picture (Fig.4e, arrowhead), the majority of the nephrin positive cells were segregated from the Thy-1 positive cells, indicating that the podocyte-like cells have not form a functional filtration barrier.

Following the glomerulus-like structure, the proximal tubule-like structures were present, where the cells in the tubular structure stained positive for aquaporin-1 [23], a proximal cell marker (compare Fig. 5b with Fig. 5g, l). In the thinner tubular segment, some tubules were positive for Na-K-Cl cotransporter 2 [24], a marker for the loop of Henle cells (compare Fig. 5c with Fig. 5h, m), and some were positive for Tamm-Horsfall glycoprotein (compare Fig. 5d with Fig. 5i, n) [25], a marker for distal-collecting duct cells, and others were positive for aquaporin-2 (compare Fig. 5e with Fig. 5j, o) [26], a collecting duct cell marker. Interestingly, expression levels of these tubular markers in the kidney-like structures appear closer to those in the adult kidney than the E17 kidney. Low magnification immunostaining images revealed that the cells at the tips, in the glomerulus-like structures, were different from the cells in the proximal tubule-like structures (Fig. supplementary Fig. S3a-d). Similar results were obtained for the collecting duct-like structures and distal tubule-like structures (Fig. supplementary Fig. S3e-g). However, we were unable to say that loop of Henle and distal tubules are formed successively as distinct segments.

It is of note that very little vWF or CD31 (an endothelial marker) expression was observed in the cells of the glomerulus-like structures (Fig. supplementary Fig. S4a-c). These endothelial cells were observed in the glomerulus of the adult kidney (positive control; Fig. supplementary Fig. S4d-e).

When the expression levels of glomerulus- (nephrin), proximal tubule-(aquaporin 1) and collecting duct-(aquaporin 2) specific gene were majored in the kidney-like structures at different time points, they were increased at the later time points (Fig. 6), consistent with the idea that the cells in the kidney like structures were in the process of differentiation/maturation.

A single clone of KS cells can develop into the kidney-like structures.

We have shown previously that more than certain numbers of KS cells were required to undergo full-blown morphogenesis to occur. Then one could argue that only

a part of KS cells possess tissue stem cell like characteristics. To counter this argument we showed here that kidney-like structures could be developed from a single KS cell. We established 5 different clones of KS cells by limiting dilution (C1-5; Fig. 7a-e), and these cells were expanded in 2-dimensional culture. Cell clusters of each clone were generated by the hanging drop method, followed by 3-dimensional ECM gel culture in the presence of growth factors, as described. Four of the 5 clones formed kidney-like structures (Fig. 7f-j and k). Although it is not clear when the KS cells begin to differentiate or how differentiation progressed, many, if not all, KS cells have the ability to reconstitute different kidney epithelial cells. This observation, together with the fact that KS cells retain their characteristics after several passages [12], suggests that these cells are adult kidney stem cells.

Discussion

Diep et al. reported that adult nephron progenitor cells can produce new nephrons in adult zebrafish *in vivo* [27], and Rinkevich Y et al. reported that the adult mammalian kidney undergoes continuous tubulogenesis via expansions of fate-restricted clones [28]. A cluster of KS cells was shown to differentiate into a kidney-like structure without requiring embryonic primordial cell types, such as metanephric mesenchyme and ureteric bud cells, suggesting that organogenesis beginning from adult tissue stem/progenitor cells may follow a different developmental program than organogenesis during embryonic development. In addition, these researchers reported that cell aggregates, rather than single stem cells, are necessary to regenerate the kidney, which is very similar to the results reported here.

We further characterized KS cells with different markers. Because KS cells are derived from S3 segment of kidney proximal tubular cells, they should originate from nephron progenitor cells present in the metanephric mesenchyme. Recently, Kobayashi et al reported such cells are positive for Six2 and such cells are reported to be absent in the adult kidney [8]. KS cells were positive for Six2 (Fig 1b), suggesting that these cells may retain embryonic nephron stem cell characteristics. How Six2 became positive in adult kidney-derived KS cells is an interesting topic for further investigation. Sagrinati et al reported that CD133 positive parietal epithelial cells of the Bowmann's capsule are adult kidney stem cells [14] and recently Lindgren et al reported that such cells are also reside in the proximal tubule [10]. They used CD133 and Vimentin as markers of possible kidney adult stem cells. KS cells were positive for these markers (Fig 1). To our knowledge, there is no single bona fide marker specific for adult kidney stem cells at present. Given the fact that KS cells were positive for embryonic nephron tissue stem cell marker Six2 and possible adult kidney tissue stem cell marker combination CD133/Vimentin, KS cells are likely to be very close, if not identical, to adult kidney stem cells. It should be underscored that KS cells could differentiate into collecting duct-like cells or mesangial-like cells, which are not derived from nephron progenitors.

Eiraku et al. reported the reconstitution of a 3-dimensional retinal structure from ES cells *in vitro* [29] by inducing ES cells to differentiate into a cluster of neural lineage cells followed by 3-dimensional culture. Together with our results, this finding suggests that tissue progenitor cells likely possess an intrinsic program to produce the 3-dimensional structure of the organ from which they originate.

The kidney-like structures could not show the vascularization and make urine. The further examinations are required to elucidate the issues. However, we believed

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that these kidney-like structures should be attractive for in vitro kidney regeneration and could contribute for not only the elucidation of kidney regeneration but also for *in vitro* study toward alternatives to animal experiments and custom-made medicine.

In summary, we have identified adult kidney stem/progenitor cells in rat that can regenerate a kidney-like structure from a single cell *in vitro*. Although the physiological roles of such cells are currently unclear, analogous cells in the adult human kidney would be a valuable resource for the regeneration of kidneys *in vitro*.

Interest Declaration.

We declare that the establishment of rKS56 cells and the potential for their therapeutic application to renal disorders were filed and submitted to the Japan Patent Office (Issue No. 2003-071029: Kidney stem cells, isolation methods to separate kidney stem cells, therapeutic approach for kidney diseases) in March 2003. The methods used to reconstitute organ structures were filed and submitted to the Japan Patent Office in August 2009 (Issue No. 2010-063659: Process for production of bioartificial organ). This study was partly funded by Organ Techonogies Inc.. Co-author, Hiroaki Asai is employed by Organ Technologies Inc.. There are no further patents, products in development or marketed products to declare.

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Figure Legends

Fig. 1
Kidney tissue stem cell like characteristics of KS cells.
a)Nephron structure: G, glomerulus; proximal tubule, S1, PCT, S2, S3 and loop of Henle; DT, distal tubule; CD, collecting duct. b-g)Characteristics of KS cells compared to 17-day-old embryonic rat kidneys (E17K).b) Green, Six2 ; blue, DAPI. c) Green, CD133; blue, DAPI. d) Green, Vimentin; blue, DAPI. Compared to 17-day-old embryonic rat kidneys. e) Green, Six2 ; red, Dolichos biflorus agglutinin, blue, DAPI. f) Green, CD133; red, Dolichos biflorus agglutinin, blue, DAPI. g) Green, Vimentin; red, Dolichos biflorus agglutinin, blue, DAPI. Representative cell staining photomicrographs from 4-5 independent experiments and embryonic kidney staining photomicrographs from 2-3 independent experiments.
Scar bar: 5 µm.

Fig. 2

Development of kidney like structures from KS cells in vitro.

a) KS cells on type IV collagen. b) KS cell sheet. DMEM/F12, 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 250 ng/dl BMP-7 and 500 ng/ml EGF. c) The method used for 3-dimensional culture. A KS cell cluster was placed into half Matrigel using a combination of culture conditions. d-o) KS cell clusters were cultured under various conditions for 3 weeks. d) KS cell cluster in half Matrigel. e) DMEM/F12 and 10% FCS. f) DMEM/F12, 10% FCS and 250 ng/ml GDNF. g) DMEM/F12, 10% FCS and 250 ng/ml b-FGF. h) DMEM/F12, 10% FCS and 250 ng/ml HGF. i) DMEM/F12, 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF and 250 ng/ml HGF. j) DMEM/F12, 10% FCS and 500 ng/ml EGF. k) DMEM/F12, 10% FCS and 250 ng/ml BMP-7. l) DMEM/F12, 10% FCS, 250 ng/dl BMP-7 and 500 ng/ml EGF. m) DMEM/F12, 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 500 ng/ml EGF and 250 ng/ml BMP-7. n) DMEM/F12, 10% FCS and 250 ng/ml activin A. o) DMEM/F12, 10% FCS and 250 ng/ml follistatin. All cultures contained antibiotics (1% penicillin + streptomycin). h-i) Representative pictures were presented after KS cell cluster culture for 3 weeks from 3-4 independent experiments.

Scar bar: 100 μm.

Fig.3

The relationship between cell number in the cluster and the ability to reconstitute a kidney-like structure.

Red bar: cyst formation, Blue bar: long distinct tubules formation, Green bar: tubules with ball-like structures at the tip. Horizontal axis represents KS cell number (6.25 to 200 × 10^3 cells)/cluster. The KS cell clusters were cultured for 3 weeks. Representative photomicrographs were from 3 independence experiments.

Scar bar: 100 μm.

Fig. 4

Morphological analysis of the kidney like structure.

a) A KS cell cluster formed a kidney-like structure (KLS) after 4 weeks of incubation using DMEM/F12 plus 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 250 ng/dl BMP-7 and 500 ng/ml EGF. Glomerulus-like structures were formed at the tips of the tubular structures, proximal-like tubules, distal-like tubules, collecting duct-like tubules and renal pelvis-like structures. G, glomerulus-like structure; Pr, proximal tubule-like structure; H, loop of Henle-like structure; D/C, distal tubule-like structure or collecting duct-like structure; Pe, renal pelvis-like structure. b) Light microscopy of a glomerulus-like structure. c) A section of the glomerulus-like structure, as viewed using Toluidine blue staining. d) Electron microscopy of a glomerulus-like structure (×800). Me, mesangial-like cell; Po, podocyte like cell; Bo, Bowman Capsule epithelial-like cell; L, lumen. e) Glomerulus-like structure (×3000). f) Glomerulus of a 17-day-old embryonic kidney (×2000), En: endothelial cell. g) Glomeruli of the adult kidney (×2000). h) Light microscopy of proximal-like tubules and the loop of Henle-like structure. P, proximal-like tubule; H, loop of Henle-like structure. i) Tubules stained with Toluidine blue. The tubular structure consists of several cubic-columnar epithelial cells. j) Proximal tubular-like cells. brush border (BB)-like structure: (arrow) (×10000). k) Proximal tubular cells of E17 kidney. brush border (BB) structure. Arrow: brush border (BB) (×8000). l) Proximal tubular cells of adult kidney. brush border (BB) structure. Arrow: brush border (BB) (×9000). m) Primary cilia on distal tubule-like cells (X10000). Arrow: primary cilia (PC).

G, glomerulus-like structure; Proximal, proximal tubules-like structure; LOH, loop of Henle-like structure; Distal, distal tubules-like structure; CD, collecting duct-like structure. Representative photomicrographs were from over 5 independent experiments.

Scar bar: 100 µm.

Fig. 5

Segment specific marker protein expression of the kidney-like structure.

a-e: Staining of each segment of the kidney-like structures. f-j: staining of an adult rat kidney (8-12W rats). k-o: staining of an embryonic 17-days old kidney.

Staining of sections of the glomerulus-like structures or glomeruli.

a, f, k) Green, nephrin; red, Thy-1; blue, DAPI.

Staining of the tubule-like structures or tubules.

b, g, l) Red, aquaporin-1 (AQP-1); blue: DAPI.

c, h, m) Red, Na-K-Cl cotransporter 2(NKCC2); blue, DAPI.

d, I, n) Green, Tamm-Horsfall glycoprotein (THP); blue, DAPI

e, j, o) Red, aquaporin-2 (AQP-2); blue, DAPI.

Scar bar: 10μm.

Representative photomicrographs were from 6-10 independent experiments.

Fig.6

Time course of the expression of kidney-specific genes in KLSs. a) Each gene expression band was scanned and subjected to densitometry. Each kidney-specific gene was expressed more strongly as the time in culture increased, paralleling the days of embryonic kidney development. (N=3, representative data shown). b) the AQP-1 / GAPDH, AQP-2 / GAPDH, and Nephrin / GAPDH were measured. AQP-1, aquaporin-1; AQP-2, aquaporin-2; KS, kidney stem/progenitor cell;1W, kidney-like structures cultured for 1 week;3W, kidney-like structures cultured for 3 weeks; E13, 13-day-old embryonic kidney; E17, 17-day-old embryonic kidney; AK, adult kidney (8-12 weeks). Representative results obtained from 2 independent experiments. Data represent mean ± standard deviation values.

Fig.7

KLS formation from single KS cell clones. Five randomly selected single KS cell clones were established and named, C1-5.

Light microscopic photographs. C1; a, C2; b, C3; c, C4; d, C5; e.

KLSs were formed from each KS single-cell clone, C1-5.

Light microscopic photographs. C1; f, C2; g, C3; h, C4; i, C5; j.

k: A comparison of the number of tubules produced by individual KS cell clone; each cell revealed a slightly different ability to reconstitute a kidney-like structure. *p<0.05.

Representative data from 3 independent experiments.

Scar bar: 100 μm.

supplementary Fig. S1

Differentiation of KS cells in 2-dimensional culture condition. Musahi-1 positive cells decreased when we cultured the KS cells under differentiated culture condition. Aquaporin-1 altered the expression and distribution partially when we cultured KS cells under differentiated culture condition. The distribution of Aquaporin-1 changed from cytoplasm to cell membrane. KS cells were cultured under undifferentiated condition (a, c) and differentiated culture condition (b, d).

a, b) Musashi-1 staining. Green: Musashi-1

c, d) Aquaporin-1 staining. Green: Aquaporin-1

Scar bar: 10μm.

supplementary Fig. S2

a-c) The comparison for reconstituting ability with immature cells. a) KS cell cluster budding after 1 week in half Matrigel with DMEM/F12 plus 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 250 ng/dl BMP-7 and 500 ng/ml EGF. b) The culture of a metanephric mesenchymal cell (MM cell) cluster from a 13-day-old embryonic kidney did not result in budding or tubule formation after 3 weeks of incubation in DMEM/F12 plus 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 250 ng/dl BMP-7 and 500 ng/ml EGF. c) A ureteric bud cell (UB cell) cluster from a 13-day-old embryonic kidney did not form tubules but instead formed many cystic structures after 3 weeks of incubation in DMEM/F12 plus 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 250 ng/dl BMP-7 and 500 ng/ml EGF. Representative KS, MM, and UB cell cluster culture pictures were selected from 6 independent head-to head experiments. Scar bar: 100 μm.

d-g) Another tissue stem cell, a PDX-1-positive cell line, did not reconstitute kidney-like structures under the culture conditions employed for reconstituting the kidney-like structures with KS cell clusters.

d) PDX-1-positive cells. Green, PDX-1; blue, DAPI. Scar bar:50 μm. e) RT-PCR analysis of PDX-1-positive cells. f) A PDX-1-positive cell cluster did not undergo morphogenesis after 2 weeks of culture in DMEM/F12 plus 10% FCS, 250 ng/ml GDNF, 250 ng/ml b-FGF, 250 ng/ml HGF, 250 ng/dl BMP-7 and 500 ng/ml EGF. g) A PDX-1-positive cell cluster produced tubules and cell aggregates after 2 weeks of culture in DMEM/F12 plus 10% FCS without adding exogenous growth factors . Representative PDX-1 positive cell staining photomicrographs were from 2 independent experiments. PCR figure was a representative for 2 independent experiments and PDX-1 positive cell cluster photomicrographs were from 3-4 independent experiments. f,g) Scar bar: 100 μm.

supplementary Fig. S3

Low magnification immunostaining pictures spanning more than one segment.

a-d) the glomerular-like structure and the proximal tubule-like structures were stained.

a) Green, nephrin, b) red, AQP-1, c) blue, DAPI, d) merge a-c.

e-g) the distal tubule-like structure and the collecting duct- like structure staining. e) red, AQP-2, f) blue, DAPI, g)merge I and j. Scar bar: 10μm.

Representative photomicrographs were from 3-4 independent experiments.

supplementary Fig. S4

Endothelial cell specific staining of the kidney-like structure

a-c) glomerulus-like structure, a: Green: von Willebrand factor (vWF), b: red: CD31, c: blue: DAPI.

d-f) glomerulus of the adult kidney, d: Green: von Willebrand factor (vWF), e: red: CD31, f: blue: DAPI.

Scar bar: 50μm.

　　Representative photomicrographs were from 3-4 independent experiments.

supplementary Table. 1

Primers used to amplify each gene.