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Induction of steady-state glomeruloid sphere by self-assembly from human embryonic kidney cells

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ABSTRACT

The glomerulus is a network of capillaries known as a tuft, located at the beginning of a nephron in the kidney. Here we describe a novel method for the induction of a macroscopically visible three-dimensional glomerulus-like sphere (GLS). This procedure did not require any additional cytokines and completed the formation of spheres within 24 h. After the formation was complete, GLS maintained a steady state for at least five days without proliferation and without a decrease in viability. Therefore, this procedure assists various assays for a prolong period of time. Overall, our protocol allows for a very simple mixing of cells from different sources to obtain fine-grained and highly dispersed GLSs. The kidney filtration barrier is a unique structure characterized by a complex three-dimensional framework of podocytes and endothelial cells. GLS exhibited the induction of many podocyte-specific gene profiles similar to those in adult human kidneys, suggesting that the sphere formation process is important for the maturation of podocytes. Focal segmental glomerulosclerosis (FSGS) is one of the major causes of steroid-resistant nephrotic syndrome, and some circulating permeability factors in the patient's serum FSGS have been implicated in the pathogenesis of the disease. Serum from patients with FSGS induced the collapse of GLS, which imitates the appearance of glomerulosclerosis in patients. In conclusion, the investigation and use of GLS may provide a novel method to elucidate the molecular mechanisms underlying complicated and unexplained events in glomeruli in a similar condition in adult kidneys.

1. Introduction

Self-assembly is the autonomous organization of components, from an initial state into a final pattern or structure without external intervention. In nature, cells self-assemble into complex three-dimensional architectures which comprise tissues. Recently, three-dimensional ex vivo cellular cultures, created through cellular self-assembly under natural extracellular matrix cues, or through biomaterial-based directed assembly, have been shown to physically resemble and recapture some functionality of target organs. These “organoids” hold enormous potentialities for their applications in modeling human development and disease, drug screening, and future therapy design or even organ replacement. Thus far, a major focus of organoids research has been on embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). When grown in a 3D microenvironment, these stem cells self-organize and acquire physiologically relevant cellular patterning, to develop into several endoderm- and ectoderm-derived tissues, often mimicking their in vivo counterparts [1–4]. Stem cell differentiation is the process by which a more specialized cell is formed from a stem cell, leading to loss of some of the stem cell's developmental potential. On the other hand, there are fundamental issues related to the reproducibility of organoids derived from stem cells. Because organoids recapitulate a large number of biological events including stepwise differentiation, cell-cell interactions, and certain physiological functions, it is amenably required that extended cultivation and various kinds of manipulation using chemicals and cytokines to generate organoids [5]. Moreover, there is little to no control over how cells self-organize into the organoids. Therefore, current protocols are unable to guarantee exact replications of organoid spheres of the same dimensions (i.e., size and shape), cellular composition, and phenotypic and molecular characteristics [6].

The control of organism and organ size is a central question in biology. The human body includes mechanisms that help regulate the organs, glands, tissues, and cells. The development of a functional organ requires not only patterning mechanisms that confer

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proper identities for its constituent cells, but also growth-regulatory mechanisms that specify the final size of the organ. The developmental regulation of final body and organ size is fundamental to generating a functional and correctly proportioned adult. The adjusting of these regulatory systems enables the body to constantly be in a steady state. Although most tissues are subject to a continuous loss of cellular mass due to wear and tear [7], their size is maintained approximately constant for the lifetime of the organism and, in many cases, can be restored in response to severe injury. This second property suggests the existence of a self-regulated steady state or 'homeostasis' which maintains a number of cells through a balance of cell loss and proliferation. Thus, the precise control of organ size is a key feature and a crucial process during animal development and regeneration. In general, cell number is dependent on the balance between cell proliferation and cell death, whereas cell size is dependent on cell growth. However, at present, our understanding of how adult organ size is determined and maintained is still incomplete.

The most advanced nephrons occur in the adult kidneys, or metanephros, of land vertebrates, such as reptiles, birds, and mammals. The capsule and glomerulus together constitute the renal corpuscle. Increasing evidence suggests that glomerulopathies are frequently caused by primary lesions in the renal podocytes. The molecular mechanisms underlying podocyte development have been extensively studied in mice. However, because of limited access to human embryos, relatively little is known regarding the molecular profiles for the maintenance of homeostasis of podocytes and glomerulogenesis in humans. Podocyte precursors are derived from the metanephric mesenchyme during kidney development. Following maturation they establish their complex cell architecture and become highly terminally differentiated with a very limited regenerative capacity [8,9]. Owing to the extraordinary characteristics of podocytes, most studies on podocytes have been performed in 2D culture with genetic modulation of proliferation and differentiation, even though podocytes are terminally differentiated renal cells that lack the ability to regenerate by proliferation. Although studies thus far reflect functions partially, elucidation of the natural steady state of podocytes in glomerulus in three-dimensional environments has been left unsolved.

Primary focal segmental glomerulosclerosis (FSGS) is one of the major causes of steroid-resistant nephrotic syndrome, and renal prognosis in patients with steroid-resistant FSGS is poor [10]. It has long been speculated that circulating permeability factors should be implicated in the pathogenesis of the disease because a substantial portion of the patients with primary FSGS experience recurrence shortly after transplantation [11]. Additional information about renal responses in FSGS has been derived from studies of isolated glomeruli from experimental animals with sera from patients with FSGS, suggesting the presence of a substance in the circulation causing collapsing phenomena in FSGS [12–14]. However, the effects of sera from patients with FSGS on human podocytes in a static state have not been determined. To fully understand the events inside the human kidney, three dimensional (3D) cell culture systems composed of human kidney cells in a static state are required. Our glomerulus-like sphere (GLS) as a 3D cell culture system may be a pivotal model for drug screening, investigating personalized medicine, and understanding human organ development.

2. Materials and methods

2.1. Patients

This study was in accordance with the Declaration of Helsinki, and we obtained approval from the institutional review board. All patients gave their written informed consent. Twenty patients with overt or heavy proteinuria were enrolled in this study. FSGS and MCNS were proven through renal biopsy.

2.2. Cell culture

hMSCs (Lonza), HUVECs (Lonza), and HEK were maintained in Endothelial Growth Medium (EGM; Lonza), MSC Growth Medium (Lonza), and 10% FCS, respectively. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. To build 3-dimensional construction in vitro, we suspended 5×10^4 hMSCs, 2×10^5 HUVECs, and 5×10^4 HEKs in 2 ml of media, before seeding the cells in a 96-well culture plate.

2.3. Immunofluorescent staining of spheroids

To perform immunocytochemistry in spheres, we transferred the spheres into iPGell (GenoStaff, Tokyo, Japan), then embedded the spheres in Optimal Cutting Temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The spheres were stained with the anti-Podocin (Santa Cruz) antibody, followed by incubation with the appropriate fluorescent secondary antibodies.

2.4. RNA purification and RT-PCR

Purified human fetal kidney and adult kidney total RNA (25 μg) were purchased from Agilent Technologies Inc. (Santa Clara, CA) and diluted to 250 ng/μl. Spheres were collected, and total RNA was extracted from the spheres using TRIzol reagent (Invitrogen). The RNA was reverse-transcribed with oligo(dT) primer using a SuperScript first-strand synthesis kit (Invitrogen) to generate the first-strand cDNA, followed by PCR to detect the expression of podocyte-specific genes and GAPDH. The sequences of the PCR primers were as follows: 5′-GGACATAGTCTGCACTGTCGAT-3′ and 5′-GGCAAATCTGACAACAAGACG-3′ for Nephrin; 5′-GTACGAGAGCGATAACCACACA-3′ and 5′-GGCTTTTCACCTGTATGAGTCC-3′ for WT1; 5′-AAAACCAAAGAAACCACCACCT-3′ and 5′-GGCATCTTTGGTCTATTTGCAG-3′ for CD2AP; 5′-CTATCCCTGGCTACACCTTCAC-3′ and 5′-CTCGGCATATCAGTGAGATCAA-3′ for Podxl; 5′-GCTACTACCGAATGGAAAATGC-3′ and 5′-AGTTCTGTTGCTGGGAGAAGAC-3′ for Podocin; 5′-GAAGCTACAGGTGTCTGGCTCT-3′ and 5′-TGGCTTCTTTCAGATCCTTCTC-3′ for Synpo; 5′-TGCACAAAATCAACAATGTGAA-3′ and 5′-TTCTCGTAGTCCTCCATCAGGT-3′ for Actn4; 5′- -3′ and 5′-GTTTTAAACGGCTGAGATTTGG-3′ for TRPC6; 5′-TAACCCTGCTACCCTCATGTCT-3′ and 5′-TCCACAGTGATGTCTCCATAGG-3′ for PTPRO; and 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ for GAPDH. The reaction products were separated on 1.5% agarose gel and stained with ethidium bromide.

2.5. Cell viability and cell number

The size of GLS was microscopically measured. Cell viability was assessed using the AlamarBlue assay as described previously (31). In brief, medium was removed from the wells and 1 ml 10% AB in CCM added. After a 4-h incubation, samples of AB/CCM were removed and measured on a Fluroskan Ascent FL (Thermo Labsystems, UK) fluorescent plate reader (Thermo Fisher Scientific, Waltham, MA, USA) (excitation 530 nm, emission at 620 nm). Three independent experiments were performed in duplicate. The mean and standard deviation were calculated. A live/dead viability staining assay was performed using the live/dead viability/cytotoxicity kit (Biotium) according to the manufacturer's instructions.

2.6. Serum-induced collapse assay

Human GLSs were cultured in serum-free medium in 96-well optical plates (PerkinElmer, Waltham, MA). GLSs were incubated for 48 h at 37 °C in isolation media containing a 1:50 dilution of serum from healthy subjects or patients with MCNS or FSGS to a final concentration of 5%. The number of dropout cells from GLSs was counted in each group.

2.7. Statistical analysis

The data were expressed as means ± S.E., and were analyzed by an unpaired Student's *t*-test.

3. Results

3.1. Spontaneous self-assembly of glomerulus-like sphere

To recapitulate the initial cellular interactions of organogenesis in vitro, endothelial and mesenchymal cells were cocultivated with human embryonic kidney cells. We set out to develop a method by which glomerulus-like microspheres could be constructed from a suspension of individual cells, so that fine-grained chimeras could be produced easily and reliably. HEK were cocultivated with hMSCs and HUVECs in a microwell culture plate (Fig. 1A). After 24 h incubation without addition of any cytokines or reagents, the formation of aggregates of cells and microspheres was observed (Fig. 1B and Supplementary Movie S1). In contrast, HEK single culture showed the formation of a few twisted shapes (Fig. 1B). Generated glomerulus-like microspheres exhibited their good spherical form in microwell plates (Fig. 1C).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.11.160

3.2. GLS maintained a steady state in vitro

In normal adult organs, these tissues and apparatus are in a kind of steady-state condition and preserve their size. To confirm whether the GLS is in a steady-state condition, we first observe the morphological alterations of GLSs. The morphological appearance was maintained during the observation period of 5 days (Fig. 2A). Correspondingly, the sphere size in GLSs showed no significant changes (Fig. 2B). Next, we performed the cell viability assay in GLSs. Live/Dead assays demonstrated no sign of diminishing cell viability (Fig. 2C). In addition, we carried out a quantitative assay using a well-established Alamar blue assay. Results showed that cell metabolism affecting viability was not significantly changed up to 5 days after the formation of GLSs (Fig. 2D).

3.3. Podocyte-specific genes were induced in the formation of GLS

Podocyte differentiation is required for normal glomerular filtration barrier function and is regulated through the transcription factor WT1. After a tightly orchestrated differentiation program, podocytes develop foot processes and assemble specialized adherens junctions, the slit diaphragm which mediates contact between adjacent cells. To further characterize the phenotype of the podocytes within the GLS, we performed RT-PCR analysis on mRNAs of GLS. The results demonstrate that podocytes within the GLS retain a constitutive expression level of typical transcriptional markers such as WT1, as well as important structural markers such as podocin, nephrin, and so on (Fig. 3A). The quantitative analysis

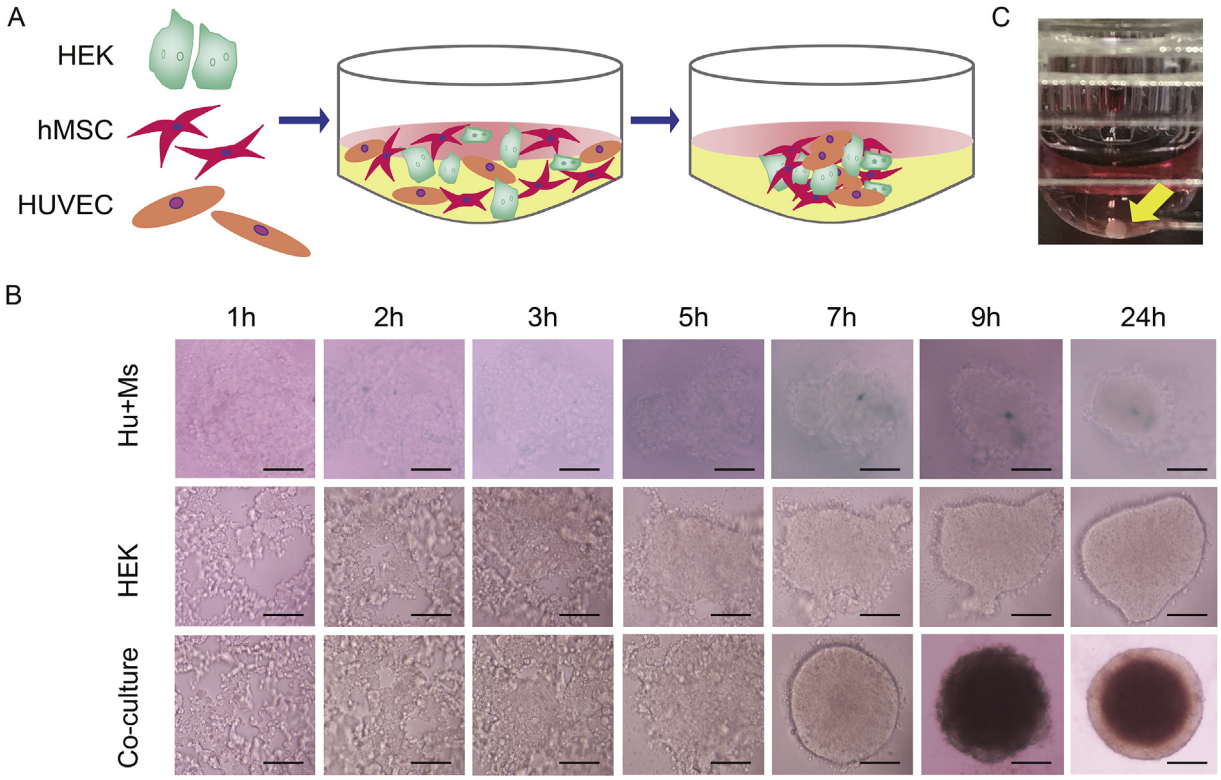

Fig. 1. Spontaneous self-assembly of human glomerular-like microspheres in vitro. (A) Schematic representation of co-culture method. (B) Self-assembly of three-dimensional human embryonic kidney cells in co-cultures with hMSC and HUVEC. Time-dependent formation of human GLS in 96-well plates. Phase contrast images showing the cell morphologies for the factorial-designed experiment. Scale bars, 100 μm. (C) Side view of human GLS (yellow arrow) in 96-well plates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

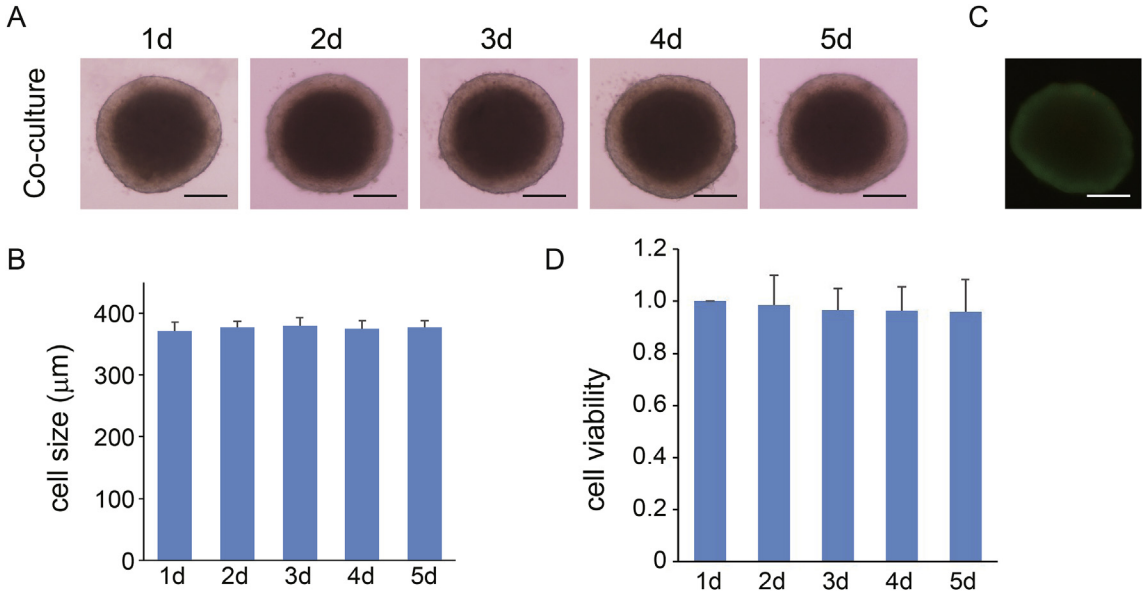

Fig. 2. The maintenance of steady state of GLS. (A) Morphological stabilization for 5 days is shown as phase contrast images. Scale bars, 100 μm. (B) Time course of the cell size after sphere formation is shown. Data points represent mean cell viability ± SE (n = 12). (C) Live-dead staining assay for GLS. Cells were treated with 2 μM calsein AM/4 μM EthD-III for staining. The red staining indicates dead cells while the green staining showed live cells. (D) Time course of the cell viability after sphere formation is shown. Cell viability was determined by Alamar blue staining assay (AbD Serotec, Oxford, UK). The fluorescent intensity was measured with TECAN Infinite® M200Multi-mode Microplate Reader at 590 nm emission (560 nm excitation).

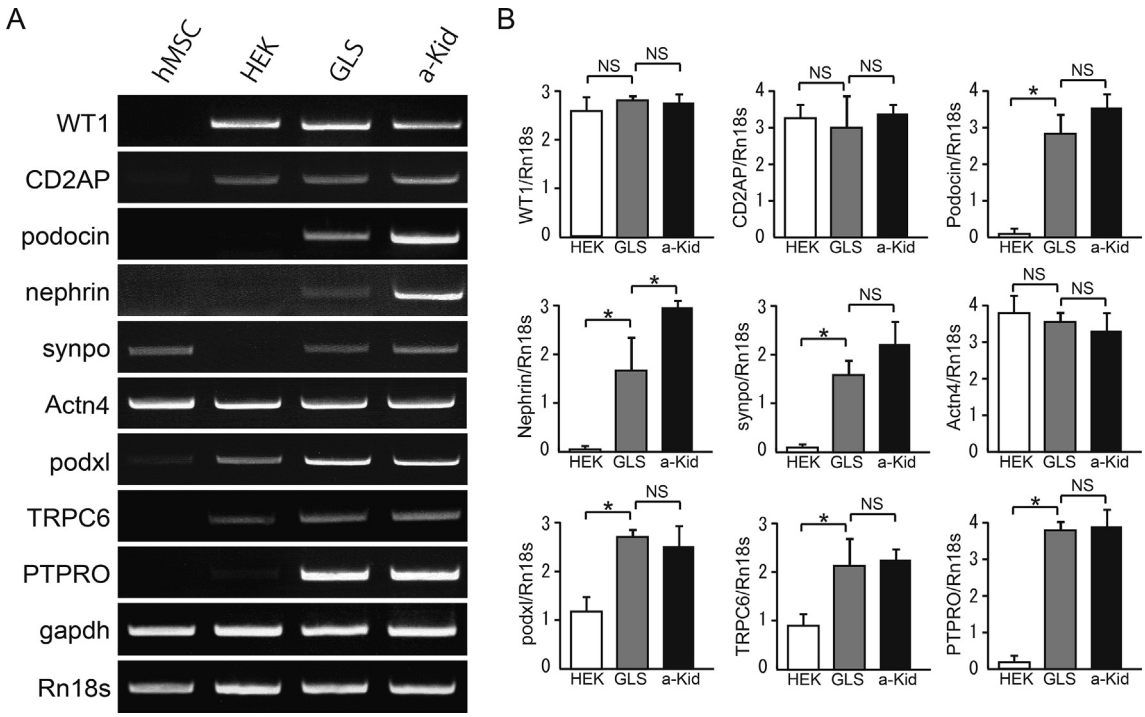

Fig. 3. Expression profiling of podocyte marker genes in GLS and each cell line. (A) RT-PCR analysis of podocyte marker genes for mRNA obtained from hMSC, HEK, GLS and human adult kidneys. (B) The expression levels of mRNA of podocyte marker genes in the above four groups. They were analyzed by qPCR and normalized to the expression of Rn18s. The values are expressed as the mean ± S.D. (NS, not significant, *, $p < 0.05$).

through qPCR also demonstrated clearly the similarity in the gene expression profile between GLS and adult kidneys (Fig. 3B).

3.4. GLS exhibited morphological changes in response to the stimulation of patients' sera

FSGS is the most common primary glomerular disease underlying ESRD in the United States and is increasing in incidence globally. FSGS results from podocyte injury, yet the mechanistic details of disease pathogenesis remain unclear. To examine the effects of circulating permeability factors from patients with FSGS on podocyte injuries, we observed the morphological changes in GLS. GLS exposed to serum from patients with FSGS showed collapsing changes and thereby dropout of cells from GLS (Fig. 4A). These phenomena were shown slightly in the examination using serum from patients with MCNS and not shown in healthy subjects.

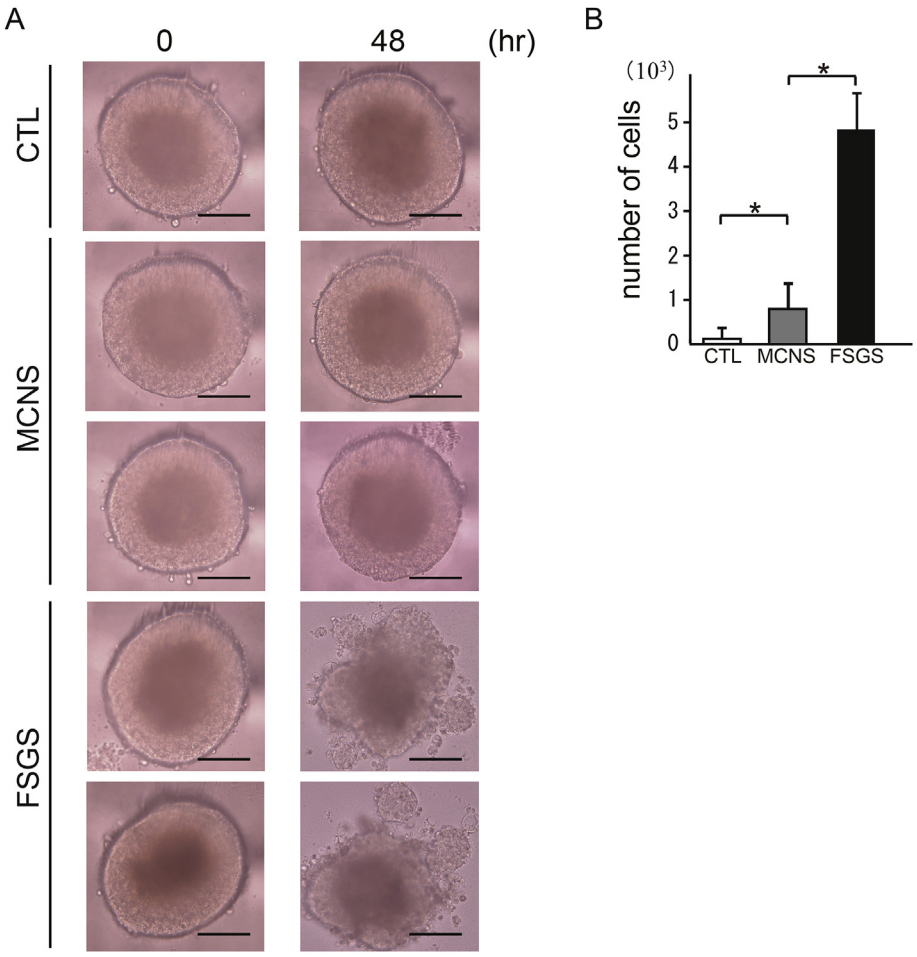

Fig. 4. Effects of patient sera with kidney diseases on the stability of GLS. (A) Morphological alteration 48 h after addition of serum from healthy subjects and patients with MCNS or FSGS. Representative bright-field images are shown. Scale bars, 100 μm. (B) Quantitative estimation of cell dropout from GLS by stimulation of serum as the number of cells in each well. Serum was obtained from healthy subjects (n = 5) and patients with MCNS (n = 10) or FSGS (n = 10).

With regard to the number of dropout cells from each GLS, the significant difference in the count of dropout cells was compatible with the morphological changes in GLS (Fig. 4B). These data suggest that the GLS system can be used as an in vitro model that mimics the phenomena happenings in glomeruli in patients with FSGS.

4. Discussion

The nephron is the microscopic structural and functional unit of the kidney. Each nephron has a single glomerulus, which is located in the outer part of the kidney (the cortex) and is responsible for the ultrafiltration of blood. The glomerular tuft is a spherical structure in the renal cortex that filters plasma water and solute to form urine. To understand how the glomerular tuft works with stability, it is necessary to understand the cellular mechanisms underlying the formation and maintenance of the microstructure of the glomerulus.

In this study, we demonstrated the formation and steady maintenance of the spherical microstructure through the spontaneous self-assembly of three kinds of cells. Well-known biological examples of self-assembly are viral shell formation [15] and microtubule assembly [16] at the molecular level. At the tissue level, classic examples are the dissociation-reassembly studies of sea sponges [17]. Regarding kidneys, work published by Grobstein over half a century ago suggested that it is possible to combine intact metanephrogenic mesenchyme tissue with an intact ureteric bud and induce development of both nephrons and ureteric buds [18]. Similarly, Auerbach and Grobstein showed that it was possible to combine disaggregated metanephrogenic mesenchyme with a surrogate inducer (spinal cord rather than ureteric bud) and still obtain nephrons [19]. However, mechanisms of self-assembly of an apparatus in kidney remains unknown. Here, we demonstrated the process of formation of a spherical structure of glomerulus. This process did not require any specific cytokines or reagents. In addition, the GLS was built in a standard medium within 24 h, suggesting that cell-cell interaction may drive the formation of spherical microspheres. However, there is still an important question that remains unsolved for this three-dimensional GLS. Similar to other kinds of organ organoids including kidney organoids, the formation of microvessels in organoids or microspheres remains to be achieved.

How organs grow to be the right size for the animal is one of the central mysteries of biology. Similarly, the process of various apparatus achieving a reproducible size during development is also shrouded in mystery. Because the kidneys are essential for the homeostasis (maintaining a constant internal environment) of the body's extracellular fluids, maintenance of the microstructure of glomeruli is absolutely necessary. However, with respect to this mechanism, there are many unsolved questions. In this study, we found the maintenance of the size and cell viability of GLS was probably through self-assembly, suggesting that GLS would be in a steady state. Therefore, it is a critical point that GLS as a model of an apparatus in kidneys maintains at least the microstructure in a steady-state.

Glomerular diseases are serious, and increasing problems are found all over the developed world [20]. A full-scale adult kidney is an anatomically complicated structure that contains >60 cell types [21]. Among them, the loss of podocytes appears to play a pivotal role in the progression of glomerular disease and the development of glomerular sclerosis [22,23]. The Wilms' tumor gene 1 (WT1) is a zinc finger transcription factor whose function is required for normal nephrogenesis and podocyte differentiation [24–27]. We confirmed the expression of WT1 in HEK cells. However, important podocyte-specific genes such as nephrin and podocin which constitute the slit diaphragm contributing to the filtration process in the kidney, were not expressed in HEK cells. In the process of self-assembly, these absolutely necessary podocyte-specific genes expressions were induced. This induction may be due to cell-cell interactions and some liquid factors from MSC or HUVEC, because no cytokines or reagents were added during this process.

Podocyte depletion leads to nephron degeneration and subsequent glomerulosclerosis which is closely correlated with the decline in renal function in most kidney diseases [22,23]. This has resulted in an unmet clinical need for cell-specific therapy in the treatment of FSGS and other proteinuric kidney diseases. The growing recognition during the past decade of collapsing glomerulopathy as an important cause of ESRD is ascribed to true increases in its incidence [28]. Collapsing focal segmental glomerulosclerosis is most often seen in association with HIV infection [28]. However, the pathogenesis of collapsing FSGS in patients not infected with HIV is still unclear. Except for HIV infection, circulating factors in the serum of collapsing glomerulopathy patients produce podocyte damage, whereas such factors are not present in noncollapsing FSGS [29]. On the other hand, very little is known on the molecular nature of the reparative mechanisms which defend the podocyte against various types of environmental stress. To address these questions, it is necessary to develop an in vitro model which closely mimics the in vivo state. In this study, we demonstrated a method in which a glomerulus-like microsphere was simply self-assembled without using iPS or ES cells. The microsphere formed a good spherical body resembling glomerulus, and exhibited a steady state. These microspheres and organoids share common problems of absence in vascular structures. However, on the eve of the emergence of the mature glomerulus in the embryonic kidney, the nephron waits to receive blood flow. In this context, the GLS may be in just shortly before glomerulus maturation.

This new approach is gaining popularity in cell culture research because growing cells in three-dimensional space rather than on a flat, two-dimensional plate surface more accurately represents the in vivo condition. These assays are potentially attractive for high-throughput screening (HTS) adaptation. Such 3D kidney-like structures would, for example, meet the needs of the pharmacological and toxicological industry for drug screening. This would be a very valuable method for testing the cell autonomy of any mutations or knockdowns, or for assessing the potential of different types of stem and other cells to contribute to the different renal tissues.

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Transparency document

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