Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

An optimal serum-free defined condition for *in vitro* culture of kidney organoids

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ARTICLE INFO

Article history:
Received 8 May 2018
Accepted 15 May 2018
Available online xxx

Keywords:
Defined medium
Nephron progenitor cell
Organoid
Metanephros
Kidney development

ABSTRACT

Kidney organoid is an emerging topic of importance for research in kidney development and regeneration. Conventional culture systems for kidney organoids reported thus far use culture media containing serum, which may compromise our understanding and the potential clinical applicability of the organoid system. In our present study, we tested two serum-free culture conditions and compared their suitability for the maintenance and growth of kidney organoids in culture. One of the serum-free culture conditions was the combination of keratinocytes serum free medium (KSFM) with knockout serum replacement (KSR) (KSFM + KSR), and the other was the combination of knockout DMEM/F12 (KD/F12) and KSR (KD/F12 + KSR). With cell aggregates derived from E12.5 mouse embryonic kidneys, we found that KD/F12 + KSR was superior to KSFM + KSR in promoting the growth of the aggregate with expansion of $Six2^{+}$ nephron progenitor cells (NPC) and elaborated ureteric branching morphogenesis. With KD/F12 + KSR, we found that lower concentrations of KSR at 5–10% were superior to a higher concentration (20%) in promoting the growth of aggregates without affecting the expression levels of NPC marker genes. We also found that NPC in aggregates retained their differentiation potential to develop nephron tubules through mesenchyme-to-epithelial transition (MET), after being maintained in culture under these conditions for up to 7 days. In conclusion, we have identified a defined serum-free culture condition suitable for the maintenance and growth of kidney organoids that retain the differentiation potential to develop nephron structures. This defined serum-free culture condition may serve as a useful platform for further investigation of kidney organoids *in vitro*.

1. Introduction

The kidney is a complex organ consisting of more than 20 different types of cells organized into a complex structure that is crucial for its proper function in the body, including clearance of metabolic waste, maintenance of volume, electrolyte and pH homeostasis and various endocrinologic functions [1]. Despite this complexity, the kidney develops from a relatively simple structure in the embryo, called metanephros. The metanephros develops mainly from three lineages of progenitor cells, i.e., nephron progenitor cells (NPC), which make up the glomerulus and most of the epithelial tubule system, starting from proximal tubule down to distal tubule; ureteric progenitor cells (UPC), which differentiate into the collecting duct and ureter; and stromal progenitor cells (SPC), which form the supportive stromal tissues [2,3]. After the invasion of UPC into the nephrogenic zone at embryonic day 10.5 (E10.5) in mouse embryos, these three lineages of progenitor cells undergo self-renewal and differentiation processes, and through their mutual interactions, they develop into a mature organ [4–8].

In recent years, boosted by the advancement in our capability to effectively induce kidney progenitor cells from embryonic stem cells and human induced pluripotent stem cells, the kidney organoid has emerged as a useful tool for research in kidney development, drug screening, disease modeling and potentially kidney regeneration [9–14]. Although the original methods described to

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https://doi.org/10.1016/j.bbrc.2018.05.098
0006-291X/

generate kidney organoids led to the formation of preliminary structures that are devoid of proper excretory function of the kidney [15–18], refinements in preparation methods have recently been reported that allowed for the development of an anatomically more realistic organization where nephrons were connected to a single collecting duct tree ending with a ureter [12,19]. These studies illustrated the potential of the kidney organoid as a valuable tool for research in kidney development and regeneration. However, in these studies, the use of culture media containing undefined factors such as serum could put limitations on further investigation and the potential clinical applicability of kidney organoids. It is therefore desirable to develop a defined culture condition that allows the growth and maintenance of organoids in culture.

In our present study, we report a serum-free defined culture condition, i.e., the combination of knockout DMEM/F12 (KD/F12) and knockout serum replacement (KSR), that is suitable for the maintenance and growth of kidney organoids in culture. Upon induction of differentiation, these organoids retained their potential to develop nephron tubules.

2. Materials and methods

2.1. Mice

Hoxb7$^{myr\text{-}Venus/+}$ transgenic mice [20] were obtained from Jackson Laboratory (Bar Harbor, Main) and maintained with C57BL/6J background and used in our present studies according to the approved animal protocol.

2.2. Dissection, dissociation and re-aggregation of mouse embryonic kidney cells

The morning of the discovery of a vaginal plug was considered as E0.5, and embryonic kidneys were dissected from embryos at E12.5, dissociated, and re-aggregated as previously described with some modification [21]. In brief, embryonic kidneys were dissected free-hand under a dissecting microscope (Olympus, Center Valley, PA) in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY), and Hoxb7-Venus transgenic embryonic kidneys were separated from wild type embryonic kidneys under a fluorescent microscope (Meiji Techno America, San Jose, CA). The embryonic kidneys were then washed with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) and incubated in TrypLE reagents (Thermo Fisher scientific, Waltham, MA) for 10 min at room temperature. After adding ice-cold DMEM with 10% FBS, single cells were dissociated by trituration and filtered through a 70 μm cell strainer (BD Bioscience, San Jose, CA). To re-aggregate the dispersed single cells, a total of 2×10^4 cells were placed into a 96 well ultra-low attachment round bottom dish (Corning, Tewksbury, MA), centrifuged at 650 g for 2 min, and cultured overnight in the culture media as described.

2.3. Organoid culture

The re-aggregated pellets were carefully collected and placed on top of a 5 μm isopore poly-carbonate filter (Millipore, Billerica, MA), and cultured at 37 °C and 5% CO_2 in the combinations of components as shown in Table 1. The chemicals and reagents used for organoid culture include: keratinocyte serum-free medium (KSFM; Invitrogen), knockout DMEM/F12 (KD/F12; Thermo Fischer Scientific), knockout serum replacement (KSR; Life Technologies, Carlsbad, CA), FBS (Invitrogen), ROCK inhibitor Y27632 (10 μM) (Abcam Biochemicals, Cambridge, MA), 2-mercaptoethanol (110 μM) (2-ME; Invitrogen), γ-secretase inhibitor DAPT (5 μM), GSK3β inhibitor CHIR99021 (3 μM) (Cellagen Technology, San Diego, CA), penicillin, streptomycin (Sigma-Aldrich), GlutaMax (Thermo Fischer Scientific). For maintenance, aggregates were cultured in media consist of either the combination of KSFM and KSR (KSFM + KSR) or the combination of KD/F12 and KSR (KD/F12 + KSR), containing the above-mentioned chemicals. To induce NPC differentiation, both Y27632 and DAPT were removed from the above medium.

Table 1
Media formulation. Aggregates were maintained in media consisting of either the combination of KSFM and KSR (KSFM + KSR) or the combination of KD/F12 and KSR (KD/F12 + KSR), containing the listed chemicals. NPC differentiation was induced by removing Y27632 and DAPT from KD/F12 + KSR medium (Differentiation).

Undefined	Defined	
KSFM + KSR	KD/F12 + KSR	Differentiation
KSFM	KD/F12	KD/F12
KSR	KSR	KSR
DAPT	DAPT	
Y27632	Y27632	
2-ME	2-ME	2-ME
GlutaMAX	GlutaMAX	GlutaMAX
P/S	P/S	P/S

2.4. Quantitative PCR

The total RNA from each aggregate was extracted by Trizol reagent (Invitrogen) and reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) as per manufacturers' instructions. Real time PCR was performed with MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad), where initial denaturation was at 95 °C for 5.5 min followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C. The results were normalized with that of Gapdh. The sequences of primers used are as following (forward/reverse):

Gapdh (TGAACGGATTTGGCCGTATTG/ACCATGTAGTTGAGGTCA ATGAAG);
Six2 (CAAGTCAGCAACTGGTTCAAGA/ACTGCCATTGAGCGAGGA);
Pax2 (GTTCCCAGTGTCTCATCCAT/GGCGTTGGGTGGAAAGG);
Eya1 (ATGGAAATGCAGGATCTAAC/AACTTCGGTGCCATTGGGAG);
Cited1 (CCGTACCTCAGCTCCTGTG/AGCTGGGCCTGTTGGTCT);
Wnt4 (AGTGGAGAACTGGAGAAGTG/TGTCAAGATGGCCTTCCTG)
Fgf8 (TGGAAGCAGAGTCCGAGTTC/ATACGCAGTCCTTGCCTTTG);
Podxl1 (TCCTTGTTGCTGCCCTCTAC/TTCCAAGGTTGGGTTGTCAT);
Slc5a1 (CTTGATCATCTCCTTCCTCACC/ATTGTGTCCTTGGAGTCCT CTG);
Lrp2 (CAATGTATGCAGCCAAAGACAG/GGCTCTGGAACTATCTCA GAAGG);
Nkcc2 (GATGCAGAACTGGAAGCAGTC/GGCTCTGGAGTGTTCCTGT AAG);
Slc12a3 (ATGATGGCTTCAAGGACGAG/TCCCGAGAGTAATCCAGC AG);
Wnt7b (TACCTAAGTTCCGCGAGGTG/AGGCTTCTGGTAGCTGCGTA);
Wnt11 (GTGAAGTGGGGAGACAGGCT/CACGTCCTGGAGCTCTTGC);
Ret (TTCTGAAGACAGGCCACAGGA/CACTGGCCTCTTGTCTGGCT);
Aqp2 (CATCCTCCATGAGATTACCCCTG/GCTCATCAGTGGAGGCA AAGAT);
Upk1a (TCGGACAGGCAACTTCATCC/CAACATCACAGGGAGGGTCC);
Foxd1 (TTCGGATTCTTGGACCAGAC/CAAGTCAGGGTTGCAGCATA);
Raldh2 (AGATGCTGACTTGGACTACG/TCTGAGGACCCTGCTCAG TT);
Sfrp1 (GAAGCCTCTAAGCCCCAAGG/CCCCAGCTTCAAGGGTTTCT);
Acta2 (CCTTCGTGACTACTGCCGAG/TATAGGTGGTTTCGTGGA TGCC);
Pdgfrb (CCATGGGTGGAGATTCGCAG/TCCATCGGATCTCATAGC GTG);
Renin1 (ACACGCACCGCTACCTTTG/AATACGCCCCATTCAGCACT)

Please cite this article in press as: M. Nishikawa, et al., An optimal serum-free defined condition for *in vitro* culture of kidney organoids, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.098