Different morphologies of human embryonic kidney 293T cells in various types of culture dishes

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Received: 17 July 2019 / Accepted: 16 December 2019 / Published online: 21 December 2019
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Abstract Human embryonic kidney 293T (HEK293T) cells are used in various biological experiments and researches. In this study, we investigated the effect of cell culture environments on morphological and functional properties of HEK293T cells. We used several kinds of dishes made of polystyrene or glass for cell culture, including three types of polystyrene dishes provided from different manufacturers for suspension and adherent cell culture. In addition, we also investigated the effect of culturing on gelatin-coated surfaces on the cell

morphology. We found that HEK293T cells aggregated and formed into three-dimensional (3-D) multicellular spheroids (MCS) when non-coated polystyrene dishes were used for suspension culture. In particular, the non-coated polystyrene dish from Sumitomo bakelite is the most remarkable characteristic for 3-D MCS among the polystyrene dishes. On the other hand, HEK293T cells hardly aggregated and formed 3-D MCS on gelatin-coated polystyrene dishes for suspension culture. HEK293T cells adhered on the non- or gelatin-coated polystyrene dish for adherent culture, but they did not form 3-D MCS. HEK293T cells also adhered to non- or gelatin-coated glass dishes and did not form 3-D MCS in serum-free medium. These results suggest that HEK293T cells cultured on non-coated polystyrene dish may be useful for the tool to analyze the characteristics of 3D-MCS.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10616-019-00363-w>) contains supplementary material, which is available to authorized users.

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Keywords HEK293T · Cell culture · Polystyrene dish · Glass dish · Three-dimensional culture · Spheroid

Introduction

Two-dimensional (2-D) cell cultures have been used for over 100 years as in vitro models in cellular and molecular biological studies. However, the properties of 2-D cells (monolayer cells) are different from those of cells in tissues and organs (Antoni et al. 2015). The

cells are usually formed in a three-dimensional (3-D) architecture and functions in organs. 3-D multicellular spheroids (MCS) have a same property with the cells in tissues and organs. The 3D culture model is superior to 2D monolayers in the search for new therapeutic targets (Melissaridou et al. 2019). Recently, three-dimensional cell culture is emerging as a new tool for drug discovery and development (Edmondson et al. 2014; Fang and Eglén 2017). A large number of tools for 3-D cell culture have been shown (Lin and Chang 2008; Souza et al. 2010). Normal human mammary epithelial (MCF-10a) and breast cancer (MCF-7) cells exhibit transient aggregate formation on fluoro-silica surface in a serum-dependent manner (Nicklin et al. 2014).

Various kinds of cultured cells can form into 3-D MCS when cultured in suspension or in non-adhesive conditions (Lazzari et al. 2017; Lin and Chang 2008). NA8 melanoma cells are formed into 3D multicellular tumor spheroids (MCTS) in poly-hydroxy methacrylate coated plates (Ghosh et al. 2005). The kinetics of cell proliferation in MCTS is significant slower than in monolayer (2D) cultures. The cancer cells formed into 3-D MCTS exhibit higher resistance to chemotherapy than cells cultured in 2D (Daster et al. 2017). Specific media promote 3D structure formation, and extracellular matrix supports 3D culture of renal cancer cells and renal cancer stem cells (Maliszewska-Olejniczak et al. 2019).

Human embryonic kidney 293 (HEK293) cells are HEK cells that have been transformed by exposing them to sheared fragments of adenovirus type 5 DNA (Graham et al. 1977). To date, various derivatives of HEK293 cells have been established, HEK293T (DuBridge et al. 1987), HEK293S (Stillman and Gluzman 1985), HEK293SG (Reeves et al. 2002), and HEK293FTM (Reeves et al. 2002). HEK293T cells are derived from HEK293 cells expressing the simian virus 40 (SV40) large T antigen, which allows for the replication of plasmids containing the SV40 origin of replication, leading to high expression of protein. For these reasons, HEK293T cells grow well and are able to be transfected with protein expression vectors well (Ooi et al. 2016).

HEK293 cells treated with arginine-glycine-aspartic containing elastin-like proteins are formed into 3D spheroid in Eagle's Minimum Essential Medium with 10% FBS (Kim and Jeon 2013). HEK293 cells can be formed into spheroid by using liquid overlay

technique in Dulbecco's Modified Eagles Medium with 10% FBS (Blumlein et al. 2017). HEK293T cells can be cultured as spheres in serum-free and stem cell-promoting culture conditions (Debeb et al. 2010). Some reports have shown that HEK293T cells exhibit stem cell properties in serum-free culture conditions (Ryu et al. 2004; Wang et al. 2014). Suspended HEK293T cells can be cultured by using fibronectin-adsorbed polymer nanospheres in serum-free medium (Ryu et al. 2004). HEK293T cells exhibiting overexpressed miR302/367 cluster are reprogrammed into mirPS cells in serum-free N2B27-based medium (Wang et al. 2014). Method of 3D-formation of HEK293T cells in a normal medium condition has rarely been shown. In addition, HEK293 and HEK293T cells are used for neuroscience-related studies because HEK293 cells express significant amounts of protein and mRNA for neurofilament (NF) subunits (NF-M, NF-L, and α -internexin) (Shaw et al. 2002). However, the precise mechanism of morphological and functional changes of HEK293T cells is not clear.

In the present study, we investigated the effect of cell culture environments on morphological and functional properties of HEK293T cells. We found that HEK293T cells aggregated and formed 3-D MCS on specific polystyrene dishes for suspension culture in complete medium. In contrast, HEK293T cells adhere to the polystyrene dishes for adherent culture, and do not form 3-D MCS. These results suggest that HEK293T cells change their bio-properties according to the culture environment.

Materials and methods

Reagents

All other chemicals (except when and where stated) were from Wako Pure Chemical Industries (Tokyo, Japan) or Sigma (St. Louis, MO, USA).

Culture dishes used in this study

Polystyrene culture dishes for suspension culture were purchased from Sumitomo bakelite CO (Cat.# MS-1135R) (dish-S), Corning (Cat.# 430588) (dish-C), or Thermo Scientific Nunc (Cat.# 171089) (dish-T). Polystyrene culture dish for adherent culture was

purchased from Thermo Scientific Nunc (Cat.# 130180) (dish-TA). Glass dish was purchased from Tokyo Garasu Kikai (Cat.# 792-02-12-04) (dish-G). Glass bottom dish was purchased from Iwaki (Cat.# 3910-035) (dish-GB). The spheroid-organoid culture plates with very low-binding characteristics were purchased from Thermo Scientific Nunc (Cat.# 174930 or 145387).

Geratin-coating of culture dish

The method of coating cell culture dish with gelatin was performed as following. 0.2% gelatin solution was added into each dish. The dishes were covered with the gelatin solution and plated at 37 °C to polymerize for 30 min. The excessive gelatin solution was aspirated and plated until drying.

Cell lines

HEK293T (RCB2202) cells were provided from RIKEN BRC. SUIT-2 cells (TKG0705) were purchased by the Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan). SUIT-2 and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) 64 µg/mL penicillin (Nacalai Tesque, Kyoto, Japan), 25 µg/mL streptomycin (Nacalai Tesque, Kyoto, Japan), and 0.25 µg/mL amphotericin B (GE Healthcare Life Sciences, Tokyo, Japan) at 37 °C in 5% CO₂. SCC-TC (TKG 0646) and DLD-1 (TGK 0379) cells were provided from Cell Resource Center for Biomedical Research Cell Bank. SCC-TC and DLD-1 cells were cultured in RPMI1640 medium supplemented with 10% FBS, 64 µg/mL penicillin (Nacalai Tesque, Kyoto, Japan), 25 µg/mL streptomycin (Nacalai Tesque, Kyoto, Japan), and 0.25 µg/mL amphotericin B (GE Healthcare Life Sciences, Tokyo, Japan) at 37 °C in 5% CO₂.

Morphological changes on various culture dishes

HEK293T, SCC-TC, DLD-1, or SUIT-2 cells (0.5×10^5 cells/mL) were plated on the various plates. The cells were then cultured for the indicated times and observed using a microscope (Leica DM IL LED; Leica microsystems, Wetzlar, Germany).

Cell death assay for HEK293T cells cultured on various culture dishes

HEK293T cells were cultured for 7 days on various dishes. For both cell detachment and obtaining a single cell suspension, the cells were treated with Accutase (Innovative Cell Technologies, Inc., San Diego, CA) for 1 h. Cell viability was measured by co-staining the cells with Hoechst 33342 (2 µg/mL) and propidium iodide (1 µg/mL) followed by observing them under a fluorescence microscope (Leica DM IL LED; Leica microsystems, Wetzlar, Germany).

Contact angle measurement

Contact angle was measured to determine the degree of hydrophilicity and hydrophobicity of a dish surface. The water contact angles were measured on the surfaces of various dishes using a contact angle gauge (LSE-ME3, Nick, Saitama, Japan) according to the Japanese Industrial Standards (JIS) R 3257. Ultrapure water was used as a sample solution and 1 µL of it was added dropwise on the surface of a dish to measure the contact angle. An average contact angle value was obtained by measuring 5 different positions of the same dish. The average contact angle value for all the dish types was calculated based on the average values of three different dishes.

X-ray photoelectron spectroscopy (XPS)

The surface chemical bonding state and composition were evaluated using XPS (PHI Quantera IITM, ULVAC-PHI Inc., Kanagawa, Japan). C1s and O1s XPS spectra were collected using an X-ray with Al K α radiation. Photoelectron spectroscopy analysis software (PHI MultiPakTM Version 9, ULVAC-PHI Inc., Kanagawa, Japan) was used to analyze the spectra. Each spectrum was charge corrected with respect to C1s at 284.8 eV, and the peak area of each element was obtained after the Shirley-type background subtraction.

Statistical analysis

The data shown in the figures represent mean values $\pm$ standard deviation (SD) of 3 or 4 independent experiments. The data were statistically

evaluated by Student's *t*-test. The statistical significance at different *P*-values is indicated in each figure.

Results

HEK293T cells cultured on polystyrene plate for suspension culture

Several kinds of culture dishes made of polystyrene or glass were used for the cell cultures. We used three kinds of polystyrene dishes procured from different manufacturers for suspension cell culture. Additionally, we investigated the effect of using gelatin-coated surface for cultures on the cell morphology. HEK293T cells aggregated and formed into 3-D MCS in the non-coated polystyrene dishes for suspension culture. In particular, the non-coated polystyrene dish (dish-S) shows the most remarkable characteristic for aggregation among the polystyrene dishes. HEK293T cells hardly aggregated on the gelatin-coated polystyrene dishes for suspension culture. As shown in Fig. 1, HEK293T cells formed 3-D multicellular spheroids on the non-coated polystyrene dishes for suspension culture.

HEK293T cells could not aggregate on non- or gelatin-coated polystyrene dish for adherent culture

We investigated whether HEK293T cells aggregated on non-coated polystyrene dishes for adherent culture. As shown in Fig. 2a, HEK293T cells adhered on both the non- and gelatin-coated polystyrene dish for adherent culture and did not form 3-D MCS (Fig. 2a). Additionally, as shown in Fig. 2b and c, HEK293T cells adhered to the bottom of the glass dishes. These results suggest that HEK293T can adhere to the bottom of glass dishes as well as polystyrene cell culture plates, which inhibits the formation of 3-D MCS.

HEK293T did not aggregate on the glass dish in serum-free DMEM

Next, we investigated the effect of serum on the formation of 3-D MCS of HEK293T cells. As shown in Fig. 3a, HEK293T cells adhered on the non-coated glass dishes in DMEM supplemented with 0, 1, 10%

HEK293T cells Polystyrene dish for suspension culture

Fig. 1 HEK293T cells cultured on polystyrene dishes for suspension culture. HEK293T cells were cultured on non-coated or gelatin-coated suspension culture dishes (Sumitomo bakelite (dish-S), Corning (dish-C), or Thermo Scientific Nunc (dish-T)) for 18 h. The cells were observed using phase contrast microscopy

FBS. HEK293T cells should the round shape and adhered to the bottom of the non-coated glass dish in a serum-free medium. They did not form 3-D MCS. It was also observed that some of the HEK293T cells died in the serum-free condition. As shown in Fig. 3b, HEK293T cells could adhere both to the non- and to

the gelatin-coated glass dishes in DMEM supplemented with 1% FBS. These results suggest that

Fig. 2 HEK293T cells cultured on polystyrene dishes for adherent culture and glass dishes. **a** HEK293T cells were cultured on non-coated or gelatin-coated polystyrene dishes from Thermo Scientific Nunc for adherent culture for 18 h. **b** HEK293T cells were cultured on non-coated or gelatin-coated glass dishes procured from Tokyo Garasu Kikai for 18 h. **c** HEK293T cells were cultured on non-coated or gelatin-coated glass-bottom dishes from Iwaki for 18 h

HEK293T cells are not able to form 3-D MCS on the glass dish even in serum-free medium.

The formation of 3-D MCS on the polystyrene dish for suspension culture

As shown in Fig. 4a, HEK293T cells strongly adhered to the bottom of non- or gelatin-coated polystyrene dish for adherent culture when cultured for 4 days (Fig. 4a). Interestingly, HEK293T cells formed 3-D MCS on the non-coated polystyrene dish for suspension culture (Fig. 4b and Fig. S3), as well as on the super-low cell attachment surface dish from Thermo Scientific Nunc (Cat. # 130180) (Fig. 4c). Another low cell binding 24-well plate for 3-D cell culture (Cat. #145387) was used in this study. HEK293T cells also aggregated on this dish and formed 3-D MCS (Fig. S2).

SCC-TC cells formed 3-D MCS on the polystyrene dish for suspension culture

Human rectal carcinoma cell line, SCC-TC, normally adhered to the bottom of dish for adherent culture and exhibited a round shaped morphology (Fig. 5a). Next, we investigated whether SCC-TC cells formed 3-D MCS on the polystyrene dish for suspension culture. As expected, SCC-TC cells, like HEK293T, formed 3-D MCS on the non- and gelatin-coated polystyrene dish for suspension culture (Fig. 5b).

Chemical properties of culture dish surface

As shown above, HEK293T and SCC-TC cells formed 3-D MCS on the non-coated polystyrene dishes used for suspension cultures. The electrical charge on the cell surface of cultured cancer cells was negative (Chen et al. 2016; Le et al. 2019). The more hydrophilic (*i.e.* the less hydrophobic) the surface of material film coating a dish is, the more cell adhesion

Fig. 3 Effect of FBS on the adherent property of HEK293T cells.

a HEK293T cells were cultured on a glass dish in DMEM containing 0, 1, 10% FBS for 18 h.

b HEK293T cells were cultured on non-coated or gelatin-coated glass dishes in DMEM containing 1% FBS for 18 h

is observed on the dish surface (Chang and Wang 2011). The surface hydrophobicity can be assessed by measuring the contact angle formed by spreading a water droplet on the surface. We examined the water contact angle of all types of the culture dishes used in this study. As shown in Fig. 6, the water contact angles of dishes used for suspension culture (dish-S, -C, and -

T) were higher than those of the dish used for adherent culture (dish-TA) or glass dishes (dish-G and -GB). Accordingly, in the dishes used for suspension culture, the water contact angle of dish-S, but not of dish-C and -T, was greater than 90°. As shown in Table 1, the atomic percentage of oxygen in dish-S was the lowest among the used dishes, suggesting that the surface of

HEK293T cells**(A) Polystyrene dish for adherent culture** **(B) Polystyrene dish for suspension culture** **(C) Thermo Scientific Nunc 174930 Nunclon™ Sphera**

this dish type is the most hydrophobic among all the suspension culture dish types used in this study. These results also indicated the dependency of 3-D MCS

Fig. 4 Spheroid formation of HEK293T cells on polystyrene dishes for suspension culture. **a** HEK293T cells were cultured on non-coated or gelatin-coated polystyrene dishes procured from Thermo Scientific Nunc for 4 days. **b** HEK293T cells were cultured on non-coated or gelatin-coated suspension culture dishes (dish-S) for 4 days. **c** HEK293T cells were cultured on 3D cell culture plates for 2 days

SCC-TC cells**(A) Polystyrene dish for adherent culture** **SCC-TC cells****(B) Polystyrene dish for suspension culture**

Fig. 5 Spheroid formation of SCC-TC cells on polystyrene dishes for suspension culture. HEK293T cells were cultured on non-coated or gelatin-coated suspension culture dishes (dish-S) for 4 days

formation on the surface hydrophobicity of a culture dish.

DLD-1 and SUI-2 cells did not form 3-D MCS

We studied the propensity of human colorectal adenocarcinoma cells, DLD-1, and human pancreatic cancer cells, SUI-2, to form 3-D MCS. We found that the DLD-1 cells aggregated on the polystyrene dishes for suspension. However, the DLD-1 cells did not form 3-D MCS on these dishes. In addition, we observed that the SUI-2 cells did not aggregate on these dishes (Fig. S1).

Fig. 6 Water contact angle of various culture dishes. Water contact angle on suspension culture dishes [Sumitomo bakelite (dish-S), Corning (dish-C), or Thermo Scientific Nunc (dish-T)], adherent culture dish purchased from Thermo Scientific Nunc (dish-TA), glass dish (dish-G) and glass bottom dish (dish-GB) was measured. *** $P < 0.001$ compared with dish-S

Table 1 Ratios (atomic%) of carbon, and oxygen atoms in various culture dishes

	Carbon	Oxygen
dish-S	99.2	0.8
dish-C	98.5	1.5
dish-T	98.0	2.0
dish-TA	84.9	15.1

Discussion

In this study, we assessed the morphologies of human cultured cells on several different types of dishes. We demonstrated that HEK293T and SCC-TC cells aggregated and formed spheroid structures on specific culture dishes for suspension culture in the serum-containing medium. In addition, HEK293T cells adhered to the bottom of polystyrene dishes for adherent cultures, and to glass dishes. Our results suggest for the first time that HEK293T cells aggregated and formed spheroids on the normal polystyrene dishes in culture medium in the presence of 10% FBS.

Serum for cell culture media includes the hormones, attachment and spreading factors, growth factors, cytokines, fatty acids, lipids, vitamins, trace elements, carbohydrates, and non-protein nitrogens (Brunner et al. 2010). Attachment and spreading

factors, such fibronectin, laminin, and serum spreading factor, are important for cell growth and adherence. Therefore the observation that sphere formation takes place in the serum-containing conditions is very interesting. The methods for detachment of cultured cells from the dish surface are useful for cell biology. In this study, HEK293T cells aggregated on the surface of polystyrene culture dish for suspension culture in the presence of FBS. However, the molecular mechanisms underlying the aggregation and 3-D MCS formation in HEK293T and SCC-TC cells are not clear. Further studies are required to clarify these molecular mechanisms.

HEK293T and SCC-TC cells could form 3-D MCS on the non-coated polystyrene dishes used for suspension culture. However, DLD-1 and SUIT-2 cells could not form 3-D MCS under identical conditions. The electrical charges on the surfaces of DLD-1 and SUIT-2 cells may differ from those of HEK293T and SCC-TC cells.

Cadherins are important transmembrane proteins that mediate cell–cell adhesion. The protein levels of cadherins are variable during the formation of spheres (Riedl et al. 2017; Powan et al. 2017). Phospho-ERK1/2 levels are significantly changed, and phospho-PKC and E-cadherin expression are induced in the 3D spheroid culture as compared to the 2D culture (Riedl et al. 2017). E-cadherin expression is seen to be elevated in the 3D spheroids, whereas N-cadherin

expression was decreased in human non-small-cell lung cancer H460 cells (Powan et al. 2017). However, the gene expression of cell adherent proteins, such as cadherin and carcino-embryonic antigen cell adhesion molecules, did not increase in HEK293T cells forming spheres on the polystyrene dish (data not shown).

The formation of 3-D MCS plays a role in cancer progression and the metastatic process. We found that the HEK293T and SCC-TC cells aggregate and form 3-D MCS on the gelatin-free polystyrene dishes for suspension culture. These methods for cell aggregation and sphere formation might be useful in stem cell cancer research.

In this study, various human cells were observed to aggregate and form 3-D MCS when cultured on polystyrene dishes for suspension cultures. Our results suggest a simple and novel 3-D cell culture method using these dishes. In addition, HEK293T cells cultured on non-coated polystyrene dish may be useful for analyzing the characteristics of 3D-MCS.

Acknowledgements We would like to thank our corroborators in our laboratory.

Authors' contributions KI, KO, KH, YS, TN and HH conceived and designed the experiments, and contributed to manuscript preparation. KI, KO, KH, YS, and KS performed the experiments. All authors read and approved the final manuscript.

Funding This research was funded by the Grant-in-Aid for Scientific Research of Seikei University (Grant No. 2018) and Scientific Research from the Japan Society for the Promotion of Science (18K11001 to KI).

Conflict of interest The authors declare that there are no conflicts of interest related to this article.

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