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Effects of glucose, lactate and basic FGF as limiting factors on the expansion of human induced pluripotent stem cells

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Pluripotent stem cells (PSCs) are one of the promising cell sources for tissue engineering and drug screening. However, mass production of induced pluripotent stem cells (iPSCs) is still developing. Especially, a huge amount of culture medium usage causes expensive cost in the mass production process. In this report, we reduced culture medium usage by extending interval of changing culture medium. In parallel, we also increased glucose concentration and supplied heparan sulfate to avoid depletion of glucose and bFGF respectively. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses showed that reducing medium change frequency increased differentiation marker expressions but high glucose concentration downregulated these expressions. In contrast, heparan sulfate did not prevent differentiation marker expressions. According to analyses of growth rate, cell growth with extended medium change interval was decreased in later stage of log growth phase despite the existence of high glucose concentration and heparan sulfate. This result and culturing iPSCs with lactate showed that the accumulation of excreted lactate decreased the growth rate regardless of pH control. Conclusively, these experiments show that adding glucose and removing lactate are important to expand iPSCs with reduced culture medium usage. This knowledge should be useful to design economical iPSC mass production and differentiation system.

[**Key words:** Induced pluripotent stem cells; Glucose; Lactate; Basic fibroblast growth factor; Heparan sulfate; Cell expansion; Mass production; Regenerative medicine]

Pluripotent stem cells (PSCs) such as embryonic stem cells and induced pluripotent stem cells (iPSCs) are one of the most promising cell sources for tissue engineering and drug screening. For industrial use of PSCs, designing a stable mass production system is required (1,2) and reducing running cost is important. For example, pancreatic islet transplantation for diabetes care requires approximately 6×10^9 beta cells, which requires approximately more than 1 L of culture medium volume. Thus, the estimated cost just for PSC expansion is 5000 USD per patient (3).

Daily feeding culture medium is a major cause of high cost for mass production of PSCs. PSCs need a large amount of glucose for their metabolism, which requires frequent medium change. During culture, PSCs also producing lactate as a waste of anaerobic respiration (4–6), whose accumulation causes decreasing pH and cell death (7). Therefore, the culture medium of PSCs should be replaced frequently in order to exchange nutrients and wastes. Furthermore, human PSCs are known to require to some growth factors for self-renewal (8–10). Especially, basic fibroblast growth factor (bFGF) degrades quickly in 37°C (10–12) thus frequent medium change is also important in terms of the bFGF supplement. Therefore, the major purpose of the frequent medium change is glucose and bFGF supplementation, lactate removal and adjusting pH. In the case of large-scale expansion, there are two popular feeding strategies: batch and perfusion culture. According to previous research, perfusion culture system realized higher cell yields than batch culture after 7 days of stirred suspension culture when using the same volume of culture medium (13). Despite these reports on feeding strategies, there are few reports on what is the dominant limiting factor for PSC expansion with higher cell density.

In this article, we tried to reduce the medium usage by extending medium change interval. In parallel, we added heparan sulfate, which prolongs the half-life of bFGF dramatically (11,12), and increased glucose concentration to compensate for the shortage of those factors. Through these experiments, we clarified what is a dominant limiting factor for iPSC expansion for reducing culture volume.

MATERIAL AND METHODS

Maintenance of human iPSCs A human iPS cell line, TkDN4-M was provided by Stem Cell Bank, Centre for Stem Cell Biology and Regenerative Medicine, The University of Tokyo (14). They were cultured on 6-well tissue culture treated plate coated with vitronectin fragment (VTN-N, Life Technologies, Carlsbad, CA, USA) in Essential 8 (E8, Life Technologies). Culture medium was changed every 24 h. Calcium and magnesium free phosphate buffered saline (PBS, Wako Pure Chemical Industries, Osaka, Japan) containing 0.02% EDTA (PBS-EDTA) was used to subculture cells on VTN-N. The passage number was lower than 50 for the experiments.

Preventing bFGF degradation by adding heparan sulfate 1×10^4 cells/cm^2 of hiPSCs were seeded on VTN-N coated 12 well plate with Y-27632 (Wako Pure

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Chemical Industries) and cultured for 48 h with medium change every 24 h. After 48 h culture, the culture medium was replaced with E8 medium containing different concentrations of heparan sulfate (Iduron, Cheshire, UK). After replacing medium, 20 μL medium samples were collected every 12 h without changing medium for quantifying bFGF. Enzyme-linked immunosorbent assay (ELISA) for bFGF was established by DY233 human FGF basic DuoSet and DY008 DuoSet Ancillary Reagent Kit 2 (R&D Systems).

Culturing hiPSCs with extended interval 1×10^4 cells/cm^2 of hiPS cells were seeded on VTN-N coated 6 well or 12 well plate with E8 medium containing Y-27632. In addition to conventional daily medium change (every 24 h), hiPSCs were cultured in four different types of culture medium with extended medium change interval (every 48 h): original E8 medium, E8 medium containing 5 μg/mL of heparan sulfate, E8 medium with twice higher glucose concentration (30 mM), and E8 medium with both heparan sulfate and twice higher glucose concentration (Table 1).

Cells were passaged every 4–6 days and gene expressions were analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) after three passages.

Growth analyses of iPSCs in various mediums with extended interval Pictures of hiPSC colonies were captured by microscope (Leica Microsystems, Wetzlar, Germany) every 24 h. The area of colonies was measured by image J software (National Institutes of Health, Bethesda, MD, USA) and the surface coverage of colonies was calculated. To calculate the relationship between the surface coverage and actual cell number, actual cell numbers were counted by trypan blue (Wako Pure Chemical Industries) exclusion test immediately after taking pictures.

By calculating cell number (N) from surface coverage, we measured cellular growth without a destructive evaluation from pictures. From the calculated cell number, the specific growth rates (μ) on two different time periods (48–72 h and 72–96 h) were calculated by Eq. 1.

$$\mu_i = \frac{\ln(N_{i+1}/N_i)}{t_{i+1} - t_i} \quad (1)$$

RT-qPCR analyses Collected cells were dissolved in Trizol reagent (Life Technologies). RNA samples were isolated by adding chloroform and the centrifugation of 15,000 *g* for 15 min. RNA samples were purified by adding 2-propanol (Wako Pure Chemical Industries) and the centrifugation of 15,000 *g* for 15 min. Following washing with 75% ethanol (Wako Pure Chemical Industries), purified RNA samples were reverse-transcribed by Super Script III First strand (Life Technologies). After reverse transcription, transcribed complementary DNA samples were quantitated by Taqman gene expression assays (Life Technologies) and StepOne Plus (Life Technologies). Target genes were Oct4 (Hs00742896_s1), Nanog (Hs02387400_g1), GATA4 (Hs99999904_m1), CD34 (Hs00990732_m1), PAX6 (Hs00240871_m1), Actinβ (Hs99999903_m1), and bFGF (Hs00266645_m1). As a control, gene expressions of cells before experiments were measured.

RESULTS

Effect of high glucose condition to iPSC culture with extended interval IPSCs showed good growths during three passages in the conventional culture medium and medium change schedule (Fig. 1A1). However, some cells outgrowing from colonies appeared in the group of extended medium change interval with conventional culture medium (Fig. 1A2), which implies the possibility of de-undifferentiation. Those outgrowing cells did not appear in the group of extended medium change interval with high glucose medium (Fig. 1A3).

The RT-qPCR analyses also supported this tendency. Although there was no significant difference of pluripotency marker gene expressions among the groups, differentiation marker expressions were increased in the group of extended medium change interval with conventional culture medium (Fig. 1B).

TABLE 1. Name and details of iPSC culture medium used in this article.

Name	Description	Glucose (mM)	Heparan sulfate (μg/mL)
E8	Essential 8	15	–
HS	E8 **with heparan sulfate**	15	**5**
HG	E8 **with high glucose**	**30**	–
HSHG	E8 **with heparan sulfate and high glucose**	**30**	**5**

Bold style indicates modified components from Essential 8.

According to glucose concentration measurement, glucose concentration was kept more than 10 mM until 96 h (Fig. 1C). Therefore glucose supplementation was enough during 96 h culture. Especially, there were few differences of glucose concentrations and specific consumption rates (Fig. S1) between the groups of two different feeding intervals, thus the upregulation of differentiation markers gene expressions seemed not to be caused by the difference of glucose supplementation. In the group of extended feeding interval with high glucose medium, glucose concentration was kept more than 25 mM, higher than original glucose concentration of the medium.

Growth estimation from colony surface coverage According to the plot of iPSC colony surface coverage versus actual cell density (Fig. S2a), there was a linear relationship between iPSC colony coverage and actual cell density, thus estimating cell density from colony coverage is valid non-destructive growth analysis. This fact indicates that iPS cell size is stable in 2D culture and the colony size depends on the number of cells. According to growth curve from colony surface coverage, iPSCs were in log growth phase from 24 to 96 h after seeding (Fig. S2b).

Lactate accumulation and the effect on the growth Daily medium change protocol could keep lower lactate concentration than that with extended medium change interval (Fig. 2A). Notably, iPSCs with additional glucose produced more than 15 mM during 48–96 h.

Comparing growth rate between in early stage (48–72 h) and later stage (72–96 h) of the log growth phases, the growth rates in the later stage was lower than those in the early stage. Notably, in the group with extended medium change interval, the growth rate in the later stage was significantly lower than that of daily medium change (Fig. 2B).

To investigate the effect of lactate, we calculated growth rates with various lactate concentrations. Without pH adjustment, more than 15 mM of lactate was critical for iPSCs because of too low pH (round markers in Fig. 2C). From this result, 15 mM of lactate was the maximum limitation for hiPSCs in E8 medium without any pH adjustment. Although they survived with 25 mM lactate with pH adjustment, the growth rate decreased with higher lactate concentration (squares in Fig. 2C).

Preserving bFGF by heparan sulfate and the effect on iPSC undifferentiation ELISA analyses showed that supplementation of more than 200 ng/mL of heparan sulfate prevented bFGF degradation, and bFGF concentration increased during iPSC culture with more than 1000 ng/mL heparan sulfate (Fig. 3). The fact implies the possibility that iPSCs not only consumed and also secreted bFGF during culture. qPCR analyses also showed high gene expression and supported this tendency (data not shown). From this result, 5000 ng/mL seemed enough concentration for preventing self-digestion of bFGF.

According to morphological analysis, some cells outgrew their colonies and showed de-undifferentiated morphology in the group of extended medium change interval with heparan sulfate (Fig. 4A1: indicated by white arrow). These outgrowing cells did not appear when culturing with both heparan sulfate and high glucose concentration (Fig. 4A2).

Contrary to expectation, in terms of RT-qPCR analysis, supplementing heparan sulfate triggered rather an upregulation of differentiation markers (Fig. 4B). This upregulation was suppressed by increasing the glucose concentration.

DISCUSSION

In this paper, we reduced the usage of culture medium by extending medium change interval to understand what is the limiting factor for the expansion of hiPSCs. There are some possible

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