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Oxygen consumption of human heart cells in monolayer culture

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ABSTRACT

Tissue engineering in cardiovascular regenerative therapy requires the development of an efficient oxygen supply system for cell cultures. However, there are few studies which have examined human cardiomyocytes in terms of oxygen consumption and metabolism in culture. We developed an oxygen measurement system equipped with an oxygen microelectrode sensor and estimated the oxygen consumption rates (OCRs) by using the oxygen concentration profiles in culture medium. The heart is largely made up of cardiomyocytes, cardiac fibroblasts, and cardiac endothelial cells. Therefore, we measured the oxygen consumption of human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs), cardiac fibroblasts, human cardiac microvascular endothelial cell and aortic smooth muscle cells. Then we made correlations with their metabolisms. In hiPSC-CMs, the value of the OCR was 0.71 ± 0.38 pmol/h/cell, whereas the glucose consumption rate and lactate production rate were 0.77 ± 0.32 pmol/h/cell and 1.61 ± 0.70 pmol/h/cell, respectively. These values differed significantly from those of the other cells in human heart. The metabolism of the cells that constitute human heart showed the molar ratio of lactate production to glucose consumption (L/G ratio) that ranged between 1.97 and 2.2. Although the energy metabolism in adult heart *in vivo* is reported to be aerobic, our data demonstrated a dominance of anaerobic glycolysis in an *in vitro* environment. With our measuring system, we clearly showed the differences in the metabolism of cells between *in vivo* and *in vitro* monolayer culture. Our results regarding cell OCRs and metabolism may be useful for future tissue engineering of human heart.

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1. Introduction

Heart failure is a major cause of morbidity and mortality [1]. Despite progress in pharmacological and surgical therapies and device-based treatment, the survival rate of patients with severe heart failure remains poor. For severe heart failure, heart transplantation has been the only treatment, until now. The transplantation procedure is associated with various problems, including limited donor supply, the prolonged need for immunosuppression, and the risk of organ rejection [2]. In contrast, regenerative therapy has the potential to treat severe heart failure without the complications of transplantation. Recent research has shown that various heart tissue engineering methods used in regenerative therapy offer potential new approaches in the treatment of heart failure [3]. Further progress in the field of cardiovascular regeneration therapy requires the development of an efficient oxygen supply system for cardiac cells, because those cells require more oxygen for growth and maturation during culture. In order to develop such a system, it is very important to study the oxygen consumption and metabolism of cardiac cells in culture. Cellular viability and proliferation [4], protein synthesis [5], and carbohydrate metabolism [6] have all been reported to change according to the pericellular oxygen concentration. Therefore, the metabolism and function of cells is crucially dependent on the local oxygen environment. Several authors have reported on measurements of cellular oxygen consumption under *in vitro* culture conditions. For example, OCRs of renal mesangial and hepatic epithelial cells were estimated by measuring the pericellular oxygen concentration in a monolayer culture [7]. The OCR of mammalian cardiomyocytes was also measured in a monolayer culture which showed that oxygen consumption of beating cardiomyocytes was higher, by approximately 50%, than that of non-beating cardiomyocytes [8]. In another report, the oxygen concentration and cell viability within an engineered cardiac construct, based on neonatal rat cardiomyocytes cultured on collagen scaffolds, were measured

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and found to decrease linearly with the distance from the construct surface [4].

In measuring the OCR of cardiomyocytes in culture, animal cells have commonly been used as substitutes for human cells [9]. The establishment of hiPSC-CMs has made it possible to culture beating human cardiomyocytes *in vitro*, and to use them for specific experiments. In several recent papers, the OCR and extracellular acidification rate (ECAR) of hiPSC-CMs seeded on a specialized plate were measured simultaneously by using an extracellular flux analyzer to estimate the OCR/ECAR ratio to assess the relative contribution of glycolysis and mitochondrial respiration in energy generation [10,11]. In these studies, the OCR was measured for the purpose of high throughput screening, and thus lacked any detailed quantitative comparisons between different cell types. The aim of the present study was, therefore, to conduct a kinetic analysis of the OCRs of different cardiac cell types.

We developed an oxygen measurement system equipped with an oxygen microelectrode sensor. The system was used to measure the dissolved oxygen concentrations of culture media in a dish with a micro-scale. We were able to estimate cellular oxygen consumption based on the local oxygen concentration profile and thereby investigate the correlation between metabolism and oxygen consumption. These findings are expected to provide useful information to aid in the future advancement of cardiac tissue engineering.

2. Materials and methods

In this study, all procedures using animals were performed in accordance with the guidelines outlined by the Institutional Animal Committee of Tokyo Women's Medical University.

2.1. Cell culture

Human induced pluripotent stem cells (hiPSCs,253G1) [12], which were provided by RIKEN BRC (Tsukuba, Japan) through the National Bioresources Project of MEXT, Japan, were cultured, and hiPSCs-derived cardiomyocytes (hiPSC-CMs) were obtained using the bioreactor system, developed in our laboratory, as previously described [13]. Immunocytochemistry showed that approximately 80% of the cells were positive for cardiac troponin T. Following cardiac differentiation, the cells were dissociated with 0.05% trypsin/EDTA. By using a strainer (BD Bioscience, San Jose, CA), the cell aggregates were removed and single cells were reseeded at a density of 5.2×10^4 cells/cm^2. HiPSC-CMs were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO), supplemented with 10% FBS, and 1% penicillin–streptomycin (PS). Meanwhile, human cardiac fibroblasts (HCF, PromoCell, Heidelberg, Germany) were cultured in fibroblast growth medium (FGM 3, PromoCell), the cells were seeded at 5×10^3 cells/cm^2. Human cardiac microvascular endothelial cells (HMVEC-C, Lonza, Basel, Switzerland) were cultured in endothelial cells growth medium (EGM-2MV, Lonza), the cells were seeded at 5×10^3 cells/cm^2. Human aortic smooth muscle cells (HASMC, Kurabo, Osaka, Japan) were cultured in smooth muscle cell growth medium (HuMedia-SG2, Kurabo), the cells were seeded at 2.5×10^3 cells/cm^2. We also measured the OCR and metabolism of rat cardiomyocytes (rat-CMs) because they are frequently used in tissue engineering of heart. Rat-CMs were isolated from the hearts of 1-day-old Sprague–Dawley rats and were cultured as described in our previous report [14]. Isolated cardiomyocytes were cultured in a medium containing 6% fetal bovine serum (FBS), 40% Medium 199 (Invitrogen), 0.36% PS, 3.0 mM glucose, and 54% balanced salt solution containing 116 mM NaCl, 1.0 mM NaH_2PO4, 0.8 mM $MgSO_4$, 1.2 mM KCl, 0.8 mM $CaCl_2$, and 26.2 mM $NaHCO_3$. The isolated cardiac cells were seeded at a density of 2.5×10^5 cells/cm^2. Each type of cell was cultured as a monolayer in 35 mm-diameter dishes and maintained at 37 °C in humidified air containing 5% CO_2. The hiPSC-CMs were cultured for 7 or 8 days before oxygen measurement, while the other cell types were grown to confluence. The medium was changed every 2 or 3 days, and again 1 day prior to the oxygen measurement. We confirmed that hiPSC-CMs and rat-CMs were beating just before the oxygen measurements.

2.2. Measurement of the oxygen concentrations

As shown in Fig. 1, the oxygen concentration measurement system was composed of a microelectrode, a Clark-type oxygen microsensor with a 8–12 μm-diameter tip made of fragile glass (OX-10, Unisense, Denmark) connected to a sensitive picoammeter (PA2000, Unisense), *X*-, *Y*- and *Z*-axes linear actuators (Unisense) for manipulating the sensor, and a high-precision electronic balance (HTR-220, Shinko Denshi, Japan) for detecting the position of the tip of the electrode where came in contact with the bottom of the dish. The environmental conditions during measurement were maintained in a glove box hypoxia workstation ($INVIVO_2$ 300, Ruskinn Technologies), with a humidified atmosphere (with 65–80% humidity) containing 21% oxygen and 5% CO_2, maintained automatically. The position of the electrode and the signal from the

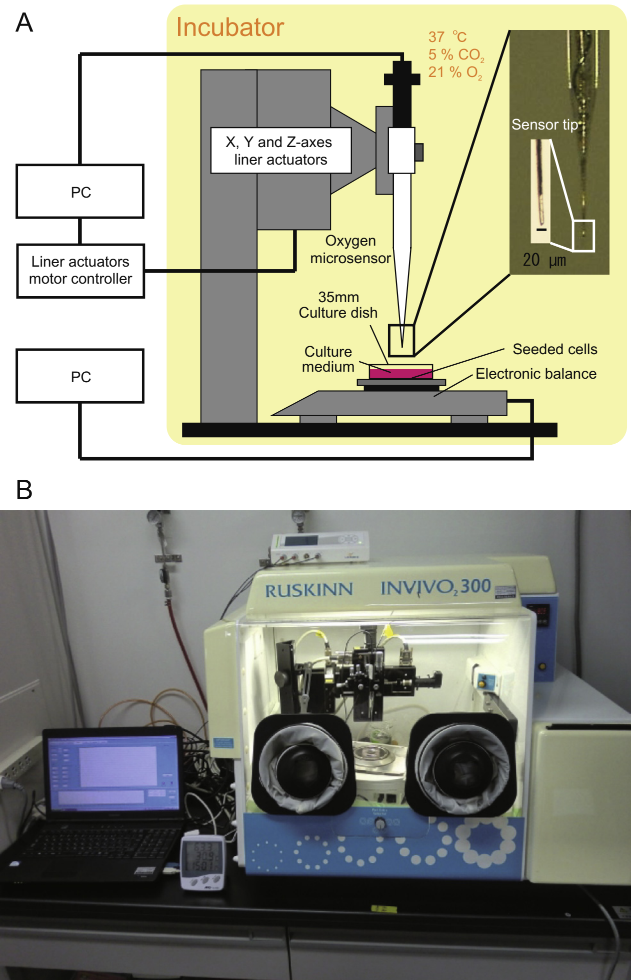

Fig. 1. Developed oxygen measurement system. The schematic illustration (A) and the over-view (B) of the system. See Section 2 for the details.

balance were monitored by computers running the appropriate software (Unisense Profix 3.05 software program, Unisense, and RTS for Windows ver.1.9, Shinko Denshi, Japan). The position of the sensor was manipulated within an accuracy of 1 μm in the *X*-, *Y*-, and *Z*-axes. The experiment was performed as follows. The sensor was moved downward by the actuator at intervals of 3 μm, and contact by the sensor tip on the bottom of the dish was detected by the increase in weight data on the balance. The oxygen concentration was then measured at intervals of 40 μm by raising the sensor from the bottom of the dish. Prior to the measurement, a two-point calibration of the microsensor was performed for each experiment (usually once a day). A zero reading was obtained by immersing the device in approximately 10% (w/v) sodium sulfite solution. A second calibration point was obtained via immersion in water at equilibrium with the atmosphere in a glove box with bubbling air. An increase in the oxygen concentration versus the height from the bottom of the dish was used to calculate the OCR [15]. To estimate oxygen consumption per cell, the number of living cells was counted using trypan blue and a hemocytometer after the oxygen measurement.

2.3. Calculation of the oxygen consumption rate

Calculation of the OCR was based on Fick's laws of diffusion, and was described in a previous study [16]. Briefly, the flux of oxygen (J_{O_2} [g/m^2/s]) is estimated from the gradient of oxygen concentration along the height of the culture media ($grad_{O_2}$ [g/m^4]) as $J_{O_2} = -D * grad_{O_2}$, where D [m^2/s] is the diffusion coefficient of oxygen. When cells consume oxygen, the flux has a negative sign. J_{O_2} can also be expressed as $J_{O_2} = -\mathrm{OCR} * \sigma$, where OCR [g/s/cell] is the OCR of the cell, and σ [cells/m^2] is the surface density of the cells. Thus, the OCR can be derived by the equation $\mathrm{OCR} = D * grad_{O_2}/\sigma$.

To estimate $grad_{O_2}$, all oxygen concentration data measured from the bottom, except the concentration data obtained at the bottom, were fitted to the linear function of $DO(z) = A * (z - z_{max}) + DO_{sat}$, where z_{max} is the height of the medium and DO_{sat} is the saturation DO concentration. By least-square fitting, the parameter A was obtained as $grad_{O_2}$.

2.4. Analysis of the quantity of mitochondria-DNA in each cell using quantitative transcriptase-polymerase chain reaction

The quantity of mitochondria in each of the hiPSC-CMs, HASMC, HCF and HMVEC-C was assessed by the quantity of mitochondrial DNA in each cell, as determined with quantitative PCR. Total DNA was obtained from each cell using a genomic DNA isolation kit (Zymo Research, Irvine, CA). In addition, 0.08 μg of DNA was used for the quantitative PCR primer pairs, and Taqman® MGB probes were designed for three mitochondrially encoded genes [16S ribosomal RNA, nicotinamide adenine dinucleotide (NADH) dehydrogenase 1, and NADH dehydrogenase 3] and a chromosomal gene [pyruvate kinase (muscle)], which was used as a reference gene, using the Taqman® gene expression assay™ (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with a 7300 Real-Time PCR System (Applied Biosystems). Relative quantities of mitochondrial DNA in hiPSC-CMs were adjusted to 1, and then the relative values were calculated for each cell.

2.5. Biochemical analysis

The glucose and lactate levels in culture media were measured according to the hexokinase UV method and lactate oxidase method, respectively (SRL Inc., Tokyo, Japan).

2.6. Data analysis

The data were presented as the mean ± SD. The statistical analyses were performed using the *t*-test between two groups, and performed using Dunnett's test for more than three groups to obtain multiple comparisons. A significant difference was defined as a value of $p < 0.05$.

3. Results

3.1. Oxygen consumption rates and metabolism

The oxygen profiles of the five cell types showed different degrees of gradient. The gradient of hiPSC-CMs was the steepest, while those of HASMC, HCF, and HMVEC-C were relatively gradual (Fig. 2). The OCRs were obtained for each sample by calculating the gradient of the oxygen concentration against the height. The calculated OCRs of hiPSC-CMs, HASMC, HCF, HMVEC-C, and rat-CMs were 0.71 ± 0.38, 0.12 ± 0.02, 0.19 ± 0.03, 0.22 ± 0.10, and 0.29 ± 0.08 pmol/h/cell, respectively (Fig. 3A). The results showed that the OCR for hiPSC-CMs was higher than those of the other types of human heart cells ($p < 0.05$). Furthermore, the OCR of hiPSC-CMs was approximately two to three times as high as that of the rat-CMs.

Glucose consumption and lactate production rates during one-day culture are shown in Fig. 3B. The glucose consumption rates of hiPSC-CMs, HASMC, HCF, HMVEC-C, and rat-CMs were 0.77 ± 0.32, 0.29 ± 0.02, 0.57 ± 0.04, 0.19 ± 0.08, and 0.13 ± 0.02 pmol/h/cell, respectively. Meanwhile, the lactate production rates of hiPSC-CMs, HASMC, HCF, HMVEC-C, and rat-CMs were 1.61 ± 0.70, 0.59 ± 0.07, 1.12 ± 0.06, 0.42 ± 0.18, and 0.19 ± 0.06 pmol/h/cell, respectively. The glucose consumption and lactate production of the hiPSC-CMs were higher than those of other human heart cells and rat-CMs. The estimated molar ratios of lactate production to glucose consumption (L/G ratio) of hiPSC-CMs, HASMC, HCF, HMVEC-C, and rat-CMs were 2.08, 2.02, 1.97, 2.28, and 1.56, respectively. These ratios were used to quantitate the dominance of anaerobic glycolysis in the cells. There were no correlations between the OCRs and L/G ratios of any of the five cell types.

3.2. Analysis of the quantity of mitochondrial DNA

To investigate whether the relative quantity of mitochondria in each cell of any type was correlated with the OCRs obtained in this study, quantitative real-time PCR was performed by using four human cells (hiPSC-CMs, HASMC, HCF, HMVEC-C). The relative quantities of mitochondria DNA in those cells were calculated by using three mitochondrially encoded genes and those three values showed similar tendencies (Fig. 4). The hiPSC-CMs showed the highest value of all the cells (Fig. 4).

4. Discussion

There are several methods available to estimate the OCR of cells in static culture. In one method, OCR is measured by monitoring the decrease in oxygen concentration of the medium, including cells attached to the bottom of the dish or plate, in a closed chamber [7]. This method has been used in many previous studies, including recent ones using a commercially available extracellular flux analyzer [10,11]. For high throughput screening, this method of measuring OCR is appropriate because it is relatively fast (within 3–5 min) [17]. On the other hand, in the present study, we used the open-air method for measuring OCR, which is based on the diffusion theory described in the method Section 2.3 as well as in

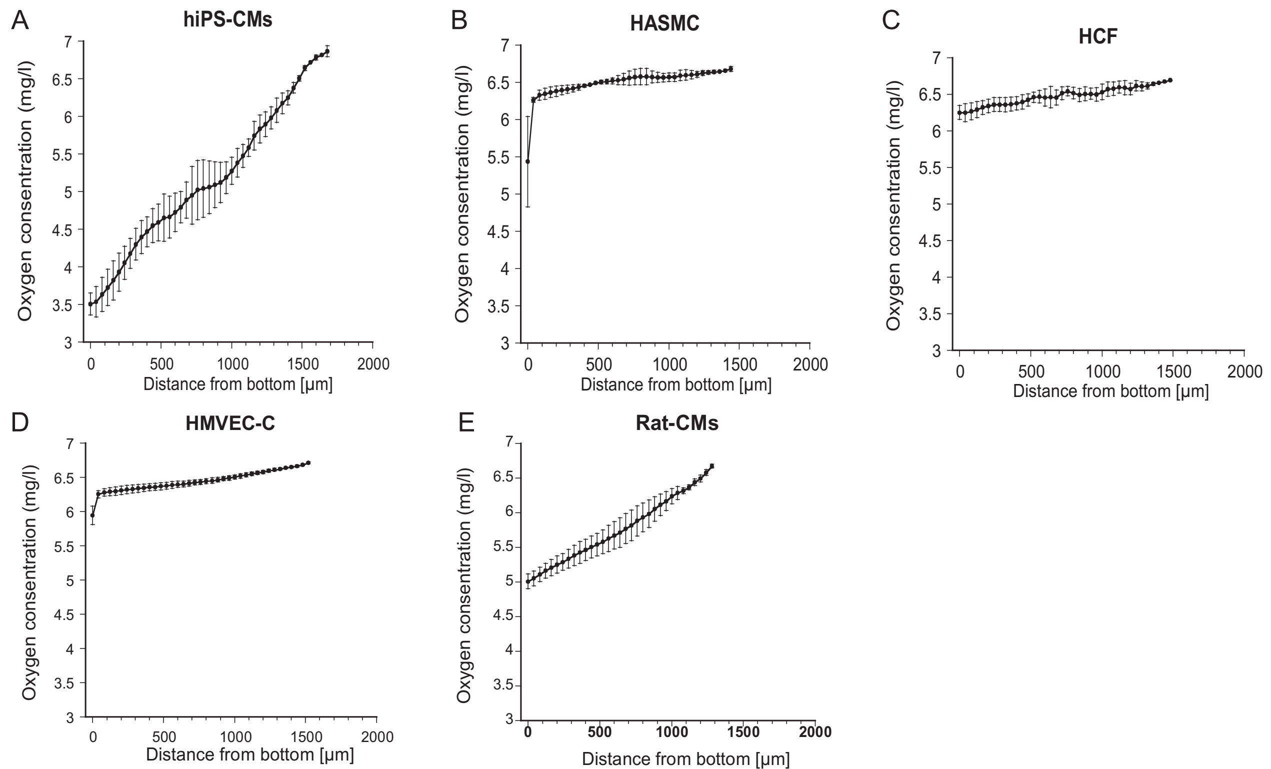

Fig. 2. Example of the oxygen concentration profiles plotted against the height from the bottom of the dish used for culturing hiPSC-CMs (A), HASMC (B), HCF (C), HMVEC-C (D), and rat-CMs (E). The oxygen profiles of the five cell types showed different degrees of gradient. The gradient of hiPSC-CMs was the steepest, while those of the other cells were relatively gradual.

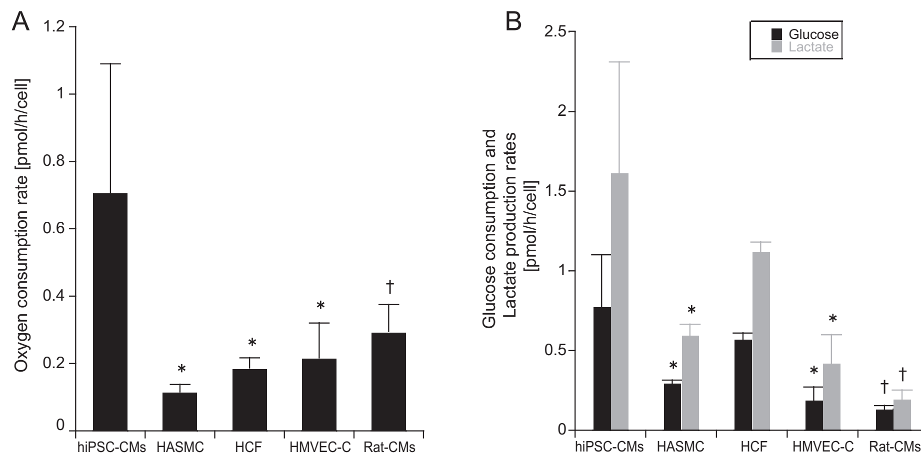

Fig. 3. (A) Oxygen consumption rates (OCRs) are the average of the obtained data [n = 10 (hiPSC-CMs), n = 8 (rat-CMs) and n = 6 (others)]. The OCR for hiPSC-CMs was higher than those of the other types of human heart cells. (B) Glucose consumption (black) and lactate production (gray) rates are the average of the obtained data [n = 3 (HCF) and n = 6 (others)]. The glucose consumption and lactate production rates in the hiPSC-CMs were higher than those of the other human heart cells. Error bars represent the SD. Asterisk (∗) and dagger (†) marks represent significant differences from the hiPSC-CMs data based on the Dunnett's test and the t-test, respectively ($p < 0.05$).

previous studies [16], and differs from the method mentioned above which requires a closed space. This method enables us to measure the oxygen consumption dynamics under steady states over a longer time period, because, in principle, this method does not change the local environment of culturing cells during measurements. In addition, it also enables us to measure "local" OCRs within a sample (within about 10 μm from the tip of the microelectrode). Thus, when the sample is heterogeneous for cell density, cell type, or cell activity, the open-air method can detect local differences of OCR within a given sample, whereas the closed chamber method cannot detect these differences.

The most constituents of the heart are cardiomyocytes, cardiac fibroblasts, cardiac endothelial cells. The cardiomyocytes occupy approximately 90% of the heart volume, but the whole heart consists of approximately 30% cardiomyocytes and 70% nonmyocytes in cell count [18,19]. Therefore it is important to characterize the individual cells of a human heart before attempting to fabricate a human heart with tissue engineering technologies. However, to the best of our knowledge, the various types of human cardiac cells, except for hiPSC-CMs, have never been characterized. In this study, we measured the OCRs of cells that constitute the human heart and compared the obtained data between the cell types.

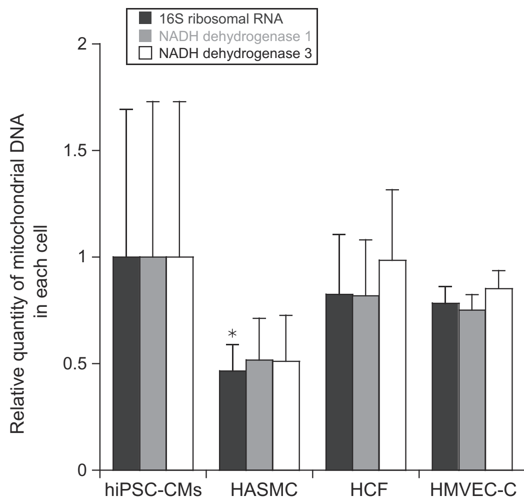

Fig. 4. Measurement of relative quantity of mitochondrial DNA in each cell by quantitative real-time PCR. The relative values were the average of the obtained data ($n = 3$). Relative quantities of mitochondrial DNA in hiPSC-CMs were adjusted to 1, and then the relative values were calculated for each cell. The relative quantities of mitochondrial DNA in hiPSC-CMs and HASMC showed the highest and lowest values in all the cells and the error bars were shown ($n = 3$). Asterisk (*) marks represent significant differences from the hiPSC-CMs data based on the Dunnett's test, ($p < 0.05$).

Estimated OCR, glucose consumption and lactate production rates of the hiPSC-CMs were significantly higher than the other types of human heart cells. In order to elucidate the reasons why the hiPSC-CMs consumed so much more oxygen than the other cell types, we compared the quantity of mitochondrial DNA between the cell types. The relative quantity of mitochondria and OCRs in hiPSC-CMs showed the highest values among the four human cells (Figs. 3A and 4). One of the reasons for the high OCR of hiPSC-CMs may be the large quantity of mitochondria. In addition, we suppose that the other reason is a higher turnover frequency of oxidative phosphorylation in the mitochondria of hiPSC-CMs than other cell types, because of their very high demand for energy for spontaneous contraction. Actually, Yamada et al. reported that the OCR of beating cardiomyocytes was almost 50% higher than quiescent cardiomyocytes [8].

In addition to OCR, glucose consumption and lactate production were measured for each cell sample to investigate the metabolic differences between the cell types (Fig. 3B). Glycolysis is a metabolic pathway which converts glucose into pyruvic acid by a chemical reaction to produce ATP. The molar ratio of lactate produced to glucose consumed (L/G) was used as an index of the state of being aerobic or anaerobic. When all of the pyruvate molecules are converted to lactate in anaerobic respiration, L/G is two. If enough oxygen is supplied to the cells, part of the pyruvic acid enters the mitochondrion to be fully oxidized by the Krebs cycle, and thus the L/G ratio becomes less than two. In the present study, the measured L/G ratios of the four human cells ranged between 1.97 and 2.2. It appeared that glycolysis via anaerobic respiration was a dominant pathway in the cells, whereas the metabolism of most human heart cells *in vivo* is aerobic. In the hiPSC-CMs, the cause of anaerobic respiration was suggested to be a shortage of oxygen, because the oxygen concentration of the hiPSC-CMs just above the cell layer ranged between 1.54 mg/L (36 mmHg) and 3.50 mg/L (83 mmHg). It was a low level in comparison to the arterial blood pO_2 (90–110 mmHg). On the other hand, the oxygen concentration of the other human heart cells, just above the cell layer, ranged between 5.09 mg/L (120 mmHg) and 6.65 mg/L (157 mmHg). It indicates that the energy metabolism of these cells is anaerobic despite enough oxygen. Thus oxygen demand is quite different between cardiomyocytes and other cell types in the heart.

Several previous studies showed that an appropriate level of tissue oxygen concentration is one of the important factors for the maturation of cardiomyocytes and subsequent structural development [20,21]. Therefore, control of the local oxygen concentration during the development of cardiac tissue constructs is a critical factor. As discussed above, *in vitro* pericellular oxygen concentrations of the hiPSC-CMs were thought to be insufficient for optimal metabolism, whereas a higher oxygen concentration exceeding the homeostatic level induces cellular oxidative stress [22]. On the other hand, *in vivo*, cells obtain oxygen from the hemoglobin in red blood cells delivered by arterial blood flow in a hypoxic environment. Thus, perfusion culture circulating medium with oxygen carriers may be the ideal biomimetic system to provide the optimal level of oxygen. In future studies, based on the data obtained in this study, we now plan to develop human cardiac tissue constructs using the perfusion systems developed previously for culturing stacked cardiac cell sheets [23,24], and appropriate oxygen carriers.

In conclusion, our new oxygen measurement system clearly showed the differences in the metabolism depending on cell types and may contribute to future myocardial tissue engineering.

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