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Optimal Population of Embryonic Stem Cells in "Hanging Drop" Culture for in-vitro Differentiation to Cardiac Myocytes

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Abstract: Pluripotent embryonic stem (ES) cells differentiate to cardiac myocytes in vitro by many other previous reports demonstrated "hanging-drop" method. In this study, the number of ES cells in each hanging-drop plays an important role in the cultivation of cardiac myocytes. We examined the optimal hanging-drop size to obtain embryonic stem cell-derived cardiac cells (ESCMs) in vitro using specific labeled mouse ES cells (hCGP7) which were stably transfected with the enhanced green fluorescent protein (GFP) under transcriptional control of the cardiac myocyte-specific promoter with fluorescence-activated cell sorting (FACS) analysis. GFP-positive cells were sorted efficiently in the hanging-drop cell number of approximately 800~1600 cells/drop. The present data will be an important element to establish the optimal methods of ES cellular differentiation in vitro.

Key words: embryonic stem cell, fluorescence-activated cell sorting

Myocardinal reconstruction with cardiac myocytes derived from embryonic stem (ES) cells is attracting a great deal of attention of cardiologists as a potential therapeutic option in future for serious heart diseases. To make this regenerative therapy feasible a much more efficient culture method must be exploited to obtain a large number of ES cell-derived cardiac myocytes (ESCMs).

In the "hanging-drop" method reported by Wobs AM *et al*,[1)] undifferentiated ES cells are cultured for a couple of days in a small drop of medium hanged from lids of culture dishes. This result in a development of embryonic bodies composed of three primitive germ layers, endoderm, ectoderm and mesoderm.[2)] Spontaneous beating cells, which appear in the mesoderm, are collected fore further differentiation to ESCMs.

The present study was designed to obtain more information for improvement of efficiency of the "hanging-drop" method. We examined optimal population of ES cells in each culture "drop" for the highest harvest of ESCMs. To select ESCMs with fluorescence activated cell sorter (FACS) analysis, murine ES cells were knocked in with a cardio-specific homeobox gene, *Nkx2.5* assembled with green fluorescent protein (GFP) gene.

Materials and Methods

Nkx2.5-GFP knock-in ES Cells (hCGP7)

A cell line of *Nkx2.5-GFP* knock-in ES cells (hCGP7) was established as described elsewhere (Hidaka et al, in preparation). Briefly, one of the murine *Nkx2.5* loci was knocked-in with a promoter-less EGFP gene (Clontech) and a puromycin resistance gene driven by the Pgk1 promoter. The parental ES strain, ht7, was provided by Dr. Niwa, Osaka University.

Cell culture

Undifferentiated ES cells ht7 or hCGP7 were digested with trypsin-EDTA and suspended in Glasgow minimum essential medium (GMEM: Sigma) supplemented with 10% FBS (Gibco), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM 2-mercapto ethanol. The ES cells were then cultured in small drop hanged from the lid of culture dish ("hanging-drop") to aggregate and form EBs for 2 days. Each drop (20 μl) contained 100, 400, 800, 1600, 3000 or 10000 cells/drop were further cultured as "suspended". The ES cells were cultured as suspended to form EBs, and further cultivated for 8 days.

FACS analysis

Single ES cells were prepared by dispersing EB culture (day 2+8) with trypsin-EDTA (0.25%). Flow cytometric analysis was performed using a dual laser FACS Calibur (Becton Dickinson). Cells were excited with 488 nm Argon laser and a 530 nm bandpass filter was used to detect GFP positive cells.

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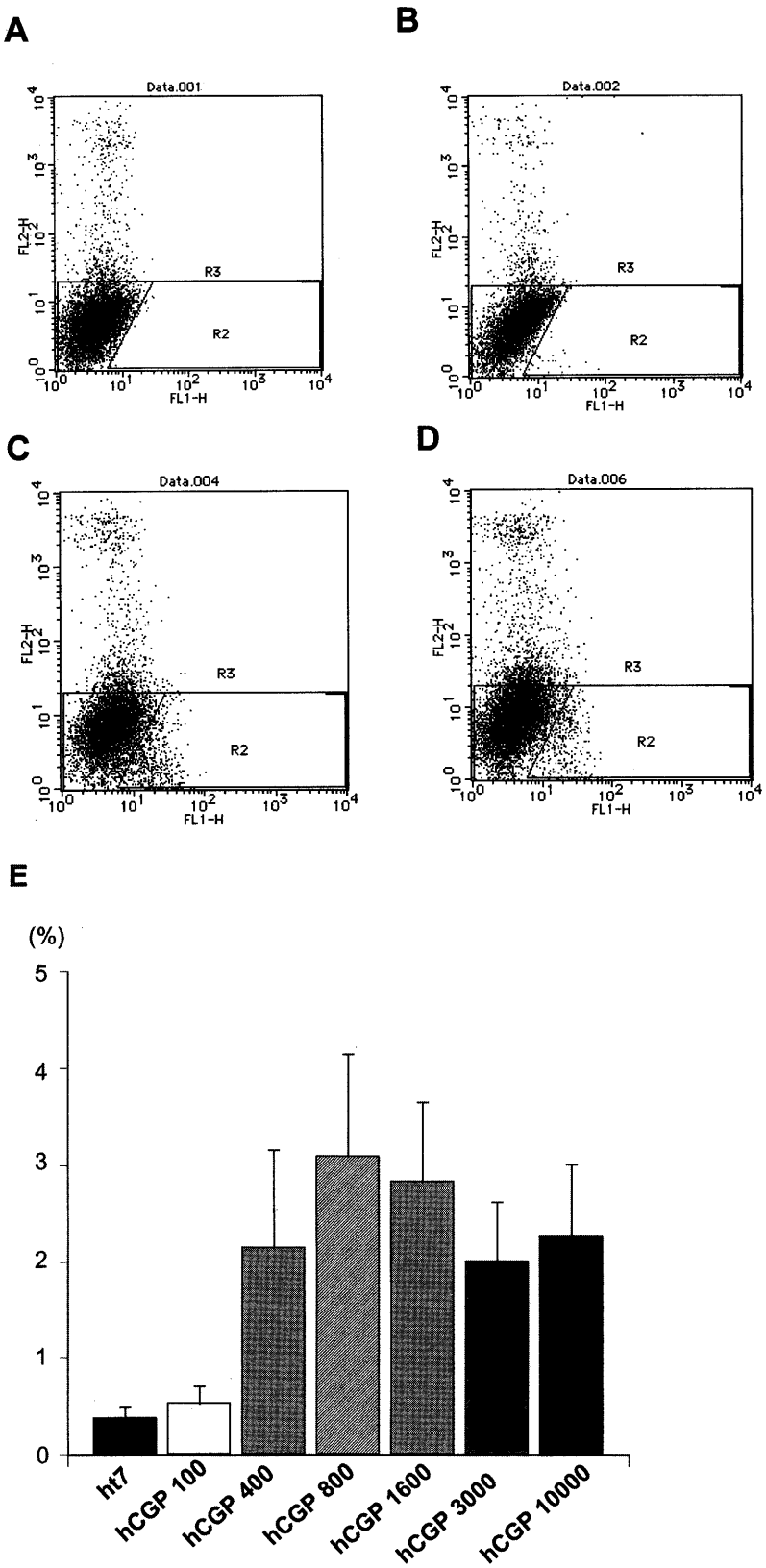

Fig. 1 ACS analysis of single cell preparation from embryoid bodies.
EBs were enzymatically digested at the stage of day 2+8. (A) Control (ht7 with 800 cells/drop in hanging-drop), (B) hCGP7 100 cells/drop, (C) hCGP7 800 cells/drop, (D) hCGP7 3000 cells/drop. R2 area represents GFP-positive and PI-negative cells. (E) Summary of GFP positive fraction among PI negative cells (GFP-positive and PI negative cells) at each size of hanging-drop.

Data were recorded using the CellQuest acquision software (Becton Dickinson)[4]. ES cells derived from ht7 were used as control.

Results and Discussion

Spontaneously beating cells appeared in each EBs after 5 or 6 days of "suspended" culture. In FACS analysis, ESCMs were recognized as GFP-positive and low propidium iodide (PI) cells. Figure 1 (A~D) shows representative scatter dots of FACS analysis. Abscissas and ordinates indicate intensities of intensities of GFP and PI respectively. The fraction of high PI intensity (>20) indicate deal cells (R3 area). Viable ESCMs should be included in the GFP-positive and low PI intensity zone (R2 area). In labeled control (ht7-derived EBs), the amount of GFP-positive viable cells in R2 area was only 0.2% of the total viable cells (Fig. 1A). In labeled (hCGP7-derived) EBs, the amount of GFP-positive viable cells was much greater (>0.5% of total viable cells), and it depends on the cell population in each culture drop. The peak amount (6.7%) was obtained at 800 cells/drop (Fig. 1C), and it decreased at either lower cell population (0.6% at 100 cells/drop: Fig. 1B) or at higher cell population (6.1% at 1600 cells/drop and 4.4% at 3000 cells/drop: Fig. 1D). The data obtained from EBs for each condition of cell population are summarized in Fig. 1E.

The present data have shown that ESCMs are most efficiently obtained in the hanging-drop with the cell population of approximately 800~1600 cells/drop. One possible explanation for the result is that aggregated cells in hanging drop culture grow in a similar manner with embryo in uterus at the early stage of gestation. Because it has been known that the differentiation of the embryonic visceral endoderm is affected by curvature radius, it is also conceivable that the differentiation into the embryonic heart might also be dependent on the radius. Experiments employing the hanging-drop culture with known sizes or curvature radius would be helpful to clarify the points.

There will be increasing demands for ESCMs as a source for cellular therapy of myocardial reconstruction. The present study will provide useful information to establish the optimal methods to obtain ESCM in an efficient manner.

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