Prion Protein and Stage Specific Embryo Antigen 1 as Selection Markers to Enrich the Fraction of Murine Embryonic Stem Cell-Derived Cardiomyocytes

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ABSTRACT

Background The prion protein (PrP) might be useful as a tool to collect cardiac progenitor cells derived from embryonic stem (ES) cells. It is also possible that PrP^+ cells include undifferentiated cells with a capacity to develop into tumors.

Methods PrP^+ cells isolated from embryoid bodies (EB) formed by mouse AB1 ES cells were examined using RT–PCR analysis and clonogeneic cell assay. To assess their potential to differentiate into cardiomyocytes, $Nkx2.5^{GFP/+}$ (hcgp7) cells, another ES cell line that carries the GFP reporter gene in the Nkx2.5 loci, were used.

Results PrP^+ cells isolated from EB of day 7 and 14 did not express pluripotency markers, but expressed cardiac cell markers, while PrP^+ cells isolated from EB of day 21 expressed pluripotency markers. Cultured PrP^+ cells isolated from EB of day 21 expressed pluripotency markers to form colonies, whereas those isolated from EB of day 7 and 14 did not. To exclude proliferating cells from PrP^+ cells, stage specific embryo antigen 1 (SSEA1) was employed as a second marker. $PrP^+/SSEA1^-$ cells did not proliferate and expressed cardiac cell markers, while $PrP^+/SSEA1^+$ did proliferate.

Conclusion PrP^+ cells isolated from EB included undifferentiated cells in day 21. $PrP^+/SSEA1^-$ cells included cardiomyoctes, suggesting PrP and SSEA1 may be useful as markers to enrich the fraction of cardiomyocytes.

Key words cell differentiation; embryonic stem cells; prion protein; stage-specific embryonic antigens

Embryonic stem (ES) cells are characterized by their capacity for self-renewal and pluripotency. They spontaneously differentiate into cardiomyocytes through the formation of embryoid bodies (EB).[1–4] ES cells-derived cardiomyocytes are a potential source for cell-transplantation therapy in patients with a damaged heart.[5, 6] A prerequisite for transplantation of these cells is their purity, because undifferentiated ES cells are capable of producing tumors.[7] A sophisticated method is necessary to isolate cardiac progenitors from among ES cells and achieve a fraction of high purity. The normal prion protein (PrP) belongs to the glycosylphosphatidyl-inositol-anchored protein family, and is expressed in adult tissues.[8–11] The PrP is anchored to the outer surface of neurons, lymphocytes and other cells that express *Nestin*[9, 12] and promotes the differentiation of ES cells into neuronal progenitor cells.[9, 12, 13] The PrP was reported to be expressed in the intestine during embryonic development[9, 14, 15] as well as in the heart and neurons.[9, 14–16] The PrP was reported to form a complex with stress induced phosphoprotein 1 (STIP1) to regulate neuronal development.[9, 17] The PrP is known to be involved in iron uptake[9, 18] and Ca^{2+} homeostasis[9, 19] and also to play a pivotal role in cellular responses against reactive oxygen species (ROS) to form superoxide dismutase (SOD) via binding to copper.[9, 11] Thus, the PrP is essential to responses to the environment by cells of the three germ layers. An abnormal PrP causes neurodegenerative diseases such as Creutzfeldt-Jakob disease, fatal familial insomnia and Gerstmann-Sträussler-Scheinker syndrome.[10, 11]

Hidaka et al. reported that the PrP was a marker of cardiac progenitor cells in the early phase of EB formation.[16] We also found that most of PrP^+ cells differentiated into cardiomyocytes.[20] Since the PrP is expressed in various tissues, it is possible that PrP^+ cells from ES cells include cells different from cardiac progenitor cells. Recently, the PrP was reported to enhance the expression of the transcription factor *Nanog* suggesting that PrP^+

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Abbreviations: DMEM, Dulbecco's-modified Eagle's medium; EB, embryoid bodies; ES cells, embryonic stem cells; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; GMEM, Glasgow minimum essential medium; HBSS, Hank's balanced salt solution; LIF, leukemia inhibitory factor; PE, phycoerythrin; PrP, prion protein; ROS, reactive oxygen species; SOD, superoxide dismutase; SSEA1, stage specific embryo antigen 1; STIP1, stress induced phosphoprotein 1

cells can proliferate and form tumors after transplantation.[7, 9, 21] This might indicate the harmfulness of PrP^+ cells as a cell source for transplantation. However, it has never been tested whether PrP^+ cells from EB include undifferentiated cells. In the present study, we attempted to characterize PrP^+ cells derived from EB formed by mouse ES cells. We found that PrP^+ cells from EB of days 21, but not those of day 7 and 14, expressed pluripotency markers and were capable of proliferation. Combining the PrP with stage specific embryo antigen 1 (SSEA1) as the second marker enabled us to enrich the fraction of cardiomyocytes that do not proliferate.

MATERIALS AND METODS

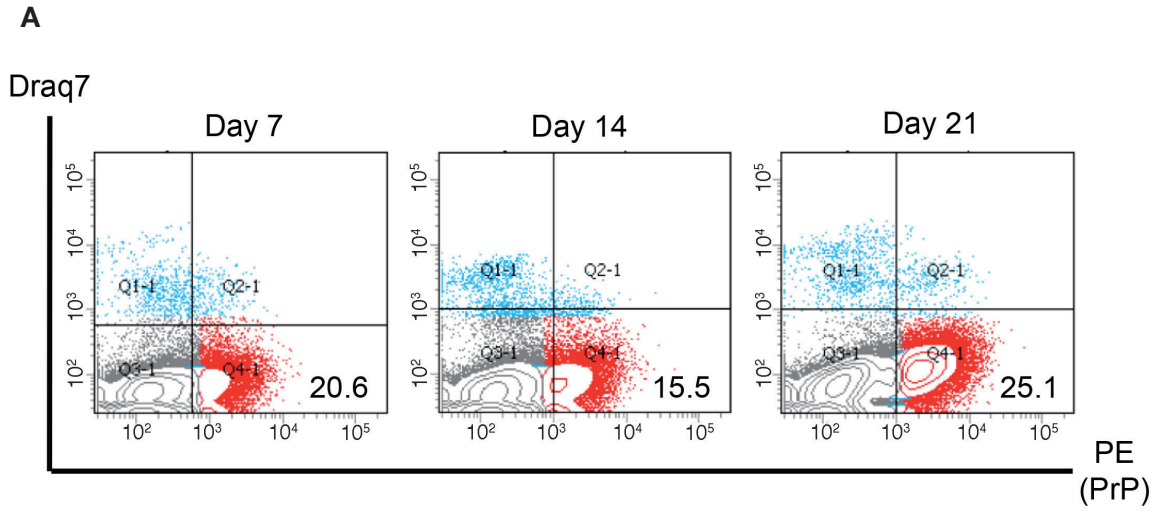
Cell culture and differentiation

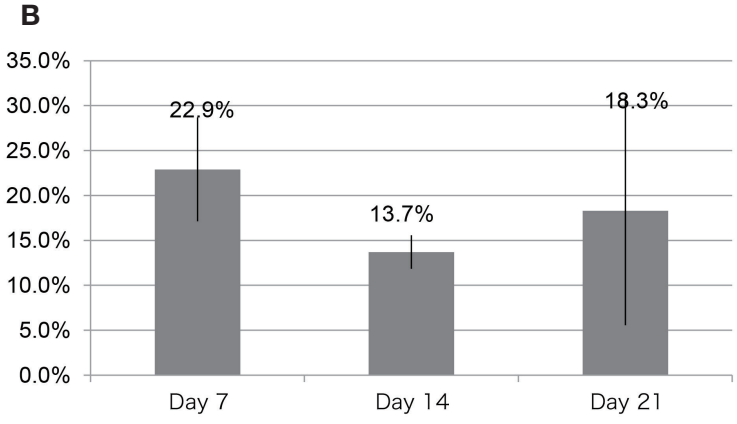
AB1 ES cells derived from 129SV/EV mice were kindly provided by Dr. Shimotsuke (Riken CDB, Kobe, Japan). They were cultured on SNL feeder cells treated with mitomycin C (Sigma-Aldrich, St Louis, MO). SNL cells were derived from STO mouse embryonic fibroblasts with a forced expression of *leukemia inhibitory factor* (LIF) and *neomycin resistance genes*. AB1 cells were grown and maintained in Dulbecco's-modified Eagle's medium (DMEM; Wako Pure Chemical, Osaka, Japan) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Corning, Corning, NY), 1 × penicillin-streptomycin-L-glutamine solution (Wako Pure Chemical), 1 × MEM non-essential amino acid solution (Wako Pure Chemical), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich) and 1,000 units/mL LIF (ESGRO; Merck KGaA, Darmstadt, Germany).

Dr. Morisaki (National Cerebral and Cardiovascular Center, Suita, Japan) provided ht7 ES cells. These cells carry the *hygromycin resistance gene* in one of the *Oct4* loci.[22] Derived from the ht7 cells, hcgp7 ($Nkx2.5^{GFP/+}$) ES cells carry the *GFP reporter gene* in one of the *Nkx2.5* loci.[23] Both ht7 and hcgp7 cells were grown and maintained on gelatin-coated dishes in Glasgow minimum essential medium (GMEM; Wako Pure Chemical) supplemented with 10% heat-inactivated FBS (Corning), 1 × penicillin-streptomycin-L-glutamine solution (Wako Pure Chemical), 1 × MEM non-essential amino acid solution (Wako Pure Chemical), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 1 mM sodium pyruvate (Wako Pure Chemical), and 1,000 units/mL LIF (Merck KGaA), without feeder cells. Differentiation of ES cells into cardiac progenitors was induced via formation of EB. Briefly, EB were generated by plating 20 μL of cell suspension (2.5–10 × 10^4 cells/mL) in DMEM (Wako Pure Chemical) supplemented with 10–20% heat-inactivated FBS (Corning), 1 × penicillin-streptomycin-L-glutamine solution (Wako Pule Chemical), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich) (EB medium) on the lid of a dish, followed by incubation in hanging drops for 2 days. EB were transferred into the medium and cultured as floating EB or attached out-growth cells for indicated days until analysis.

Table 1. Primer list in gene expression analysis

Gene	Forward	Reverse	Annealing temperature	No. cycles
Zfp42	TTCACGGAGAGCTCGAAACT	CCATCCCCTTCAATAGCTCA	60	30
Nanog	CACCCACCCATGCTAGTCTT	ACCCTCAAACTCCTGGTCCT	60	28
Oct4	ATTCTCGAACCTGGCTAAGCT	ATGGTGGTCTGGCTGAACACCTTT	60	28
Pax6	CAGAAGACTTTAACCAAGGGC	TGGGCTATTTTGCTTACAACTT	60	30
NeuroD1	AAGCCATGAATGCAGAGGAGGACT	AGCTGCAGGCAGCCGGCGACC	60	30
Afp	ACATCAGTGTCTGCTGGCAC	AGCGAGTTTCCTTGGCAACAC	60	30
Sox17	AAGAAACCCTAAACACAAACAGCG	TTTGTGGGAAGTGGGATCAAGAC	60	30
T	GTCTTCTGGTTCTCCGATGT	CCAGGTGCTATATATTGCCT	60	30
Mesp1	CAGAATCGTGGGACCCAT	CGGCGTCCAGGTTTCTAG	60	30
Tbx5	ATGGTCCGTAACTGGCAAG	TTTCGTCTGCTTTCACGATG	60	30
Nkx2.5	ACCGTCGCTACAAGTGCAA	CCATAGGCATTGAGACCCA	60	30
Anp	TGGGCTTCTTCCTCGTCTT	TTCTACCGGCATCTTCTCCT	60	30
Myl7	TGACCCAGGCAGACAAGTTC	CGTGGGTGATGATGTAGCAG	60	30
Myl2	AAGGTGTTTGATCCCGAGGG	GGGAAAGGCTGCGAACATCT	60	30
Prnp	CTGAAGCATTCTGCCTTCCT	GCCGACATCAGTCCACATAG	60	30
Gapdh	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	60	25





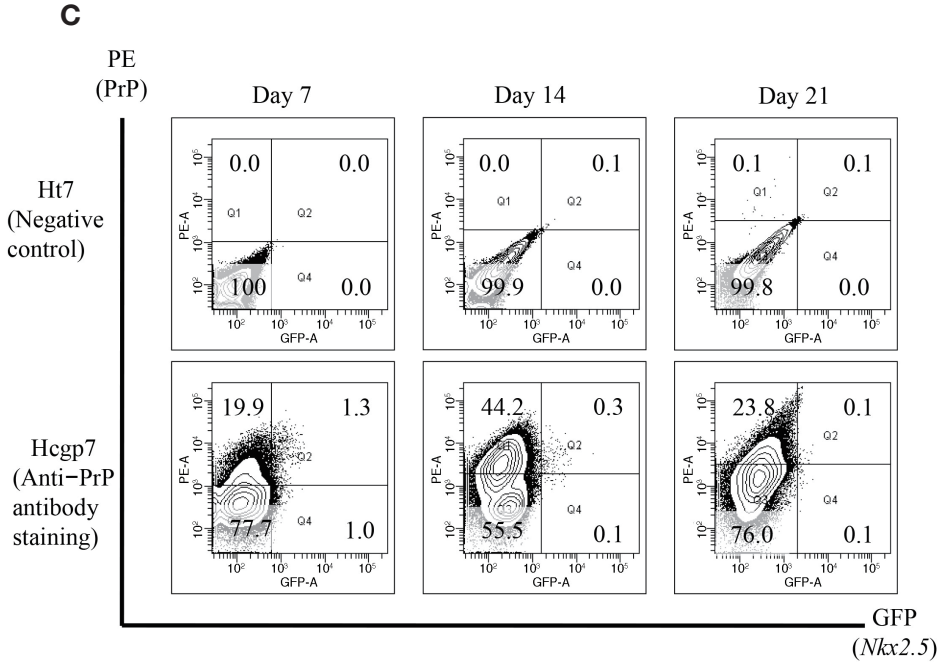


Fig. 1. PrP$^+$ cells isolated from EB involved the cardiac myocytes.
A: Flow cytometry analysis of cell surface PrP in EB derived from AB1 ES cells. Ordinate indicates the prevalence of DRAQ7-posisitve cells as dead cells and abscissa indicated the prevalence of PrP$^+$ cells. The cells within a gated area show the population of living PrP$^+$ cells.
B: Summary of the flow cytometry analysis. The graph shows the mean and the standard deviation of the PrP$^+$ rate in Day 7, 14 and 21 after a differentiation induction ($n = 3$).
C: Prevalence of *Nkx2.5*/GFP-positive cell in PrP$^+$ cells isolated from EB of hcgp7 at Day 7, 14 and 21. The ordinate showed the prevalence of PrP$^+$ cells and the abscissa indicated the expression level of *Nkx2.5*/GFP-positive cells. The upper panel showed the flow cytometry analysis of ht7 cells, as negative control and the lower panel showed the flow cytometry analysis on hcgp7 cells.
EB, embryoid body; ES cells, embryonic stem cells; GFP, green fluorescent protein; PE, phycoerythrin; PrP$^+$, prion protein positive.

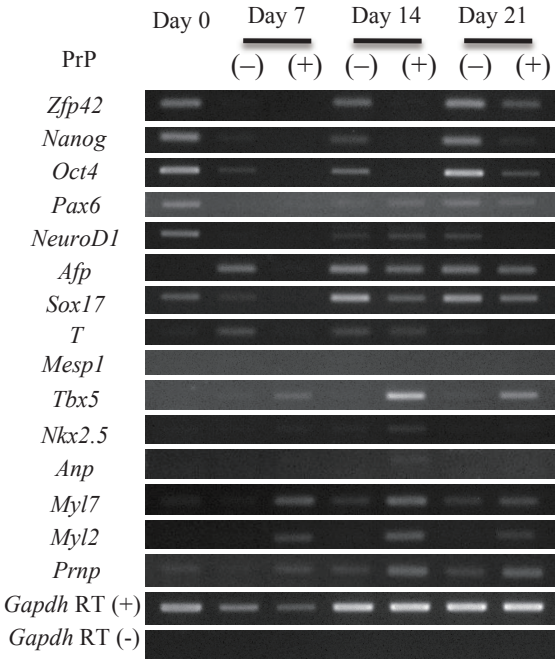


Fig. 2. mRNA level of pluripotent, ectodermal, endodermal, mesodermal and cardiac cell markers in PrP$^+$ cells from EB at day 7, 14 and 21. Cells were isolated by FACS, and then mRNA was extracted from them. Each set corresponds to the transcript amplified using the indicated primers. (+) : PrP$^+$ cell fraction, (–) : PrP$^–$ cell fraction. PCR of *Gapdh* from RNA sample without reverse transcriptase [*Gapdh* RT (–)] did not show any fragment, indicating no contamination of cDNA by genomic DNA.
EB, embryoid body; FACS, fluorescence activated cell sorting; mRNA, messenger RNA; PrP$^–$, prion protein negative; PrP$^+$, prion protein positive; RT, reverse transcription.

Flow cytometry

Cells were dissociated from EB at day 7 ± 1, 14 ± 1 and 21 ± 1 by Collagenase type II (Worthington, Lakewood, NJ) with gentle pipetting, followed by a treatment with Cell Dissociation Buffer (enzyme-free, Hanks'-based; Thermo Fisher Scientific, Waltham, MA) for 5–8 min. Cells were stained with phycoerythrin (PE) -conjugated anti-PrP (mouse monoclonal clone SAF83; Funakoshi, Tokyo, Japan) labeled with the PE Labeling Kit-NH2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Dead cells were excluded with Draq7 (Biostatus, Shepshed, England).

The percentage of cells positive for PrP or GFP was determined by flow cytometry (BD FACS Canto II; BD, Franklin Lakes, NJ). They were resuspended in Hank's balanced salt solution (HBSS, Wako Pure Chemical) containing 2% FBS and Draq7, diluted 100 times, and subjected to cell sorting (Moflo XDP, Beckman Coulter, Brea, CA) with Summit software to collect either PrP$^+$ or GFP$^+$ cells.[20]

Clonogenic cell assay

PrP$^+$ cells were isolated from EB at day 7, 14 and 21 by FACS. 1,000 or 10,000 cells were seeded on gelatin-coated dish and cultured in EB medium for 7 to 17 days. Colonies fixed with 100% ethyl alcohol were stained with Giemsa.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from EB using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA samples were treated with DNaseI (Promega Corporation, Fitchburg, WI) to eliminate genomic DNA and cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan). PCR amplifications were performed using Emerald Amp Max polymerase (Takara Bio) with primers listed in Table 1.

Statistical analysis

Data are expressed as mean ± SD.

RESULTS

PrP$^+$ cells differentiated into cardiac myocytes

Figure 1A shows representative flow cytometry data indicating the prevalence of PrP$^+$ cells in EB of AB1 cells. And Fig. 1B shows the summary of the flow cytometry analysis. The PrP$^+$ rate were 22.9, (s = 5.8. n = 3), 13.7, (s = 1.9. n = 3) and 18.3, (s = 12.7. n = 3) (%) in EB of day 7, 14 and 21, respectively. PrP$^+$ cells were not detected in EB before day 4 of differentiation induction (data not shown). The prevalence of PrP$^+$ cells expressing *Nkx2.5*/GFP was studied using mouse *Nkx2.5*$^{GFP/+}$ ES cells. As shown in Fig. 1C, the prevalence of PrP$^+$ cells expressing *Nkx2.5*/GFP on days 7, 14 and 21 was 1.3, 0.3 and 0.1%, respectively, indicating a decline in the number of cardiac progenitor cells among PrP$^+$ cells.

Heterogeneity of PrP$^+$ cells isolated from EB at various periods post differentiation induction

To verify whether PrP$^+$ cells contained undifferentiated cells, mRNAs levels of pluripotency markers were examined in PrP$^+$ cells isolated from EB. Figure 2 shows mRNA levels of pluripotency markers (*Zfp42, Nanog* and *Oct4*), ectoderm cell markers (*Pax6, NeuroD1*), endoderm cell markers (*Afp, Sox17*), mesoderm cell markers (*T, Mesp1*) and cardiac cell markers (*Tbx5, Nkx2.5, Anp, Myl7* and *Myl2*) expressed in PrP$^+$ cells isolated from EB of days 7, 14 and 21. PrP$^+$ cells from EB of day 7 predominantly expressed mRNAs of cardiac cell markers. PrP$^+$ cells from EB of day 14 expressed mRNAs of ectodermal and endodermal cell markers besides mesodermal cell and cardiac cell markers. Surprisingly,

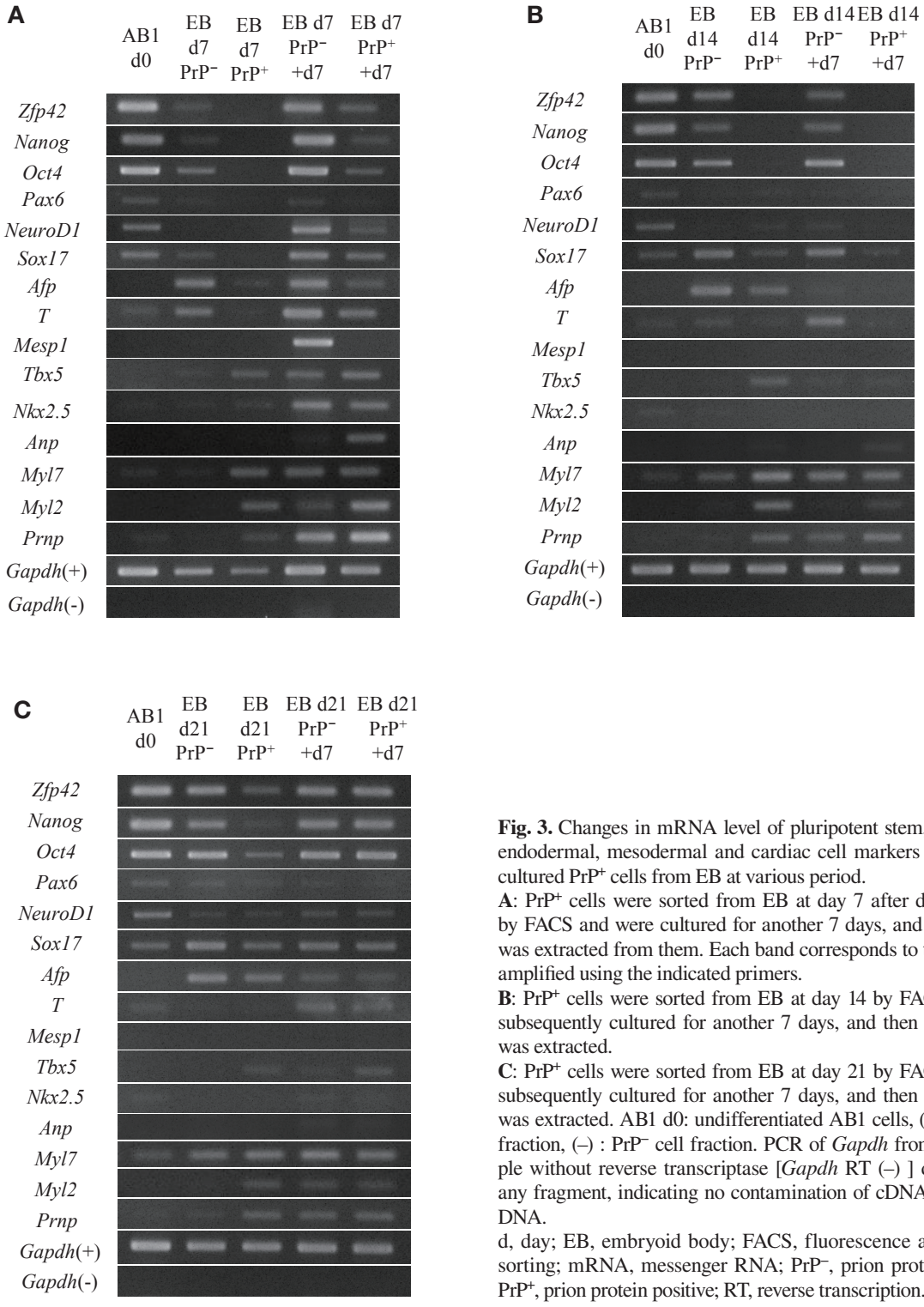


Fig. 3. Changes in mRNA level of pluripotent stem, ectodermal, endodermal, mesodermal and cardiac cell markers expressed in cultured PrP^+ cells from EB at various period.

A: PrP^+ cells were sorted from EB at day 7 after differentiation by FACS and were cultured for another 7 days, and then mRNA was extracted from them. Each band corresponds to the transcript amplified using the indicated primers.

B: PrP^+ cells were sorted from EB at day 14 by FACS and were subsequently cultured for another 7 days, and then their mRNA was extracted.

C: PrP^+ cells were sorted from EB at day 21 by FACS and were subsequently cultured for another 7 days, and then their mRNA was extracted. AB1 d0: undifferentiated AB1 cells, (+) : PrP^+ cell fraction, (–) : PrP^- cell fraction. PCR of *Gapdh* from RNA sample without reverse transcriptase [*Gapdh* RT (–)] did not show any fragment, indicating no contamination of cDNA by genomic DNA.

d, day; EB, embryoid body; FACS, fluorescence activated cell sorting; mRNA, messenger RNA; PrP^-, prion protein negative; PrP^+, prion protein positive; RT, reverse transcription.

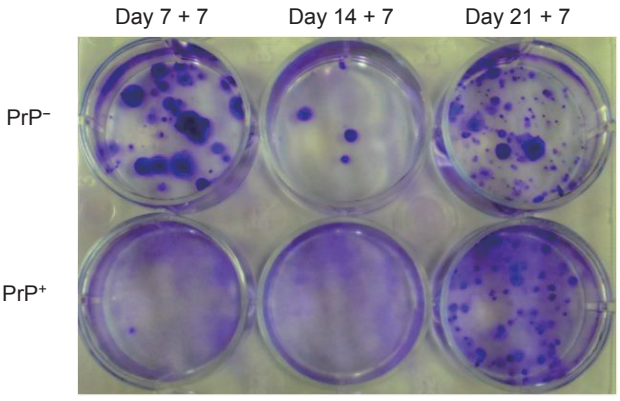


Fig. 4. Proliferation of cultured PrP$^+$ cells derived from EB at day 7, 14 and 21.
PrP$^+$ cells were sorted from EB at day 7, 14 and 21 by FACS, and were subsequently cultured for 7 days. Each cell was stained by Giemsa's solution. The upper panels: colony formation of PrP$^-$ cells derived from EB at the various periods. The lower panels: colony derived of PrP$^+$ cells derived from EB at the various periods.
EB, embryoid body; FACS, florescence activated cell sorting; PrP$^-$, prion protein negative; PrP$^+$, prion protein positive.

PrP$^+$ cells from EB of day 21 expressed mRNAs of pluripotency markers as well as those of cells of the three germ layers.

PrP$^+$ cells from EB included undifferentiated cells capable of proliferation

Expression of pluripotency markers suggested the prevalence of undifferentiated cells among PrP$^+$ cells from EB of day 21. Since undifferentiated cells proliferate in a short-term culture, we examined the expression of pluripotency markers in PrP$^+$ cells cultured for 7 days after isolation from EB. As shown in Fig. 3A, PrP$^+$ cells from EB of day 7 expressed mesodermal, cardiac cell markers. But, they also expressed pluripotent, ectodermal and endodermal cell markers, slightly. PrP$^+$ cells from EB of day 14 did not express pluripotency markers, ectodermal or endodermal cell markers after a 7-day culture, but they expressed cardiac cell markers in Fig. 3B. PrP$^+$ cells from EB of day 21 expressed pluripotency markers after a 7-day culture without changes in mRNA levels of mesodermal, cardiac, ectodermal and endodermal cell markers in Fig. 3C. Next, we examined the capability of PrP$^+$ cells from EB to proliferate by means of clonogenic cell assay. As shown in Fig. 4, PrP$^+$ cells isolated from EB of day 21 formed colonies, while cultured PrP$^+$ cells from EB of day 7 and 14 did not. These results indicated that PrP$^+$ cells from EB of day 21 contained undifferentiated cells.

Use of PrP and SSEA1 as selection markers to enrich the fraction of differentiated cardiomyocytes

Stage specific embryo antigen 1 (SSEA1) is a surface marker of pluripotent stem cells.[24] Figure 5A shows expression of SSEA1 in PrP$^+$ cells isolated from EB of days 7, 14 and 21. The prevalence of PrP$^+$/SSEA1$^+$ cells was 0.3%, 0.1% and 0.3%, respectively. Figure 5B shows colony formation by PrP$^+$ /SSEA1$^-$ cells and PrP$^+$ /SSEA1$^+$ cells from EB of day 21 cultured for 10 and 17 days, respectively. Cultured PrP$^+$ /SSEA1$^-$ cells did not form any colony, but cultured PrP$^+$ /SSEA1$^+$ cells did, indicating SSEA1 is a useful marker to exclude proliferating cells from among PrP$^+$ cells. Figure 5C shows cardiac cell markers (*Tbx5* and *Myl2*) expressed in PrP$^+$ /SSEA1$^-$ cells isolated from EB of day 21. The level of mRNA corresponding to cardiac cell markers was comparable between PrP$^+$ cells and PrP$^+$ /SSEA1$^-$ cells.

DISCUSSION

In the present study, we found that PrP$^+$ cells isolated from EB of day 7 and 14 did not express pluripotency markers but expressed cardiac cell markers, and that PrP$^+$ cells isolated from EB of day 21 expressed pluripotency markers as well as those of the three germ layers; that PrP$^+$ cells isolated from EB of day 21 formed colonies and expressed pluripotency markers, whereas those from EB of day 7 and 14 did not form colonies; and that PrP$^+$/SSEA1$^-$ cells expressed cardiac cell markers and did not proliferate, while PrP$^+$/SSEA1$^+$ did proliferate.

Pluripotent stem cells such as ES or induced pluripostent stem cells are a potential cellular source for cell transplantation therapy of damaged hearts.[16, 20, 25] However, pluripotent stem cells-derived cells include undifferentiated cells. Several groups tried to purify cardiomyocytes among differentiated ES cells by marker gene transduction or fluorescence-based purification methods. Yet, for clinical use, fast, effective and scalable purification methods with no genetic modification are essential. A few surface markers that can be used for isolation of cardiomyocytes have been reported.[16, 23] *Flk1* is a marker of cardiovascular progenitors: common progenitors for cardiac, smooth muscle and endothelial cells.[5] *c-Kit* is reported to be a cardiovascular stem cell marker of adult and embryonic heart.[26, 27] *Pdgfra* is widely expressed in the mesoderm, including the cardiac lineage.[26] *Vascular cell adhesion molecule-1* (VCAM-1) has been reported as useful to isolate embryonic cardiomyocytes with 98% purity. VCAM-1 positive cells have been reported to specify committed cardiomyocytes,[28] and have been confirmed in human ES cells.[29] Recently, Hidaka et al. reported the PrP served to predominantly enrich the fraction of cardiomyocytes expressing *cTnI*,

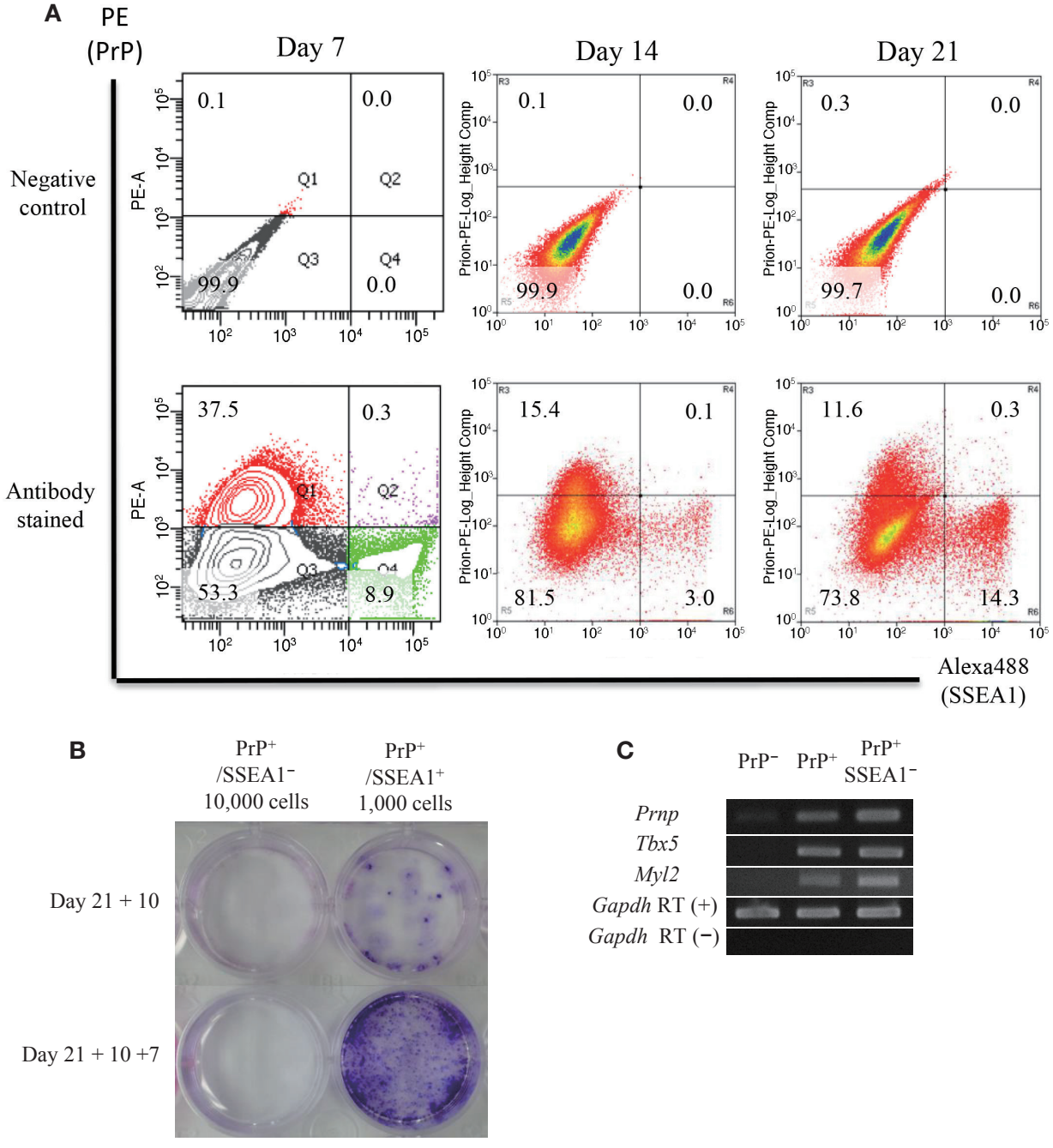


Fig. 5. Characterization of PrP$^+$/ SSEA1$^-$ cells isolated from EB at various periods.
A: Prevalence of PrP$^+$/ SSEA1$^+$ cells derived from EB at the various periods. PrP$^+$ cells were sorted from EB at Day 7, 14 and 21 by FACS. The ordinate showed the prevalence of PrP$^+$ cells and the abscissa indicated the prevalence of SSEA1$^+$ cells. The upper control board showed the flow cytometry analysis as negative control and the lower control board showed the flow cytometry analysis of PrP$^+$/ SSEA1$^+$ cells.
B: Colongenic cell assay of cultured PrP$^+$/ SSEA1$^-$ cells from EB at day 21. Either PrP$^+$/ SSEA1$^+$ or PrP$^+$/ SSEA1$^-$ cells of 10,000 cells were isolated from EB at day 21 by FACS and were subsequently cultured for another 10 and 17 days. Upper panels: colonies of cultured PrP$^+$/ SSEA1$^+$ or PrP$^+$/ SSEA1$^-$ cells from EB at day 21 for another 10 days stained with Giemsa's dye. Lower panels: colonies of cultured PrP$^+$/ SSEA1$^+$ or PrP$^+$/ SSEA1$^-$ cells from EB at day 21 for another 17 days stained with Giemsa's dye.
C: Enrichment of cardiac myocytes in PrP$^+$/ SSEA1$^-$ cells from EB at day 21. PrP$^-$ cells, PrP$^+$ cells and PrP$^+$/ SSEA1$^-$ cells were sorted from EB at day 21 by FACS and were subjected to RT–PCR using indicating primers. PCR of *Gapdh* from RNA sample without reverse transcriptase [*Gapdh* RT (–)] did not show any fragment, indicating no contamination of cDNA by genomic DNA.
EB, embryoid body; FACS, fluorescence activated cell sorting; mRNA, messenger RNA; PE, phycoerythrin; PrP$^-$, prion protein negative; PrP$^+$, prion protein positive; RT, reverse transcription, SSEA1$^-$, SSEA1 negative; SSEA1$^+$, SSEA1 positive.

and cultured PrP$^+$ cells from EB have been reported to differentiate into atrial and ventricular myocytes.[16] Hidaka et al. also demonstrated that PrP$^+$ cells from EB could proliferate, because PrP$^+$ cells included cardiac progenitors.[16] Nevertheless, in the present study, PrP$^+$ cells from EB of days 21 expressed pluripotency markers and PrP$^+$ cells from EB of day 21 proliferated *in vitro*. In contrast, PrP$^+$ cells from EB of day 14 did not express pluripotency markers and did not proliferate. The mechanism of re-expression of pluripotency markers in PrP$^+$ cells from EB of day 21 remains unclear. The PrP has been reported to activate *Nanog* expression through Integrin signaling.[9, 25] PrP is known to influence the fate of cells and cell cycle besides their pluripotency, suggesting that prolonged expression of PrP in cultured cells may reactivate pluripotency[9, 30] markers leading thereby to cell proliferation. Further experiments are necessary to examine this possibility.

Since individual markers are commonly expressed by cells of multiple lineage, a single marker may not be sufficient to distinguish cells of the cardiac lineage. Combination of two surface markers has been reported to be useful for isolation of cells of the cardiac lineage. Hidaka et al. used PDGFRa and PrP as markers to improve the purity of the cardiomyocyte fraction.[16] The present data indicated that PrP$^+$ cells from EB of days 21 included undifferentiated cells. Thus, for clinical use, it is necessary to exclude undifferentiated cells. SSEA1 is expressed in mouse pluripotent stem cells and is their authentic marker. In the present study, PrP$^+$ /SSEA1$^-$ cells isolated from EB of day 21 included cardiomyocytes but not undifferentiated cells. Taken together, use of the PrP with SSEA1 might enable us to enrich the fraction of differentiated cardiomyocytes.

Clinical implication of the present study might be obvious. The transplantation of both *c-Kit*$^+$ cells isolated from the right atrial appendage[31] and cardiac progenitor cells from endomyocardial biopsies obtained from the right ventricular septum[32] had been reported to improve the cardiac function in patients with ischemic heart disease, whereas other stem-cell based therapies including human bone marrow stem cells or mesenchymal stem cells failed to do so.[33] This indicated the importance of enriching cardiac progenitor cells to exert positive effects in the clinical setting. The present provided a novel method to collect the cardiac progenitor cells from pluripotent stem cells and to exclude the undifferentiated cells, ensuring the feasibility as well as safety on the implantation of the cardiac progenitor cells from pluripotent stem cells.

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The authors declare no conflict of interest.

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