Increased mesodermal and mesendodermal populations by BMP4 treatment facilitates human iPSC line differentiation into a cardiac lineage

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Abstract

Human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) have attracted attention as a novel tool for drug safety screening and several differentiation protocols of hiPSC lines into cardiomyocytes have been reported; the standardization of these protocols will expand their applications for safety assessments such as “clinical safety trial-on-dish”. Bone morphogenetic protein 4 (BMP4) is an important factor in promoting mesoderm differentiation and BMP4 treatment has been used at the early stage of cardiac differentiation into different hiPSCs. In the present study, we evaluated the effects of BMP4 treatment at the early stage of cardiac differentiation. We performed gene expression profiling of the germ layer during mesoderm differentiation of hiPSCs derived from three different donors. The expression of T (a mesoderm marker) and GATA6 (an endoderm marker) increased and that of PAX6 (a neuroectoderm marker) decreased in pooled embryoid bodies (EBs) after BMP4 treatment. Single-cell gene expression analysis revealed that mesodermal and mesendodermal populations increased in EBs derived from 253G1. Finally, BMP4 treatment increased mesodermal and mesendodermal populations compared with that without BMP4 in two other hiPSC lines, confirming the reproducibility of multiple hiPSC lines. Thus, our results suggest that BMP4 treatment increases mesodermal and mesendodermal populations at the early stage of cardiac differentiation in different hiPSC lines.

Key Words: BMP4; iPS cell line; Mesoderm; Mesendoderm; Single-cell analysis

Introduction

Cardiotoxicity is a leading cause of failure in the clinical development of new drugs or withdrawal of approved drugs from the market[1]. In drug development, immortalized cell lines and animal models have provided useful information for predicting the risk of serious adverse events such as QT-prolongation and arrhythmia[2-3]. However, only a few models recapitulate human cardiac physiology; this can be attributed to the lack of native functions of human cell lines and differences between animals and humans. To overcome this issue, human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) have attracted attention as a novel system for drug safety screening, especially cardiotoxicity, because these cells possess contractile ability and express cardiac-specific genes similar to those of normal heart tissue[4-7]. hiPS-CMs are useful in assessing and predicting drug-induced arrhythmia or abnormal contractility[8-10]. Moreover, several teams, including ours, have recently demonstrated that hiPS-CMs from different donors can recapitulate the difference in susceptibility among individuals to a drug-induced QT-prolongation response[11, 12], facilitating a potential application of “clinical safety trial-on-dish” using hiPS lines derived from individuals with different drug susceptibilities. To meet the expectations of drug safety regulations using hiPS-CMs, cardiomyocytes must be reliably and effectively derived from multiple hiPSC lines. Several protocols have been reported for differentiation of different iPSC lines into cardiomyocytes; however, each protocol is different based on the treatment with different growth factors involved in major embryonic developmental processes[13, 14]. However, the contribution of each growth factor during cardiac differentiation has not been completely investigated.

Cardiomyocytes are derived from the mesoderm[15, 16] and for cardiac differentiation of hiPSCs, it is important to produce mesodermal cells at an early stage of the differentiation process[16]. Bone morphogenetic protein 4 (BMP4) is an important factor that promotes mesoderm differentiation[17, 18], and has been used in several previous protocols[19-23]. However, the treatment period, concentration, and/or cardiac differentiation rate were different in each protocol for differentiation of iPSC lines into cardiomyocytes. For instance, Kattman et al. demonstrated cardiac differentiation of four hiPSCs using BMP4 at 0.5–10 ng/mL concentrations in days 2–5 (without supplementing additional growth factors for 48 h after dissociation) of differentiation and the differentiation rate into cardiomyocytes was different among the cell lines (range: 10%–60%)[19]. In addition, Ren et al., demonstrated cardiac differentiation using BMP4 at 25 ng/mL concentrations in days 0–4 of differentiation, and the cardiac differentiation rate was approximately 40%[20]. Therefore, it is important to understand the contribution of BMP4 to the differentiation of multi hiPSC lines with different susceptibility and to provide insights on differentiation into mesoderm or cardiomyocytes.

Single cell analyses have attracted considerable attention because of their potential in identifying differences between individual cells in a seemingly homogeneous population[24, 25]. During mesoderm formation in mouse, the endoderm is also derived from the epiblast.
via gastrulation. After the formation of primitive streak (PS), it can be divided into the mesoderm and endoderm,[38] suggesting that individual hiPSCs induce the formation of mesoderm and endoderm in a heterogeneous population during a process recapitulating PS formation. Thus, to understand the effect of BMP4 on differentiation into mesoderm, it is important to investigate the population of cells, which differentiate into a cardiac lineage.

In the present study, we focused on the relationship between BMP4 and early differentiation of iPSCs into the three germ layers and investigated the expression of three germ layer marker genes in single cells to identify the type of cell population increased in the three germ layers of EBs treated with BMP4.

Materials and Methods

Human iPSC culture, cardiac differentiation, and isolation of simultaneously beating embryonic bodies

Three hiPSCs lines were used in this study. One hiPSC line (253G1) from the Kyoto University was maintained as described previously.[26, 27] The other hiPSCs (K20 and A3N) were generated from human blood, as described previously.[11] All donors provided written informed consent before participating in this study, and the study protocol was approved by the institutional review board of the Takeda Pharmaceutical Company Limited. All hiPSCs were maintained in Essential 8TM medium (E8, Invitrogen, CA). hiPSCs were induced into cardiomyocytes as previously described with slight modifications (Figure 1A).[28] Briefly, hiPSCs were isolated in 0.5 M EDTA (WAKO, Kanagawa, Japan) and they formed EBs during suspension culture in prime surface 90-mm dishes (Sumitomo Bakelite, Japan) with E8 containing 10 μM/ L Y-27632 (WAKO, JP) and 0–100 ng/mL BMP4, depending on the experiment (R&D systems, MN, USA) on day 0. After two days, the culture medium was changed to StemPro34 (Invitrogen, CA) containing 2 mM glutamine, 0.4 mM monothioglycerol (MTG) and 50 μg/mL ascorbic acid (Sigma, MO, USA). The following cytokines were also used at each differentiation stage in the same StemPro34 basic medium described above: days 2–3, 0–100 ng/mL BMP4, 5 ng/mL basic FGF (bFGF; ReproCell) and 3 ng/mL activin A (Peprotech); days 4–6, 150 ng/mL CKI-7 dihydrochloride (CKI-7; Sigma) and 10 ng/mL vascular endothelial growth factor (VEGF; Humanzyme, IL, USA); day 7 onwards, 10 ng/mL VEGF, 150 ng/mL CKI-7 and 5 ng/mL bFGF. During differentiation, the medium was replaced every 3–4 days. Cultures were maintained in a 5% CO2/95% air environment, and on day 30, beating EBs were counted under a microscope on a stage warmed to 37°C. This experiment was conducted repeatedly with three independent experiments for each hiPSC line.

mRNA expression in pooled EBs after BMP4 treatment in 253G1

RNA was isolated from EBs on day 2 using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm using a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the RNA samples were stored at -80°C until further assay. Reverse transcription (RT) into complementary DNA and the subsequent PCR were performed using 50 ng of total RNA from each sample using a high capacity cDNA RT kit (Life Technologies, Japan). Real-time PCR was performed using the Applied Biosystems 7900 fast real-time PCR (RT-PCR) system and Taqman gene expression assay kits (Applied Biosystems) for β-actin (ACTB: Hs09999903_m1), T (Hs00610080_m1), GATA binding protein 6 (GATA6: Hs00232018_m1), and paired box 6 (PAX6: Hs00240871_m1).

The RT-PCR cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles. mRNA expression level was analyzed using the comparative cycle threshold method with β-actin as the internal control and expressed as expression levels relative to those of the controls. This experiment was conducted repeatedly with three independent experiments for each hiPSC line.

mRNA expression in single cells after BMP4 treatment in 253G1, K20, and A3N

On day 2, EBs were dissociated using trypsin EDTA. Single cells were isolated using the C1 Single-Cell Auto Prep System (Fluidigm, South San Francisco, CA, USA) followed by RT and pre-amplification, according to the manufacturer’s instructions. The cells were loaded onto C1 Single-Cell Auto Prep IFC, and single-cell gene expression experiments were performed using the Applied Biosystems 7900 fast real-time RT-PCR system and Taqman gene expression assay kit, as described above. The results of cells not expressing ACTB were removed from the analysis. Genes that were not expressed were assigned a Ct of 40. Data were plotted as a heatmap of the Ct values without normalization. It is known that the expression of genes including that of the internal control gene in individual cells varies by 1000-fold. Therefore, in the present study, we used the Ct value without normalization to avoid misleading. These experiments were conducted repeatedly with three independent experiments for each hiPSC line.

Statistical analysis

The data were analyzed using Dunnet or Tukey test. The results with p values of < 0.05 were considered statistically significant. All statistical analyses were performed using SAS system version 8.2 (SAS Institute, Cary, NC)

Results and Discussion

Gene expression profile of hiPSCs differentiated into the three germ layers by BMP4 treatment in 253G1

Mesodermal cells are produced during cardiac differentiation of hiPSCs at the early stage of differentiation[16] and in the present study, BMP4 treatment was performed at the early stage of differentiation. To evaluate the effect of BMP4 treatment on differentiation of hiPSCs into mesoderm, we first investigated the expression level of germ layer marker genes in EBs at day 2 of differentiation from 253G1. BMP4 at 10 ng/mL concentration produced the highest percentage, 51.0 ± 10.4 %, of spontaneously beating EBs (Figure 1B). The size and shape of EBs were comparable between treatments with and without BMP4 (Figure 1C). Next, we investigated the expression of the marker genes for the three germ layers. The gene expression of T, a transcription factor transiently expressed in early mesoderm, and GATA6, an early endodermal lineage marker, was the highest in the 10 ng/mL group, which was the optimal concentration for differentiation into beating EBs as described above (Figure 1D and E). In contrast, the gene expression of PAX6, an ectoderm marker, was lower in the 0–1 ng/mL BMP4 treatment group than that in the 10–100 ng/mL BMP4 treatment group (Figure 1F). These results suggest that BMP4 induces the differentiation into both mesodermal and endodermal cells and inhibits the differentiation into ectodermal cells.
BMP4 facilitates human iPSC line differentiation into a cardiac lineage via increasing mesodermal/mesendodermal populations

Figure 1: Mesodermal differentiation after BMP4 treatment of 253G1. (A) The differentiation protocol for cardiomyocytes. (B) EB beating rate at 30 days after the initialization of differentiation with BMP4 treatment. (C) Phase contrast image of embryoid bodies (EBs) at 2 days after the initial BMP4 treatment. Bars = 500 μm. Gene expression levels of T (D), GATA6 (E), and PAX6 (F) at 2 days after BMP4 treatment. Gene expression data were normalized relative to 0 ng/mL. Data were then analyzed using Dunnett’s test. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 vs. 0 ng/mL. Data are presented as mean ± SD of independent experiments (n = 3).
Cell population in the three germ layers of EBs formed with BMP4 in 253G1

Next, to investigate whether T and GATA6 are expressed in a single cell or in a population of cells expressing T or GATA6, single-cell analyses were conducted. Interestingly, cell populations expressing T and/or GATA6 were observed in the BMP4 treatment group (Figure 2A). The number of cells expressing both T and GATA6 increased on day 2 of differentiation, whereas the population expressing PAX6 was comparable with that in the population not treated with BMP4 (Figure 2B). We calculated the proportion of the populations representing mesoderm, endoderm, mesendoderm, and ectoderm based on the expression of the germ layer markers. The mesodermal and mesendodermal populations were 31.1% and 35.8%, respectively, after BMP4 treatment, whereas no such population was detected without BMP4 treatment (Figure 2C). During the differentiation of human ES cells into mesoderm and definitive endoderm, an intermediate stage called mesendoderm appears recapitulating PS formation[16]. Moreover, in mouse development, mesodermal cells in PS co-express T and GATA6 on embryonic day 6.5[29]. Therefore, BMP4 treatment at early differentiation from hiPSCs increases the mesendodermal population, which co-expresses T and GATA6 and is important for efficiently inducing cardiac differentiation. Conversely, the differentiation into other cell types in BMP4 treatment was not clear.

Contribution to management of mesendoderm differentiation after BMP4 treatment in two hiPSC lines (K20 and A3N)

To evaluate the applicability of BMP4 as a key factor in increasing mesendodermal population for cardiac differentiation from hiPSCs, we investigated the relationship between effective differentiation and increasing mesendodermal population in two other iPSC lines. BMP4 treatment promoted efficient differentiation into spontaneously beating EBs in both lines. The optimal BMP4 concentrations for efficient cardiac differentiation were 2 and 7 ng/mL for K20 (66.8% ± 4.9%) and A3N (55.5% ± 28.8%) lines, respectively, and bell-shaped responses to BMP4 concentration were observed in both lines (Figure 3A). Each experiment to investigate the sensitivity to BMP4-induced cardiac differentiation was performed in replicates, and the results revealed that the reproducibility of efficient differentiation by optimal BMP4 was observed for each hiPSC line. Therefore, these results suggest that the BMP4 concentrations at the initial cardiac differentiation phase need to be optimized for each hiPSC cell line. Cell populations from both lines also expressed T and GATA6 as single or double positively with optimal BMP4 treatment (Figure 3B). Single-cell analysis demonstrated that the number of cells expressing T and GATA6 increased with BMP4 treatment at the optimal concentrations of 2 and 7 ng/mL in K20 and A3N lines, respectively, whereas the expression of PAX6 was comparable with that in cells without BMP4 treatment (Figure 3C). The proportion of BMP4-treated cell populations from K20 and A3N lines was 19.6% and 22.4% mesoderm and 32.1% and 53.4% mesendoderm, respectively (Figure 3D).

These results suggested that BMP4 contributed to key initial molecular events for differentiation into cardiomyocytes in each hiPSC line. Therefore, the concept could be employed for the differentiation of multiple hiPSC lines into cardiomyocytes.

*Figure 2: Mesodermal and mesendodermal population changes after BMP4 treatment of 253G1. (A) Heat map of single-cell gene expression patterns after BMP4 treatment. (B) Density dot plot of single-cell gene expression in embryoid bodies (EBs) at 2 days after BMP4 treatment. (C) Classification of populations after BMP4 treatment. Data are presented as three independent experiments. The total cell number in the 0 and 10 ng/mL treatment groups was 103 and 106 cells, respectively.*
Moreover, this finding might help efficiently define optimal BMP4 concentrations; an initial short-term screening of BMP4 can be completed in a 48-h protocol and used to initiate a full-scale (i.e., 10 days) differentiation protocol using the identified optimal BMP4 concentration. On the contrary, it was not clear whether the differentiation of cells not expressing any genes was induced, and it has been reported that native differentiation propensity is different for each pluripotent stem cell[30]. Further studies to explain the differences in optimal BMP4 concentrations are required to elucidate the mechanism of cardiac differentiation using a large number of hiPSC lines.

Conclusion

We demonstrated that increasing mesodermal and mesendodermal populations with BMP4 treatment could contribute to early cardiac differentiation of different iPSC lines by single-cell gene expression profiling. An in-depth evaluation with other approaches such as single-cell RNA sequencing might provide a deeper understanding of the mechanism for different differentiation propensities of individual cells.

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Abbreviations

BMP4 : Bone morphogenetic protein 4
EB : Embryoid body
hiPS-CM : Human induced pluripotent stem cell-derived cardiomyocyte
Conflicts of Interests

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Additional Information

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