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GSK3 β inhibition activates the *CDX/HOX* pathway and promotes hemogenic endothelial progenitor differentiation from human pluripotent stem cells

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Abstract

WNT/ β -CATENIN signaling promotes the hematopoietic/endothelial differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs). The transient addition of a GSK3 β inhibitor (GSKi) has been shown to facilitate *in vitro* endothelial cell differentiation from hESCs/hiPSCs. Since hematopoietic and endothelial cells are derived from common progenitors (hemogenic endothelial progenitors [HEPs]), we examined the effect of transient GSKi treatment on hematopoietic cell differentiation from hiPSCs. We found that transient GSKi treatment at the start of hiPSC differentiation induction altered the gene expression profile of the cells. Multiple *CDX/HOX* genes, which are expressed in the posterior mesoderm of developing embryos, were significantly upregulated by GSKi treatment. Further, inclusion of the GSKi in a

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Contributions

K.K. conceived the study, performed most of the experiments, and analyzed the data. M.K., M.N. assisted with *in vitro* differentiation experiments. M.K., A.D., J.T., M.K.S., M.T., and A.U. established hiPSC lines in this study. K.K. and T.H. co-directed the project and wrote the manuscript.

Conflict of interest disclosure

The authors declare no conflicts of interest.

serum- and stroma-free culture with chemically defined medium efficiently induced HEPs, and the HEPs gave rise to various lineages of hematopoietic and endothelial cells. Therefore, transient WNT/ β -CATENIN signaling triggers the activation of the *CDX/HOX* pathway, which in turn confers hemogenic posterior mesoderm identity to differentiating hiPSCs. These data enhance our understanding of human embryonic hematopoietic/endothelial cell development and provide a novel *in vitro* system for inducing the differentiation of hematopoietic cells from hiPSCs.

Keywords

pluripotent stem cells; hematopoietic differentiation; GSK3 β inhibitor

Introduction

Human induced pluripotent stem cells (hiPSCs) are a promising source of cells for regenerative medicine, drug screening, and pathogenetic studies of incurable diseases [1]. In addition, hematopoietic cells derived from hiPSCs may be used for transfusion and immunotherapy. To support these applications, an efficient and reliable *in vitro* differentiation induction system for producing hematopoietic cells from hiPSCs is needed.

WNT/ β -CATENIN signaling promotes the hematopoietic differentiation of human embryonic stem cells (hESCs) [2]. Recent reports showed that endothelial differentiation from hESCs/hiPSCs was enhanced by transient treatment with a GSK3 β inhibitor (GSKi) [3,4]. However, the roles of WNT/ β -CATENIN signaling in hematopoietic/endothelial cell differentiation from hESCs/hiPSCs remain to be clarified. During gastrulation, epiblasts ingress through the primitive streak (PS) and give rise to mesoderm cells via the epithelial-to-mesenchymal transition (EMT) [5,6]. PS formation and EMT induction are severely impaired in mouse embryos lacking the Wnt/ β -catenin pathway [5,6]. Therefore, we hypothesized that WNT/ β -CATENIN signaling enhances the hematopoietic/endothelial differentiation of hESCs/hiPSCs by facilitating PS formation and EMT induction.

Here, we demonstrate that the transient addition of CHIR99021, a GSKi, greatly improved the differentiation of hiPSCs into hemogenic endothelial progenitors (HEPs) and hematopoietic cells. GSKi treatment also resulted in the upregulation of *CDX/HOX* genes, suggesting that WNT/ β -CATENIN signaling triggers the activation of the *CDX/HOX* pathway, which promotes hematopoietic/endothelial cell differentiation from hiPSCs.

Material and methods

The hiPSC lines are listed in Table S1. *In vitro* differentiation protocol of hiPSCs has been previously described [3] with some modifications (Fig. 1A). First, single cell suspension of hiPSCs (10^4 to 10^5) were put onto 60 mm culture dishes coated with growth factor-reduced Matrigel® (BD Biosciences, San Jose, CA) in mTeSR™1 (STEMCELL Technologies, Vancouver, BC, Canada) with 10 μ M rock inhibitor (rocki) (Y-27632, WAKO, Tokyo) (day-4). Two days later, medium was replaced to mTeSR™1 without rocki. On day 0, the cells were washed twice with PBS and cultured in STEMdiff APEL medium (STEMCELL Technologies) with or without 5 μ M GSKi (CHIR99021, WAKO). On day 1, the cells were

washed twice with PBS and cultured in STEMDiff APEL medium with 25 ng/mL human bone morphogenic protein 4 (BMP4, R&D systems, Minneapolis, MN). Next day, 40 ng/mL human vascular endothelial growth factor 165 (VEGF, R&D systems) was added. The effects of WNT inhibition were analyzed by addition of 150 ng/mL Dickkopf-related protein 1 (Dkk1, Peprotech Rocky Hill, NJ). On day 4 and 8, medium was replaced to STEMDiff APEL containing 300 ng/mL human stem cell factor (SCF, R&D systems), 300 ng/mL human Fms-related tyrosine kinase ligand (FLT3L, R&D systems), 10 ng/mL human interleukin-6 (IL-6, Peprotech), 10 ng/mL human interleukin-3 (IL-3, Peprotech), 50 ng/mL human granulocyte colony stimulating factor (G-CSF, Peprotech) and 25 ng/mL human BMP4. Use of this cytokine combination was originally described by Chadwick *et al.* [7].

The differentiated cells were harvested by Accumax cell dissociation reagent (Innovative Cell Technologies, San Diego, CA) and analyzed by FACSARIAIII (BD Biosciences) and LSRFortessa™ X-21 (BD Biosciences). The cells were also analyzed by culturing in MethoCult H4034 (STEMCELL Technologies). OP9 cells were used as described previously [8]. The cytokines and antibodies used are listed in Tables S2–S4. A microarray analysis was carried out using a 3D-Gene® Human Oligo Chip 24k (Toray, Tokyo, Japan). Detailed methods are provided in Supplemental Information.

Results and discussion

Upregulation of EMT-related genes and CDX/HOX genes by early GSKi treatment

First, we confirmed the nuclear accumulation of β -CATENIN in GSKi-treated hiPSCs (029L2) (Fig. 1B). Next, the GSKi was removed on day 1 of differentiation induction and BMP4 was added for mesoderm induction (Fig. 1A). We noticed morphological changes in the hiPSC colonies on day 2. At this time point, the GSKi-treated hiPSC colonies became loose and the cells at the periphery of each colony were scattered, whereas the cells in the untreated hiPSC colonies remained tightly packed (Fig. 1C).

To examine the gene expression profile of differentiated hiPSCs after early GSKi treatment, we performed a microarray analysis on day 2 of differentiation induction. Compared to the untreated cells, 217 genes, including EMT-related genes [9], were significantly upregulated (> 5.0 fold) in the GSKi-treated cells (Fig. 1D, Table S5). On the other hand, 148 genes, including ESC markers [10], were significantly downregulated (< 5.0 fold) by GSKi treatment (Fig. 1E, Table S6). The induction of EMT by GSKi treatment was confirmed based on downregulation of CD326 (EpCAM) and induction of CD56 (NCAM) (Fig. 1F).

Strikingly, all *CDX* genes (*CDX1*, 2, and 4) and six *HOX* genes were strongly upregulated by GSKi treatment (Fig. 2A). Since *Cdx* genes are expressed in the posterior mesoderm [11], early GSKi treatment appeared to induce posterior mesoderm cells from hiPSCs. Anterior *HOX* genes (*HOXB2* and *HOXA1*), cardiac lineage-related genes (*TBX3*, *HAND1*, and *NKX2-5*), and multiple mesoderm/mesendoderm markers were also upregulated by early GSKi treatment, while axial mesoderm markers such as *GSC*, *CER1*, *CHRD*, and *FOXA2* [12] were unaffected (Fig. 2B). Given the finding that GSKi treatment induces robust cardiac differentiation of hESCs [13], early GSKi treatment induced not only posterior mesoderm cells but also anterior/cardiac mesoderm cells.

The Wnt effector *Lef1* has been shown to regulate *Cdx* gene expression [14–16]. According to our data, four *WNT* family genes and *LEF1* were significantly upregulated by GSKi treatment (Fig. 2C). Given the finding that *Wnt* family genes are expressed in the posterior region of embryos [17], it is possible that early GSKi treatment induced the formation of posterior mesoderm cells from hiPSCs via the *LEF1-CDX* axis. Once the hiPSCs acquired posterior mesoderm cell identity, *WNT* expression was initiated; thus, canonical WNT/ β -CATENIN signaling could be activated even in the absence of a GSKi.

Hematopoietic differentiation following early GSKi treatment

The hematopoietic differentiation of hESCs/hiPSCs *in vitro* proceeds from CD34⁺CD43[−] HEPs to CD34⁺CD43⁺ hematopoietic progenitor cells, followed by the appearance of CD43⁺CD45⁺ hematopoietic cells [18,19]. To determine whether early GSKi treatment affects HEP differentiation from hiPSCs, 029L2 hiPSCs were treated with or without a GSKi (day 0–1, 0–2, or 0–4), BMP-4 (day 1–4), and VEGF (day 2–4). Intriguingly, on day 4, the percentage of CD34⁺CD43[−] HEPs increased dramatically from 0.2 to 19.8% following transient GSKi treatment (day 0–1) (Fig. 2D). GSKi treatment on day 0–2 was also effective for HEP induction, whereas continuous GSKi treatment was not (Fig. 2D). Consistent with previous data [20], continuous GSKi treatment augmented CD184 (CXCR4)⁺ endodermal progenitor production (Fig. 2D). Addition of a WNT inhibitor, Dkk1, inhibited the GSKi-mediated HEP differentiation and EMT (Fig. 2E), suggesting that WNT upregulation by GSKi is responsible for the HEP and EMT induction.

We next examined the effects of GSKi treatment (day 0–1) on the hematopoietic differentiation of hiPSCs. On day 4, a growth factor cocktail containing BMP4, SCF, FLT3L, IL-6, IL-3, and G-CSF [7] was added. On day 12, well-differentiated CD43⁺ cells and a large number of CD45⁺ cells were observed following GSKi treatment (Fig. 3A). In contrast, CD43⁺ cells were barely detectable without GSKi treatment (Fig. 3A). Next, we examined whether the transient GSKi treatment is effective in other hiPSC lines. Expectedly, the GSKi treatment (day 0–1) significantly enhanced CD34⁺CD43[−] HEP differentiation on day 4 and CD43⁺ hematopoietic cell differentiation on day 12 in four different hiPSC lines including TkDNSeV-2 [21] (Fig. 3B).

Colony assays revealed that 029L2 hiPSCs were differentiated into hematopoietic progenitor cells by GSKi (Fig. 3C). Notably, enucleated erythrocytes were detected, implying that these cells were derived from definitive hematopoiesis. These data proved that the transient GSKi treatment greatly promotes the hematopoietic differentiation of hiPSCs.

The hemogenic potential of GSKi-induced HEPs

To verify the hematopoietic/endothelial differentiation potential of the GSKi-induced CD34⁺CD43[−] HEPs, we isolated these cells on day 4 and cultured them on OP9 stromal cells in the presence of various growth factors (Fig. 3D). When IL-6 and SCF were added, the HEPs produced CD43⁺ hematopoietic cells (Fig. 3D). On the other hand, CD31⁺ endothelial cells were produced from the HEPs when VEGF was added (Fig. 3D). Furthermore, when GSKi-induced HEPs were cultured on OP9 cells in the presence of erythropoietin (EPO), thrombopoietin (TPO), or granulocyte/macrophage colony stimulating

factor (GM-CSF), CD235⁺ erythroid cells, CD41⁺ megakaryocytes, and CD11b⁺ myeloid cells were produced (Fig. 3D). Similar results were obtained when we used SM28 hiPSC line. Therefore, the GSKi-induced HEPs are capable to produce endothelial cells as well as multi-lineage hematopoietic cells.

According to the literature, enforced expression of *Cdx4* enhanced hematopoietic differentiation of mouse ESCs and in zebrafish [22,23]. In addition, it was recently shown that *HOXA9* promotes the hematopoietic differentiation of hESCs [24]. Thus, it is likely that these molecules are involved in the efficient induction of HEPs from hiPSCs by the transient GSKi treatment. In conclusion, we demonstrated that early GSKi treatment promotes the differentiation of hiPSCs into posterior mesoderm cells and activates *CDX/HOX* signaling, which facilitates the differentiation of hiPSCs into hematopoietic/endothelial cells. Our data highlight the pivotal role of WNT/ β -CATENIN signaling in human embryonic hematopoietic development. Our *in vitro* hematopoietic induction system for hiPSCs faithfully recapitulates early human hematopoietic development, including PS, EMT, and posterior mesoderm induction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effects of GSKi treatment on EMT of hiPSCs

(A) Schematic diagram of *in vitro* differentiation protocol of hiPSCs. hiPSCs were cultured in STEMDiff APEL with or without GSKi (day 0–1). GSKi was then removed and BMP4 (day 1–4) and VEGF (day 2–4) were added. On day 4 and day 8, medium was changed to STEMDiff APEL containing BMP4, SCF, FLT3L, IL-6, IL-3 and G-CSF. (B) Nuclear accumulation of β-CATENIN by GSKi. 029L2 hiPSCs were differentiated in STEMDiff APEL with or without GSKi for 1 day. Microscopic images are shown in left panels. In right panels, relative fluorescence intensities of β-CATENIN signals and Hoechst stains (white lines in left panels) were quantified. (C) Morphology of 029L2 hiPSC colonies treated with or without GSKi (day 0–1) followed by 1 day culture with BMP4. Scale bar = 200 μm. (D) EMT-related upregulated genes in the GSKi-treated hiPSCs. On day 2, RNA was collected from the differentiated 029L2 hiPSCs treated with GSKi (day 0–1) or without GSKi and subjected to microarray analysis. (E) ESC marker genes downregulated in the GSKi-treated 029L2 hiPSCs. (D, E) Fold changes in relative expression level (+GSKi/-GSKi) were shown. (F) 029L2 hiPSCs were differentiated in STEMDiff APEL with or without GSKi (day 0–1) followed by 3 day culture with BMP4 and VEGF as shown in Fig. 1A. On day 4, expressions of CD326 and CD56 were analyzed by FACS. Average percentages of CD56⁺ cells ($n = 4$) ± standard deviation (SD) were shown in the right panel (*, $P < 0.005$).

Figure 2. Effects of GSKi treatment on gene expression and HEP induction

(A) Upregulation of *CDX/HOX* genes in the GSKi-treated hiPSCs. (B) Expression of mesoderm/mesendoderm markers and axial mesoderm markers in the GSKi-treated hiPSCs. (C) Expression levels of *WNT* related genes in the GSKi-treated hiPSCs. (A–C) 029L2 hiPSCs were treated with GSKi (day 0–1) or without GSKi followed by BMP4 treatment. On day 2, RNA was collected and subjected to microarray analysis. Fold changes in relative expression level (+GSKi/-GSKi) were shown. (D) Induction of CD34⁺CD43⁻ HEPs by the transient GSKi treatment. 029L2 hiPSCs were differentiated in STEMDiff APEL with or

without GSKi, as indicated. On day 1 and day 2, BMP4 and VEGF were added as shown in Fig. 1A. On day 4, expressions of CD34, CD43, and CD184 were analyzed by FACS. (E) Effects of WNT inhibitor Dkk1 on the GSKi-induced HEP differentiation and EMT. 029L2 hiPSCs were differentiated in STEMDiff APEL with or without GSKi (day 0–1) followed by 3 day culture with BMP4 and VEGF as shown in Fig. 1A. Dkk1 was added as indicated. On day 4, expressions of CD34, CD43, and CD56 were analyzed by FACS. In D and E, average percentages ($n = 3$) $\pm$ SD were shown (*, $P < 0.005$).

Figure 3. Effects of GSKi treatment on hematopoietic cell differentiation of hiPSCs
(A) Effect of GSKi on hematopoietic cell differentiation of 029L2 hiPSCs. On day 4, growth factor cocktails were added and analyzed on day 8 and 12. (B) Percentages of CD34⁺CD43⁻HEPs on day 4 and CD43⁺ hematopoietic cells on day 12, differentiated from various hiPSC lines with/without transient GSKi treatment. 029L, 029G2 (a different clone of 029), SM28 (cord blood-derived), and TkDNSeV-2 (TkDN, embryonic fibroblast-derived) were used. Averages ($n = 3$) $\pm$ SD were shown (*, $P < 0.005$). (C) Hematopoietic colony assays. 029L2 hiPSCs were differentiated with/without transient GSKi treatment. Whole cells were collected and cultured in MethoCult H4034. After 10 to 14 days, hematopoietic colonies were scored. Average numbers of colonies from 10^5 cells ($n = 3$) $\pm$ SD were shown (*, $P < 0.005$). Some colonies from day 12-induced cells were collected and stained with DiffQuick solution. Arrowhead indicates enucleated erythrocytes. (D) Analyses of differentiation potentials of day 4 CD34⁺CD43⁻ HEPs. On day 4, HEPs from 029L2 and SM28 hiPSCs were isolated and cultured with OP9 cells for 10 to 16 days in the presence of cytokines as indicated.