SHORT REPORT

Collaboration between WNT and BMP signaling promotes hemoangiogenic cell development from human fibroblast-derived iPS cells

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Abstract

Induced pluripotent stem (iPS) cells are generated by nuclear reprogramming of mature cells to a pluripotent state, and show biological properties of embryonic stem (ES) cells. The observation that human (h)ES cells generate hemoangiogenic progeny, defined by their high-level expression of KDR and low-level expression of PDGFRα (KDR+PDGFRαlo) via WNT and BMP signaling during 5-8 days of differentiation in a serum-free environment led us to address how hiPS cells give rise to hemoangiogenic progeny. In the presence of WNT3a, four hiPS cell lines derived from human skin fibroblasts commonly generated KDR+ and/or PDGFRα+ progeny by day 8 of differentiation. Endogenous BMP signaling was required for the WNT3a-directed upregulation of hemoangiogenic cell development and the hemoangiogenic activity was confined in all cases to the KDR+PDGFRαlo fraction. Thus, iPS cells derived from human skin fibroblasts resemble hES cells in the generation of hematopoietic and endothelial cells in vitro.

Introduction

Only a year after Dr. S. Yamanaka and his colleagues first reported the induction of pluripotent embryonic stem (ES) cell-like cells from mouse fibroblasts by application of their unique gene-transfer-based nuclear reprogramming technology, and provided the name ‘induced pluripotent stem’ (iPS) cell (Takahashi and Yamanaka, 2006), human fibroblasts were successfully reprogrammed to pluripotent stem cells (Yu et al., 2007; Takahashi et al., 2007). Furthermore, by application of removable, non-viral and/or non-integrative vectors for gene transfer, or by replacement of (some) cDNAs with proteins and/or small molecules, the technology that depended initially on stable integration of four cDNAs has been developed to the stage where the iPS cells contain no transgenes (Okita et al.,...
hemogenic activity was invariably enriched in the although the overall efficiency and exogenous factor-
hematopoietic differentiation of all iPS cells tested, fibroblasts ( Yu et al., 2007 ). WNT signalling increased (foreskin)1, 2 and 4 from primary neonatal foreskin iPS(IMR90)2 from the fetal fibroblast cell line IMR90, and iPS established from fibroblasts at different stages of ontogeny: This question is addressed here with hiPS cell lines. However, it has not been demonstrated whether hiPS cells entirely hemoangiogenic activity generated at this stage. Like ES cells, iPS cells are capable of self-renewal and differentiation towards mature cells originating from all three germ layers, including cardiac, neural, and erythro-myeloid cells ( Hanna et al., 2007; Mauritz et al., 2008; Narazaki et al., 2008; Choi et al., 2009; Hirami et al., 2009; Karumbayaram et al., 2009; Niwa et al., 2009; Senju et al., 2009; Zhang et al., 2009). We have previously demonstrated the essential role of the WNT-BMP signalling network in directing hES cell differentiation toward precursor of hematendothelial lineages under serum-free conditions ( Wang and Nakayama, 2009 ). The KDR+PDGFRα lo precursors develop from days 3-5 of differentiation and represent the entire hemoangiogenic activity generated at this stage. However, it has not been demonstrated whether hiPS cells respond to WNTs and BMPs like hES cells during differentiation. This question is addressed here with hiPS cell lines established from fibroblasts at different stages of ontogeny: iPS(IMR90)2 from the fetal fibroblast cell line IMR90, and iPS (foreskin)1, 2 and 4 from primary neonatal foreskin fibroblasts ( Yu et al., 2007 ). WNT signalling increased hematopoietic differentiation of all iPS cells tested, although the overall efficiency and exogenous factor-dependency differed among clones. Furthermore, the hemogenic activity was invariably enriched in the KDR+PDGFRα lo progeny population. WNT effects depended on the activation of endogenous BMP signaling during differentiation.

Results

Exogenous WNT3a but not BMP4 promotes the development of hemogenic cells from hiPS cells

Based on the results with hES cells ( Wang and Nakayama, 2009 ), we hypothesized that WNT signaling would direct hiPS cells to differentiate to hematendothelial cells. Human iPS cells were differentiated to form an embryoid body (EB) with and without BMP4 or WNT3a, and the resulting EB cells were subjected to the OP9 co-culture (Fig. 1A). As for EBs derived from HES3 cells (Fig. 1C), those derived from iPS(foreskin)1 cells in the presence of WNT3a gave rise to CD45+CD33+ myeloid-like non-adherent cells on OP9 in two weeks (Fig. 1B). However, the EBs from hiPS cells differed in that a low level of CD45+CD33+ cells accumulated even when the EBs were formed in the absence of exogenous factor (Fig. 1B).

The quantitative effect of exogenous factor on hematopoietic differentiation of hiPS cells was determined by a hemogenesis assay: a brief co-culture on OP9 followed by counting of erythroid-myoeloid (EM) colony-forming cells (CFCs) ( Wang and Nakayama, 2009 ) (Fig. 1D). The hiPS-derived EBs formed in the presence of WNT3a (W) generated 148±28.6 EM-CFCs/105 EB cells. When BMP4 (B) was present during differentiation (Fig. 1D, middle panel), only 16.7±1.76 EM-CFCs/105 EB cells were produced. Similarly, the hES-derived EB cells gave rise to 117±10.3 (W) and 11.7±5.93 (B) EM-CFCs/105 EB cells, respectively (Fig. 1D, bottom panel). In contrast, consistent with the long-term OP9 co-culture experiments (Figs. 1B, C), the iPS-derived EBs formed in the absence of factor (None) generated more EM-CFCs (21.0±4.58/105 EB cells) than the hES-derived EBs (6.33±4.48/105 EB cells). Nevertheless, exogenous WNT3a, but not BMP4, upregulated the level of hemogenic activity during iPS(foreskin)1 cell differentiation. iPS(foreskin)4 cells produced a similar level of hemogenic cells when differentiated in the presence of WNT3a, although spontaneous generation was more pronounced (Supplemental Figure [S. Fig.] 1). These results indicate that fibroblast-derived hiPS cells resemble hES cells in their efficient generation of hemogenic cells in response to exogenous WNT3a.

WNT3a stimulates the generation of KDR+PDGFRα lo EB cells from hiPS cells

The hemoangiogenic activity developed from hES cells is confined to the KDR+PDGFRα lo EB cell fraction ( Wang and Nakayama, 2009 ). Therefore, we determined whether WNT signaling stimulates the generation of KDR+PDGFRα lo progeny from hiPS cells.

As for HES3 cells, the undifferentiated hiPS cells expressed KDR very weakly, and a minor fraction of them expressed PDGFRα (Figs. 2A and B, and S.Fig. 2, "Day 0"). Upon differentiation, KDR+PDGFRα + (black) and KDR+PDGFRα - (red) cells were upregulated/generated in the absence of exogenus protein factors ("None"). However, the emergence of the KDR+PDGFRα lo progeny (K’Plo, green) was minimal. When WNT3a was added from day 0 of differentiation, the KDR+PDGFRα lo fraction was preferentially upregulated from all hiPS and hES cell lines tested, albeit at varying levels ("W"). In this sense, the response of hiPS cells to exogenous WNT3a during differentiation was similar to that of hES cells. However, consistent with the results from hemogenic cell analysis (S.Fig. 1), iPS(foreskin)4 cells gave rise to a significantly higher level of the KDR+PDGFRα lo progeny spontaneously (S.Fig. 2 "None").

In the case of hES cells, the developmental stage for the hemogenic mesoderm/endothelium is between days 3 and 5 of differentiation. However CD45+ hematopoietic progenitor cells are not detected, even on day 8 when CD34+CD31+ cells are evident ( Wang and Nakayama, 2009 ). Similarly, by day 8 of differentiation of iPS(foreskin)1 cells in the presence of WNT3a, CD34+CD31+ EB cells were apparent (Fig. 2C, left panel), but CD45+ cells were never observed (right panel).
Figure 1  WNT3a promotes hematopoietic cell development from hiPS cells. (A) Scheme for detection of hemogenic activity using total EB cells. IPS(foreskin)1 cells and HES3 cells were differentiated in the presence of no factor (None), BMP4 (B), Noggin (N), WNT3a (W), or WNT3a+Noggin (WN) for 8 days. The resultant EB cells were sorted for PI-excluded cells (Live cells), which were then individually cultured on OP9 cells. (B, C) Hematopoietic cell development from the hiPS-derived EB cells (B) and HES3-derived EB cells (C) on OP9. Non-adherent hematopoietic cells that preferentially emerged from WNT3a-treated EB cells in 14 days (right photos) were collected and analyzed by flow cytometry for CD33 and CD45 (left panels). (D) Emergence of hemogenic activity. The OP9 co-culture was initiated with 2 × 10^5 EB cells, and all cells from each co-culture were subjected to the EM-CFC analysis on day 4. All erythro-myeloid colonies (arrow heads top photo) were scored. The numbers of EM-CFCs were averaged (independent experiment number [n] = 3) and plotted with error bars indicating±SEM, * P < 0.05, ** P < 0.005. Corresponding data obtained from iPS(foreskin)4 cells are shown in S.Fig. 1. (E) Induction of hemoangiogenic mesoderm gene expression. RNAs isolated from day 6 iPS(foreskin)1 EBs were directly subjected to real-time RT-PCR analysis using the designated primers. The relative expression levels were averaged (n = 4) and plotted with error bars indicating SEM, * P < 0.05, ** P < 0.005. Time-dependent expression of CDH5 from differentiating IPS(foreskin)1 and HES3 cells is shown in S.Fig. 1.
Figure 2  WNT3a promotes generation of the KDR^PDGFR\alpha^lo progeny from hiPS cells. (A, B) Flow cytometric analysis of Day 8 EBs. Left panels; iPS(foreskin)1 cells, iPS(IMR90)2 cells and HES3 cells were directly subjected to FACS analysis (Day 0), or were differentiated for 8 days in the absence (None) or presence (W) of WNT3a. EB cells from the latter were then subjected to FACS analysis. The KDR^PDGFR\alpha^+, KDR^PDGFR\alpha^- and KDR^PDGFR\alpha^lo cell populations are represented as black, red and green dots, respectively. The percentage of total EB cells in each fraction is shown in the same respective color. Right panels; the percentage of KDR^PDGFR\alpha^lo (K^P^lo) cells generated on day 0 (Day 0), and on day 8 of differentiation with no factor (None) or with WNT3a (W) is representative of 2–4 independent experiments. Corresponding data obtained from iPS(foreskin)4 cells are shown in S.Fig. 2. (C) Generation of CD34^CD31^CD45^ EB cells from hiPS cells. iPS(foreskin)1 cells differentiated in the presence of WNT3a for 8 days were subjected to FACS analysis for CD31, CD34 and/or CD45 expressing cells. (D) Effect of Noggin on the KDR^PDGFR\alpha^lo cell generation from hiPS cells. The iPS(foreskin)1 and HES3 cells differentiated in the presence of no factor (None), Noggin (N), WNT3a (W) and WNT3a+Noggin (WN) for 8 days were subjected to FACS analysis for KDR and/or PDGFR\alpha expressing cells. The percentage of K^P^lo cells in total EB cells was normalized against that with no added factor (None), then averaged (n=5 for iPS(foreskin)1, n=3 for HES3) and plotted as fold-induction with error bars indicating SEM, *P<0.05.
The WNT3a effects on differentiating hiPS cells depend on endogenous BMP signaling

BMP signaling mediates the stimulation by WNT of KDR⁺PDGFRα⁻ hemoangiogenic cell development from hES cells (Wang and Nakayama, 2009). Therefore, we addressed whether the WNT3a effects on hiPS cells also depended on endogenous BMP signaling. When the BMP inhibitor Noggin was added with WNT3a during iPS(foreskin)1 and iPS(foreskin)4 cell differentiation, the hemogenic activity induced by WNT3a alone was virtually abolished (Fig. 1D and S.Fig. 1, ‘WN’ and the WNT-upregulated portion of the KDR⁺PDGFRα⁻ cell population was reduced to the basal level by Noggin (Fig. 2D and S.Fig. 2, ‘WN’). Furthermore, Noggin completely inhibited the induction by WNT3a of the hemoangiogenic cell marker transcripts, CDH5 and TAL1 (Fig. 1E, ‘WN’). These data indicate that the effects of WNT3a on differentiating hiPS cells require the involvement of BMP signaling, probably activated by endogenous BMPs.

Gene expression profile of the hiPS cell-derived KDR⁺PDGFRα⁻ progeny

The lineage of cells included in the KDR⁺PDGFRα⁻ fraction developed in the presence of WNT3a from hiPS cells was determined by fluorescence-activated cell sorting (FACS) and reverse transcription-polymerase chain reaction (RT-PCR). We compared the levels of lineage-specific transcripts in each of the four sorted fraction: KDR⁺PDGFRα⁻ (double negative, or ‘DN’), KDR⁺PDGFRα⁺ (K⁺P⁺), KDR⁺PDGFRα⁻ (double positive or ‘DP’) and KDR⁺PDGFRα⁻ (K⁺P⁻), derived from differentiated HES3 cells (Fig. 3 and S.Fig. 3, white bar), iPS(foreskin)1 cells (black bar), and iPS(foreskin)2 cells (dark gray bar). The POU5F1 (OCT4) transcript, a marker for undifferentiated pluripotent stem cells, was detected at high levels in undifferentiated hES (‘ES’) and hiPS (‘iPS’) cells, and very low levels in all other sorted fractions (S. Fig. 3). The early mesoderm transcripts T and MESP1, and the lateral plate/extraembryonic mesoderm transcript FOXF1 were found in all KDR⁺ and/or PDGFRα⁻ cells with
significantly higher expression in the DP cell fraction (Fig. 3). These results suggest that WNT3a induces differentiation of hiPS cells towards mesodermal lineages. In addition, the expression of hemoangiogenic cell marker genes such as TAL1, CDH5, RUNX1 and GATA2 (Fig. 3), and MYB and CD34 (S. Fig. 3) was detected almost exclusively in the KDR⁺PDGFRα⁻ fraction, suggesting that this fraction is enriched in the hemogenic activity. Essentially the same results were obtained from iPS(foreskin)4 cells (S.Fig. 3C). Thus, the gene expression profile of the iPS-derived KDR⁺and/or PDGFRα⁺ progeny is almost identical to that of the hES-derived progeny when generated in the presence of WNT3a.

**The hemoangiogenic potential of the WNT3a-induced KDR⁺PDGFRα⁻ progeny from hiPS cells**

Transcript analyses predicted that WNT3a-induced KDR⁺PDGFRα⁻ progeny from hiPS cells could generate hematopoietic progenitor cells (Fig. 3). The FACS-purified EB fractions derived from iPS(foreskin)1, 2 and 4 cells were therefore individually subjected to EM-CFC and hemogenesis assays (Wang and Nakayama, 2009). Only a few erythro-myeloid colonies arose from any of the fractions tested (Fig. 4A right panels and S.Fig. 4, white bar). In contrast, the KDR⁺PDGFRα⁻ cell fraction showed the exclusive ability to give rise to many erythro-myeloid colonies after the 4-day culture on OP9 (black bar). Thus, while the absolute numbers of the EM-CFC derived from the WNT3a-induced KDR⁺PDGFRα⁻ progeny varied among different hiPS cell lines, the unique potency of the KDR⁺PDGFRα⁻ progeny to generate hematopoietic progenitors was preserved.

Finally, we asked whether the hiPS cell-derived KDR⁺PDGFRα⁻ progeny were able to generate endothelial cells in culture. The KDR⁺PDGFRα⁻ cells generated from iPS (foreskin)1, 2 and 4 cells in the presence of WNT3a were individually cultured on OP9 cells and endothelial cell colonies were visualized via the marker CD31 and CDH5 (Fig. 4B and S. Fig. 4). The number of positive colony was scored to quantify the endothelial cell precursors (colony forming unit-endothelial cell, or CFU-End). The data clearly showed that the WNT3a-induced-KDR⁺PDGFRα⁻ progeny from all hiPS cell lines generated endothelial cell precursors, despite variation in the absolute numbers of CFU-End among hiPS cell lines.

**Discussion**

We have shown that hiPS cells established from human skin fibroblasts by stable integration of four reprogramming genes: OCT4, NANOG, SOX2 and LIN28 (Yu et al., 2007) resemble hES cells in differentiating toward precursor cells of the hematoendothelial lineage. Blood cell formation from miPS and hiPS cells has been reported (Hanna et al., 2007; Choi et al., 2009; Niwa et al., 2009). However, we have
demonstrated for the first time that the signaling requirement for hiPS cells to generate hemoangiogenic progeny is similar to that for hES cells; WNT signaling promoted the process in cooperation with endogenously activated BMP signaling (Figs. 1 and 2) (Wang and Nakayama, 2009).

The dependency of hiPS cells on exogenous WNT3a was, however, less stringent than that for hES cells, as seen by the more pronounced spontaneous generation of the KDR/PDGFRα progeny during differentiation in the absence of exogenous protein factor (Figs. 1 and 2) with hiPS cells than the hES cells tested. In agreement with Choi, et al (2009), we found that production of hemogenic cells in response to exogenous factors such as WNT3a also differed significantly among the hiPS cell lines tested, even though they were derived from essentially the same fibroblast cell type and maintained and differentiated by the same methods. We suggest that among the major causes of the spontaneous differentiation and clonal variation is the difference in the level of WNTs and/or BMPs produced endogenously during differentiation, as is the case for hES cells where the spontaneous differentiation as evident by the PDGFRα cell genesis is markedly reduced by Noggin or DKK1 (Wang and Nakayama, 2009). However, the capacity for factor-independent differentiation may also contribute to these differences, and may already have been determined at the stage of maintenance culture. In fact, the effect of Noggin on spontaneous genesis of the PDGFRα progeny from hiPS cells was not marked (data not shown).

The differences between iPS cells and ES cells are attributable, at least in part, to reactivation of transgene expression during differentiation in the former. It remains to be determined whether differences in the integration sites, copy numbers, and expression levels of the transgenes contribute to clonal variation and influence both factor-dependent and factor-independent differentiation. Different extents of reprogramming (Mikkelsen et al., 2008), differences in the genetic and epigenetic background of cells to be reprogrammed, and/or random mutations potentially introduced during establishment of iPS cells would also cause such variations, some of which are also attributed to the clonal difference found among hES cell lines (Osafune et al., 2008). In this context, it will be interesting to examine whether iPS cell lines arising from a transgene-free reprogramming method show more uniform in vitro differentiation, and whether the lineage of cells used for reprogramming corresponds to a specific differentiation property of the resulting iPS cells.

Nevertheless, the signalling principle established with hES cells for the efficient genesis of hemoangiogenic cells seems to be applicable to hiPS cells, confirming that hES cells are a useful model not only for the study of early developmental events occurring during human embryogenesis, but also as the "gold standard" pluripotent stem cells to establish a universal differentiation protocol applicable to a variety of iPS cells.

Materials and methods

Pluripotent cells, maintenance culture, and differentiation culture

HES3 hES cells were grown in bulk culture on irradiated primary mice embryonic fibroblasts (MEFs) in SR medium supplemented with 4 ng/ml human fibroblast growth factor 2 (FGF2, R&D Systems. Minneapolis, MN) (Wang and Nakayama, 2009). The human fibroblast-derived iPS cell lines: iPS (foreskin)1, 2 and 4, and iPS(IMR90)2, were kindly provided by J. Thomson (Madison, WI), and maintained on MEFs in SR medium supplemented with 100 ng/ml FGF2 (Yu et al., 2007). Differentiation of hES and hiPS cells was induced in the Knockout Serum Replacement (KSR)-based serum-free differentiation medium containing 0.9% (w/v) methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) with various factors, and incubated at 37 °C under 5% CO2, 5% O2 (Wang and Nakayama, 2009). Human BMP4 and human/mouse Noggin-Fc (R&D), and mouse WNT3a (Australian Stem Cell Centre) were added at 10, 500 and 100 ng/ml, respectively.

Flow cytometry

EBs were harvested and dissociated into single cells that were stained with mouse anti-human PDGFRα (BD Biosciences, North Ryde, NSW Australia) and anti-human FLK1/KDR (RELIAtech, Braunschweig, Germany) monoclonal antibodies, and analyzed or sorted for PDGFRα and KDR markers (Wang and Nakayama, 2009). Live cells were pre-gated with propidium iodide (PI, Sigma-Aldrich, St Louis, MO). For total EB cell assays, live EB cells were collected by FACS as PI-excluding cells.

Hemoangiogenesis assay using OP9 stromal cells

The FACS-fractionated EB cells (2.5×10⁴) or FACS-purified total EB cells (1-2×10⁶) were plated onto 80-90% confluent OP9 cells and the co-culture was performed in the presence of human stem cell factor (hSCF, Amgen, Thousand Oaks, CA), human thrombopoietin (hTPO, Millipore, Temecula CA), human stem cell factor (hSCF, Amgen, Thousand Oaks, CA), human interleukin-3 (hIL3, Millipore), human granulocyte-macrophage colony-stimulating factor (hG-CSF, Millipore) (Wang and Nakayama, 2009). For the hemogenesis assay, the whole co-cultures were harvested by a brief TrypLE-Select (Invitrogen, Mount Waverley VIC Australia) treatment on day 4, followed by EM-CFC analysis.

Erythro-myeloid colony-forming cell (EM-CFC) analysis

Freshly isolated or OP9-co-cultured EB cells were added to serum-free CFC medium (Nakayama et al., 2003), supplemented with hIL3, hSCF, human granulocyte colony stimulating factor (hG-CSF, Millipore), hGM-CSF, hL6 and human erythropoietin (hEPO, Millipore), then divided into 2–4 35-mm bacterial-grade dishes, and maintained at 37 °C under 5% CO2, 5% O2 (Wang and Nakayama, 2009). On day 11, total colonies including colony-forming unit erythroid (CFU-E), burst-forming unit erythrocyte (BFU-E), colony-forming unit macrophage/monocyte (CFU-M), colony-forming unit granulocyte (CFU-G), and erythroid-monocyte/macrophage mixed colony-forming unit (CFU-EM) were counted. Images of colonies were acquired as described (Wang and Nakayama, 2009).
Endothelial colony-forming cell analysis

1.25 × 10^4 FACS-fractionated EB cells were seeded onto OP9 cells, and cultured for 7–10 days in the presence of hSCF, hIL3, hTPO and hGM-CSF. The cultures were fixed with ethanol for indirect immunofluorescence staining with the mouse anti-human CD31 and CDH5 antibodies (DAKO, Glostrup, Denmark), and fluorescent colonies were scored as described (Wang and Nakayama, 2009).

Real-time RT-PCR analysis

Total RNA was prepared, DNase I-treated, and reverse transcribed into cDNA. Real-time quantitative PCR was performed using the ABI 7500 (Applied Biosystems, Scoresby, VIC Australia). The eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) gene was used as a reference housekeeping gene, and the relative expression values were calculated as described (Wang and Nakayama, 2009).

Statistical analysis

Differences between groups were compared using the Student t test (KaleidaGraph, Synergy Software, Reading, PA). Results were considered not significant (ns) at P values of 0.05 or greater.

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Appendix A. Supplementary data

Supplementary data associated with this article (S.Figs) can be found, in the online version, at doi:10.1016/j.scr.2010.03.002.

References


Wang, Y., Nakayama, N., 2009. WNT and BMP signaling are both required for hematopoietic cell development from human ES cells. Stem Cell Res. 3, 113–125.