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RESEARCH ARTICLE

Prostaglandin E2 promotes hematopoietic development from human embryonic stem cells

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Abstract Recent studies have suggested that prostaglandin (PG) E2 (PGE2) and the prostaglandin pathway are essential for hematopoietic stem cell growth and development. However, similar studies on hematopoietic commitment from human embryonic stem cells (hESCs) are still limited. Here we report that the addition of PGE2 promotes hematopoietic differentiation of hESCs. The induced cells from hESCs/OP9 co-culture and in the presence of PGE2 were characterized by reverse transcription-PCR (RT-PCR), flow cytometry, colony-forming assays and Wright-Giemsa staining. Our results demonstrated that PGE2 exposure could alter the gene expression pattern and morphology of co-cultured hESCs and resulted in a robust hematopoietic differentiation with higher frequencies of $CD34^+$ and $CD45^+$ cells. Furthermore, the Smad signaling pathway may be involved in PGE2 and OP9 induced hematopoietic differentiation of hESCs. This research may improve our knowledge of stem cell regulation and hopefully lead to better stem cell-based therapeutic options.

Keywords human embryonic stem cells, prostaglandin E2, hematopoiesis, *in vitro* differentiation

1 Introduction

Prostaglandin E2 (PGE2) is the most abundant eicosanoid and a mediator of numerous physiological and pathological processes (Miller, 2006). Many studies have shown that PGE2 participates in regulation of hematopoiesis (Frisch et al., 2009; Hoggatt et al., 2009). In the synthesis of biologically active prostaglandins, cyclooxygenases (Cox-1 and Cox-2) catalyze the key step (Smith, 2000). The Cox1 and Cox2 genes were found to be highly expressed during the onset of definitive hematopoiesis, which aroused heightened interest in the involvement of prostaglandin in this process. Recently, researchers uncover a crucial role for PGE2 in hematopoietic stem cell growth and development not only in embryonic, but also in adult stem cell homeostasis in both simple and complex vertebrate systems (Liou et al., 2007; North et al., 2007). These findings have attracted much attention to the possible role of the prostaglandin pathway in human hematopoietic development. Because human embryonic hematopoietic development is inaccessible for *in vivo* study, an *in vitro* model allowing the study of this process at both the cellular and molecular levels is necessary (Lu et al., 2004; Wang et al., 2005; Srivastava et al., 2007; Liu et al., 2009). The success of hematopoietic lineage differentiation from human embryonic stem cells (hESCs) makes it possible to study early hematopoietic regulation *in vitro* (Lu et al., 2008; Ma et al., 2008). In the current study, the role that PGE2 plays in hESC hematopoiesis is explored by using the *in vitro* differentiation model.

In general, the development of myeloid cells from hESCs requires stages of differentiation: mesoderm patterning, hematopoietic stem cell differentiation and lineage derivation. Bone morphogenetic protein (BMP) and Wnt signal pathways are well-documented pathways that regulate hematopoiesis. It is known that BMPs determine early mesoderm commitment (Zhang et al., 2008), while Wnt signaling regulates hematopoietic stem cell (HSC) formation and later hematopoietic homeostasis (Naito et al., 2006). Since PGE2 has been demonstrated to regulate vertebrate hematopoiesis (North et al., 2007), it is possible that PGE2 exerts its effects through these signal pathways. Recently, the interaction between PGE2 and Wnt signaling during HSC formation has been confirmed

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(Goessling et al., 2009); however, the involvement of other signaling molecules is still mysterious. Smads are key mediators of BMP signaling. Upon activation, receptor-activated Smads (R-Smads; Smad1, 2, 3, 5, and 8) are phosphorylated, associate with the co-Smad Smad4, and translocate to the nucleus, where they activate target gene expression (Larsson and Karlsson, 2005). Previous studies have shown that, in endothelial cells, PGE2 could function by phosphorylating R-Smads (Alfranca et al., 2008). Because Smad proteins have been shown to be involved in both BMP and PGE2 signaling, the expression and activation of Smads are checked for the molecular mechanism of PGE2 functions. In the present study, we show that a long-acting derivative of PGE2, 16,16-dimethyl-PGE2(dmPGE2), promoted hematopoietic development from hESCs, and the effect was mediated via the Smad signaling pathway.

2 Materials and methods

2.1 Cell culture

Human embryonic stem cell line H9, at passages 20 and 24 (Bhattacharya et al., 2004), was obtained from the WiCell Research Institute Inc. (Madison, WI, USA). Regularly, hES cells were thawed and plated into one well of a six-well tissue culture plate (Nunc, Sigma-Aldrich, Oakville, ON, Canada) on irradiated (25 Gy) mouse embryonic fibroblast (MEF) feeder cells. The cell culture medium contained knock-out Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% serum replacer (SR), 1% nonessential amino acids (NEAA), 1 mmol/L L-glutamine (all from Invitrogen Corporation, Carlsbad, CA, USA), 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich) and 4 ng/mL human basic fibroblast growth factor (bFGF) (R&D Systems, Inc., Minneapolis, MN, USA). The hES cells were passaged approximately every 5–6 days to sustain undifferentiated growth, and were used between passages 40 and 65.

MEF cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

The OP9 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and was maintained in OP9 growth medium consisting of α-modified minimum essential media (α-MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 20% non-heat-inactivated defined fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 0.1 mmol/L NEAA, and 2 mmol/L L-glutamine (glu).

2.2 Immunofluorescence analysis

Immunofluorescence staining of tumor rejection antigens (TRA)-1-60, TRA-1-81 and the core transcription factor (Oct4) was carried out similarly as described (Bhattacharya et al., 2004) and detected with fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-labeled secondary antibodies. HESC colonies on chamber slides (Lab-Tek, Nunc, Thermo Fisher Scientific, Rochester, NY, USA) were fixed in 4% paraformaldehyde at room temperature for 15 min. The cells were then washed with PBS three times and blocked with 5% normal goat serum at room temperature for 1 h, followed by incubation with primary antibodies at 4°C overnight. For Oct4 staining, cells were permeabilized by 0.1% Triton X-100 for 20 min at room temperature before antibody incubation. Antibodies against human Tra-1-60, Tra-1-81, and Oct4 (all from Chemicon Inc., Danvers, MA, USA) were used and localized with FITC- or TRITC-conjugated secondary antibodies (Santa Cruz, CA, USA). 4',6-diamino-2-phenylindole (DAPI) (5 μg/mL) was added before acquiring images.

2.3 Alkaline phosphatase staining

Self-renewal of hESCs was determined by morphological assessment and alkaline phosphatase (AP) staining. ES cell colonies were photographed under an Olympus microscope equipped with a digital camera (Nikon). The AP activity of hESCs was detected as follows. First, cells were washed three times with ice cold PBS, fixed in 4% paraformaldehyde at room temperature for 15 min, and then stained with BCIP/NBT (Promega, Madison, WI, USA) at room temperature in the dark for 15–20 min. The undifferentiated hESC colonies that showed high levels of AP activity were scored.

2.4 Hematopoietic differentiation of hES cells

For hES cell differentiation, the OP9 cells were plated onto gelatinized 6-well plates in OP9 growth medium. After formation of confluent cultures on day 4 and 5, half of the medium was changed, and cells were treated with mitomycin C (Sigma-Aldrich) for 2 h. Undifferentiated hESCs were harvested by treatment with 1 mg/mL collagenase IV (Invitrogen) and dispersed by scraping to maintain the cells in small clumps. Concurrently, hES cell cultures growing under the same conditions were used to obtain a single-cell suspension for counting. The hES cells were added to OP9 cultures at a density of 1×10^5/2 mL per well of a 6-well plate in α-MEM supplemented with 10% FBS (HyClone) and 100 μm monothioglycerol (MTG; Sigma, St Louis, MO, USA). PGE2 (Cayman) was added at day 0 at indicated concentrations. The hES cell/OP9 co-cultures were incubated for up to 9 days at 37°C in normoxic conditions and 5% CO_2 with a half-medium change on day 2, 4, 6, and 8. Cells were harvested on certain days, and single-cell suspensions were prepared by treatment of the hES cell/OP9 co-culture with collagenase IV (Invitrogen; 1 mg/mL in α-MEM) for 15 min at 37°C.

Cells were washed twice with phosphate-buffered saline (PBS)-5% FBS, filtered through a 100 μmol/L cell strainer (BD Biosciences, Palo Alto, CA, USA), counted, and used for flow-cytometric analyses, clonogenic assays and gene expression analyses.

2.5 Gene expression analysis by RT-PCR

The expression of hematopoiesis-inductive transcription factors and hematoendothelial specific genes, including SCL, GATA-1, Runx1, Brachyury, CD34, VE-cad and vWF, were examined with reverse transcription-PCR (RT-PCR). All PCR reactions were performed as follows: 95°C for 5 min; 94°C for 40 s; annealing at various temperatures for 40 s, and 72°C for 40 s (25 cycles); 72°C for 10 min, and 4°C for 5 min. The forward and reverse primers used, the length of their PCR products and the annealing temperatures are described in Table 1.

2.6 Flow cytometric analysis

The phenotype of the hESCs and expression of indicators of differentiated hematopoietic cells, such as CD34 and CD45, were analyzed by flow cytometry. The cells were dissociated with 0.25% trypsin-EDTA and resuspended in PBS with 10% goat serum for 15 min at 4°C. The cells were then fixed with 4% paraformaldehyde at room temperature for 15 min. After fixation and wash with PBS, the hESCs were incubated with stage-specific embryonic antigen (SSEA)-4 antibody (Chemicon Inc., Danvers, MA, USA) and then with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz). The hESC-derived hematopoietic cells were incubated with FITC-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies: anti-human CD34 and anti-human CD45 (BD Biosciences, San Jose, CA, USA) at 4°C for 30 min. The cells were then washed three times with PBS and analyzed by flow cytometry analysis using the FACSCalibur (Becton-Dickinson, Mountain View, CA, USA).

2.7 Positive selection of $CD34^+$ cells by magnetic sorting

A single-cell suspension from certain days of hESC/OP9 co-culture was labeled with CD34 paramagnetic monoclonal antibodies (mAbs) using Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech, Auburn, CA, USA) as recommended by the manufacturer and processed through an LS + separation column attached to a Midi-MACS separation unit (Miltenyi Biotech) to obtain the magnet-retained fraction of purified $CD34^+$ cells. Purity of isolated $CD34^+$ cells, as determined by flow cytometry, was generally more than 90% at a single column run, and cell viability, as evaluated by trypan blue exclusion, was always higher than 95%.

2.8 Hematopoietic colony-forming assays

Hematopoietic colony-forming assays were performed in 35-mm low adherent plastic dishes using a 2 mL/dish of

Table 1 The primers for RT-PCR

gene accession no.	product/bp	primer sequence
Oct4 NM_00173531	123	5′AACCTGGAGTTTGTGCCAGGGTTT3′ forward 5′TGAACTTCACCTTCCCTCCAACCA3′ reverse
Brachyury NM_003181	300	5′ GCGGGAAAGAGCCTGCAGTA3′ forward 5′ TTCCCCGTTCACGTACTTCC3′ reverse
RUNX1 NM_001754	170	5′ATGTGGTCCTATTTAAGCCAGCCC3′ forward 5′TCATCTGGCTGAAGACACCAGCTT3′ reverse
CD34 NM_001773	201	5′CCTAAGTGACATCAAGGCAGAA3′ forward 5′GCAAGGAGCAGGGAGCATA3′ reverse
GATA1 NM_002049	197	5′ TTAGCCACCTCATGCCTTTCCCT3′ forward 5′ CCAGAGACTTGGGTTGTCCAGAAT3′ reverse
SCL NM_003189	259	5′ TCTCTCGGCAGCGGGTTCTTT3′ forward 5′ CCAGGCGGAGGATCTCATTCTT3′ reverse
γ-Globin NM_000559	370	5′ CGCTTCTGGAACGTCTGAGGTTAT3′ forward 5′ CCAGGAGCTTGAAGTTCTCAGGAT3′ reverse
Ang1 NM_001146	200	5′ GGGGGAGGTTGGACTGTAAT3′ forward 5′ GAATAGGCTCGGTTCCCTTC3′ reverse
vWF NM_000522	366	5′ CCCACCCTTTGATGAACACA3′ forward 5′ CCTCACTTGCTGCACTTCCT3′ reverse
GAPDH NM_002046	110	5′CTGACTTCAACAGCGACACC3′ forward 5′TGCTGTAGCCAAATTCGTTGT3′ reverse

MethoCult GF + H4434 semisolid medium (Stem Cell Technologies) consisting of 1% methylcellulose, 30% FBS, 1% bovine serum albumin (BSA), 50 ng/mL stem cell factor (SCF), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng/mL interleukin-3 (IL-3), 10 ng/mL interleukin-6 (IL-6), and 3 units/mL erythropoietin. Sorted $CD34^+$ cells were plated at 1×10^5/mL. Undifferentiated hES cells were tested at densities up to 5×10^5/mL, and no colony forming cells (CFCs) were found. All hematopoietic colony-forming assays were performed in duplicate. After 12–14 days, CFCs were scored according to their cellular morphology.

2.9 Cytospin and Wright-Giemsa staining

Colonies picked from methylcellulose were dropped onto slides and fixed for 20 min in 4% paraformaldehyde. Then they were stained with Wright-Giemsa reagents (Fisher Scientific) following manufacturer's instructions.

2.10 Western blotting

Co-cultured hESC/OP9 cells treated with or without PGE2 were analyzed for the Smads protein by Western blotting. Cells were lysed with RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS; Sigma) supplemented with 1 μg/mL protease inhibitors, and incubated on ice for 30 min. The samples were centrifuged for 30 min at 13 000 *g* and the supernatant was filtered and stored at −80°C. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to polyvinylidene difluoride (PDVF) membranes (Sigma). The membranes were blocked for 1 h in 5% non-fat skim milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (T-TBS) and incubated with the anti-human Smad antibody (Santa-Cruz, Biotechnology Inc., Santa-Cruz, CA, USA) overnight at 4°C. After washing in T-TBS, membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. The immunoreactive bands were visualized by enhanced chemiluminescence (Santa-Cruz).

2.11 Statistical analysis

Results were expressed as means±SD. Statistical significance was determined using an unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$.

3 Results

3.1 Culture of human ES cells

We established *in vitro* cultures of hESCs to study molecular mechanisms involved in the formation of early hematopoietic progenitors. The experiments were done using NIH-approved hES cell line H9 (code WA09). Human ES cells were maintained in the undifferentiated state by irradiated murine feeder layer as described in **Materials and methods**. In this culture condition, we obtained stably proliferating hESCs, which formed typical, well-defined hESC colonies (Fig. 1A). The undifferentiated hESC colonies exhibited high levels of alkaline phosphatase activity (Fig. 1B), and were positive for antibodies against Oct4, TRA-1-60, and TRA-1-81 (Fig. 1C). Flow cytometry analysis of hESCs showed positive expression of SSEA-4, TRA-1-81 and negative expression of CD34 and CD45 (Fig. 1D). These results showed that hESCs were maintained in an undifferentiated state.

3.2 Semisolid culture assay

As shown previously, after 9 days of hESC/OP9 co-culture, hESCs could differentiate into hematopoietic progenitors as detected by multi-lineage hematopoietic CFU potential (Vodyanik et al., 2005). To determinate the effects of PGE2 on the differentiation of hESCs, we seeded 1×10^5 cells in methylcellulose to functionally assess the frequency of hematopoietic progenitors. Addition of PGE2 at concentrations of 10 ng/mL and 20 ng/mL increased all colony forming units, although a higher dosage of PGE2 at 100 ng/mL displayed cytotoxicity. The best effect of PGE2 showed at 20 ng/mL. Under this circumstance the total number of derived CFU colonies was increased by 1.4-fold, as compared with the control colony growth (185 ± 15.3 *vs* 132 ± 12.9, $P < 0.05$) (Fig. 2A). As such, we chose to use 20 ng/mL PGE2 in all subsequent experiments. For the two groups, hematopoietic-like colonies were first found after 6 days of co-culture, reached their highest number at day 14, and then decreased. The frequency of colony-forming cells produced from hESC/OP9 treated with PGE2 was much more than that from hESC/OP9 without PGE2.

For further understanding of the characterization of the hematopoietic clusters from hESC/OP9 co-culture, Wright-Giemsa staining was performed. The CFU-GM, CFU-M, CFU-GEMM and CFU-E colonies obtained from hESC/OP9 treated with PGE2 could be seen, as shown in Fig. 2B. Different from previous reports, the number of the erythroid colonies observed was not more than the other kind of colonies (Vodyanik et al., 2005).

3.3 Exposure to PGE2 improves the survival, proliferation and differentiation of hESCs co-cultured with OP9

To determine if differentiation occurred, the hESC/OP9 co-cultures treated with or without PGE2 were observed under a light microscope, throughout the co-culture period (Fig. 3A). Human ES cells co-cultured with OP9 but without PGE2 treatment differentiated into multiple morphologically distinct cell types with multiple foci of differentiation in each colony and created complex

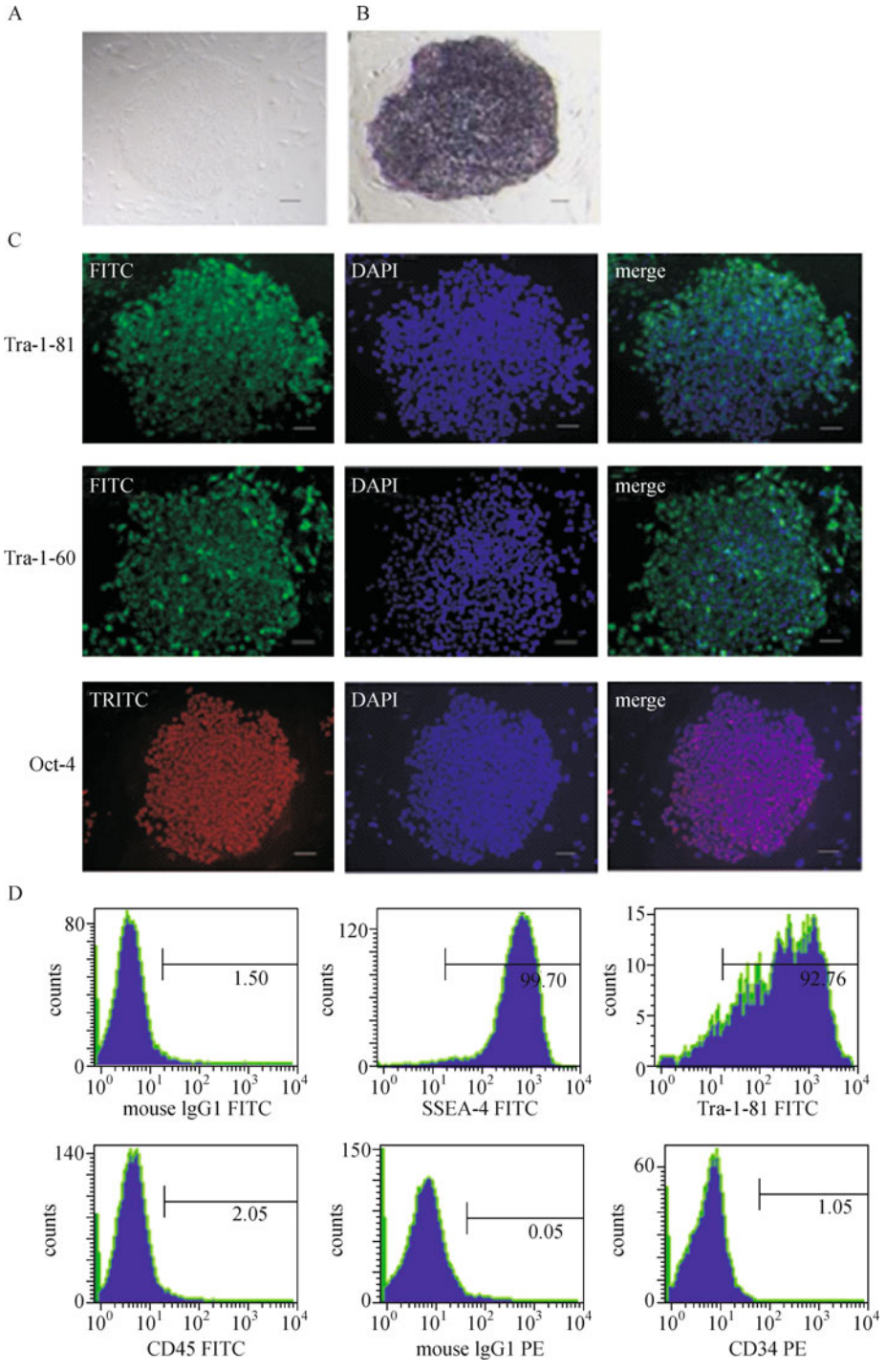

Fig. 1 Phenotype and morphology of undifferentiated hES cells. A: Morphology of hESCs; B: Colonies of hES cells were stained for alkaline phosphatase; C: Immunocytochemical analysis of stem cell markers in hES cells (scale bars: 100 μm). Immunocytochemical staining showed that the hES cells exhibited high expression of Oct4, Tra-1-60, and Tra-1-81 (scale bars: 100 μm). D: Expression of cell surface markers SSEA-4, Tra-1-81, CD34 and CD45 was analyzed by flow cytometry.

structures, in which it was difficult to discern a pattern. Observation of hESCs co-cultured with OP9 and treated with PGE2 revealed a completely different pattern of differentiation. Differentiation initiated almost exclusively from the middle of the colonies, reproducibly yielding colonies with a tripartite morphology consisting of a darkened inner center, an intermediate layer, and a white outer layer probably made of undifferentiated hESCs.

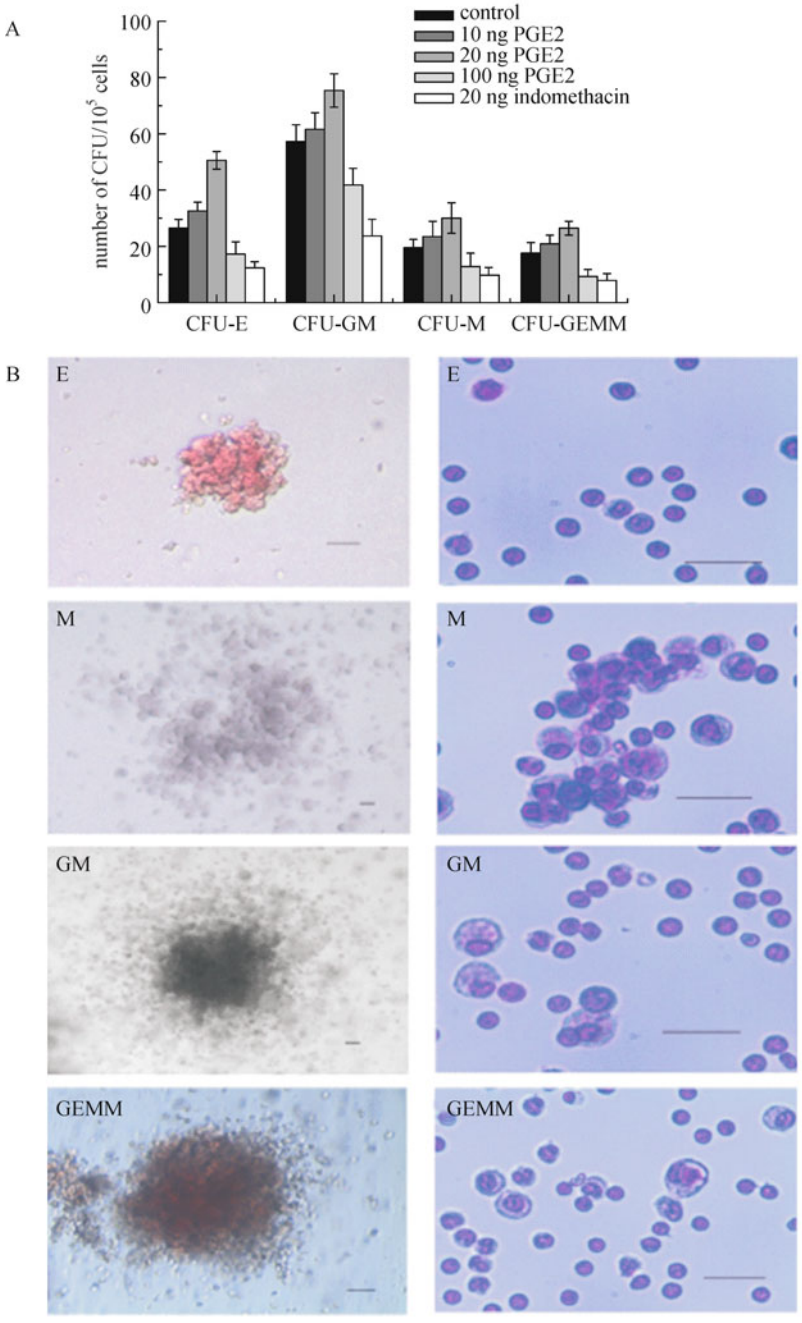

Fig. 2 Hematopoietic differentiation of human embryonic stem cells co-cultured with OP9.A: Number of hematopoietic colonies generated from human embryonic stem cells (hESCs) in different treatment groups. The best effect of prostaglandin E2 (PGE2) showed at 20 ng/mL. Under this circumstance the total number of derived colony forming unit (CFU) colonies was increased by 1.4-fold, as compared with the control colony growth (185±15.3 *vs* 132±12.9, $P < 0.05$). The data represent the mean ± SD from three experiments. B: Morphology (left column) and Wright staining of cytospins (right column) of different CFC types in hESC/OP9 co-culture.

These tripartite colonies often grew to a very large size.

To compare the numbers of cells obtained from the two groups, we dissociated the co-cultures into single-cell suspensions and counted the living cells. The numbers of cells obtained from the two groups at day 3 and 5 of culture were similar; however, subsequently the total cell number from the PGE2-treated group increased and surpassed the cells from the group treated without PGE2 (Fig. 3B).

3.4 PGE2 promotes the capacity for hematopoietic progenitor development

To further evaluate the hematopoietic potential of hESC/

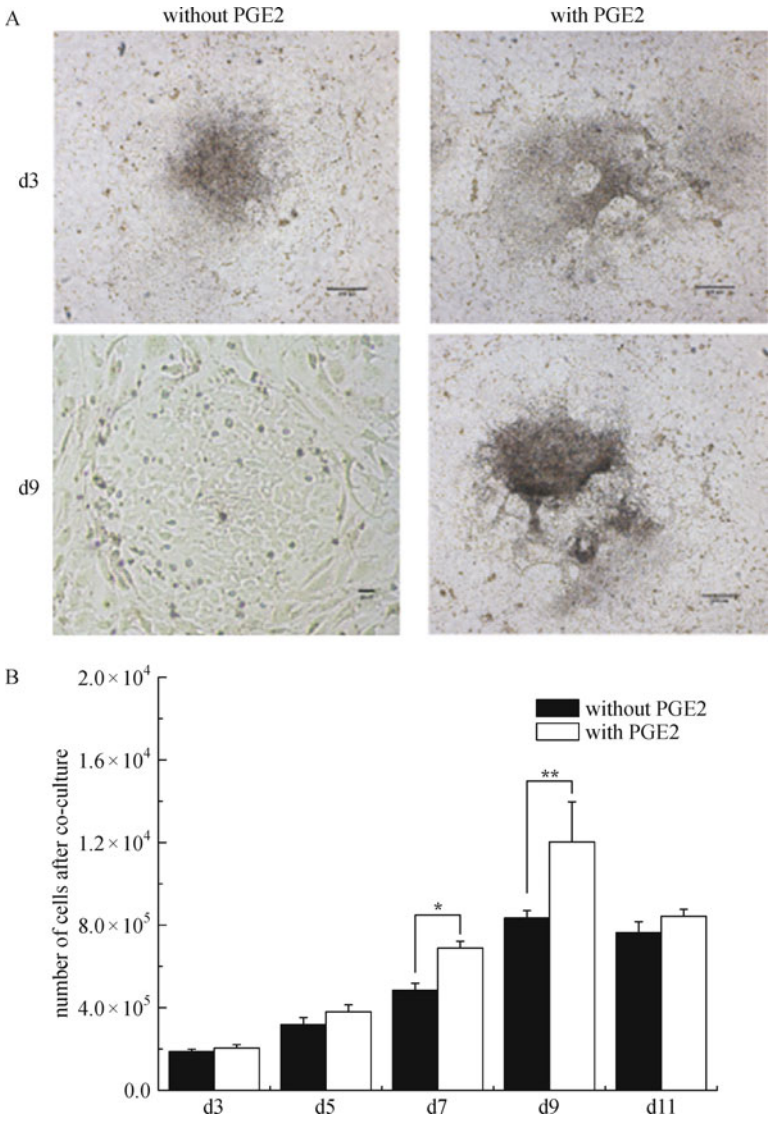

Fig. 3 PGE2 modulates proliferation and differentiation of hESCs on OP9 feeder layer. A: Clumps of hESCs were seeded on irradiated OP9 with or without prostaglandin E2 (PGE2) and photographed at low magnification after 3 or 9 days. B: Cells seeded as above were counted at various times. Co-culture on OP9 with PGE2 yielded more cells. The data represent the mean±SD from three experiments. Student's *t*-test: *: $P < 0.05$, **: $P < 0.01$.

OP9 treated with or without PGE2, we analyzed the gene expression pattern of hESC/OP9 at different days of co-culture. The expression of hematopoietic- and endothelial-associated genes, such as Brachyury, CD34, Runx1, GATA1, SCL, vWF and VE-cad, were examined with RT-PCR every other day from days 3 to 9 during the induction to chronologically monitor hematopoietic differentiation (Fig. 4A). Elevated and prompt expression of these genes suggested that PGE2 could promote both hematopoietic and endothelial progenitor formation from differentiated hESCs. And for the PGE2 treated group Oct-4 expression declined greatly and decreased to undetectable levels at 9 days co-culture.

The cells in the two groups that recovered from co-culture were then tested for expression of CD34 (a marker of primitive human hematopoietic cells) and CD45 (the marker of hematopoietic cells) by flow cytometry at different inducing time points. For hESC/OP9 treated without PGE2, the emergence of $CD34^+$ cells was consistently observed at day 5 of the co-culture, and these cells increased by day 7 and peaked at 9 days of co-culture. $CD45^+$ cells were detectable in a small population at day 5, with the number increasing greatly at day 9. For hESC/OP9 treated with PGE2, $CD34^+$ cells could be first detected at day 3 of co-culture, and $CD45^+$ cells were first found at day 5 of co-culture. The time-courses of

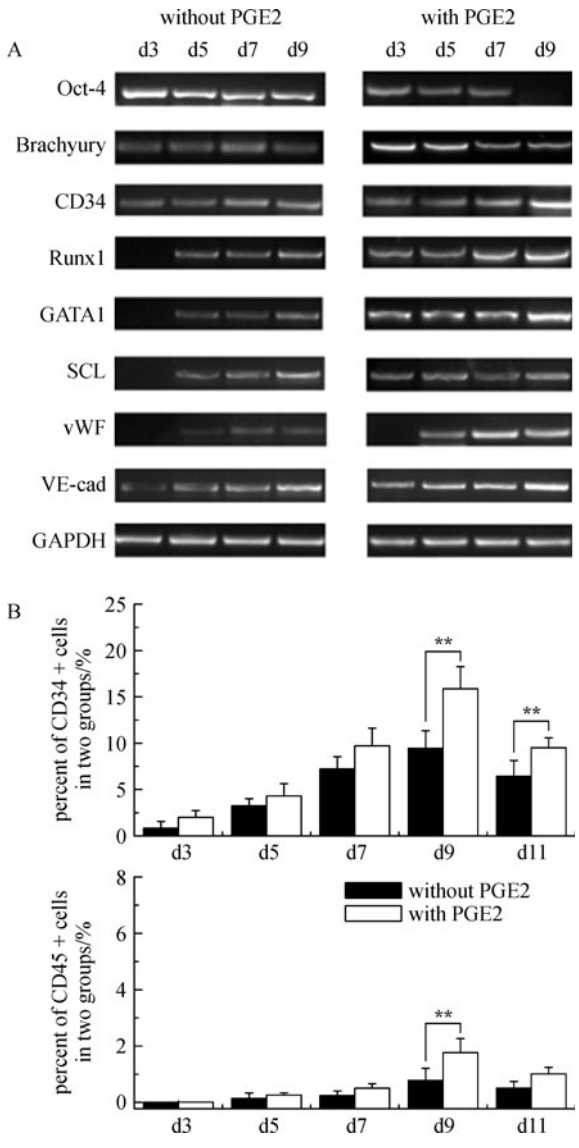

Fig. 4 Kinetic analysis of hematopoietic development from hESC/OP9 co-culture without or with prostaglandin E2 (PGE2). A: Gene expression analysis of hematopoiesis-induced transcription factors and hematoendothelial markers by reverse transcription-PCR (RT-PCR). B: Emergence of $CD34^+$ and $CD45^+$ hematopoietic cells during H9/OP9 co-culture. The data represent the mean±SD from three experiments. Student's *t*-test: **: $P < 0.01$.

appearance of $CD34^+$ and $CD45^+$ cells based on three sets of experiments are shown in Fig. 4B. Addition of PGE2 prompted the derivation of both $CD34^+$ and $CD45^+$ cells.

3.5 PGE2 modifies Smad-mediated regulation of hematopoietic development

Smad1/5 and Smad4 are the most important mediators of BMP signal in hematopoiesis (Larsson and Karlsson, 2005). To study the effects of PGE2 on Smads signal pathway, the expression and phosphorylation of Smad1/5

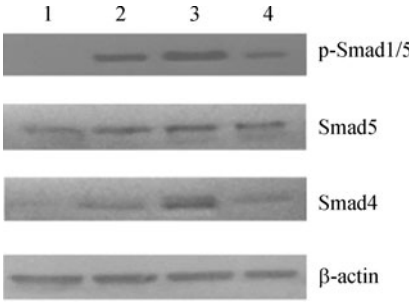

Fig. 5 Western blot analysis of the activation of Smad signal pathway during hematopoietic differentiation of hESCs. 1: H9; 2: without PGE2; 3: with PGE2; 4: Indomethacin. Human embryonic stem cells were co-cultured with OP9 feeder layer for 9 days as previously described, the expression/phosphorylation of Smad1/5 and the expression of Smad4 during the incubation process were detected respectively. Endogenous β-actin expression was set as control. The supplement of PGE2 increased Smad1/5 phosphorylation slightly, while it dramatically improved Smad4 expression. Correlatively, Smad4 level was reduced by the inhibitor of PGE2, indomethacin. The gels shown are representative of three obtained with similar results.

and the expression of Smad4 were analyzed by Western blot (Fig. 5). The expression of a housekeeping gene, β-actin, was set as control. Embryonic stem cells differentiated on OP9 feeder layer had increased Smad5 expression and phosphorylation during the 9-day incubation. PGE2 supplement only slightly increased the activation of R-Smad1/5. Interestingly, PGE2 treatment dramatically enhanced the expression of co-Smad4 compared to the non-treated counterpart. When the effect of PGE2 was blocked by its specific inhibitor, indomethacin, Smad4 expression declined correlatively, which confirmed that the expression of Smad4 can be modulated by PGE2 during hematopoietic differentiation of hESCs.

4 Discussion

Our studies support an expected role for PGE2 in regulating hematopoietic differentiation from hESCs. We demonstrate that PGE2 treatment, together with OP9 stromal cell co-culture, promotes hematopoietic development from human embryonic stem cells. The positive effect of PGE2 on HSC formation and homeostasis during zebrafish embryogenesis (North et al., 2007) and murine embryonic stem cell differentiation (Goessling et al., 2009) is conserved in supporting *in vitro* hematopoiesis of human embryonic stem cells.

Recently, the potential of hESCs to differentiate into cells of hematopoietic lineage has been achieved with two experimental methods: co-culture with stromal cells and formation of embryo bodies. The stromal cell line OP9 has been reported to efficiently induce hematopoietic (Vodyanik et al., 2005; Ji et al., 2008) and endothelial

(Hirashima et al., 1999; Choi et al., 2009) differentiation of embryonic stem cells. Although the underlying mechanism remains to be investigated, stromal cells might provide a suitable environment for self-renewal, proliferation and differentiation of hemangioblasts. In our study, the derivation of endothelial cells, as shown by endothelial marker expression (Fig. 4A), might also provide cellular contact and secrete cytokines conducive to hematopoietic differentiation.

Addition of prostaglandin E2 augments the hematopoietic induction by OP9 co-culture. PGE2 has been known to mediate numerous physiological events. For example, it behaves as a mitogen in many cell types, participates in angiogenesis by promoting endothelial cell functions (Leahy et al., 2000; Cha et al., 2005), and enhances hematopoietic stem cell homing, survival, and proliferation, even with short-term treatment (Hoggatt et al., 2009). Another study by North et al. suggested that Cox1 and Cox2, regulators of PGE2, participated in embryonic HSC induction through regulation of the stem cell niche and the HSC itself (North et al., 2007). Accordingly, we would expect that after initial differentiation provoked by OP9 cells on hESCs, PGE2 improves the survival, proliferation and differentiation of several lineages, including hematopoietic stem cells and endothelial/mesenchymal cells, which in turn provide a favorable microenvironment for hematopoietic differentiation. Our present study supports the synergistic role played by PGE2 treatment and OP9 co-culture: supplement of PGE2 prompts hematopoietic and endothelial marker's expression, increases $CD34^+$ cell population, and enhances colony forming unit frequency from hESC derivations, while abolishing PGE2 function with indomethacin reverses these effects significantly. We also set up a new hESC-HSC induction system using OP9 co-culture and PGE2 treatment.

In addition, previous studies have shown that PGE2 inhibits growth of granulocyte/macrophage colony forming cells (CFU-GM) *in vitro* and myelopoiesis *in vivo* (Gentile and Pelus, 1987), but stimulates erythroid and multilineage progenitor cells in a dose-dependent manner (Lu et al., 1984). However, in our study, PGE2 did not have an impact on specific hematopoietic lineage differentiation, the expansion of CFU-GM, colony forming unit-macrophage (CFU-M), colony forming unit-granulocyte, erythrocyte,monocyte and megakaryocyte (CFU-GEMM) and *colony* forming unit-erythroid (CFU-E) was equal. This might be due to the dominant effect of OP9 and other stromal cells in hematopoietic lineage differentiation, although the mechanism is still underexplored.

A recent publication by Goessling et al. showed that the interaction of PGE2 and Wnt signaling regulates HSC formation and hematopoietic regeneration during zebrafish embryogenesis (Goessling et al., 2009). In addition to Wnt signaling, BMPs also play key roles both during embryogenesis and through adulthood and have proven to be involved in the regulation of fate decisions of hematopoietic progenitors and stem cells (Chadwick et al., 2003; Varga and Wrana, 2005). Smads are pivotal mediators of BMP signaling (Bhatia et al., 1999; Larsson and Karlsson, 2005), and they also mediate the cooperation between BMPs and Wnt signaling pathways (Letamendia et al., 2001; Liu et al., 2006). In the present study, we also investigated the functions of the Smad signaling pathway during PGE2- and OP9-induced differentiation of hESCs to hematopoietic lineages. Phosphorylation of R-Smad1/5 and the expression of co-Smad4 could be detected during the induction course. To our surprise, addition of PGE2 only led to a weak improvement of R-Smad phosphorylation/activation, while the expression of co-Smad4 increased significantly. The special effect has also been confirmed with reduced Smad4 expression by PGE2-specific inhibitor, indomethacin. Further investigation should be addressed on how PGE2 modulates the expression of Smad4. Nevertheless, our study hints that through improving Smad4, PGE2 increases hematopoietic differentiation of hESCs co-cultured with OP9 cells.

In conclusion, PGE2, in addition to OP9 co-culture, improves hematopoiesis from hESCs *in vitro*. Our study provides a new protocol to develop transplantable blood cells. The application of this method might be one of the solutions for the shortage of transfusion medicine.

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