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The negative impact of Wnt signaling on megakaryocyte and primitive erythroid progenitors derived from human embryonic stem cells

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Abstract The Wnt gene family consists of structurally related genes encoding secreted signaling molecules that have been implicated in many developmental processes, including regulation of cell fate and patterning during embryogenesis. Previously, we found that Wnt signaling is required for primitive or yolk sac-derived-erythropoiesis using the murine embryonic stem cell (ESC) system. Here, we examine the effect of Wnt signaling on the formation of early hematopoietic progenitors derived from human ESCs. The first hematopoietic progenitor cells in the human ESC system express the pan-hematopoietic marker CD41 and the erythrocyte marker, glycophorin A or CD235. We have developed a novel serum-free, feeder-free, adherent differentiation system that can efficiently generate large numbers of CD41 + CD235+ cells. We demonstrate that this cell population contains progenitors not just for primitive erythroid and megakaryocyte cells but for the myeloid lineage as well and term this population the primitive common myeloid progenitor (CMP). Treatment of mesoderm-specified cells with Wnt3a led to a loss of hematopoietic colony-forming ability while the inhibition of canonical Wnt signaling with DKK1 led to an increase in the number of primitive CMPs. Canonical Wnt signaling also inhibits the expansion and/or survival of primitive erythrocytes and megakaryocytes, but not myeloid cells, derived from this progenitor population. These findings are in contrast to the role of Wnt signaling during mouse ESC differentiation and demonstrate the importance of the human ESC system in studying species-specific differences in development.

Abbreviations: ESC, embryonic stem cell; PSC, pluripotent stem cell; iPSC, induced pluripotent stem cell; CMP, common myeloid progenitor; GFP, green fluorescent protein; SFD, serum free differentiation media; IL-3, interleukin-3; EPO, erythropoietin; GM-CSF, granulocyte-macrophage colony stimulating factor; SCF, stem cell factor; TPO, thrombopoietin; IL-6, interleukin-6; RT, reverse transcriptase; PCR, polymerase chain reaction; EB, embryoid body; MEP, megakaryocyte-erythroid progenitor.

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Introduction

Embryology has offered important insights into key pathways regulating embryonic stem cell (ESC) differentiation and efficient induction of endoderm, mesoderm, and ectoderm (reviewed in (Gadue et al., 2005; Loebel et al., 2003)). By modulating developmentally important signaling pathways, it is possible to mimic the process of embryogenesis to drive the differentiation of stem cells through discreet developmental intermediaries (Murry and Keller, 2008). The Wnt pathway has been shown to be especially important in regulating multiple stages of development of hematopoietic cells from pluripotent stem cells (PSCs) including mesoderm formation and hematopoietic specification in both mice and humans (Gadue et al., 2006) (Gertow et al., 2013; Hwang et al., 2009; Lengerke et al., 2008; Lindsley et al., 2006; Nostro et al., 2008; Vijayaragavan et al., 2009; Woll et al., 2008).

The Wnt pathway is evolutionary conserved and diversifies into two main branches, canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) (reviewed in (Wodarz and Nusse, 1998)). Both branches play critical roles in specifying cellular fate and movement during embryonic development and adult tissue regeneration. Activation of the canonical Wnt signaling pathway occurs through ligand binding of Wnts to a Frizzled family receptor that is associated with a co-receptor belonging to the LRP-5/6/arrow family. This activation inhibits the destruction complex, APC/Axin/CK1/GSK3β, leading to the stabilization of β-catenin and its translocation to the nucleus where it interacts with TCF/LEF family transcription factors (reviewed in (Logan and Nusse, 2004)). The secreted antagonists, sFRPs, WIF-1, and DKKs, can inhibit this pathway by blocking receptor binding. In the absence of a Wnt ligand, GSK3β in the destruction complex phosphorylates β-catenin, which marks it for degradation. The non-canonical Wnt signaling pathways appear to function in a β-catenin independent manner, designated as Wnt/Calcium and Wnt/JNK in vertebrates and Wnt/planar cell polarity pathway and does not utilize the LRP co-receptors (reviewed in (Veeman et al., 2003)).

While there is consensus that Wnt signaling plays important roles in hematopoietic development from PSCs, there is conflicting data especially on the role of canonical Wnt signaling in hematopoietic specification or survival. We have previously shown that activation of the Wnt pathway in murine ESC-derived hematopoiesis is essential for establishment of the primitive erythroid, but not definitive, lineages (Nostro et al., 2008). These data are supported by a publication examining human ESC-derived hematopoiesis suggesting that the canonical Wnt family member, Wnt3a, functions by expanding ESC-derived hematopoietic progenitors (Vijayaragavan et al., 2009). In contrast, a study by Gertow et al. demonstrates that canonical Wnt signaling is inhibitory for hematopoietic colony formation from human ESCs (Gertow et al., 2013). To address these seemingly contradictory results, the role of Wnt signaling in the specification and expansion of hematopoietic cells derived from human ESCs was examined.

For differentiating PSCs into hematopoietic progenitor cells, we developed a novel 2-dimensional system that avoids the use of feeder cells, serum or embryoid body (EB) formation. We show that the earliest population of hematopoietic progenitor cells have erythroid, megakaryocyte, and myeloid potentials. The first hematopoietic cells co-express CD41 and CD235, typical markers of megakaryocyte and erythrocyte progenitors, respectively. By using this system, we examined the effect of Wnt signaling at various stages of hematopoietic development of human ESCs. Unlike the murine system, we found that canonical Wnt signaling inhibits the development and expansion of this progenitor population. This inhibitory effect also impacts the expansion of erythrocytes and megakaryocytes, but not the myeloid lineage. These studies highlight the importance of working with human ESCs as species-specific differences can be addressed when translating findings from the murine system.

Materials and methods

Human ESC line maintenance

The human ESC line used in this study was H9 (NIH code WA09 from Wicell Research Institute, Madison, WI) and the iPS cell line, CHOPWT2.2, has been described previously (Mills et al., 2013). Minor modifications were made to the basic protocols for growth and maintenance of human ESC lines that have been previously described (Amit et al., 2000). Briefly, cells were grown on irradiated mouse embryonic feeder cells in maintenance medium consisting of DMEM/F12 supplemented with a 20% knock-out serum replacement, 100 μM non-essential amino acids, 0.075% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM glutamine, 50 U/ml penicillin, 50 g/ml streptomycin (all from Invitrogen, Grand Island, NY), 10^{-4} M β-mercaptoethanol (Sigma, St Louis, MO), and 10 ng/ml human bFGF (Stemgent) in 6-well tissue culture plates at 37 °C, 5% CO_2 and atmospheric O_2. The medium was changed daily or every 2 days and colonies were typically passaged every week following size and morphology assessment. Cells were passaged to new feeders as small clusters in human ESC medium containing ROCK inhibitor (10 μM) using TrypLE (Invitrogen) and gentle scraping using a 25 cm, 2-position blade cell scraper (Sarstedt, Newton, NC). The enhanced green fluorescent protein (GFP) expressing H9 sub-line was generated using a previously published strategy to target the construct into the AAVS1 locus with a zinc finger nuclease (Hockemeyer et al., 2009). The construct contained a chicken actin promoter which drives the expression of GFP.

Differentiation of human ESCs

Prior to the induction of differentiation, cells (2–4 × 10^5/well) were feeder depleted by culturing on Matrigel coated wells (BD Biosciences, Bedford, MA) (6-well tissue culture plate, Falcon 3046) in human ESC maintenance medium for 24 to 48 h or until the cells reached ~70% confluence. When the cells were ready for differentiation, one well of cells was used for cell count and pluripotency assessment by analysis of the surface markers, SSEA3 and SSEA4 (>90% co-expression). Three different base media were used in the differentiation protocol and all were supplemented with 2 mM glutamine, 50 μg/ml ascorbic acid (Sigma, St. Louis, MO), 150 μg/ml transferrin (Roche Diagnostics), and 4 × 10^{-4} M monothioglycerol (MTG) (Sigma). The base media were RPMI (Invitrogen), StemPro-34 (SP-34) (Invitrogen), and serum free differentiation (SFD) media (Gadue et al., 2006). The cultures were maintained at 37 °C in an