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Noncanonical Wnt Signaling Orchestrates Early Developmental Events toward Hematopoietic Cell Fate from Human Embryonic Stem Cells

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SUMMARY

During human development, signals that govern lineage specification versus expansion of cells committed to a cell fate are poorly understood. We demonstrate that activation of canonical Wnt signaling by Wnt3a promotes proliferation of human embryonic stem cells (hESCs)—precursors already committed to the hematopoietic lineage. In contrast, noncanonical Wnt signals, activated by Wnt11, control exit from the pluripotent state and entry toward mesoderm specification. Unique to embryoid body (EB) formation of hESCs, Wnt11 induces development and arrangement of cells expressing Brachyury that coexpress E-cadherin and Frizzled-7 (Fzd7). Knockdown of Fzd7 expression blocks Wnt11-dependent specification. Our study reveals an unappreciated role for noncanonical Wnt signaling in hESC specification that involves development of unique mesoderm precursors via morphogenic organization within human EBs.

INTRODUCTION

Lineage specification is a highly coordinated phenomenon delineated by temporal changes in gene expression at a single-cell level that respond to changes at a multicellular level. Such coordinated events are orchestrated by key morphogenic signaling pathways (Kimelman, 2006) including the highly conserved Wnt family members (Kimelman, 2006; Logan and Nusse, 2004). Unlike invertebrate and other nonhuman models, this has been more difficult to understand in the human system, in which early developmental events cannot be experimentally manipulated. Human embryonic stem cells (hESCs) provide an invaluable approach to modeling fundamental processes of development and provide a unique opportunity to define cellular mechanisms by which complex development events are modulated and organized by inductive signaling molecules such as Wnts.

Wnts are a family of 18 secreted glycoproteins that act as ligands for the seven-pass transmembrane Frizzled receptors (Fzd) and coreceptor LDL receptor-related protein (LRP5/6) (Wu and Nusse, 2002). Fzd signaling activates β-catenin-dependent (canonical) and -independent pathways (noncanonical) (van Noort et al., 2002). Wnt/β-catenin signaling, mediated by Wnt ligands such as Wnt3a, regulates the ubiquitin-proteasome destruction complex (Axin, APC, GSK-3, CK-1), resulting in the stabilization and translocation of β-catenin to the nucleus, where it regulates gene expression (Moon, 2005; van Noort et al., 2002). β-catenin-independent Wnt signaling is mediated by Wnt ligands such as Wnt11 and acts through kinases such as c-Jun NH2-terminal kinase (JNK) and the calcium-dependent kinases (CaMKII) and PKC (Kuhl et al., 2000; Nateri et al., 2005; Topol et al., 2003; Westfall et al., 2003). Although the Wnt signaling contributes to multiple developmental events during embryogenesis and in homeostasis of adult tissues, the roles of canonical and noncanonical Wnt pathways are poorly understood and have yet to be studied in early human development.

Considerable species variation exists at very early stages of development, especially during gastrulation and morphogenesis. Accordingly, the functions of soluble factors such as Wnts can be cell and species dependent. For example, while the embryos of Wnt3 null mice fail to develop mesoderm, inhibition of the canonical signaling in zebrafish and *Xenopus* results only in the axis truncation (Liu et al., 1999; Humphrey et al., 2004; Poon et al., 2006; Tavian and Peault, 2005; Xu et al., 2002). This species variation also precludes precise extrapolations from mouse to humans in assigning specific roles for Wnts in development. Consistent with the importance of Wnt3a in mesoderm development, there is emerging evidence that temporal activation of Wnt/β-catenin signaling is crucial for cardiac and hematopoietic fate during murine and zebrafish embryogenesis (Naito et al., 2006; Ueno et al., 2007). Similarly, in the adult system, Wnt3a-mediated signals that are important for achieving a balance of proliferation, differentiation, and self-renewal of the hematopoietic stem cell (HSC) originate from

the mesoderm (Kirstetter et al., 2006; Reya et al., 2003; Scheller et al., 2006; Willert et al., 2003). In contrast, the functional roles of the β-catenin-independent Wnt pathways are less clear but may involve regulating cell movements and frequent antagonization of the β-catenin pathway (Kuhl, 2002). β-catenin-independent Wnt signaling has been implicated in ventral cell fate choices, epithelialization in the early quail mesoderm, and cardiomyocyte differentiation in a number of species, such as quail, *Xenopus*, zebrafish, and mouse ESCs (Eisenberg and Eisenberg, 1999; Eisenberg et al., 1997; Kuhl et al., 2000; Naito et al., 2006; Pandur et al., 2002; Ueno et al., 2007). In humans, noncanonical Wnt pathways have been associated with adult stages of development that control the diversification of blood cell types and augment regenerative potential of human HSCs capable of repopulating immune-compromised NOD-SCID mice (Brandon et al., 2000; Murdoch et al., 2003), an observation also made in the mouse (Nemeth et al., 2007).

The exact role of canonical versus noncanonical Wnts in human mesoderm and blood development remains to be defined. Capitalizing on the ability of hESCs to give rise to hemogenic precursors and primitive blood cells (Wang et al., 2004), we utilized hESCs as a robust in vitro model to examine the function of canonical and noncanonical Wnt activation during embryonic mesodermal and hematopoietic cell fate determination. We reveal a distinct temporal nature of canonical and noncanonical signaling to promote early human hematopoietic development and propose that these two Wnt pathways mediate their effects via distinct cellular mechanisms to augment human blood cell fate that was not predicted by other invertebrate and nonhuman models of blood development.

RESULTS

Wnt3a Affects Commitment Subsequent to Hematopoietic Precursor Development

Initial experiments were performed to provide evidence that Wnt ligands Wnt3a and Wnt11 elicit signals along the expected canonical and noncanonical Wnt cascades within hESCs. The biological activity of the Wnts produced from L cells was characterized on cells transfected with the TCF optimal promoter-GFP reporter construct (see Figures S1A–S1C available online). In addition, microarray analysis revealed the Wnt pathways are active in hESCs and that these pathways are regulated within the hematopoietic-derived hESC compartment, which were cultured under feeder-free conditions to ensure transcript detection was not disrupted by feeder cells (Figure S1D) (Wang et al., 2004). To evaluate the effects on the biochemistry of target proteins integral to canonical Wnt signaling, the phosphorylation status of β-catenin was assessed under control versus Wnt ligand treatment in hESCs. Higher total levels of β-catenin were observed under Wnt3a treatment, while reduced levels were seen with Wnt11 stimulations compared to control condition (Figure S1E). In addition, phosphorylated β-catenin (Ser33/37, Thr41) levels were lower with Wnt3a stimulation but higher under Wnt11 conditions compared to control (Figure S1E). Furthermore, accumulation of the nonphosphorylated form of β-catenin was observed with Wnt3a stimulation and was reduced with Wnt11 cotreatment (Figure S1F). These data are consistent with the notion that noncanonical Wnt signaling inhibits its canonical counterpart in hESCs and that Wnt3a and Wnt11 can modulate β-catenin-dependent and -independent Wnt signaling, respectively.

Since Wnt3a/canonical signaling has been implicated in many aspects of hematopoietic differentiation (Kirstetter et al., 2006; Reya et al., 2000; Scheller et al., 2006; Trowbridge et al., 2006), we hypothesized that the effect of Wnt3a may be temporal in nature, similar to its role during development in nonhuman species (Ang et al., 2004; Na et al., 2006; Nohno et al., 1999). To examine this, we characterized the effect of canonical Wnt3a signaling in the hESC system, where hierarchical stages of blood development have previously been characterized (Figure 1A) (Chadwick et al., 2003; Wang et al., 2004). This developmental scheme of hematopoiesis from hESCS can be divided into two stages: stages I and II. Stage I (days 0–7) encompasses hemogenic lineage specification phase, characterized by the initial appearance of the bipotential hemogenic cells to hemogenic and endothelial precursors ($CD45^{-}CD31^{+}$ cells) and by the absence of hematopoietic ($CD45^{+}$) cells and the lack of progenitor capacity. Stage I is followed by the commitment phase, stage II (days 7–15), characterized as the period in which committed hematopoietic progenitors are detected by day 10 and peak at day 15.

Treatment with Wnt3a during stage I or stage II of EB development revealed that Wnt3a increased both the hemogenic and hematopoietic compartments only when present during the later commitment stage II (7–15 days) of blood development from hESCs. Restricted to stage II, Wnt3a induced a 2.4-fold increase in hemogenic cell frequency and a 9.2-fold increase in total hemogenic precursors (Figures 1B and 1C). Similarly, Wnt3a induced a 2.8- and 3.8-fold increase in frequency of hematopoietic cells and total hematopoietic progenitors, respectively, but only when present at stage II of the hEB differentiation (Figures 1E and 1F), and not stage I (treated for 1, 3, or 7 days) (Figure S2A). In addition, Wnt3a had no effect at stage I of hematopoietic development under serum-free conditions, indicating that serum does not mask canonical Wnt3a effectiveness (Figures S2B and S2C). Treatment with the canonical Wnt inhibitor, Dkk1, reduced both the hemogenic and hematopoietic progenitors ($CD34^{+}CD45^{+}$) that were induced by Wnt3a during this phase of blood development (Figures 1D and 1G). Evaluation of the molecular activity of Wnt3a showed an increase in stable β-catenin levels that was also Dkk1 sensitive (Figure 1H). These data indicate that the effect of Wnt3a is regulated by canonical Wnt signaling that uniquely targets only committed cells contained withins day 7–15 hEBs (stage II) to augment hematopoietic differentiation and does not affect specification of blood fate from the pluripotent state that occurs at stage I (days 0–7) (Figure S2A).

Wnt3a Induces Proliferation of Committed Hematopoietic Precursors Derived from hESCs

To understand the cellular mechanism by which Wnt3a augments hematopoiesis during later stages of hEB development when cells are already destined to the hematopoietic fate, we analyzed both cell death and proliferation of committed cells. Augmentation of hemogenic and hematopoietic potential from hESCs following Wnt3a treatment at stage II could not be explained by affects on cell survival (data not shown). Using

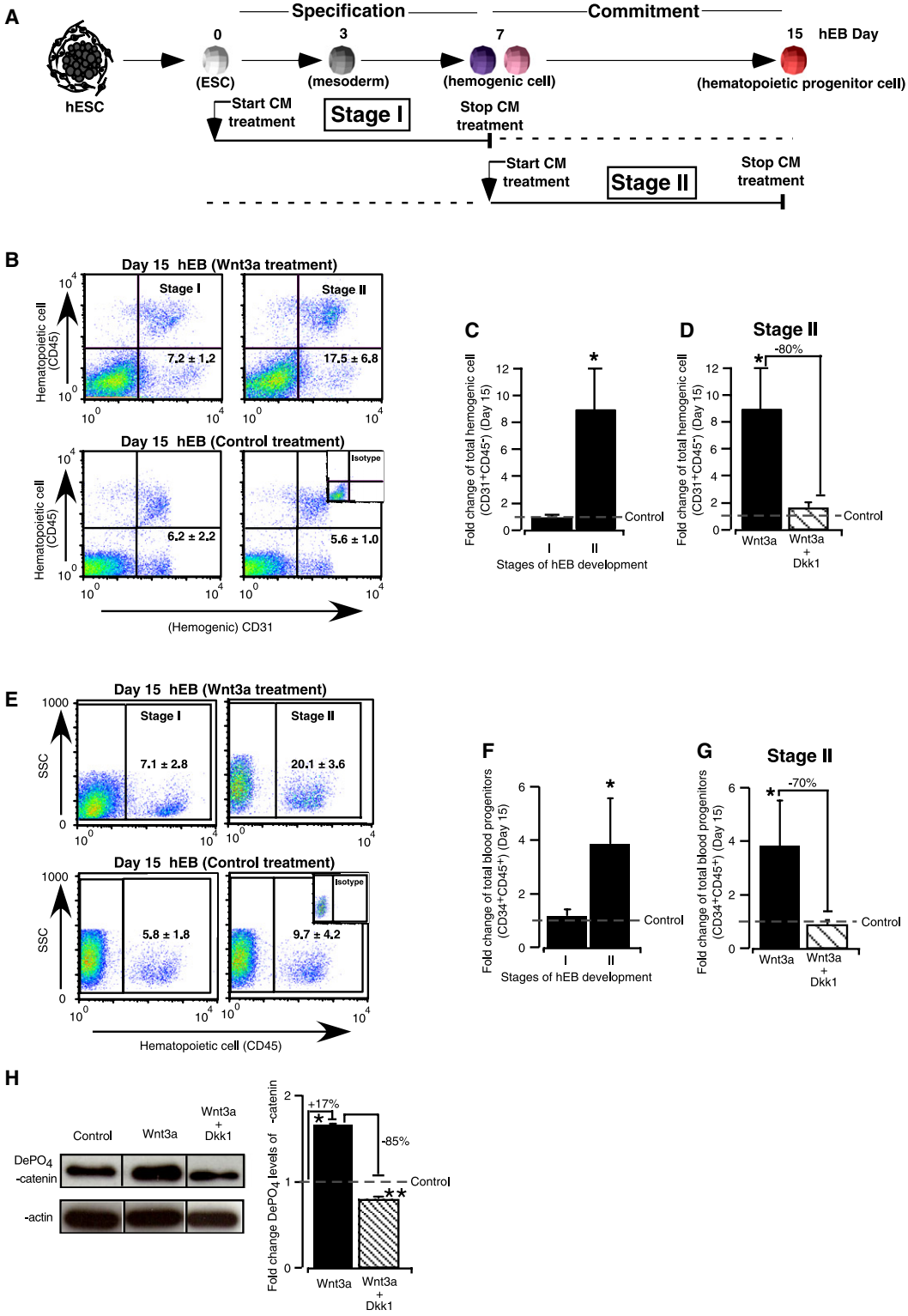


Figure 1. Wnt3a Is Important at Later Phases of hEB Development

(A) Schematic diagram of hematopoietic development from hESCs, segmented into different stages: stage I represents days 0–7 of hEB differentiation, defined by specification of hESCs (day 0) to hemogenic $CD31^{+}CD45^{-}$ precursors (day 7) and then toward emergence of blood lineage between days 7 and 15 as stage II, where commitment to hematopoietic $CD34^{+}CD45^{+}$ progenitors (day 15) occurs. hEBs were treated during either developmental stage I or stage II and analyzed for development into hemogenic and hematopoietic cells at day 15.

(B) Representative flow cytometry analysis of hemogenic cells following Wnt3a and control treatment (n = 6).

markers of proliferation, proliferation cell nuclear antigen (PCNA), and 5-bromo, 2-deoxyuridine (BrdU) incorporation, we observed a 2.3-fold increase in number of PCNA-positive hEBs upon Wnt3a stimulation as compared to control (Figures 2A–2D). Confocal images of single cells isolated from day 15 BrdU-treated hEBs show robust BrdU staining of the hemogenic ($CD31^+$) and hematopoietic cells ($CD45^+$) upon Wnt3a (Figures S3A–S3C). Quantitative analysis of proliferative status upon Wnt3a stimulation revealed that the overall cell proliferation, enumerated as a proportion of BrdU-labeled versus nonlabeled cells, was increased in the Wnt3a stage II-treated hEBs (66.5% ± 6%) compared to stage I (57.4% ± 0.1%) treated hEBs or untreated (21.4% ± 2.7%) hEBs (data not shown). Importantly, the BrdU incorporation (7ADD for DNA content) within the target hemogenic compartment ($CD31^+CD45^-$) was 4.9-fold higher during stage II treatment (Figures 2E and 2E′). Similarly, we observed a 3.1-fold increase in frequencies of cycling hematopoietic cells ($CD45^+$) (Figures 2F and 2F′) and a 5-fold increase in the levels of the canonical Wnt target gene cyclin D1, associated with cellular proliferation (Figure 2G). These data indicate that the cellular mechanism by which canonical Wnt (Wnt3a) signaling promotes hematopoietic output from hESCs is mediated by cell-cycle induction of previously committed hemogenic and hematopoietic cells.

Wnt11 Affects Specification and Precursor Progression at Early Stages of hEB Development

Given the temporal proliferative effects of Wnt3a on hESC-derived blood development, we evaluated the effects of noncanonical Wnt signals using Wnt11, based on established responses to Wnt11 in hESCs (Figure S1). In contrast to Wnt3a treatment, analysis of day 15 hEB differentiation revealed that Wnt11 increased blood formation during the initial stage of hematopoietic development from hESCs (stage I). The frequency of hematopoietic cells and hematopoietic progenitor numbers was upregulated by 2-fold and 2.5-fold, respectively, when Wnt11 was present during the specification phase (stage I) (Figures 3A and 3B). Similar to Wnt11, stimulation with Wnt5a (another noncanonical Wnt pathway ligand) during stage I of hEB differentiation also increased hematopoiesis (Figures S4A–S4D). Additionally, Wnt11 treatment during stage I of differentiation increased primitive hematopoietic programs, as indicated by ζ- and ε-globin expression, which were 16-fold and 4.6-fold higher, respectively, compared to control conditions, while definitive hematopoiesis (β-globin) was unaffected (Figure S4G). Although Wnt11 was shown to function as an inhibitor of β-catenin (Figure 3D), cotreatment of Wnt11 with Dkk1 (200 ng/ml; as optimized dose of DKK1, Figure S2D) did not affect the Wnt11 response seen at stage I of treatment (Figure 3C). Dkk1 addition was able to reduce endogenous levels of β-catenin (Figure 3E), indirectly suggesting that canonical Wnts are produced by hEB cells, but have no biological affect on hematopoietic development (Figure 3F, or at any other dose of Dkk-1 tested, Figure S2D). Together, these observations suggest that inhibition of endogenous canonical Wnt signaling cascade is not sufficient to affect hematopoietic differentiation and that Wnt11 signaling functionally enhances and temporally regulates hematopoiesis during stage I (days 0–7) of hEB development.

To further evaluate the early effects of Wnt11, hEBs were treated with Wnt11 for 1 day and analyzed at day 15. This 24 hr Wnt11 exposure was sufficient to promote blood development of both hemogenic precursors (Figure 3G) and hematopoietic progenitors (Figures 3I). Total CFU production, as a functional measure of hematopoietic progenitor capacity, was determined for $CD31^+CD45^-$ cells derived from day 10 and day 15 hEBs treated with Wnt11 (Figure 3H) and demonstrated that Wnt11 increases CFU output from this population. Interestingly, unlike the modest affects seen during continued treatment through stages I and II on hemogenic precursor output (Figures S4E and S4F), a single exposure of Wnt11 in the first 24 hr upon hEB formation was sufficient to increase the hemogenic precursor frequencies by 2.4-fold compared to control (Figure 3I). This 24 hr effect was also demonstrated under serum-free conditions, indicating that serum does not play a role in Wnt11 effects (Figures S2B and S2C). Similar to effects on hemogenic precursors, 24 hr of Wnt11 treatment resulted in a 2.2-fold increase in CFU production arising from $CD34^+CD45^+$ cells at day 15 of hEB development (Figure 3J). These data suggest that 24 hr of Wnt11 treatment (day 1 of stage I) equally augments both the hemogenic and hematopoietic progenitor phenotype and function progenitor capacity during hEB hematopoietic development.

Based on the requirements of Wnt11 during the early phase (stage I), but not the later phase (stage II) of blood differentiation, and the immediacy of Wnt11 effects (24 hr), we hypothesized that Wnt11 may promote the progression of unknown early precursors of the blood lineage and may direct control genes associated with the ground state of pluripotency (Boyer et al., 2005; Chambers et al., 2003; Niwa et al., 2000; Zeineddine et al., 2006).

Wnt11 Modulates Pluripotent Factors and Induces Expression of Early Mesoderm

Given the immediate effects of Wnt11 in induction of hematopoiesis and sustained expression of both Nanog and Oct4 within the first 2 days of hEB formation (data not shown), we examined the potential association of Wnt11 with Oct4 and Nanog transcript regulation during the 24 hr of Wnt11 treatment. Changes in Oct4 and Nanog expression were monitored over time, from

(C) Hemogenic cells ($CD31^+CD45^-$) population is upregulated exclusively by Wnt3a treatment at stage II (n = 8) and had no effect at stage I treatment (n = 6) compared to control treated.
(D) Inhibition of Wnt3a induced hemogenic differentiation at Stage II by Dkk1 (200 ng/ml; n = 4).
(E) Representative flow cytometry analysis indicating increase in hematopoietic progenitor ($CD34^+CD45^+$) cells by Wnt3a treatment at stage II versus control treatment (n = 6).
(F) Wnt3a treatment at stage II promotes total hematopoietic progenitor output (n = 8).
(G) Dkk1 inhibits stage II-induced Wnt3a increases of total hematopoietic progenitors (n = 4).
(H) Immunoblot showing stabilization of β-catenin (probed for dephosphorylated β-catenin) in hEB treated with Wnt3a or treated with Dkk1 and Wnt3a (n = 6). Controls are shown as a horizontal dashed line (*$p < 0.01$).

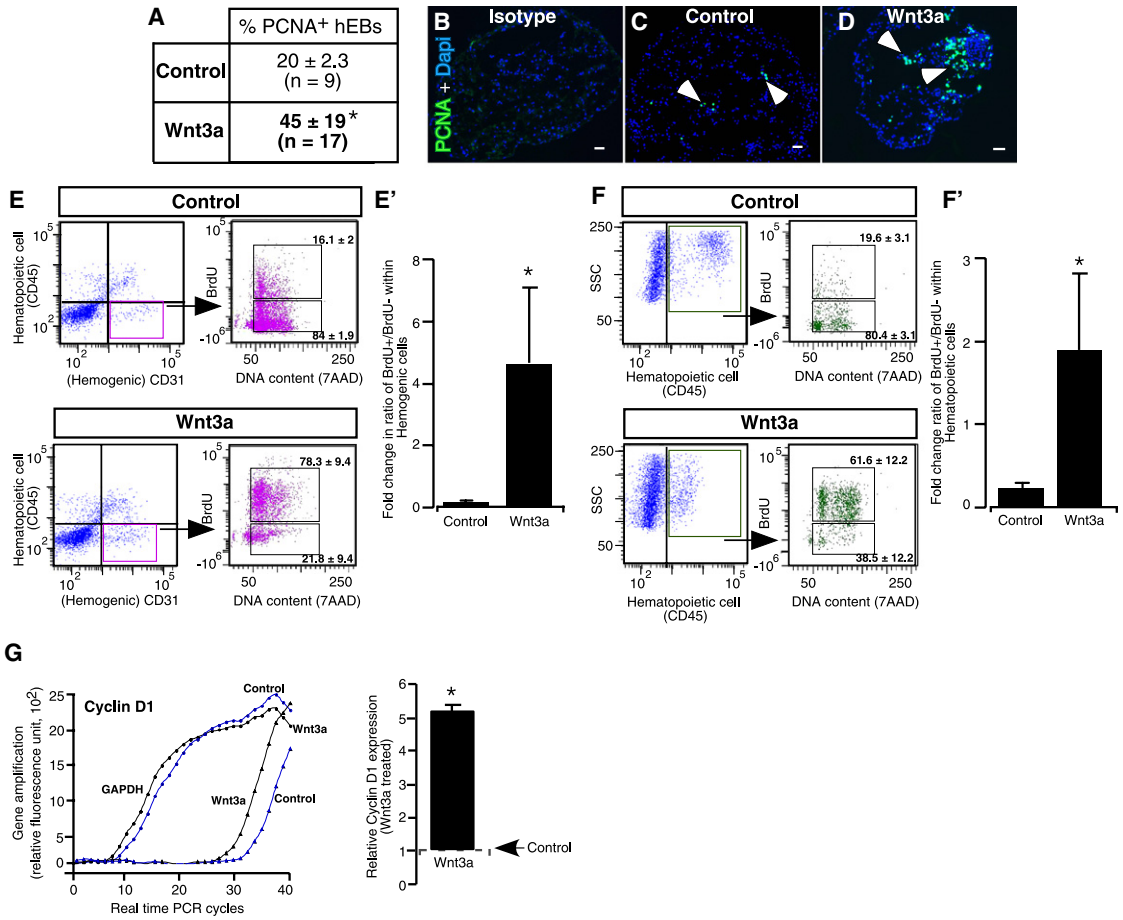


Figure 2. Wnt3a Promotes Hemogenic and Hematopoietic Cell Proliferation

(A) Percent PCNA-positive hEB at day 15 following Wnt3a stimulation compared to control treatment.

(B–D) Immunocytochemistry of hEB stained for PCNA demonstrated a higher number of PCNA-positive cells with Wnt3a treatment versus control. Scale bar, 50 μm.

(E) Representative flow cytometry of hemogenic cell distribution in different cell-cycle compartments showing that higher frequencies of hemogenic ($CD31^+CD45^-$) cells have incorporated BrdU upon Wnt3a exposure versus control conditions (n = 3). (E′) Fold change in the ratios of BrdU+/BrdU− in hemogenic cells following Wnt3a treatment compared to control (n = 3).

(F) Representative flow cytometry analysis showing higher frequencies of hematopoietic (CD45−) cells have incorporated BrdU upon Wnt3a stimulation versus control treatment (n = 3). (F′) Fold change in the ratios of BrdU+/BrdU− in hematopoietic progenitors ($CD34^+CD45^+$) following Wnt3a treatment is higher compared to control treatment (n = 3).

(G) Upregulation of cyclin D1 by Wnt3a quantified by real-time PCR compared to controls (dashed horizontal line) (n = 4; *p < 0.01).

the undifferentiated hESC state to 24 hr after hEB formation and posttreatment (Figure 4A). Oct4 transcript was increased upon hESC aggregation (Figure 4B), and rapid up- and downregulation of Oct4 was consistently observed upon Wnt11 treatment compared to control treated cells in the absence of Wnt11 (Figure 4C). While levels of Oct4 transcript were lower under control conditions by 24 hr, Oct4 expression was maintained in response to Wnt11 (Figure 4C). Furthermore, the frequency of Oct4-positive cells measured by intracellular staining for Oct4 protein, as well as western blot analysis of Oct4 protein levels, demonstrated Oct4 was regulated in response to Wnt11 stimulation compared to control conditions (Figures S5A and S5B). Given that the stability and turnover of the Oct4 protein is not well defined, the differences between the protein level and transcript expression are likely to differ (Wei et al., 2007); however, Wnt11 equally affected Oct4 protein and transcript, albeit with different kinetics due to the nature of transcript versus protein regulation. Similar to Oct4, Nanog transcript levels were found to be upregulated 24 hr after hEB formation (Figure 4D) and responded to Wnt11 treatment (Figure 4E). Our data suggest that Wnt11 regulates factors associated with pluripotent state, in addition to affecting lineage specification genes toward mesodermal development.

To test this hypothesis, we monitored changes in gene expression for factors associated with mesoderm specification, including T box and homeobox factors within the developing hEBs. The expression of stem cell leukemia factor (SCL/Tal1), important during hematopoietic development, and the homeobox gene MixL1 and the T box gene Brachyury, surrogate markers of primitive streak and blood-mesoderm development

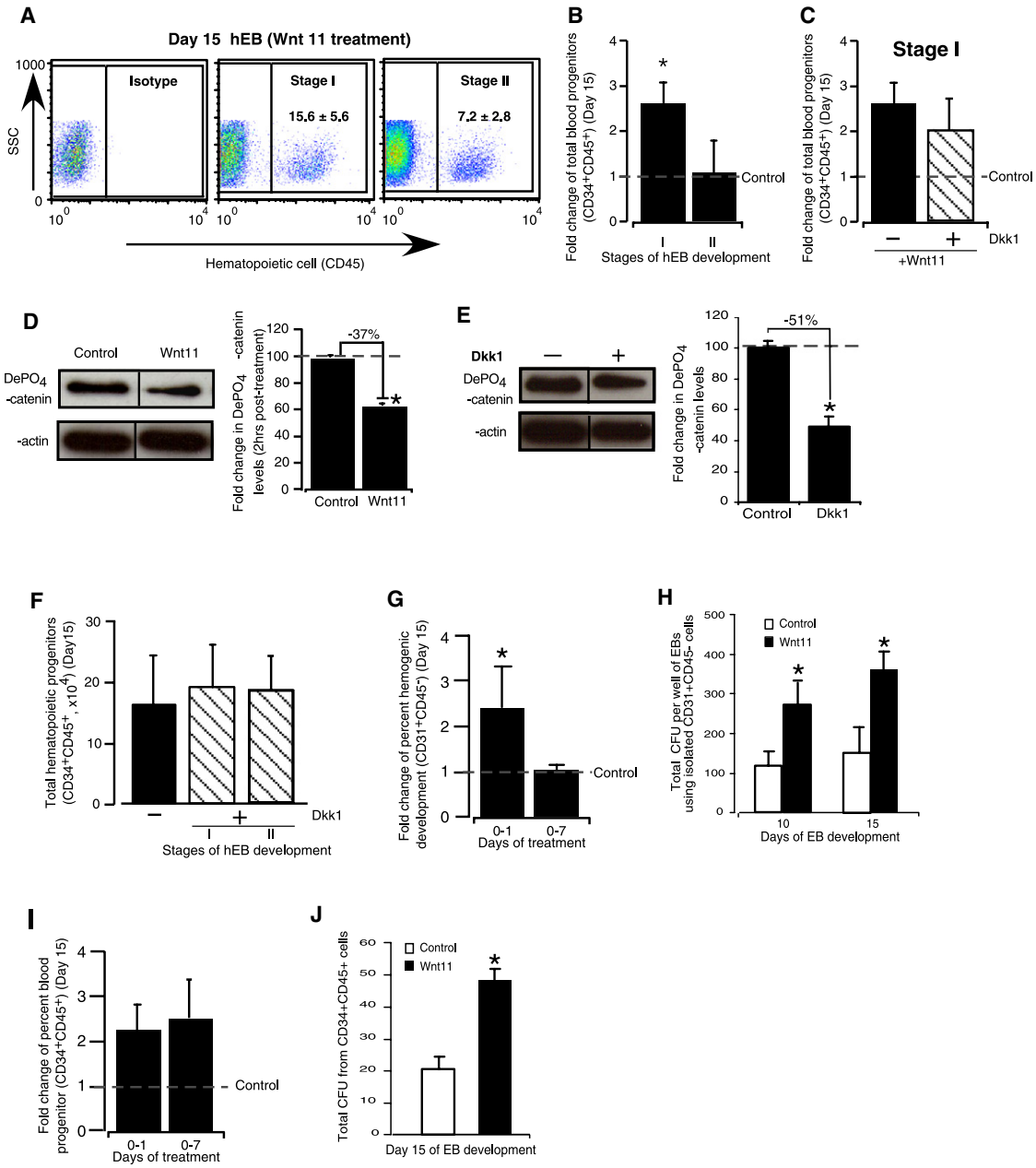


Figure 3. Wnt11 Regulates Blood Lineage Specification

(A) Representative flow cytometry analysis showing hematopoietic cell development induced by Wnt11 at stage I versus stage II of treatment (n = 7).

(B) Histogram showing upregulation of total hematopoietic progenitors ($CD34^+CD45^+$) comparing the effects of Wnt11 treatment at stage I versus stage II and to control (n = 7).

(C) Lack of effects of Dkk1 addition (200 ng/ml) in the presence of Wnt11 stimulated promotion of hematopoietic progenitors compared to controls (n = 4).

(D) Immunoblot of dephosphorylated (stable) β-catenin protein levels in day 1 hEB upon a 2 hr exposure to Wnt11 compared to control treatment and quantitated in bar graphs (n = 3).

(E) Immunoblot against dephosphorylated (stable) β-catenin in hEB-treated Dkk1 compared to control and relative protein levels quantitated in bar graphs (n = 3).

(F) Effects of Dkk1 present during stage I versus stage II of hematopoietic development. No changes in hematopoietic progenitors levels were observed when Dkk1 was present at either stage compared to basal control hematopoietic conditions (n = 3).

(G) Fold changes in hemogenic ($CD31^+CD45^-$) development following 24 hr (1 day) or 7 day Wnt11 treatment compared to control treatment at the same time points.

(H) Total CFU was compared from sorted $CD31^+CD45^-$ precursors derived from day 10 and day 15 EBs treated with Wnt11 (0–1 day) or control-CM treated.

(I) Frequency of hematopoietic progenitors ($CD34^+CD45^+$) from day 15 hEBs treated from 0 to 1 day versus 0–7 days of Wnt11 or control treatment (n = 4).

(J) Total number of CFUs from day 15 hEBs treated with Wnt11 versus control for 1 day (n = 3). Control treatments shown as horizontal dashed line (*$p < 0.01$).

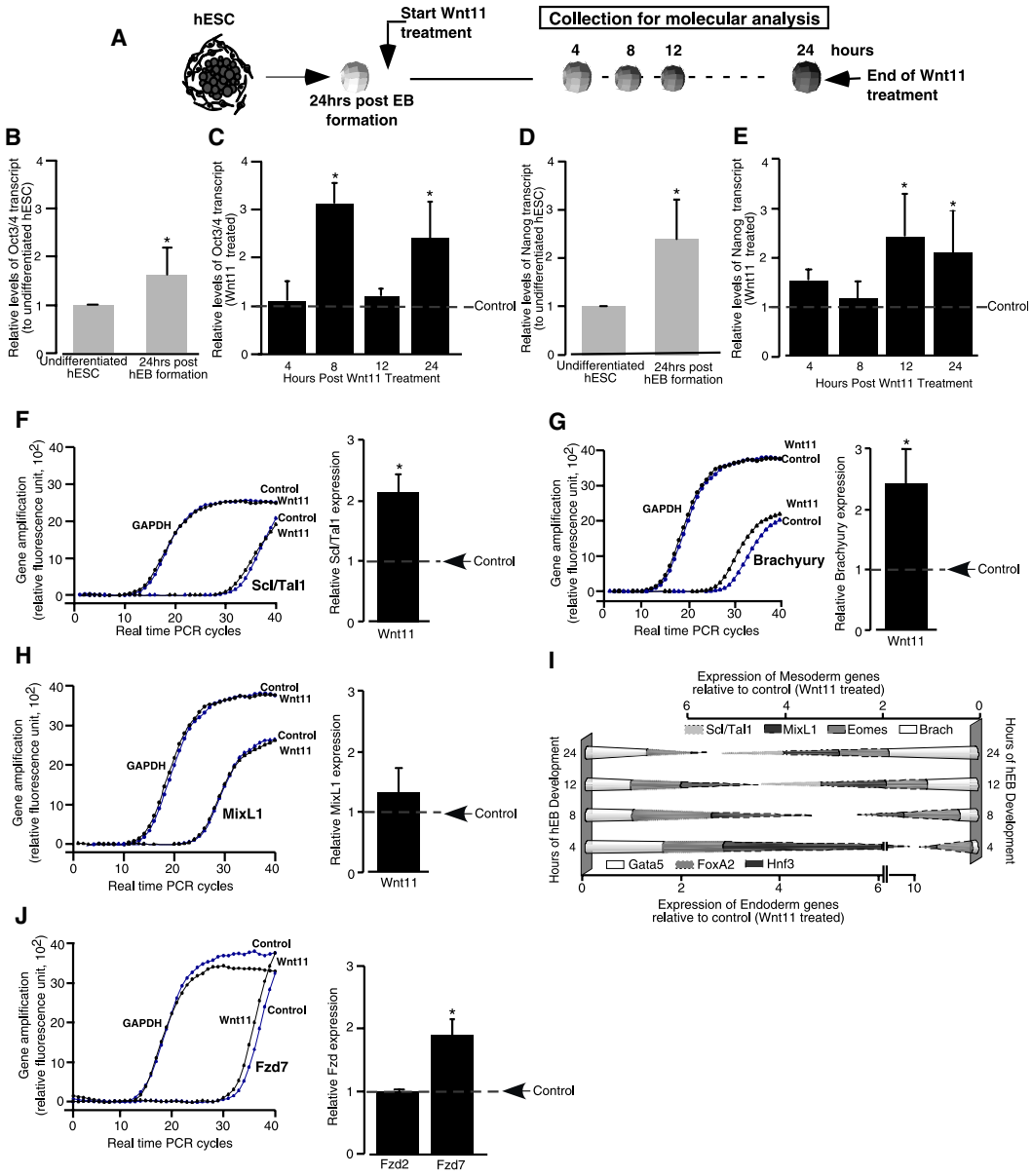


Figure 4. Regulation of Pluripotent and Differentiation Factors by Wnt11 Stimulation

(A) Experimental schema. hESCs were used to form hEBs that were stimulated with Wnt11 24 hr after EB formation (aggregation). QRT-PCR was carried on Wnt11 and control-treated hEBs isolated at 4, 8, 12, and 24 hr after treatment.

(B) Oct4 transcript levels, relative to undifferentiated hESCs, were measured 24 hr after EB formation ($n = 3$, $^*p < 0.05$).

(C) Regulation of Oct4 transcript after Wnt11 hEB treatment at 4, 8, 12, and 24 hr compared to control levels from untreated hEBs at the same time points, represented by dashed line ($n = 3$, $^*p < 0.05$).

(D) Nanog transcript levels, relative to undifferentiated hESCs, were measured 24 hr after EB formation ($n = 3$, $^*p < 0.04$).

(E) Regulation of Nanog transcript after Wnt11 hEB treatment at 4, 8, 12, and 24 hr compared to control levels from untreated hEBs at the same time points, represented by dashed line ($n = 3$, $^*p < 0.05$).

(F–H) Regulation of Scl/Tal1 ($^*p < 0.01$), Brachyury ($^*p < 0.01$), and MixL1 in day 1 hEB treated with Wnt11 (for 24 hr). Expression levels are relative to control conditions shown and are indicated by horizontal dashed line ($n = 8$).

(I) Wnt11 modulation of mesodermal genes (Scl/Tal1, MixL1, Eomesodermin [Eomes] and Brachyury [Brach]) and endodermal genes (Gata5, FoxA2, Hnf3α). Expression levels are relative to control conditions ($n = 3$).

(J) Wnt11 induced changes in Frizzled-2 and -7 expression at day 1 of hEB development as quantified by real-time PCR. Wnt11 treatment only induced Fzd7 expression. Expression levels are relative to control conditions ($n = 6$, $^*p < 0.01$).

during early embryogenesis (Huber et al., 2004; Ng et al., 2005; Shivdasani et al., 1995), were analyzed over time with Wnt11 treatment. Interestingly, undifferentiated hESCs express a wide range of mesoderm (Eomesodermin, Brachyury, MixL1, SCL/TAL1) and endoderm genes (FoxA2, Gata5, Hnf3α). Prior to treatment with the Wnt11, i.e., 24 hr post-hEB formation, endoderm

genes are downregulated while mesoderm genes like Brachyury and Mixl1 are upregulated (Figure S5C). Upon 24 hr of Wnt11 treatment, SCL/Tal1 and Brachyury expression were positively regulated by 2-fold and 2.3-fold, respectively (Figures 4F and 4G). While Brachyury expression levels peaked in day 1 hEBs, MixL1 remained largely unchanged upon Wnt11 stimulation (Figure 4H). Furthermore, Wnt11 induced Brachyury and SCL/Tal1 expression also suggested a previously associated role of Wnt signaling in mesendodermal transition (Hart et al., 2002; Maduro et al., 2005; McLean et al., 2007). To assess the specificity of Wnt11 stimulation, we analyzed the expression of surrogate genes associated with mesoderm and endoderm hours after Wnt11 treatment. Wnt11 stimulation preferentially promotes the early differentiation of mesoderm from hESCs (Figure 4I). These results indicate that Wnt11 represents a key signal that dually orchestrates genes involved in sustaining the pluripotent state, together with those involved in mesendodermal lineage induction, thereby revealing a novel role of noncanonical Wnt signals in human cell fate decisions.

Fzd7 Represents a Target of Wnt11 to Induce Mesodermal Specification

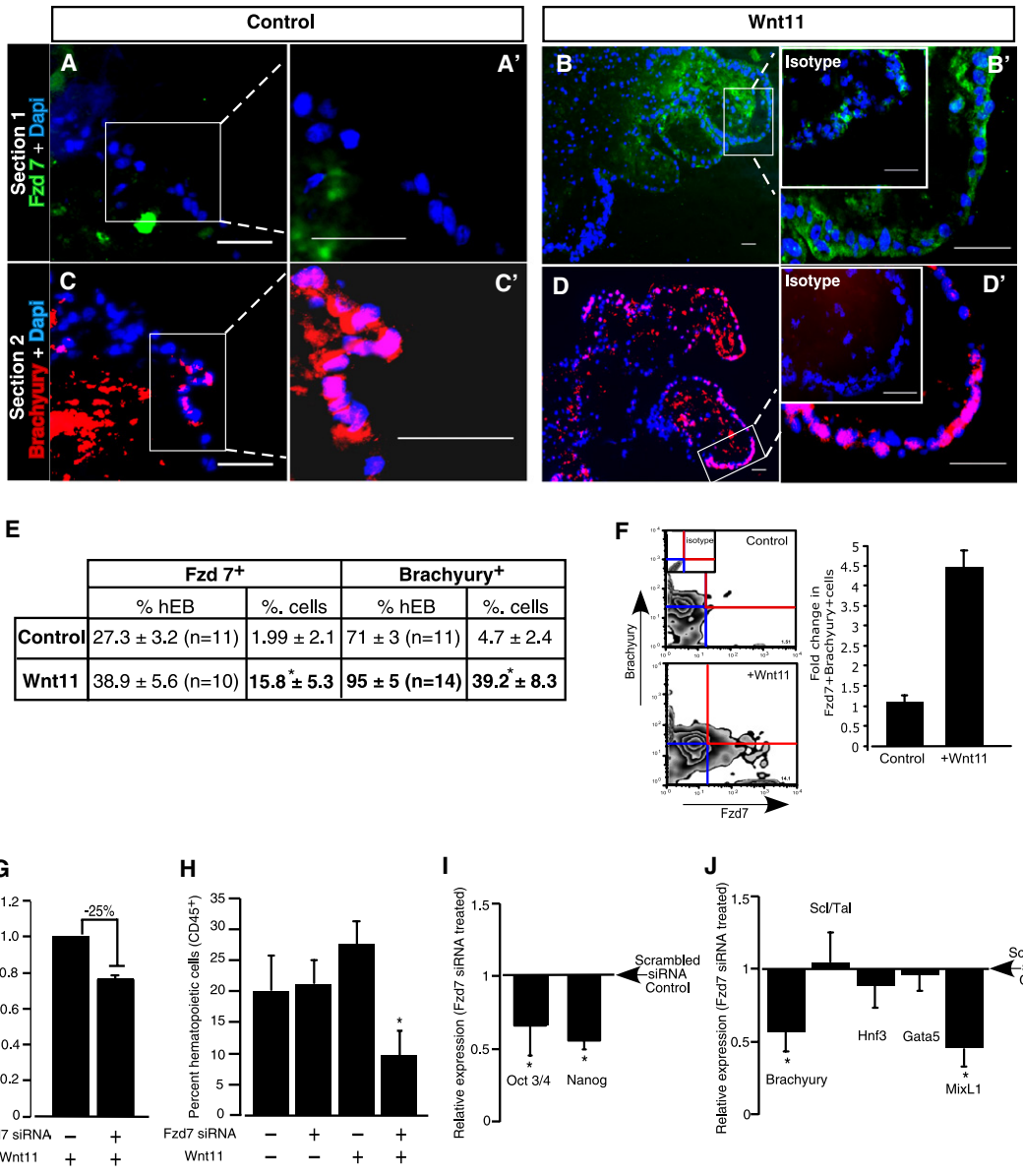
To better understand the receptor and cellular targets for Wnt11 in hESCs, we evaluated expression of candidate Fzd receptors associated with mesoderm lineage development previously linked to Wnt ligand activity in nonhuman models (Toyofuku et al., 2000; Witzel et al., 2006; Djiane et al., 2000). These included the Fzd2 and Fzd7 receptors. In comparison to Fzd2, a 2-fold upregulation in Fzd7 expression was observed with 24 hr of Wnt11 stimulation compared to control-treated hEBs (Figure 4J). This was consistent with observations in the developing mouse embryo, in which unique changes in the Fzd7 expression were induced upon Wnt11 exposure and were reflective of morphogenesis (Djiane et al., 2000; Winklbauer et al., 2001). To further examine the potential relationship between Wnt11-induced mesodermal specification and Fzd7 expression, we stained Wnt11-treated hEBs (Figures 5B–5D) with the early mesodermal marker (Brachyury) and Fzd7 and compared them to control hEBs (Figures 5A and 5C). We could only observe Fzd7 and Brachyury costained cells in the Wnt11-treated hEBs (Figures 5B–5D′). To detail this Wnt11 response, we examined cells for coexpression Fzd7 and Brachyury in control versus Wnt11-treated hEBs via immunofluorescence staining and flow cytometry analysis. Although we observed similar proportions of Brachyury$^+$ hEBs and Fzd7$^+$ hEBs in both control and Wnt11 treatment, Wnt11 stimulation caused 8.3-fold or 7.9-fold increases in percentage of cells expressing Brachyury or Fzd7 proteins, respectively (Figure 5E). Importantly, the identification of cells coexpressing Fzd7 and Brachyury was seen exclusively in the presence of Wnt11 treatment (Figure 5F and Figure S6). Based on these observations, we hypothesized that Wnt11 may act through a primitive Fzd7 receptive target population for mesoderm specification.

To understand the role of Fzd7, we used a loss-of-function approach by silencing Fzd7 during blood differentiation induced by Wnt11. The effect of the Fzd7 siRNAs was demonstrated via changes in the Fzd7+ population frequency (i.e., cell numbers). Treatment of Wnt11-hEBs with siRNA against Fzd7 effectively decreased the percentage of Fzd7$^+$ cells (which are only detectible under Wnt11 treatment) within the developing hEBs 48 hr posttransfection, and reduced the frequencies of day 15 hematopoietic cells from the Wnt11 stimulated hEBs (Figures 5G and 5H). Silencing of other frizzled receptors, such as Fzd2 that has been associated with the canonical Wnt response, did not affect hematopoietic differentiation induced by Wnt11, further supportive of the specific importance of noncanonical Wnt signaling during early hEB differentiation (Figure S7). Reductions in blood development caused by these Fzd7 siRNAs were dependent on the presence of Wnt11 (Figure 5H). Interestingly, silencing of Fzd7 within the Wnt11-stimulated hEBs also resulted in significant downregulation of the pluripotent factors Oct-4 and Nanog, mesoderm gene Brachyury and MixL1, and slight downregulation in endodermal genes Hnf3α and Gata5 but had little affect on SCL/Tal1 expression levels (Figures 5I and 5J). These data reveal that Wnt11 stimulation induces a unique Fzd7$^+$ population not present in the absence of Wnt11 stimulation.

Wnt11 Modulates E-Cadherin Expression and Patterning in Early Mesoderm from hESCs

The noncanonical Wnt signaling is known to play a pivotal role in cellular movements in the embryo proper that may be mediated by architectural scaffolds receptive to signaling cascades that promote lineage differentiation via critical cellular interactions (Dang et al., 2002; Skerjanc et al., 1994). To test if structure alters the receptivity of Wnt11 stimulation to augment hematopoietic development, we compared treatment of hESCs assembled into hEBs versus hESCs in monolayers. EBs and monolayers of hESCs were treated under identical Wnt11 or control conditions and then assayed for blood development. Hematopoietic differentiation induced by Wnt11 was only observed upon EB formation from hESCs and not monolayers cultured and treated under identical conditions (Figure 6A). Molecular analysis after 1 day of hEB formation revealed an intense upregulation of phosphorylated CaMKII activity, which was exclusive to the 30 min Wnt11-treated hEBs and was not observed in hESCs treated in monolayer cultures (Figures 6B and 6C). Undifferentiated hESCs also expressed basal CaMKII activity; however, 30 min stimulation with Wnt11 only increased CaMKII activity in hESCs in EBs and not in hESCs-assembled monolayers. The rapid response of CaMKII in hEBs suggests a direct activation of this signaling cascade by Wnt11. These data support a β-catenin-independent mechanism for Wnt11 action via CaMKII that is interconnected and dependent on cellular interactions supported in complex EB formation and architecture not available in hESCs treated under identical conditions assembled in monolayers.

Early events of mesoderm development in mammalian embryos have been associated with changes in dynamic movement and adhesion properties of nascent mesodermal progenitors (Burdsal et al., 1993; Solnica-Krezel, 2006). In addition, Wnt11 signaling has been shown to mediate E-cadherin-dependent morphogenesis (Burdsal et al., 1993; Toyofuku et al., 2000; Ulrich et al., 2005). To determine if similar mechanisms were responsible for Wnt11 responsiveness in hEBs undergoing mesodermal specification, we examined the expression patterns of E-cadherin in hEBs stimulated with Wnt11 for 24 hr compared to control. Under control conditions, E-cadherin$^+$ cells were present on the periphery of hEBs (Figure 6D, open arrow), whereas Wnt11 treatment induced clustering of E-cadherin-expressing cells



	Fzd 7+		Brachyury+	
	% hEB	%. cells	% hEB	%. cells
Control	27.3 ± 3.2 (n=11)	1.99 ± 2.1	71 ± 3 (n=11)	4.7 ± 2.4
Wnt11	38.9 ± 5.6 (n=10)	**15.8*± 5.3**	**95 ± 5 (n=14)**	**39.2*± 8.3**

Figure 5. Identification of Wnt11-Responsive Population

(A–D′) Serial section of day 1 hEB showing immunolocalization of Fzd7 (green) and Brachyury (red) exclusively in the Wnt11 treatment. Scale bar, 50 μm.

(E) Percent Fzd7- and Brachyury-positive cells in the hEBs treated with Wnt11 compared to control conditions.

(F) Histogram representing fold change in Fzd7$^+$Brachyury$^+$ cell population measured by flow cytometry analysis following 24 hr of Wnt11 treatment at stage I (day1) of hEB development (n = 3, $p < 0.01$). Side panels correspond to the histogram beside and are representative flow cytometry plots indicating that the presence of the Fzd7$^+$Brachyury$^+$ cells is Wnt11 dependent (n = 4).

(G) Percentage of Fzd7$^+$ cells within Wnt11 treated hEBs 48 hr after siRNA (scrambled siRNA versus Fzd7 siRNA) treatment.

(H) Frequencies of hematopoietic cells from day 15 hEBs following Fzd7 silencing within Wnt11 versus control-treated hEBs.

(I) Relative gene expression of pluripotent factors Oct 3/4 (n = 3) and Nanog (n = 3) 48 hr after siRNA (scrambled siRNA versus Fzd7 siRNA) treatment within Wnt11-treated hEBs.

(J) Relative gene expression of mesoderm genes (Brachyury, Scl/Tal1, MixL1) and endoderm genes (Gata5, Hnf3α) 48 hr after siRNA (scrambled siRNA versus Fzd7 siRNA) treatment within Wnt11-treated hEBs, where Brachyury and MixL1 were reduced (n = 3; *$p < 0.01$).

(Figure 6E, closed arrow). Although the percentages of E-cadherin$^+$ hEBs between the control and Wnt11 treatments were similar (data not shown), the Wnt11-treated hEBs showed a higher frequency of these clusters as compared to controls (Figure 6J). The immediate effect of Wnt11 on cellular organization within hEBs is consistent to that recently observed in murine EB development in response to Wnts (ten Berge et al., 2008). Serial sections stained for Fzd7 and E-cadherin demonstrated colocalization exclusively in the Wnt11-treated hEBs (Figures 6H–6I″) versus the control (Figures 6F–6G″). Interestingly, the

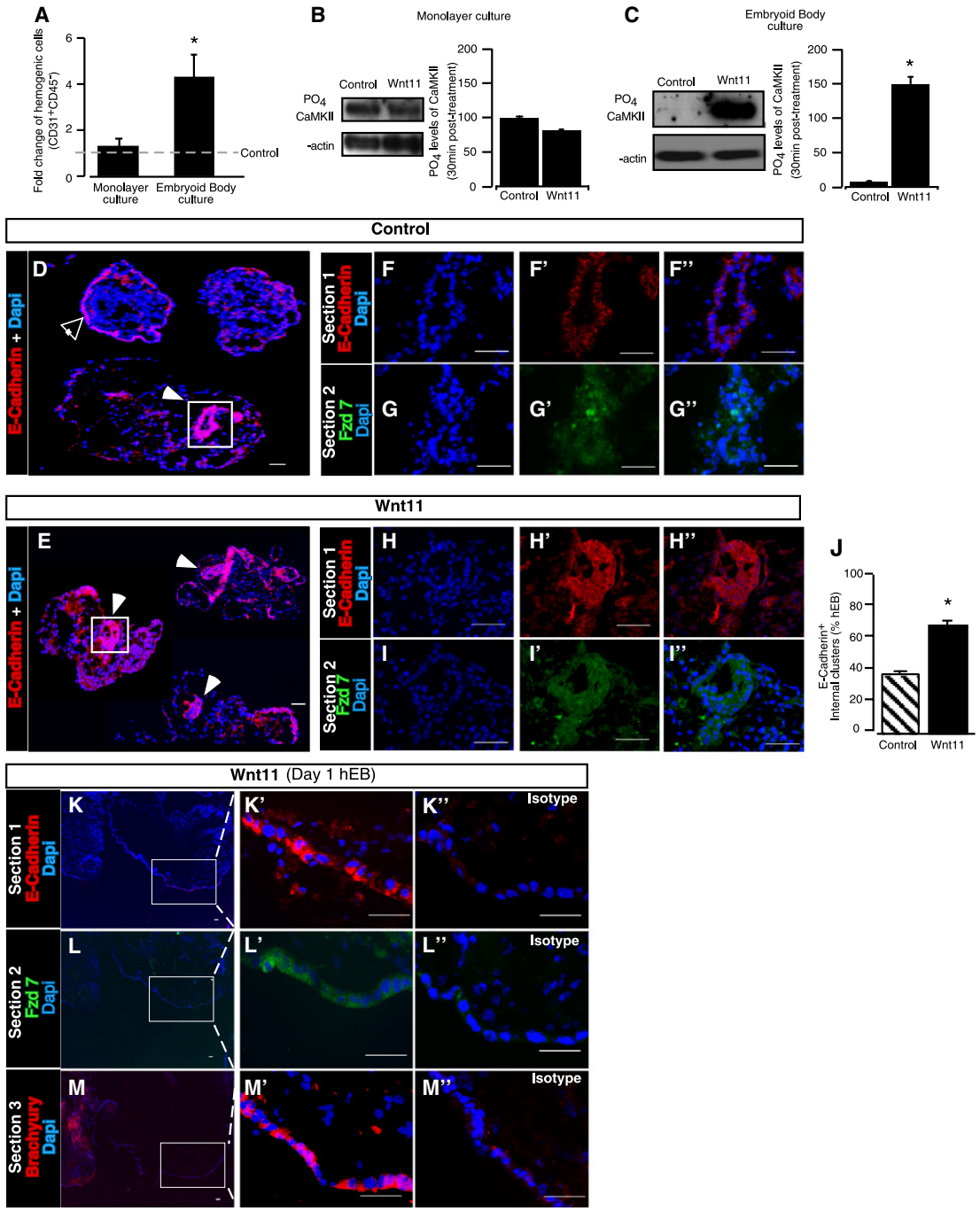


Figure 6. Structural Requirements of Wnt11 Stimulation

(A) Frequency of hemogenic precursors between hESCs assembled in monolayers versus embryoid bodies cultured under identical media conditions in the presence of Wnt11 (n = 4).

(B) Levels of phosphorylated CaMKII protein from monolayer cultures after 30 min exposure to Wnt11 versus control (n = 4).

(C) Levels of phosphorylated CaMKII protein from hEB cultures after 30 min exposure to Wnt11 versus control (n = 3).

(D) Immunostaining of E-cadherin in hEB under control conditions, showing presence of internal E-cadherin cluster (solid arrow) versus E-cadherin staining around the periphery of hEBs (open arrow) Scale bar, 50 μm (n = 4).

(E) Immunostaining of E-cadherin in hEB under Wnt11 conditions showing multiple E-cadherin-rich internal clusters (solid arrows) Scale bar, 50 μm.

(F–G″) Magnification of inset of control-treated hEBs showing lack of immunolocalization of E-cadherin and Fzd7.

(H–I″) Magnification of inset of Wnt11-treated hEB, showing immunolocalization of E-cadherin and Fzd7.

(J) Quantitative analysis of the frequencies of hEB with E-cadherin internal clusters under Wnt11 treatment compared to control (n = 4).

(K–M″) Serial section of day 1 Wnt11-treated hEB, showing immunolocalization of E-cadherin, Fzd7, and Brachyury proteins (n = 6; *p < 0.01).

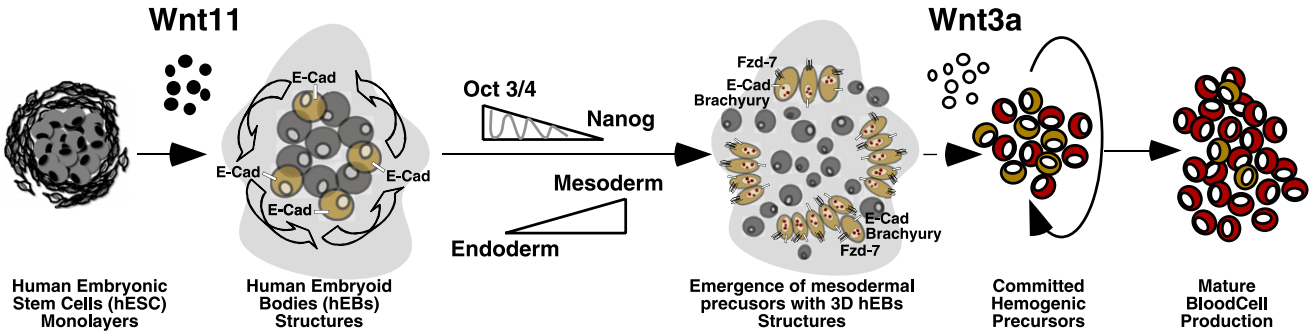


Figure 7. Schematic Representation of the Relationship between Wnts and the Hierarchical Cellular Development of Hematopoietic Cell Fate from hESCs

Noncanonical and canonical Wnt pathways play early and late roles during the sequential development of hematopoietic lineage from hESC, respectively. Under hematopoietic conducive conditions, human ESCs undergo spontaneous differentiation, initially giving rise to the bipotential hemogenic cells (represented in yellow) that can give rise to CD45+ blood cells (represented in red) and endothelial cells. β-catenin-independent (noncanonical Wnt) signaling directed by Wnt11 functions to augment hematopoietic differentiation by combined CaMKII and β-catenin regulation, and morphogenic organization (curved arrows) within the hEBs to cause the exit of pluripotency and induction of mesendoderm genes. In this schema, it is envisaged that the noncanonical Wnt signaling, directed by Wnt11, functions to augment hematopoietic differentiation by regulating mesoderm cell patterning by inducing the expression of Fzd7-, Brachyury-, and E-cadherin-positive cell populations that arise during the specification/initial stages of hEB development. The aggregation of such cell types (Fzd7/E-cadherin/Brachyury positive cells) promotes differentiation toward the blood lineage. In contrast, the canonical Wnt pathway governed by Wnt3a is important in the proliferation of the committed hemogenic and hematopoietic progenitors emerging later during the hEB development, similar to its previously defined role in somatic blood stem cells.

Wnt11-treated hEBs uniquely accumulate Fzd7$^+$, E-cadherin$^+$, and Brachyury$^+$ cells (Figures 6K–6M″). This unique population of Frd7$^+$E-cadherin$^+$Brachyury$^+$ was never detectable in the absence of Wnt11. Taken together, these results describe unique responses to noncanonical Wnt11-induced signals in hEBs that lead to the emergence of unique mesodermal population not present in the absence of Wnt11.

DISCUSSION

Understanding the cellular and molecular processes during lineage specification in vivo forms an important basis to attempt to control in vitro differentiation of hESCs. Reciprocally, hESC differentiation has been suggested as a model to map complex cellular interactions and movement that cannot be accessed in the human embryo. However, these applications have yet to be fully demonstrated in the hESC system. Using mesodermal and subsequent hematopoietic development from hESCs as a basis, our current study identifies an unpredicted role for noncanonical Wnt signaling to induce exit and hematopoietic specification of the hESCs, whereas canonical Wnt signaling was revealed to be limited to proliferation of hemogenic precursors already committed to the blood lineage. Based on our results, we propose a model by which Wnts temporally regulate hematopoietic development from hESCs via unique mechanisms known to be important in the developing embryo (Figure 7).

In vivo generation of initial blood-forming stem cells involves several developmental stages coupled to anatomical movement in the human (Tavian and Peault, 2005). Accordingly, it is likely that signaling factors governing this process are diverse and that noncanonical and canonical Wnts would affect different stages and target populations contributing to mammalian embryonic hematopoiesis similar to their effects in other lineages (Logan and Nusse, 2004). In vitro, specification events and expansion of the hematopoietic compartment can only be modeled using pluripotent cells required to make lineage choice. This cellular process has been broadly envisioned as a loss of pluripotent state and subsequent emergence of hemogenic precursors responsible for final hematopoietic differentiation and blood cell output (Ogawa et al., 2001). Although this model has been recapitulated in several species, the earlier events from pluripotent stage to mesodermal transitions and the growth factors required have not been extensively studied. Taking the current model of hESC blood development into account (Wang et al., 2004), we reveal a unique role for noncanonical Wnt signaling mediated through Wnt11 that promotes exit from the pluripotent state to mesodermal specification. The effect of Wnt11 was dependent on hESC assembly into EB structures, since no effect on hematopoietic output was induced using monolayers of hESCs (Figure 7). Wnt11 induces blood cell fate through combined CaMKII and β-catenin regulation and morphogenic organization within hEBs and causes changes in expression of pluripotent factors Oct and Nanog as mesodermal genes are rapidly induced (Figure 7). These combined processes cause the development of a unique subset of Fzd7 expressing cells that coexpress Brachyury and E-cadherin, both associated with mesodermal development and mesodermal cell movement in early mammalian development (Huber et al., 2004; Kwan and Kirschner, 2003; Winklbauer et al., 2001; Yamanaka and Nishida, 2007).

This is the first report revealing the importance of noncanonical Wnt signaling during human embryonic hematopoiesis; however, Wnt11 and similar cellular signals have been associated with hematopoietic output in other species. The presence of Brachyury$^+$, Fzd7$^+$, and E-cadherin$^+$ mesoderm progenitors as clusters within the Wnt11-treated hEBs, together with changes in mesendoderm to mesoderm gene expression, illustrate the role of the noncanonical Wnt signaling during embryonic mesoderm induction from hESCs. Association of Wnt11 to blood development has been reported in early avian and

amphibian development, in which Wnt11 influences the multilineage potential of blood cells from avian mesoderm stem cells (Eisenberg and Eisenberg, 1999; Eisenberg et al., 1997). Furthermore, a recent report by Kim et al. showed preferential expression of Wnt11 and Fzd7 within the Flk+ (hemogenic cell marker) population derived from mESCs (Kim et al., 2008). At the signaling level, the robust upregulation of phosphorylated CaMKII observed immediately upon Wnt11 exposure of hEBs correlates with the previous observation that CaMKII activation induced by noncanonical Wnt pathway in the *Xenopus* embryos is able to promote hematopoietic-associated ventral cell fates (Kuhl et al., 2000; Moon et al., 1993). Importantly, the exclusive increase of phosphorylated levels of CaMKII within the hEBs and not in treated monolayers of hESCs underscores the importance of 3D structure (Duprat, 1996; Gurdon et al., 1993; Kato and Gurdon, 1993) and cellular movement required to mediate mesodermal inductive Wnt11 effects. E-cadherin has been specifically implicated in the polarized segregation of mesodermal progenitor cells undergoing EMT at the primitive streak (Burdsal et al., 1993; Ciruna and Rossant, 2001). Taken together, our observations using the hESC system complement observed effects of Wnt11-based CaMKII signaling in other species, in which the earliest events of specification toward mesoderm can be moderated.

The role of Wnt3a to enhance blood production has been observed in several systems, such as zebrafish specification to hemogenic mesoderm (Martin and Kimelman, 2008; Shimizu et al., 2005), early mouse embryo development (Lindsley et al., 2006; Liu et al., 1999), mESC-hematopoiesis (Lengerke et al., 2008; Nostro et al., 2008), and hESC-derived hematopoiesis (Woll et al., 2007). However, the temporal nature of the signaling and cellular mechanism of Wnt3a actions was not delineated, nor were the potential interactions and effects of noncanonical pathways and associated ligands such as Wnt11. Similar to these previous studies, addition of Wnt3a augmented hematopoiesis derived from hESCs here and further revealed the action of Wnt3a was mediated via induced proliferation of cells already committed to the blood lineage and not early specification events (Figure 7). The biological effect of canonical Wnt observed here in hESCs is consistent with multiple studies showing the proliferative effects of canonical Wnt activation on mouse and human somatic blood stem cells (Kirstetter et al., 2006; Reya et al., 2003; Scheller et al., 2006; Trowbridge et al., 2006; Willert et al., 2003). Accordingly, despite the previously recognized role of canonical Wnt signals in mesodermal development, the interplay of noncanonical Wnt signaling should be examined in other development systems based on our current observations in hESCs. Moreover, interplay of canonical and noncanonical Wnt pathways in the context of human mesodermal and endodermal development cannot be ruled out, and it can set the platform for further studies, utilizing loss-of-function and gain-of-function genetic techniques, that could clarify these complex interactions.

Our study reveals that noncanonical versus canonical Wnt signaling is capable of guiding differentiation of embryonic stem cells toward mesoderm and subsequent hematopoietic fate, respectively, thereby establishing that members of the Wnt family are capable of controlling unique transitions of blood development in the human. These observations provide unique insights into early developmental events in the human, given the inability to access these processes in the human embryo. Since Wnt11 induces emergence of a unique population of Fzd7$^+$/Brachury$^+$/E-cadherin$^+$ cells, it will be important to evaluate the lineage potential of these cells that may possess broader potent developmental capacity to other mesodermal lineages such as muscle, cardiac, and bone derivatives. These capacities are currently being explored in our lab, along with defining culture methods to sustain this subpopulation in vitro. The ability to sustain these cells in culture will further allow examination of the role of Wnts in combination with others associated with ventral mesodermal fate such as BMP-4 toward a better understanding organization of signals required for hESC hematopoietic differentiation. Collectively, these findings will need to be applied to clinical goals of generating sufficient numbers of appropriately programmed hematopoietic cells from hESCs that possess HSC properties of in vivo reconstituting function similar to HSCs obtained from human bone marrow or cord blood.

EXPERIMENTAL PROCEDURES

Preparation of Soluble Wnt Proteins

Control and Wnt-expressing L cells were grown in media used to culture the hEBs. The hEB media consisted of 80% knockout Dulbecco's modified Eagle's medium (KO-DMEM; GIBCO, Burlington, ON, Canada), 20% non-heat-inactivated FBS (Hyclone), 1% nonessential amino (GIBCO) acids, 1 mM L-glutamine (GIBCO), and 0.1 mM β-mercaptoethanol (Sigma, Oakville, ON, Canada). The CM-containing active Wnt3a, Wnt11, and CM from control L cells were collected every 3 days, and two collections were carried out before use. Cell debris was removed from the CM by centrifugation at 250 g for 10 min.

hESCs Derived from Embryos and hEB Cultures

Human ESC lines H1 and H9 were cultured in MEF-CM supplemented with 8 ng/ml of bFGF (Invitrogen) as a feeder-free culture on Matrigel (BD Biosciences, Mississauga, ON, Canada). When hESC cultures have reached 80%–90% confluence and hESC colonies are dense, the cultures are disassociated using 200 U/ml Collagenase IV (GIBCO) and passaged as 1:2 ratios onto fresh Matrigel. The media were changed every day. All experiments were carried out on both the H1 and H9 cell lines. Human embryoid bodies (hEBs) were formed as previously described (Chadwick et al., 2003). The hEBs were cultured under hematopoietic conducive conditions (300 ng/ml SCF, 300 ng/ml Flt-3L, 10 ng/ml IL3 and IL-6, 50 ng/ml G-CSF, 25 ng/ml BMP-4) and supplemented with 4% vol/vol of either control Wnt3a or Wnt11 CM the day after hEB formation. Conditioned media were supplemented either during the entire hEB differentiation or during 0–7 days (stage I) or 7–15 days (stage II) of the developing hEB. Time zero thus represents the day at which the cytokines, growth factor, or CM are added. Dkk1 treatment (200 ng/ml) was done during stage I, stage II, or both stages of hEB differentiation.

Assessment of Hematopoietic Differentiation and E-Cadherin Expression by Flow Cytometry and Data Analysis

Hemogenic (recognized phenotypicially as CD45$^-$CD31$^+$) and hematopoietic cells (CD45$^+$) were identified by staining single cells (2–5 × 10^5 cells/ml) isolated from hEBs disassociated with 0.4 U/ml Collagenase B (Roche Diagnostics, Laval, QC, Canada) from day 15 with fluorochrome-conjugated monoclonal antibodies (mAb) CD31-PE and pan-leukocyte marker CD45-APC (Milteny Biotech, Germany). The mAb and their corresponding isotypes were used at 1–2 mg/ml. Frequencies of cells possessing the hemogenic and hematopoietic phenotypes were determined on live cells by 7AAD (Immunotech) exclusion, using FACSCalibur, and analysis was performed using the FlowJo software (Tree Star). Hemogenic and hematopoietic cellularity was determined by multiplying total cellular yield by their respective frequencies. For data analysis, effect on frequencies and total hemogenic and hematopoietic output by the Wnt-CMs were normalized to the control-CM within each experiment. Data presented are mean ± SEM of pooled normalized values between

experiments. Statistical significance was determined using the ANOVA-Tukey HSD test and is reported as $p < 0.05$. For E-cadherin analysis, 2 mg/ml of monoclonal anti-human APC-conjugated E-cadherin and APC-labeled mouse IgG2a isotype were used (R&D Systems). For Fzd7 and Brachyury analysis, 0.5 μg/μl anti-human Fzd7 (R&D Systems) conjugated with FITC and anti-human PE-conjugated Brachyury (R&D Systems) were used.

Hematopoietic Progenitor Assay, CFU Assay

Clonogenic blood progenitor assays were carried out by plating 10–15 × 10^3 single cells from day 15 hEBs onto methylcellulose H4230 (StemCell Technologies, Vancouver, BC, Canada) supplemented with human recombinant growth factors described previously (Wang et al., 2004). Differential colonies in the methylcellulose culture were scored based on standard morphological criteria after 14 days incubation at 37°C in a humidified chamber.

Cell-Cycle Analysis

hEBs were pulsed with 10 mM BrdU (Becton Dickinson) 24 hr before hEB disassociation. 1 × 10^6 single cells were stained for membrane CD proteins with fluorochrome-conjugated CD31-PE and CD45-FITC (Milteny Biotech, Germany) prior to processing for intracellular staining with BrdU-APC mAb (Becton Dickinson). The cells were fixed and permeabilized according to the manufacturer protocol (Becton Dickinson). Cellular proliferation was quantified by flow cytometry and qualified by Spinning Disk Confocal microscopy. For image analysis, the fluorochrome-immunostained single cells were cytospun and excited with a 488 (FITC detection), 543 (PE) or 647 nm (APC) laser path on the Lieca DMI 6000 B microscope (Bernsheim, Germany). Images were acquired with the Volocity 4 (Improvision, Coventry, UK) and analyzed with the ImageJ v1.37 (http://rsb.info.nih.gov/ij/) and ImagePro plus v6.1 software.

siRNA Transfection

For siRNA transfections, clumps of hESC colonies were obtained as previously mentioned and transferred to 6-well ultra-low attachment plates (Corning). Cell were transfected in EB differentiation medium using lipofectamine (Ambion, Inc., USA) and 50–100 nM siRNA according to the manufacturer's instructions. After 24 hr, medium was changed to EB differentiation medium supplemented with hematopoietic growth factors. All siRNAs were purchased from Ambion, Inc., and their effects were tested individually and in combination. The following siRNAs were used: Fzd2 siRNA, ID 4057, 45981, and 3962; Fzd7 siRNA, ID 4955 and 4861; Scrambled siRNA #1. siRNA transfection efficiency was assessed using a Silencer Cy3-labeled negative Control #1 siRNA (Ambion, Inc.).

Western and Immunoprecipitation

hESCs (1–3 × 10^6) or day 1 hEB cells or 2–4 × 10^5 cells of day 15 hEBs were lysed with buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 10 mg/ml protease inhibitors (Leupeptin, Aprotonin, Apeptin), 0.5 mM PMSF, and phophatase inhibitor cocktail (SetIV, Calbiochem). For western blots, equal amount of proteins (20 μg) were separated using 12%–15% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% skimmed milk for 2 hr and blotted with the primary antibody overnight at 4°C. The following primary antibodies were used: rabbit phospho-β-catenin (Ser33/37, Thr41) (1:1000, Cell Signaling), mouse monoclonal dephopho β-catenin (1:500, Alexis Biochemicals), mouse monoclonal total β-catenin (1:1000, Becton Dickinson), rabbit polyclonal phospho-CaMKII (1:1000, Cell Signaling), mouse monoclonal phospho-SAPK/JNK (1:2000, Cell Signaling), and mouse monoclonal Oct4 (1:1000, BD Transduction Lab). The membranes were washed and stained with HRP-conjugated goat anti-rabbit or anti-mouse Ab (1:10 000, Santa Cruz), and signals were detected with the enhanced chemiluminescence method (Pierce); membranes were exposed to X-ray film and UVP Bioimaging system (UVP, California), and band intensities were subsequently quantified using the ImagePro software. Immunoprecipitation (IP) was performed for detection of CaMKII and JNK activity. hESCs or hEB cell lysates (300–500 mg of total) were allowed to interact with rabbit polyclonal total CaMKII (1:250, Cell Signaling) or total JNK (1:250, Cell Signaling) immobilized antibodies (Aminolink Plus Coupling Gel, Pierce Biotechnology) according to the manufacturer's protocol. Western blots were performed on the total CaMKII and JNK IP proteins, and phosphorylated versions of the respective proteins were identified with the phospho-CaMKII and phospho-JNK Ab. Immunoblots were stripped and reblotted for either mouse polyclonal β-actin (1:5,000; Sigma), mouse GAPDH (1:10,000; Cell Signaling); or total JNK (1:1,000; Cell Signaling) as loading control.

Immunocytochemistry

Wnt- and control-treated hEBs were washed two to three times in 1 × PBS/3% FCS, fixed with 4% paraformaldehyde/PBS for 2 hr, embedded, and then snap frozen in liquid nitrogen and stored at −80°C for staining with Fzd7, E-cadherin, Brachyury, and PCNA. Cryostat serial sections (8 mM) were made for each treated specimen, and successive serial sections were single-stained with Fzd7, E-cadherin, and Brachyury proteins for detection within the same hEB region. For each single staining, three sections at an interval of seven to ten (25–30 μm) serial sections were used. The sections were hydrated with 1 × PBS and permeabilized with 0.3% Saponin/PBS for staining Fzd7 and E-cadherin, and 0.1% Triton X-100/PBS for Brachyury and PCNA. All Ab-stained sections were washed in their respective permeabilization buffer. Sections were blocked with either 10% normal goat serum (Fzd7 and E-cadherin), 10% normal donkey serum (for Brachyury), or 10% normal rabbit serum (PCNA) + 1% BSA at room temperature (RT) for 2 hr. The following primary antibody and dilutions were used: rabbit polyclonal Fzd7 (13 μg/ml, Abcam), goat affinity-purified Brachyury (10 μg/ml, R&D Systems), mouse monoclonal E-cadherin (10 μg/ml, Alexis), and mouse monoclonal PCNA-FITC (1:250, Abcam). Sections were incubated with primary antibodies overnight and subsequently secondary stained for 1 hr with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) for Fzd7, Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes) for E-cadherin, or Alexa Fluor 594 donkey anti-goat IgG (Molecular Probes) for Brachyury at 2 μg/ml. Slides were mounted and counterstained using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Labs). Sections were examined using the Olympus I × 18 microscope, and images were captured with a Photometrix Cool Snap HQ^2 camera using in vivo version 3.1.2 (Photometrix) software. Images were pseudocolored and analyzed using Image-Pro software.

Quantitative Real-Time PCR

Total RNA was isolated from hEBs using the QIAGEN AllPrep RNA Mini Kit (QIAGEN). Complementary DNA (cDNA) was made with 1–5 μg of total RNA using the first-strand cDNA synthesis kit (Amersham Biosciences), and subsequent quantitative real-time PCR (Q-rtPCR) was carried out in duplicate, using Platinum SYBR Green qPCR Super Mix-UDG on an Mx3000P Q-PCR System according to the manufacturer's instructions (Invitrogen). Amplifications were performed using the following conditions: 95°C, 10 min and 40 cycles 95°C, 30 s; 60°C, 1 min; 72°C, 30 s. All data were normalized to GAPDH, and relative gene quantification for Wnt effects was calibrated against the following control-CM effects: Wnt and control treatment Ct-Gapdh Ct = ΔCt then Wnt Δ Ct – control Δ Ct = ΔΔ Ct, and relative expression is expressed as $2^{(-\Delta\Delta\ Ct)}$. Q-rtPCR primers are listed in Table S1.

SUPPLEMENTAL DATA

The Supplemental Data include seven figures and one table and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00003-4.

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