

Wnt2 Coordinates the Commitment of Mesoderm to Hematopoietic, Endothelial, and Cardiac Lineages in Embryoid Bodies*

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Our recent gene expression profiling analyses demonstrated that Wnt2 is highly expressed in Flk1⁺ cells, which serve as common progenitors of endothelial cells, blood cells, and mural cells. In this report, we characterize the role of Wnt2 in mesoderm development during embryonic stem (ES) cell differentiation by creating ES cell lines in which Wnt2 was deleted. Wnt2^{-/-} embryoid bodies (EBs) generated increased numbers of Flk1⁺ cells and blast colony-forming cells compared with wild-type EBs, and had higher Flk1 expression at comparable stages of differentiation. Although Flk1⁺ cells were increased, we found that endothelial cell and terminal cardiomyocyte differentiation was impaired, but hematopoietic cell differentiation was enhanced and smooth muscle cell differentiation was unchanged in Wnt2^{-/-} EBs. Later stage Wnt2^{-/-} EBs had either lower or undetectable expression of endothelial and cardiac genes compared with wild-type EBs. Consistently, vascular plexi were poorly formed and neither beating cardiomyocytes nor α -actinin-staining cells were detectable in later stage Wnt2^{-/-} EBs. In contrast, hematopoietic cell gene expression was up-regulated, and the number of hematopoietic progenitor colonies was significantly enhanced in Wnt2^{-/-} EBs. Our data indicate that Wnt2 functions at multiple stages of development during ES cell differentiation and during the commitment and diversification of mesoderm: as a negative regulator for hemangioblast differentiation and hematopoiesis but alternatively as a positive regulator for endothelial and terminal cardiomyocyte differentiation.

Embryonic stem (ES)² cells have considerable potential for use in cellular therapies for many human diseases or disorders.

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² The abbreviations used are: ES, embryonic stem cell; EB, embryoid body; VEGF, vascular endothelial growth factor; Flk1, fetal liver kinase 1; BL-CFC, blast colony-forming cells; qRT, quantitative reverse transcriptase; PECAM,

platelet-endothelial cell adhesion molecule; SMC, smooth muscle cell; SM, smooth muscle; RT, reverse transcriptase; FACS, fluorescence-activated cell sorter.

However, manipulating the differentiation of pluripotent cells into a desired cell type is difficult to control. Therefore, efforts have been directed toward understanding the molecular and cellular mechanisms for maintaining ES cell pluripotency as well as for ES cell fate determination and lineage specification. Under appropriate culture conditions, ES cells will spontaneously differentiate and form embryoid bodies (EBs) that recapitulate the differentiation program of normal embryonic development. This *in vitro* experimental model is widely used to study mesoderm development, including the hematopoietic system, vascular endothelial cells, and cardiomyocytes (1–4).

Murine hematopoietic and endothelial cell development begins in the yolk sac mesoderm at approximately day 7.5 of gestation and is marked by establishment of blood islands (5, 6). The close physical relationship between these two cell populations within blood islands has led to a widely accepted hypothesis that a common precursor for both hematopoietic and endothelial cells exists, the hemangioblast (7, 8). The receptor for vascular endothelial growth factor (VEGF), fetal liver kinase-1 (Flk1), is an early molecular marker for hemangioblasts (9–12). Blast colony-forming cells (BL-CFCs) derived from EBs can generate hematopoietic and endothelial cell lineages, and represent the long-hypothesized hemangioblast (13, 14). Flk1⁺ cells may also give rise to smooth muscle cells (SMC) and cardiomyocytes under appropriate culture conditions (15–17).

Wnts are paracrine/autocrine signaling molecules that have been implicated in many aspects of normal development. Canonical Wnt signaling acts through β -catenin and T-cell factor/lymphoid enhancer factor transcriptional factors that regulate target gene expression (reviewed in Refs. 18 and 19). Developmental Wnt signaling is active during embryogenesis in the cardiac crescent and in endothelial cells during intraembryonic angiogenesis (20). Wnt ligands, receptors, and inhibitors are expressed by vascular cells and participate in both normal and pathologic vessel growth, yet the key players in these processes and their roles are not yet identified (reviewed in Ref. 21). Wnt signaling also participates in hematopoiesis by providing proliferative signals for immature hematopoietic progeni-

platelet-endothelial cell adhesion molecule; SMC, smooth muscle cell; SM, smooth muscle; RT, reverse transcriptase; FACS, fluorescence-activated cell sorter.

tors and promoting the self-renewal of hematopoietic stem cells (22, 23).

Among the various Wnts, Wnt2 is localized to sites within the heart, vasculature, and associated mesoderm during development, making it a likely candidate to regulate mesoderm differentiation and subsequent hematovascular development (24, 25). *Wnt2*^{-/-} mice have been generated and analyses of these mice indicate that Wnt2 is required for proper vascularization of extraembryonic tissues, although a complete assessment of hematovascular development was not performed (25). Remarkably, our inductive microarray studies indicate that Wnt2 is among the most highly up-regulated genes in Flk1⁺ cells during EB differentiation, suggesting that Wnt2 is the key Wnt signal for coordination of hemangioblast differentiation from primitive mesoderm. To extend these studies, we established ES cell lines carrying targeted mutations for Wnt2 to characterize mesoderm differentiation in EBs. Wnt2 plays critical roles in the commitment and diversification of mesoderm, acting as a positive signal for endothelial and cardiomyocyte differentiation but inhibiting hemangioblast differentiation and hematopoiesis, suggesting that manipulation of Wnt2 signals may be useful to tune stem cell differentiation for vascular applications.

EXPERIMENTAL PROCEDURES

Generation of Wnt2 Mutant ES Cell Lines and ES Cell Culture—The R1 ES cell line heterozygous for the Wnt2 mutation (*Wnt2*^{+/^{neo}}) was previously described (25). The hygromycin (hygro-) targeting vector was constructed using strategies (outlined in Fig. 1) similar to that for the Neo-targeting vector (25). The hygro-targeting construct was electroporated into *Wnt2*^{+/^{neo}} ES cells. Homologous recombination occurring between the hygro-targeting vector and the wild-type allele replaces exon 2 of *Wnt2* with the PGK-hygro cassette, resulting in a compound heterozygote (*Wnt2*^{hygro/^{neo}}). Hygro-resistant ES cell colonies were screened by PCR with a primer set indicated by arrows in Fig. 1. PCR-positive colonies were grown in G418-containing media and the subsequent G418-resistant colonies were analyzed by Southern blotting to confirm homologous recombination. Genomic DNA digested with SacI was probed by a 0.5-kb 3' external fragment (indicated by a bar in Fig. 1). Of 117 colonies analyzed, two *Wnt2*^{hygro/^{neo}} (hereafter referred to as *Wnt2*^{-/-}) ES cell colonies (G3 and D8) were obtained, and G3 was used in all of following experiments, although cells derived from both clones behaved identically in our assays. *Wnt2*^{+/+}, *Wnt2*^{+/-}, and *Wnt2*^{-/-} ES cells were maintained on mitomycin-treated feeder cells in the presence of LIF. Cells were transferred to gelatin plates with 5637 cell-conditioned medium as a source of LIF and passaged two times to remove feeder cells before the onset of differentiation. *In vitro* differentiation was performed as previously described (26).

FACS Analysis and Immunostaining—EBs were dissociated with trypsin and stained with phycoerythrin-conjugated anti-Flk1 monoclonal antibody (BD Pharmingen) for FACS analysis of Flk1 expression. Cells were analyzed on a FACScan (BD Biosciences) with Cytomation Summit software. For immunostaining, EBs were fixed in ice-cold methanol:acetone (50:50), blocked, and incubated with primary antibodies followed by

appropriate secondary antibodies. The primary antibodies used were rat anti-PECAM-1 (MEC13.3, BD Pharmingen), mouse anti- α -actinin (EA-53, Sigma), and mouse anti- α -smooth muscle actin (1A4, Sigma). The secondary antibodies used were Alexa 488-conjugated goat anti-rat IgG and Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes).

Reverse Transcriptase-Polymerase Chain Reaction—Semi-quantitative RT-PCR was performed as previously described (27). Quantitative RT-PCR (qRT-PCR) was performed with an Applied Biosystems 7700 Sequence Detection System using total RNA purified from EBs. Amplifications were performed in triplicate for 40 temperature cycles. Copy numbers of the mRNA template in each sample were calculated using the ABI Sequence Detector Software.

Hemangioblast Colony and Hematopoietic Progenitor Cell Assays—EBs were generated as described with some modification (28). Briefly, ES cells were plated in 6-well dishes in differentiation medium containing Iscove-modified Dulbecco's medium, 15% differentiation fetal calf serum (StemCell Technologies, Vancouver, BC, Canada), glutamine (2 mM), monothioglycerol (450 μ M), holo-transferrin (500 μ g/ml), and ascorbic acid (50 μ g/ml). ES cells were plated at 50,000 cells/ml for day 4 EBs or 5,000 cells/ml for days 5–6 EBs, with an additional 5% protein-free hybridoma medium (Invitrogen).

BL-CFCs, erythroid, and myeloid colonies were generated as described (28, 29). For BL-CFC assays, day 4 EB cells were plated at 50,000 cells/ml in 1% methylcellulose with 10% differentiation fetal calf serum, 25% D4T cell-conditioned media (13), c-kit ligand (1% conditioned medium), and VEGF (5 ng/ml, R & D Systems). Days 5 and 6 EB cells were plated at 50,000 cells/ml in 1% methylcellulose with 15% plasma-derived serum (Animal Technologies, Antech, TX), erythropoietin (20 units/ml) (Amgen, Thousand Oaks, CA), kit ligand (1%), thrombopoietin (2% conditioned medium), transferrin (500 μ g/ml), and 5% PFHM-II for primitive and definitive erythroid colony assays, or with 15% plasma-derived serum, erythropoietin (20 units/ml), c-kit ligand (1%), thrombopoietin (2%), 5% hybridoma medium, interleukin-3 (1% conditioned medium), and interleukin-11 (5 ng/ml, R & D Systems) for myeloid and mixed colony assays. Primitive and definitive erythroid colonies were counted after 4–6 days, and myeloid and mixed colonies were counted after 7–10 days.

Production of Conditioned Media—We used a *Drosophila* S2 cell line expressing Wnt2 and a control S2 cell line, which were generous gifts from Roel Nusse (Stanford University). Wnt2 S2 cells were cultured in Schneider's media supplemented with 10% fetal bovine serum under selection with hygromycin (125 μ g/ml) for 2–3 passages before collection of media.

RESULTS

Increased Numbers of Flk1⁺ Cells and BL-CFC Frequency in Wnt2-deficient EBs—In a previously described set of gene expression profiling experiments (comparing gene expression profiles in Flk1⁺ cells versus Flk1⁻ cells at different stages during ES cell *in vitro* differentiation), we found that Wnt2 expression was much higher in Flk1⁺ cells in comparison with Flk1⁻ cells (27). This expression pattern was confirmed by RT-PCR analysis, which indicated that Wnt2 was expressed in early

Wnt2 and Hematovascular Differentiation

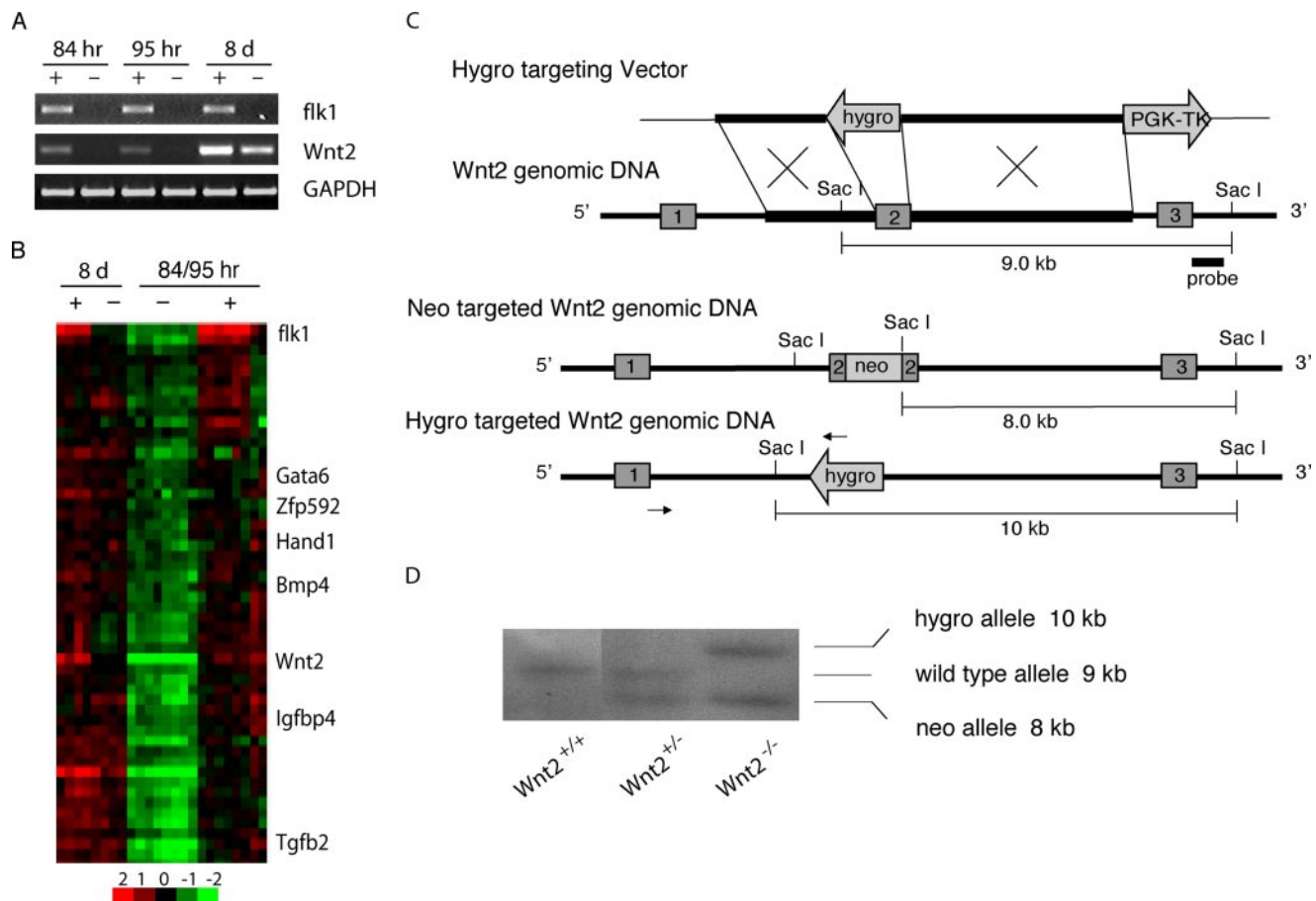


FIGURE 1. Wnt2 expression analyses and targeted mutation of the Wnt2 locus to generate $Wnt2^{hygro/neo}$ ($Wnt2^{-/-}$) ES cell lines. *A*, RT-PCR confirmation of Wnt2 expression pattern in Flk1⁺ versus Flk1⁻ cells at the indicated times during ES cell differentiation. *B*, hierarchical clustering analysis for differentially expressed genes in Flk1⁺ versus Flk1⁻ cells during ES cell differentiation indicating that Wnt2 is closely clustered with Flk1 and other mesoderm-associated genes. The color scale ranges from green for log ratio -2 to red for log ratio +2. *C*, schematic representation of the Wnt2 gene-targeting strategy. The hygro targeting vector, the wild-type Wnt2 locus including exons 1–3, the original neo-targeted Wnt2 allele (25), and the hygro-targeted Wnt2 allele are shown. PCR screening primers (arrows) and 3'-external probe (horizontal bar) are indicated. Digestion of genomic DNA with SacI and hybridization with a 3'-probe identify different band sizes: 9.0 kb for the wild-type allele, 8.0 kb for the neo allele, and 10 kb for the hygro allele, respectively. *D*, verification of homologous recombination occurring at the wild-type allele in ES cell clones by Southern blotting. The size of the DNA band is indicated in kb.

Flk1⁺ cells and undetectable in Flk1⁻ cells, and was expressed in later stage Flk1⁻ cells at much lower levels compared with Flk1⁺ cells (Fig. 1A). In a hierarchical clustering analysis for differentially expressed genes in Flk1⁺ versus Flk1⁻ cells over time, Wnt2 was clustered closely with Flk1 and other genes related to mesoderm development such as *Bmp4*, *Tgfb2*, *Hand1*, and *Gata6* (Fig. 1B).

Based on these observations, we hypothesized that Wnt2 may participate in developmental events within the mesoderm that determine vascular and/or hematopoietic differentiation. Therefore, we initially evaluated the effects of Wnt2 on the early development of Flk1⁺ cells by creating $Wnt2^{-/-}$ ES cells (Fig. 1, C and D). We compared these cells to $Wnt2^{+/-}$ ES cells, similar to approaches that have been previously described for other targeted alleles in this system (10), because the $Wnt2^{-/-}$ and $Wnt2^{+/-}$ ES cells have been through identical selection processes and $Wnt2^{+/+}$ ES cells lacking a selection marker cannot be brought through the same selection process. Nevertheless, $Wnt2^{+/-}$ ES cells differentiate in all of the lineages we characterize in these experiments with kinetics that mimic unselected $Wnt2^{+/+}$ ES cells (see below), indicating that a phenotype due to heterozygosity for Wnt2 is either absent or

extremely subtle. All the ES cells used in these studies maintained a highly undifferentiated state, as determined by Nanog expression and germ-line competency (data not shown).

Days 3–4 (72, 82, and 96 h) $Wnt2^{+/-}$ and $Wnt2^{-/-}$ EBs were analyzed by FACS for their potential to generate Flk1⁺ cells. In $Wnt2^{+/-}$ EBs, the Flk1⁺ cell population was initially detected at around 82 h and it progressively increased in abundance to about 30% at 96 h (Fig. 2, A and B). These kinetics match those of $Wnt2^{+/+}$ EBs found in our previous studies (27), arguing against a significant phenotype due to lack of a single Wnt2 allele. In contrast, $Wnt2^{-/-}$ EBs generated Flk1⁺ cells in increased numbers and with accelerated kinetics. The Flk1⁺ cell population was detected as early as 72 h and increased dramatically to about 50% at 96 h in $Wnt2^{-/-}$ EBs. Consistent with these observations, Flk1 mRNA was more abundantly expressed in days 3.5 and 4 EBs in $Wnt2^{-/-}$ ES cells as well (Fig. 2C). These experiments indicate that Wnt2 coordinates events within primitive cell populations that determine when and how mesoderm-derived cells such as those expressing Flk1⁺ appear.

To assess the specificity of Wnt2 effects on mesoderm differentiation, we analyzed the expression of genes associated with ES cells and the three germ layer lineages (ectoderm,

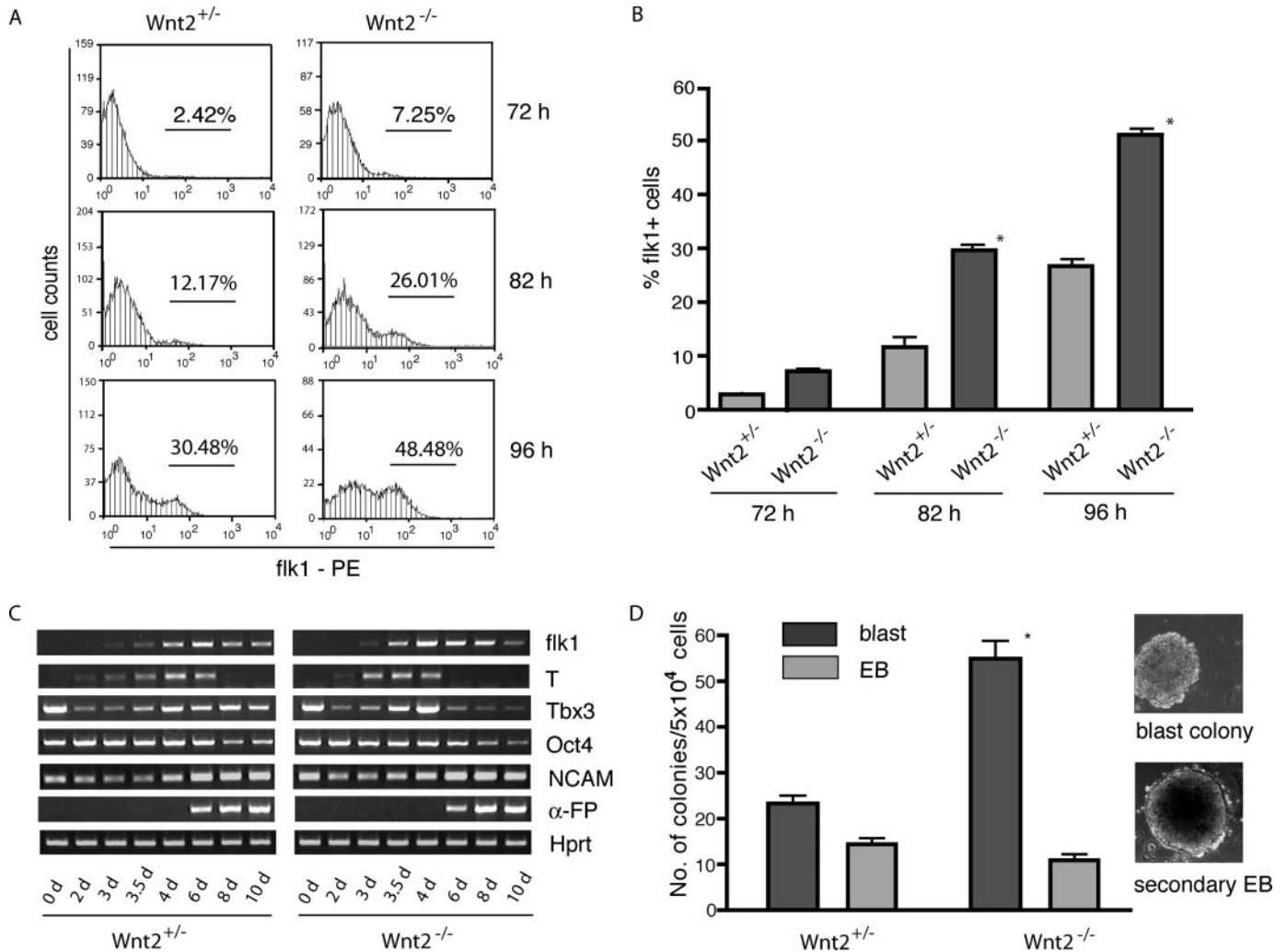


FIGURE 2. Altered developmental potential of Flk1⁺ cells and BL-CFCs in *Wnt2*^{-/-} ES cells. *A*, kinetic analysis of flk1 expression by FACS in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs at 72, 82, and 96 h during ES cell differentiation. Representative results from three experiments are shown. *B*, quantitative results of three independent experiments performed as described in *A* are shown. Data represent mean ± S.E. *, *p* < 0.05 compared with *Wnt2*^{+/-} EBs. *C*, expression analyses of the indicated genes (genes associated with ES cells and the three germ layer lineages) by RT-PCR in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs at different time points during ES cell differentiation (*n* = 3). Data shown are mean ± S.E. *, *p* < 0.05 compared with *Wnt2*^{+/-} EBs. Representative images of a hemangioblast (BL-CFC) colony and a secondary EB are shown.

TABLE 1
Quantitative RT-PCR analysis of indicated mRNAs over time in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs (*n* = 3 per condition)

	0 Days		2 Days		3 Days		3.5 Days		4 Days		6 Days		8 Days		10 Days	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
<i>Wnt2</i> ^{+/-}																
<i>Flk1</i>	1.0	0.0	1.0	0.2	1.4	0.3	2.5	0.7	18.3	2.1	24.9	3.7	14.4	5.3	9.3	3.4
<i>T</i>	1.0	0.0	2.1	0.1	2.9	0.5	6.9	1.2	16.7	3.1	9.1	2.0	1.4	0.2	1.1	0.2
<i>Oct4</i>	3.2	0.7	3.9	0.8	3.8	0.5	3.6	0.4	3.6	0.8	3.3	0.6	1.2	0.3	1.0	0.0
<i>Wnt2</i> ^{-/-}																
<i>Flk1</i>	1.0	0.0	1.1	0.2	2.1	0.4	21.3	5.3	37.7	4.8	31.6	3.9	28.5	1.9	6.9	2.0
<i>T</i>	1.0	0.0	1.9	0.4	18.8	1.3	22.2	3.7	15.0	1.8	1.8	0.2	1.6	0.3	1.3	0.1
<i>Oct4</i>	3.8	1.0	4.0	0.6	3.3	0.6	3.1	0.7	3.0	0.6	2.6	0.5	1.1	0.2	1.0	0.0

endoderm, and mesoderm) in *Wnt2*^{-/-} and *Wnt2*^{+/-} EBs during different stages of ES cell differentiation (Table 1, Fig. 2C). Oct4, which is expressed in the inner cell mass and in undifferentiated ES cells, was down-regulated with similar kinetics during differentiation in both *Wnt2*^{-/-} and *Wnt2*^{+/-} EBs. The mesoderm marker gene, *Brachyury* (*T*), was expressed at higher levels at day 3 in *Wnt2*^{-/-} EBs (18.8-fold increase compared

with mRNA levels in undifferentiated cells) compared with *Wnt2*^{+/-} EBs (2.9-fold increase), and was also down-regulated more rapidly in *Wnt2*^{-/-} EBs. Flk1 and another T-box gene that has been implicated in yolk sac development, *Tbx3* (31), were similarly up-regulated early, but greatly reduced at later stages of *Wnt2*^{-/-} EBs compared with *Wnt2*^{+/-} EBs. In contrast to those mesoderm-associated genes, no significant differ-

TABLE 2

Quantitative RT-PCR analysis of endothelial mRNAs over time in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs (n = 3 per condition)

	0 Days		2 Days		3 Days		3.5 Days		4 Days		6 Days		8 Days		10 Days	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
<i>Wnt2</i> ^{+/-}																
PECAM1	3.8	0.5	1.9	0.4	1.4	0.1	1.2	0.2	1.0	0.0	3.0	0.6	3.2	0.4	4.6	0.9
VE-cad	1.0	0.0	1.0	0.1	1.2	0.1	1.1	0.1	1.2	0.2	10.8	2.1	14.0	1.9	17.9	2.1
Tie2	1.8	0.1	1.4	0.1	1.2	0.2	1.0	0.0	1.1	0.1	10.1	3.4	16.3	1.7	28.1	2.9
<i>Wnt2</i> ^{-/-}																
PECAM1	4.3	0.5	1.6	0.2	1.6	0.3	1.2	0.2	1.0	0.0	5.6	1.0	3.9	0.8	1.7	0.3
VE-cad	1.0	0.0	1.2	0.2	1.2	0.2	1.2	0.1	2.0	0.2	24.3	3.6	19.9	2.8	9.8	3.0
Tie2	2.0	0.1	1.5	0.1	1.1	0.1	1.0	0.0	1.9	0.5	24.8	3.5	17.3	1.9	6.1	1.3

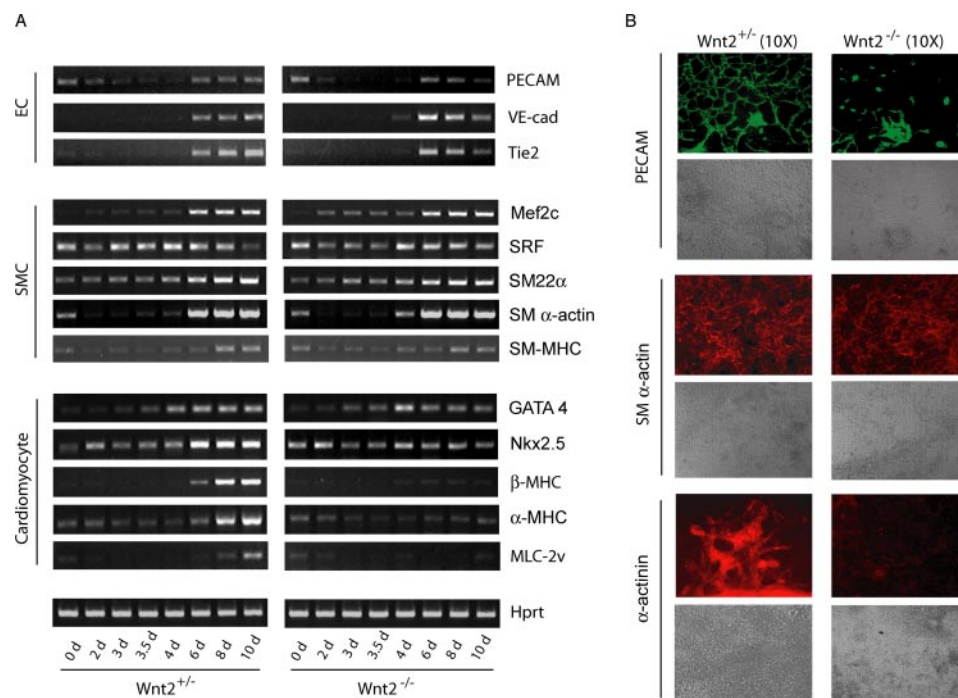


FIGURE 3. The impact of Wnt2 deficiency on the commitment of ES cells to endothelial, SMC, and cardiomyocyte lineages. A, expression analyses of the indicated genes by RT-PCR in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs at different time points during ES cell differentiation are shown. Genes are grouped according to their specificity for EC, SMC, and cardiomyocyte lineages as indicated on the left. Hprt was included as a loading control. B, immunostaining for PECAM, SM α-actin, and α-actinin in *Wnt2*^{+/-} and *Wnt2*^{-/-} EB at day 8 during ES cell differentiation. Specific antibodies stained images as shown on the top and corresponding phase-contrast images are on the bottom. Experiments were repeated 3 times independently.

ence was found for the ectoderm-associated marker neural cell adhesion molecule or the endoderm-associated gene α-fetoprotein in *Wnt2*^{-/-} EBs compared with *Wnt2*^{+/-} EBs. Therefore, the effects of Wnt2 on ES cell differentiation appear to be largely restricted to the mesoderm, and deficiency of Wnt2 promotes the early differentiation of mesoderm and dysregulates its terminal differentiation.

The BL-CFC is an *in vitro* equivalent of the hemangioblast and is able to generate primitive and definitive hematopoietic cells as well as endothelial cells when cultured with appropriate growth factors (13, 14). To further confirm the effects of Wnt2 on the differentiation of the early mesoderm-associated hemangioblast, we performed hemangioblast colony assays. Because maximum numbers of BL-CFCs are reached between days 3.25 and 4.25 during EB differentiation (13), we plated cells of *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs at day 4.0 in methylcellulose cultures in the presence of VEGF and c-kit ligand. BL-CFCs can be readily identified morphologically in distinction to second-

ary EBs (Fig. 2D). Compared with *Wnt2*^{+/-} ES cells, the generation of BL-CFCs from *Wnt2*^{-/-} ES cells was significantly enhanced. The production of secondary EBs from *Wnt2*-deficient cells was slightly, but not significantly, decreased. The increase in BL-CFC frequency in *Wnt2*-deficient ES cells coincides closely with the increase of Flk1⁺ cells by FACS analysis (Fig. 2A). Taken together, these experiments indicate that deficiency of Wnt2 enhances the differentiation and proliferation of ES cells to hemangioblasts.

Wnt2 Affects the Differentiation of EBs to Endothelial Cells but Not SMC—Because Flk1⁺ hemangioblasts serve as vascular progenitors, we next investigated the role of Wnt2 in vascular development. Flk1 expression levels were reduced in *Wnt2*^{-/-} EBs at day 10 (Fig. 2C), which coincides with attainment of the mature vascular phenotype and primitive vascular plexus formation in EBs (26), indicating the possible impairment of terminal vascular

development. Therefore, we performed RT-PCR for endothelial-specific genes including PECAM, VE-cadherin, and Tie2 at different time points during differentiation in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs (Table 2, Fig. 3A). These three endothelial markers were up-regulated with similar kinetics during differentiation over time (from day 6 to 10) in *Wnt2*^{+/-} EBs, with peak levels at day 10 when the endothelial phenotype is maturing. However, this expression pattern was reversed in *Wnt2*^{-/-} EBs, with peak levels 2-fold or greater above those seen in *Wnt2*^{+/-} EBs in day 6 EBs and levels less than half of those seen in *Wnt2*^{+/-} EBs at day 10 (Table 2). To further confirm the effect of Wnt2 on endothelial development, day 8 EBs were stained with a PECAM antibody to demarcate the vascular network. In *Wnt2*^{+/-} EBs, well organized vascular plexi were formed, whereas only a few, scattered PECAM-positive cells were seen in *Wnt2*^{-/-} EBs (Fig. 3B). These data indicate that the terminal differentiation of ES cells to endothelial cells is impaired in *Wnt2*^{-/-} EBs.

TABLE 3

Quantitative RT-PCR analysis of cardiomyocyte mRNAs over time in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs (n = 3 per condition)

	0 Days		2 Days		3 Days		3.5 Days		4 Days		6 Days		8 Days		10 Days	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
<i>Wnt2</i> ^{+/-}																
<i>GATA4</i>	1.0	0.0	1.0	0.0	1.2	0.1	1.8	0.4	3.1	0.7	3.0	0.4	2.8	0.3	2.9	0.4
<i>Nkx2.5</i>	1.0	0.0	1.4	0.2	1.1	0.2	1.2	0.2	1.3	0.2	3.0	0.7	2.8	0.6	3.0	0.5
β -MHC	1.1	0.2	1.1	0.1	1.0	0.1	1.0	0.0	1.2	0.4	3.9	1.1	19.7	4.2	27.3	3.6
α -MHC	2.1	0.3	2.2	0.3	1.7	0.4	1.4	0.6	1.0	0.0	2.8	0.4	14.7	3.1	23.6	3.3
<i>MLC-2v</i>	1.3	0.1	1.0	0.1	1.0	0.2	1.0	0.1	1.0	0.0	1.4	0.6	1.9	0.2	4.0	0.5
<i>Wnt2</i> ^{-/-}																
<i>GATA4</i>	1.0	0.0	1.2	0.2	1.8	0.3	2.0	0.3	3.5	0.5	2.4	0.6	2.2	0.2	2.0	0.4
<i>Nkx2.5</i>	1.0	0.0	1.5	0.1	1.0	0.0	1.0	0.1	1.4	0.2	1.4	0.2	1.5	0.3	1.3	0.1
β -MHC	1.3	0.2	1.0	0.1	1.1	0.2	1.0	0.0	1.3	0.3	1.3	0.1	1.2	0.1	1.1	0.3
α -MHC	2.6	0.6	2.0	0.4	1.6	0.2	1.3	0.1	1.0	0.0	1.7	0.2	1.7	0.4	1.9	0.2
<i>MLC-2v</i>	1.5	0.3	1.3	0.3	1.0	0.1	1.1	0.2	1.0	0.0	1.0	0.1	1.1	0.2	1.3	0.2

SMC are periendothelial cells that are essential for vascular maturation and stabilization. Recent studies have shown that Flk1⁺ cells in EBs may also give rise to SMC under appropriate conditions (15). Therefore, we also performed expression analyses using molecular markers for SMC, including transcription factors involved in regulation of SMC differentiation (Mef2c and serum response factor) and contractile proteins smooth muscle (SM) α -actin, SM 22 α , and SM myosin heavy chain, the latter of which is probably the most SMC-restricted gene. All of these markers showed very similar expression patterns, and no consistent differences were detected between *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs during ES cell differentiation (Fig. 3A). To further confirm this observation, day 8 EBs were stained with a SM α -actin antibody. In support of the gene expression analyses, we saw no difference for SM α -actin staining between *Wnt2*^{+/-} and *Wnt2*^{-/-} ES cells (Fig. 3B). Therefore, Wnt2 is necessary for hemangioblast development as well as further differentiation to endothelial cells but probably not for SMC differentiation, whether or not these SMCs derive from Flk1⁺ cell populations.

Wnt2-deficient EBs Have Impaired Cardiomyocyte Differentiation—Cardiomyocytes are also mesoderm-derived and recent data indicate that Flk1⁺ cells may give rise to some populations of cardiomyocytes (16, 17). In addition, Wnt2 expression is detected in precardiac mesoderm and derived structures such as the pericardium from as early as 7.5 days post-conception forward (24, 25), and Wnt signaling is generally required for normal cardiogenesis (32, 33). Therefore, we investigated whether Wnt2 had any effect on *in vitro* cardiomyocyte differentiation in the ES differentiation model, where the differentiation and formation of beating cardiomyocytes occurs in later stage EBs (days 8 and 10). First, we analyzed *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs at different time points for their expression of specific cardiomyocyte markers: the cardiogenic transcription factors, GATA4 and Nkx2.5, and cardiac-specific proteins, α - and β -myosin heavy chain and ventricular-specific myosin light chain-2v. In *Wnt2*^{+/-} EBs, these genes were all up-regulated over time during differentiation (Table 3, Fig. 3A). In comparison, GATA4 and Nkx2.5 were expressed, but at reduced levels, after day 6 in *Wnt2*^{-/-} EBs. Moreover, whereas we readily detected greater than 20-fold induction of cardiac-specific proteins α - and β -myosin heavy chain and myosin light chain-2v in later *Wnt2*^{+/-} EBs (days 6 to 10), minimal quantitative induction of these genes was detected in later *Wnt2*^{-/-}

EBs (Table 3). Consistent with a major defect in the terminal stages of cardiomyocyte differentiation, we did not observe the formation of spontaneously beating clusters in any *Wnt2*^{-/-} EBs after 8–10 days culture, whereas these beating clusters were easily detected in all *Wnt2*^{+/-} EBs. Similarly, when we stained day 8 EBs with an antibody for α -actinin, a marker for cardiomyocytes, we found α -actinin-positive cardiomyocytes in *Wnt2*^{+/-} EBs but no stained cells were found in *Wnt2*^{-/-} EBs (Fig. 3B). Taken together, these data indicate that Wnt2 may have a subtle regulatory role in early stages of cardiomyocyte differentiation, terminal cardiomyocyte differentiation in EBs is critically dependent on Wnt2.

Wnt2 Deficiency Affects the Developmental Potential of Primitive and Definitive Hematopoiesis—Because Wnt2 deficiency promotes the differentiation of hemangioblasts but impairs their terminal differentiation to endothelial cells, we questioned the fate of the increased number of hemangioblasts during early mesoderm differentiation from *Wnt2*^{-/-} ES cells. Because Flk1⁺ cells or hemangioblasts also give rise to primitive and definitive hematopoietic cells (11, 12), we examined whether Wnt2 contributed to hematopoiesis as well. We initially performed expression analyses of hematopoietic genes, including three hematopoietic transcriptional factors (EKLF, GATA1, and Scl/Tal1), the embryonic and adult hemoglobin genes (β -H1 and β -major), and the hematopoietic cell surface marker tyrosine phosphatase CD45. β -H1 and β -major globin genes are used as markers for erythroid cells and CD45 is a marker for nucleated hematopoietic cells. We observed that the expression of all three hematopoietic cell-specific transcriptional factors, EKLF, GATA1, and Scl/Tal1, were more highly expressed in days 6 and 8 *Wnt2*^{-/-} EBs (Table 4, Fig. 4A). Similar expression patterns were obtained for the other hematopoietic cell genes. β -H1 expression was up-regulated in days 6 and 8 *Wnt2*^{-/-} EBs. β -major and CD45 expression were greatly increased in days 8 to 10 *Wnt2*^{-/-} EBs (Table 4). Notably, day 10 is around the time when definitive hematopoiesis occurs in this system. The expression pattern of these hematopoietic cell-specific genes suggests that hematopoietic cell differentiation and specification from hemangioblasts is expanded or accelerated in the absence of Wnt2.

To confirm the results obtained from the gene expression analyses, we performed hematopoietic progenitor cell assays, which provide an easy and direct way to assess primitive and definitive hematopoiesis. On days 5 and 6 of differentiation,

TABLE 4

Quantitative RT-PCR analysis of hematopoietic mRNAs over time in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs (n = 3 per condition)

	0 Days		2 Days		3 Days		3.5 Days		4 Days		6 Days		8 Days		10 Days	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
<i>Wnt2</i> ^{+/-}																
<i>CD45</i>	1.0	0.0	1.0	0.1	1.0	0.0	1.0	0.1	1.1	0.2	1.2	0.2	1.5	0.4	8.9	2.0
<i>β-major</i>	1.0	0.0	1.0	0.2	1.1	0.2	1.3	0.2	1.5	0.3	10.5	0.8	8.7	2.1	8.5	1.9
<i>GATA1</i>	6.1	0.4	1.3	0.3	1.2	0.3	1.0	0.0	3.0	0.5	13.1	0.9	3.8	0.3	3.3	0.9
<i>Wnt2</i> ^{-/-}																
<i>CD45</i>	1.0	0.0	1.0	0.2	1.0	0.1	1.1	0.2	1.0	0.2	1.1	0.1	8.0	1.2	19.4	3.1
<i>β-major</i>	1.0	0.0	1.1	0.3	1.0	0.3	1.2	0.4	1.2	0.3	32.8	5.0	27.2	3.5	11.7	2.3
<i>GATA1</i>	5.3	0.7	1.5	0.1	1.0	0.0	1.2	0.3	28.9	4.4	16.7	1.6	15.1	1.9	5.8	0.7

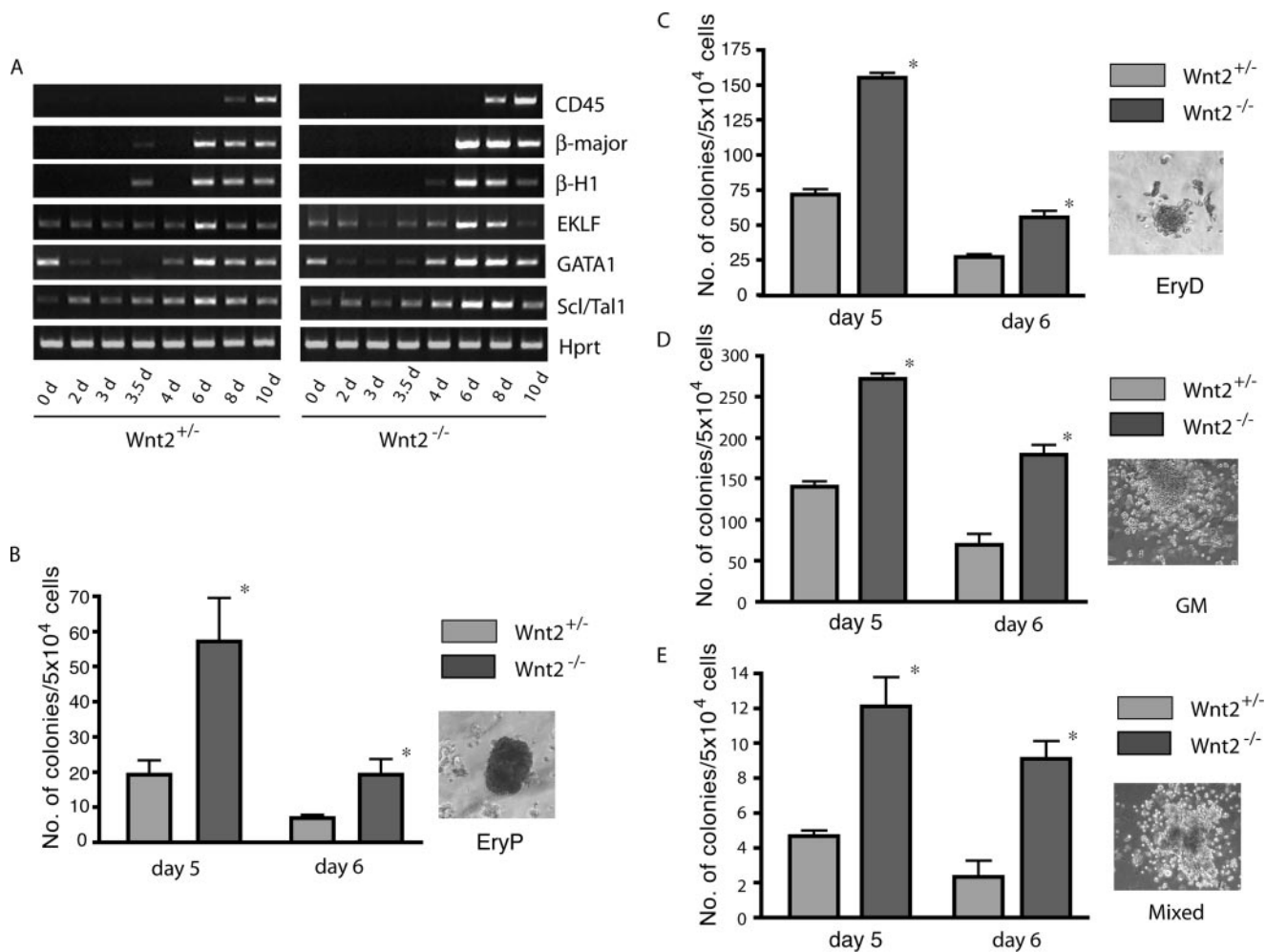


FIGURE 4. The impact of *Wnt2* deficiency on the hematopoietic differentiation potential of ES cells. *A*, expression analyses of the indicated genes specific for hematopoietic cell lineages in *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs as determined by RT-PCR at different time points during ES cell differentiation are shown. *Hprt* was included as a loading control. *B–E*, hematopoietic progenitor assays for days 5 and 6 *Wnt2*^{+/-} and *Wnt2*^{-/-} EBs. Results shown are mean ± S.E. from three independent experiments, cultures were plated in triplicate. *, *p* < 0.05 compared with *Wnt2*^{-/-} EBs. *B*, primitive erythroid colony (*EryP*) and its representative image. *C*, definitive erythroid colony (*EryD*) and its representative image. *D*, myeloid colonies, including granulocyte-macrophage (*GM*), granulocyte only, megakaryocyte, erythroid-macrophage, and erythroid-megakaryocyte colonies. Image shown is representative of *GM* colonies. *E*, mixed hematopoietic colonies and a representative image are shown.

primary EBs were collected and dissociated to single cells, and then replated into culture conditions supporting either erythroid or myeloid cell growth, respectively. Colonies including primitive erythroid (*EryP*), definitive erythroid (*EryD*), mixed (*CFC-mix*), and other committed myeloid progenitors (*CFC*, mostly granulocyte-macrophage progenitors) were counted. Maximal numbers of hematopoietic progenitor colonies were obtained at day 5 and significantly greater differentiation to both erythroid and myeloid pro-

genitor colonies was observed in *Wnt2*^{-/-} EBs (Fig. 4, *B–E*). Compared with day 5 EBs, a dramatic decrease in all hematopoietic progenitor colonies was found in day 6 EBs. However, the number of those colonies was still increased in day 6 *Wnt2*^{-/-} EBs when compared with *Wnt2*^{+/-} EBs at the same time. These progenitor assay results, in combination with our gene expression analyses, provide strong evidence that deficiency in *Wnt2* results in expansion or acceleration of hematopoietic cell differentiation.

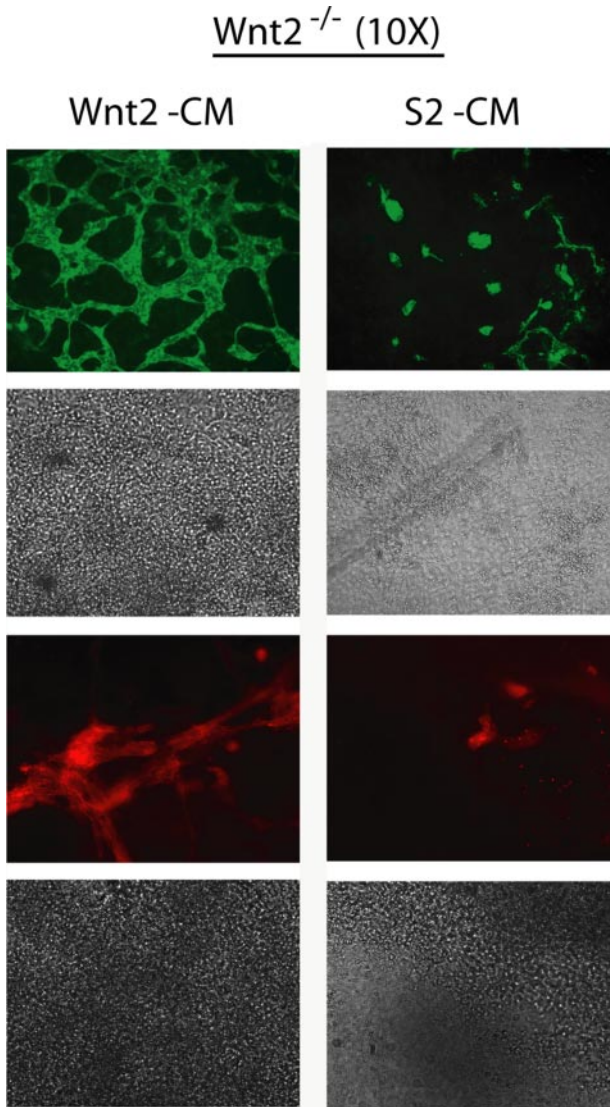


FIGURE 5. **Wnt2-conditioned media rescue the phenotypes of $Wnt2^{-/-}$ ES cell lines.** Immunostaining for PECAM and α -actinin in $Wnt2^{-/-}$ EB at day 8 during ES cell differentiation, in the presence of Wnt2-conditioned media (Wnt2-CM) or control media (S2-CM) for the final 5 days. Specific antibody stained images are shown on the top and corresponding phase-contrast images are on the bottom. Experiments were repeated 3 times independently.

Wnt2 Rescues the Phenotypes of $Wnt2^{-/-}$ ES Cells—To confirm that our observations in $Wnt2^{-/-}$ ES cells is indeed due to Wnt2 deficiency, we tested whether addition of Wnt2 to differentiation medium would rescue the failure of endothelial and cardiomyocyte differentiation. We used conditioned media from S2 cells expressing Wnt2 (Wnt2-CM) or from control S2 cells (S2-CM) in these experiments, as we have previously described (27). Wnt2-CM markedly increased the development of both PECAM-(+) structures and α -actinin-positive cells in day 8 $Wnt2^{-/-}$ embryoid bodies (Fig. 5), indicating that Wnt2 is indeed required for both endothelial and terminal cardiomyocyte differentiation in this system.

DISCUSSION

The experiments presented here define a previously unsuspected role for Wnt2 in coordinating development within the

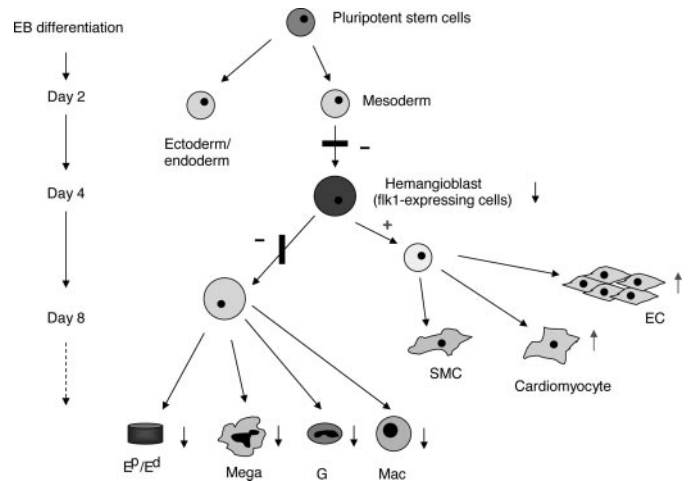


FIGURE 6. **Proposed model depicting the role of Wnt2 in hemangioblast, endothelial, cardiomyocyte, SMC, and hematopoietic cell commitment during ES/EB differentiation.** Vertical or horizontal bars and down arrows indicate the negative effects of Wnt2 seen on the committed mesoderm cell numbers, and positive (+) markers and up arrows represent the increase number of mesoderm progeny.

pre-cardiac and hematovascular mesodermal niches in EBs, which corresponds closely with its expression in early mesodermal subpopulations and developing cardiovascular structures (25). Based on these studies, we have identified specific sequential regulatory roles for Wnt2 in this differentiation process. The expansion of hemangioblasts in $Wnt2^{-/-}$ EBs indicates that Wnt2 has a suppressive or coordinating role in initial differentiation within the hematovascular lineage, and the marked suppression of terminal cardiomyocyte differentiation in these EBs suggests that there may be a competitive relationship between these lineages within the mesoderm, such that Wnt2 favors cardiomyocyte differentiation at the expense of hematovascular development. Subsequently, Wnt2 also has a regulatory role in maturation of hemangioblasts, with our experiments in $Wnt2^{-/-}$ EBs indicating that Wnt2 is required for normal endothelial maturation and vascular plexus formation, but that Wnt2 suppresses early and definitive hematopoietic differentiation (Fig. 6).

Wnt signaling has been generally associated with several key steps in vascular development. For example, studies of transgenic Wnt/ β -catenin reporter mice have demonstrated the activation of canonical Wnt signaling in endothelial cells during intraembryonic angiogenesis (20), and the requirement of Wnt signaling for correct vascular patterning and maintenance of vascular integrity has been confirmed by specific inactivation of β -catenin in endothelial cells (34). Nevertheless, the specific Wnts and any developmental role that they may play within the hematovascular compartment have remained open questions. $Wnt2^{-/-}$ mice are known to have impaired vascularization of extraembryonic tissues that results in incompletely penetrant perinatal lethality of mice (25). However, other cardiovascular defects were not reported in these mice, and until now little focus has been placed on the role for Wnt2 in hematovascular development. In retrospect, it is important to consider the possibility of a broader developmental role for Wnt2 than what was inferred from these earlier studies. The mice reported in these studies were outbred, were evaluated at a time when many

Wnt2 and Hematovascular Differentiation

molecular markers and provocative studies of cardiovascular development were not available, and did not undergo a thorough analysis of cardiac morphogenesis or hematopoietic development. In addition, redundancy or compensation by other Wnt family proteins (in particular, Wnt5a (35)) coupled with difficulties in studying Wnt proteins due to the lack of functional recombinant proteins and antibodies, have hampered our ability to understand which Wnt family members play key roles in cardiovascular development. The present studies help to clarify the mechanisms leading to perinatal lethality in *Wnt2*^{-/-} mice and indicate a broader specific role for Wnt2 in cardiovascular and hematopoietic development than has previously been recognized.

Wnt proteins typically act as autocrine factors or have spatially confined paracrine effects (18, 19). Further studies will be needed to determine which other components of the Wnt signaling pathway act downstream of Wnt2 to mediate its autocrine/paracrine effects within the mesodermal-derived compartments defined in our studies. Fzd5 is the most likely candidate receptor for Wnt2 in cells with hemangioblast potential. Fzd5 interacts functionally with Wnt2 when ectopically expressed, and mice that are null for Fzd5 have defects in both yolk sac and placental vascularization that overlap with those of *Wnt2*^{-/-} mice (35). Ishikawa and colleagues (35) did not detect Wnt2 expression in the yolk sac, and suggested that Wnt2 was the most likely candidate Wnt to mediate the placental defects observed in their studies, but that another Wnt might be responsible for the signaling events that accounted for the yolk sac hemangioblast defects they observed. However, we found that Wnt2 mRNA is easily detected within the yolk sac (data not shown), and it is more likely that the partial discordance between Wnt2 and Fzd5 knock-out phenotypes is due to factors such as differences in the mouse strains used or to complementation by other Wnts in the yolk sac.

The molecular mechanisms that determine how Wnt signaling affects vascular development are not well characterized. β -Catenin accumulation has been linked to the rate of proliferation in endothelial cells (36). VEGF, a potent stimulator for vasculogenesis and angiogenesis, can be regulated by Wnt/ β -catenin and is a target gene of Wnt signaling in cancer (37, 38). Conversely, the GSK3 β / β -catenin signaling axis can promote angiogenesis in endothelial cells via VEGF signaling (39). Cox2, another potent stimulator of angiogenesis, is up-regulated in vascular cells in response to Wnt signaling (40). Our recent studies indicate widespread activation of genes that regulate Wnt signaling within the endothelial lineage as differentiation proceeds and delineate a role for Wnt activation in proliferation of endothelial progenitors (27). However, the present studies define more precise effects for Wnt/GSK3 β / β -catenin activation generally, and Wnt2 signaling in particular, that extend beyond maturing endothelial cells and indicate precise coordination of multiple events during differentiation of mesoderm-derived tissues that determine early steps in vascular development.

The consequences of Wnt2 deletion on hematopoietic development in our studies are potent and unanticipated. Wnt activation has varied effects within the hematopoietic compartment: Wnt proteins function as hematopoietic growth factors

and stimulate hematopoietic progenitors in some experiments, and modulation of Wnt activity affects the ratio of differentiated to mature hematopoietic cells (22, 41). Wnt signaling also leads to self-renewal of hematopoietic stem cells, preventing their differentiation and promoting proliferation (23). Although these data indicate roles for Wnt signaling in immature hematopoietic progenitors, our data from *in vitro* differentiation of *Wnt2*^{-/-} EBs suggest that Wnt2 has a specific and heretofore unanticipated role in hematopoietic cell lineage commitment.

Wnt2 is also expressed in the heart in adults (24) and in precardiac mesoderm and derived tissues (25). The present studies indicate an additional role for Wnt2 during terminal cardiomyocyte differentiation. Several lines of evidence delineate contributions by Wnt signaling in cardiac development. Inhibition of Wnt signaling by Dkk-1 induces heart-specific gene expression in posterior lateral plate mesoderm, and Wnt signals (Wnt3a and Wnt1) from the neural tube block heart formation in the adjacent paraxial mesoderm (32, 33). However, noncanonical Wnt signaling mediated by Wnt11 is required for heart formation in *Xenopus* embryos (42), and promotes differentiation of both ES cells and circulating progenitors to cardiomyocytes (30, 43). These studies suggest that canonical Wnt signaling is an inhibitory signal, whereas non-canonical Wnts may positively regulate cardiogenesis. Our data demonstrate that Wnt2 promotes cardiomyocyte differentiation, in contrast with other Wnts that activate the canonical pathway. This difference suggests that different Wnt ligands may play different roles during development: whereas Wnt3a and Wnt1 may provide negative signals, Wnt2 and Wnt11 act as positive regulators of cardiogenesis. Further studies, perhaps using compound mutants in mice, will be needed to explore the precise roles of these proteins in cardiac differentiation.

In summary, the observations in this report reveal unique roles for Wnt2 during ES differentiation and fate specification. Wnt2 functions at multiple stages of development in this model. Wnt2 is a negative regulator for hemangioblast commitment, and later on, a positive regulator for endothelial cell and terminal cardiomyocyte differentiation, but a negative regulator for blood cell commitment. Although involvement of Wnt signaling has been demonstrated in different aspects of mesoderm development, our report is the first one that suggests that Wnt2 affects multiple differentiation stages and cell lineages. These data also support the complexity in cell lineage specification and commitment and the need for defined balance of negative and positive regulators for accurate and reproducible development.

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**Wnt2 Coordinates the Commitment of Mesoderm to Hematopoietic, Endothelial,
and Cardiac Lineages in Embryoid Bodies**

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