# Wnt5a Is Required for Endothelial Differentiation of Embryonic Stem Cells and Vascularization via Pathways Involving Both Wnt/β-Catenin and Protein Kinase Cα

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***Abstract***—In this study, we examined the signaling pathways activated by Wnt5a in endothelial differentiation of embryonic stem (ES) cells and the function of Wnt5a during vascular development. We first found that *Wnt5a*$^{-/-}$ mouse embryonic stem (mES) cells exhibited a defect in endothelial differentiation, which was rescued by addition of Wnt5a, suggesting that Wnt5a is required for endothelial differentiation of ES cells. Involvement of both β-catenin and protein kinase (PK)Cα pathways in endothelial differentiation of mES cells requiring Wnt5a was indicated by activation of both β-catenin and PKCα in *Wnt5a*$^{+/-}$ but not in *Wnt5a*$^{-/-}$ mES cells. We also found that β-catenin or PKCα knockdowns inhibited the Wnt5a-induced endothelial differentiation of ES cells. Moreover, the lack of endothelial differentiation of *Wnt5a*$^{-/-}$ mES cells was rescued only by transfection of both β-catenin and *PKCα*, indicating that both genes are required for Wnt5a-mediated endothelial differentiation. Wnt5a was also found to be essential for the differentiation of mES cells into immature endothelial progenitor cells, which are known to play a role in repair of damaged endothelium. Furthermore, a defect in the vascularization of the neural tissue was detected at embryonic day 14.5 in *Wnt5a*$^{-/-}$ mice, implicating Wnt5a in vascular development in vivo. Thus, we conclude that Wnt5a is involved in the endothelial differentiation of ES cells via both Wnt/β-catenin and PKC signaling pathways and regulates embryonic vascular development. **(*Circ Res*. 2009;104:372-379.)**

**Key Words:** Wnt5a ■ embryonic stem cells ■ β-catenin ■ PKCα ■ endothelial differentiation

The Wnt family of proteins comprises a large family of cysteine-rich secreted proteins that control multiple processes, including embryonic patterning, growth, migration, and cell differentiation.[1] Wnts are known to activate several different pathways. One of them, the canonical pathway, is characterized by in stabilization of β-catenin as a result of the transmission of the signal through cell surface receptors and in subsequent transcriptional activation of target genes. In other pathways, often called noncanonical pathways, Wnt proteins function via cell surface receptors to stimulate the Wnt/$Ca^{2+}$ pathway through the activation of protein kinase (PK)C[2] or the Wnt/PCP pathway through the activation of c-Jun N-terminal kinase (JNK).[3,4] Wnt5a has been reported to function through the both noncanonical pathway involving PKC[5] and the canonical pathway involving β-catenin.[1,6] At a functional level, Wnt5a has been implicated in the regulation of development, proliferation, and cell differentiation.[7–12] During development, Wnt5a is involved in the differentiation of chondrocytes,[13] as well as dopaminergic neuron differentiation of ventral midbrain.[14–16] Wnt5a is also highly expressed in human primary endothelial cells[17] and Flk-$1^+$ cells[18] and induces proliferation, migration, and survival of endothelial cells.[19] However, the role of Wnt5a in endothelial differentiation of ES cells and its signaling mechanism was unclear.

In this study, we investigated the role of Wnt5a in endothelial differentiation of ES cells by generating and analyzing W*nt5a*$^{-/-}$ ES cells. We found that the failure of endothelial differentiation in *Wnt5a*$^{-/-}$ ES cells was rescued by *Wnt5a*-retroviral infection or extracellular treatment with recombinant Wnt5a. Interestingly, we also observed that both β-catenin and PKCα were required for recovery of endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells. The abilities of *Wnt5a*$^{-/-}$ ES cells resupplemented with Wnt5a to differentiate into endothelial progenitor cells (EPCs) and to form vascular tubes indicate a role for Wnt5a in endothelial differentiation. Finally, we demonstrate a function of Wnt5a in vascular development in vivo, as evidenced by a defect in the vascularization of embryonic day (E)14.5 *Wnt5a*$^{-/-}$ mouse embryos. Moreover, here, we address the intricate relationship between noncanonical Wnt/PKC and canonical Wnt/β-catenin pathways during Wnt5a-mediated endothelial differentiation. Thus, we provide the first evidence for a

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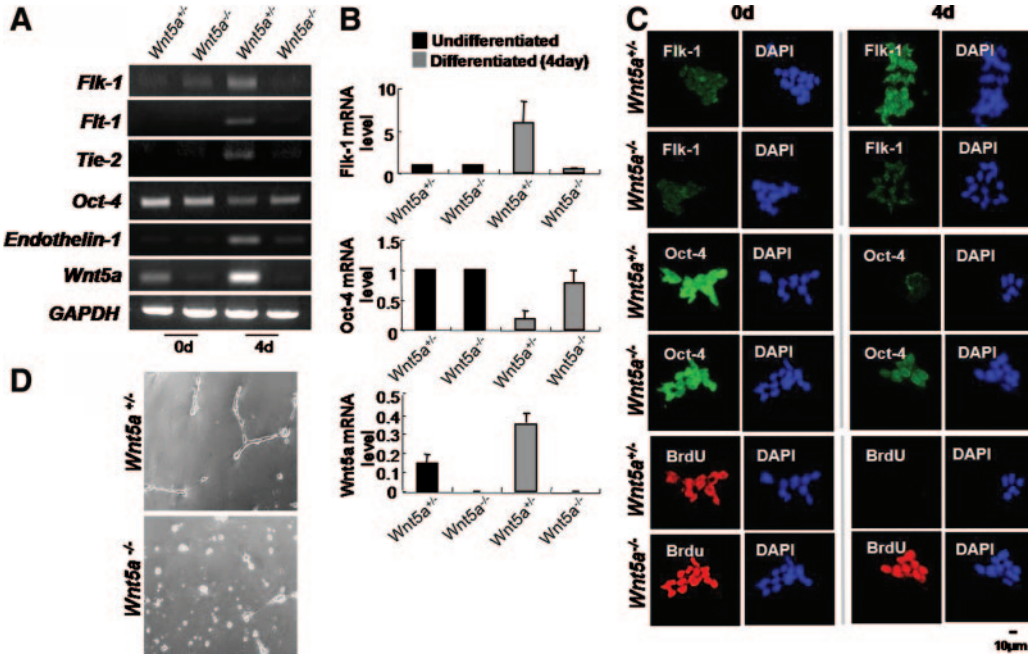


**Figure 1.** Effects of *Wnt5a* knockout on the endothelial differentiation of ES cells and in vitro tube formation. The undifferentiated (0 days) and differentiated (4 days) $Wnt5a^{+/-}/Wnt5a^{-/-}$ ES cells were grown as described in Materials and Methods. The mRNA levels of *Flk-1*, *Flt-1*, *Tie-2*, *Oct-4*, endothelin-1, *Wnt5a*, and *GAPDH* were measured by RT-PCR (A) or real-time RT-PCR (B) analyses of total RNA for the undifferentiated and differentiated $Wnt5a^{+/-}/Wnt5a^{-/-}$ ES cells. Error bars show SD. C, The protein levels of Flk-1 and Oct-4 were monitored by immunocytochemical analyses as described in Materials and Methods. Scale bar=10 $\mu$m. D, $Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ ES cells were differentiated for 4 days and then transferred on a Matrigel-coated plate. Tube formation was observed after 24 hours. The images were captured using a light microscope equipped with a digital charge-coupled device camera. All of the images are $\times$200 magnifications.

function of Wnt5a in endothelial differentiation of ES cells and in the vascularization of the mouse embryo via pathways involving both Wnt/$\beta$-catenin and PKC$\alpha$.

## Materials and Methods

### Culture of ES Cells

$Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ mouse ES cells were generated as described previously.[20]

### EPC Culture

To collect EPCs, the Sca-$1^+$ cells were sorted from 4-day differentiated ES cells by magnetic labeling cell sorting, as described in detail in the expanded Materials and Methods section, available in the online data supplement at http://circres.ahajournals.org.

### Immunohistochemistry

$Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ E14.5 embryos were fixed, embedded, sectioned, and stained as described in detail in the online data supplement.

## Results

### Generation and Characterization of $Wnt5a^{-/-}$ ES Cells

The role of Wnt5a in endothelial differentiation of ES cells was indicated by a step-wise increase of *Wnt5a* mRNA during endothelial differentiation of R1 mouse ES cells and a concomitant increase and decrease in mRNA levels of *Flk-1*, an endothelial marker, and of *Oct-4*, a stem cell marker, respectively (Figure I, A, in the online data supplement). A $Wnt5a^{-/-}$ ES cell line was generated from a $Wnt5a^{-/-}$ mouse blastocyst.[20] The $Wnt5a^{-/-}$ ES cells exhibited similar morphology to the $Wnt5a^{+/-}$ ES cells when the ES cells were grown on mouse embryo fibroblast feeder cells (supplemental Figure I, B). The knockout of *Wnt5a* in $Wnt5a^{-/-}$ ES cells was validated by the absence of *Wnt5a* mRNA (supplemental Figure I, C). Moreover, $Wnt5a^{-/-}$ ES cells displayed stem cell characteristics as evidenced by the expression of high *Oct-4* mRNA levels, which were similar to those in $Wnt5a^{+/-}$ ES cells (supplemental Figure I, C). To evaluate the pluripotency of the $Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ ES cells in vivo, we induced teratomas by injection of the ES cells. Nude mice transplanted with either $Wnt5a^{+/-}$ or $Wnt5a^{-/-}$ ES cells produced teratomas (d=8$\approx$20 mm) after 4 weeks (supplemental Figure II, A and B). The teratomas formed by both cell types displayed a heterogeneous differentiation potential with cells of ectodermal (supplemental Figure II, C and F), mesodermal (supplemental Figure II, D and G), and endodermal (supplemental Figure II, E and H) lineages. The differentiation potentials of the $Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ ES cells were further confirmed in vitro by the expression of increased mRNA levels of Brachyury (*T*), a mesodermal marker, and *Afp* and *Gata-4*, endodermal markers, in embryoid bodies (supplemental Figure III). However, we only observed increased mRNA levels of *Flk-1* during embryoid body formation in $Wnt5a^{+/-}$ but not $Wnt5a^{-/-}$ ES cells (supplemental Figure III).[21]

### Wnt5a Is an Important Factor for Endothelial Differentiation of ES Cells and Vascularization

*Flk-1*, *Flt-1*, and *Tie-2* mRNA levels increased in $Wnt5a^{+/-}$ but not $Wnt5a^{-/-}$ ES cells after 4 days of culture in endothelial differentiation media (Figure 1A). Conversely,

*Oct-4* mRNA markedly decreased during the differentiation process of *Wnt5a*$^{+/-}$ ES cells but only modestly in *Wnt5a*$^{-/-}$ ES cells (Figure 1A). Moreover, endothelin-1 mRNA, a marker of endothelial activation,[22] was also induced in differentiated *Wnt5a*$^{+/-}$ but not *Wnt5a*$^{-/-}$ ES cells (Figure 1A). The loss of endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells was more convincingly shown by quantitative RT-PCR analyses (Figure 1B). Fluorescence-activated cell sorting analyses indicated that significantly greater numbers of Flk-1$^{+}$ cells were present during endothelial differentiation of *Wnt5a*$^{+/-}$ ES cells compared to *Wnt5a*$^{-/-}$ ES cells (53.12% versus 0.46%, respectively; supplemental Figure IV). The role of Wnt5a in endothelial differentiation of ES cells was further confirmed by immunocytochemical staining of *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ ES cells (Figure 1C). The intensity of Flk-1 staining significantly increased in *Wnt5a*$^{+/-}$ ES cells after 4 days of endothelial differentiation, but this increase was not observed in *Wnt5a*$^{-/-}$ ES cells (Figure 1C, upper images). Simultaneously, Oct-4 clearly decreased during the differentiation process of *Wnt5a*$^{+/-}$ ES cells but did not decrease as much in*Wnt5a*$^{-/-}$ ES cells (Figure 1C, middle images). Proliferation of ES cells, as monitored by bromodeoxyuridine staining, was reduced during endothelial differentiation of *Wnt5a*$^{+/-}$ ES cells but maintained in *Wnt5a*$^{-/-}$ ES cells (Figure 1C, lower images). We examined the ability of *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ ES cells to form vascular-like structures in vitro to further characterize the role of Wnt5a in vascularization. Whereas *Wnt5a*$^{+/-}$ ES cells formed rod-shape endothelial tubes on a Matrigel after 4 days of culture in differentiation condition, *Wnt5a*$^{-/-}$ ES cells did not form such tubes (Figure 1D).

To further investigate the role of Wnt5a in endothelial differentiation, we tested whether the defect in endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells could be rescued by supplementation of Wnt5a. Whereas the mRNA levels of *Flk-1*, *Flt-1*, and *Tie-2* remained low in *Wnt5a*$^{-/-}$ ES cells infected with *LacZ* retroviruses, they significantly increased after infection with *Wnt5a-LacZ* retroviruses (Figure 2A). The recovery of endothelial differentiation by *Wnt5a* supplementation was also confirmed by immunocytochemical analyses (Figure 2B). The loss of stem cell characteristics in Wnt5a-treated *Wnt5a*$^{-/-}$ ES cells was also indicated by reductions of Oct-4 mRNA and protein levels (Figure 2A and 2B). The lack of endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells could also be rescued by administration of purified Wnt5a protein to the cultures (Figure 2C). Furthermore, we observed induction of Flk-1 protein in Wnt5a-transfected *Wnt5a*$^{-/-}$ ES cells (Figure 2D). Overall, our data demonstrates that Wnt5a is an essential factor for endothelial differentiation of ES cells.

## Both the β-Catenin– and the PKCα-Mediated Pathways Are Required for Endothelial Differentiation of ES Cells by Wnt5a

To identify the signaling pathways involved in the Wnt5a-induced endothelial differentiation of ES cells, we monitored changes in the status of β-catenin and PKC during this process. The levels of β-catenin, as well as its reporter activity,[23] were highly upregulated in differentiated

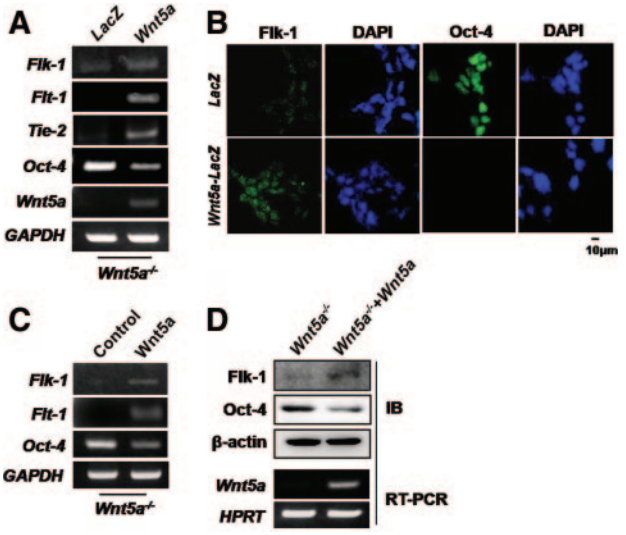


**Figure 2.** Effects of Wnt5a supplementation on endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells. A and B, *Wnt5a*$^{-/-}$ ES cells were grown for 1 day on collagen IV–coated dishes in differentiation medium and then infected with the retroviruses for *LacZ or Wnt5a-LacZ* for 12 hours. The infected cells were then differentiated for an additional 3 days and subjected to RT-PCR analyses for *Flk-1*, *Flt-1*, *Tie-2*, *Oct-4*, *Wnt5a*, and *GAPDH* (A) and immunocytochemical analyses (B). Scale bar=10 μm. C, *Wnt5a*$^{-/-}$ ES cells were cultured as described in Figure 2A, and 100 ng/mL recombinant Wnt5a was added every day during the differentiation process. The mRNA levels of *Flk-1*, *Flt-1*, *Oct-4*, and *GAPDH* were measured by RT-PCR analyses. D, *Wnt5a*$^{-/-}$ ES cells were grown as described in Figure 2A and transfected with *Wnt5a.* Transfected cells were subjected to immunoblotting analyses to detect Flk-1, Oct-4, and β-actin proteins (upper gels) or were subjected to RT-PCR analyses to detect *Wnt5a* and *HPRT* mRNAs (lower gels).

*Wnt5a*$^{+/-}$ ES cells compared to *Wnt5a*$^{-/-}$ ES cells (Figure 3A and 3B, respectively). To avoid any possibility that the activation of the Wnt/ β-catenin signaling might be caused by secondary effects rather than direct effects of Wnt5a, we measured the immediate activation of β-catenin by recombinant Wnt5a. The level of β-catenin in *Wnt5a*$^{-/-}$ ES cells started to increase at 30 minutes after treatment with recombinant Wnt5a and then continued to increase until 8 hour after stimulation (supplemental Figure V), indicating that Wnt5a activates the Wnt/β-catenin pathway in these cells. PKC activity, as assessed by phosphorylated PKC (p-PKC) at Thr-500, Thr-641, and Ser-660,[24] was significantly increased after 4 days of differentiation of *Wnt5a*$^{+/-}$ ES cells, but that increase was not observed in *Wnt5a*$^{-/-}$ ES cells in identical culture conditions (Figure 3A). The phosphorylation of the JNK, a downstream target of PKC signaling,[25,26] did not change during endothelial differentiation of ES cells regardless of *Wnt5a* genotype. To further investigate the involvement of the β-catenin and PKC pathways in ES cell endothelial differentiation through Wnt5a, we measured the mRNA levels of proteins involved in those signaling pathways (Figure 3C). As expected, we did not observe any significant induction of β-catenin mRNA levels. Interestingly, *PKCα* mRNA was induced during endothelial differentiation of *Wnt5a*$^{+/-}$ ES cells but not *Wnt5a*$^{-/-}$ ES cells. However, we did not observe any change in the mRNA level

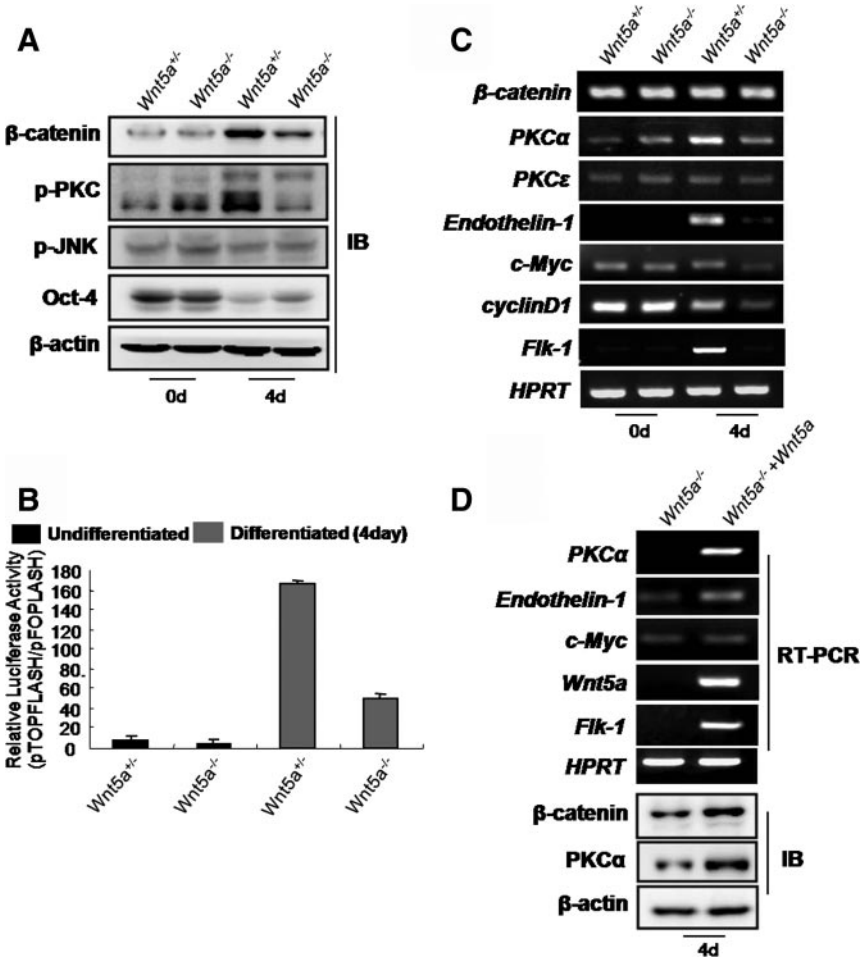


**Figure 3.** Effects of *Wnt5a* knockout on the activations of PKC and β-catenin during endothelial differentiation of $Wnt5a^{+/-}$ ES cells. A, Cell lysates of $Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ ES cells were subjected to immunoblotting with anti–β-catenin, anti–Pan-p-PKC, anti–p-JNK, anti–Oct-4, or anti–β-actin antibody. B, $Wnt5a^{+/-}$ and $Wnt5a^{-/-}$ ES cells were transfected and luciferase activities were measured as described in Materials and Methods. Error bars indicate SD of 3 independent reporter analyses. C, Total RNA was subjected to RT-PCR analyses of *Flk-1*, β-catenin, *PKCα*, *PKCε*, endothelin-1, c-Myc, *cyclinD1*, and *HPRT*. D, For transient transfection, $Wnt5a^{-/-}$ ES cells were cultured as described in Figure 2A and then transfected with 1.0 μg of pcDNA3.1 or Wnt5a-pcDNA3.1. Transfected cells were subjected to RT-PCR analyses to detect the mRNA levels of *Flk-1*, *PKCα*, endothelin-1, *c-Myc*, *Wnt5a*, and *HPRT* (upper gels) or were subjected to immunoblotting analyses to detect β-catenin, PKCα, and β-actin proteins (lower gels).

of *PKCε* following endothelial differentiation of $Wnt5a^{+/-}$ ES cells (Figure 3C). The mRNA level of endothelin-1, a known transcriptional target of Wnt/β-catenin signaling,[27] also increased during the differentiation process in a Wnt5a-dependent manner (Figure 3C). The role of Wnt5a in the transcriptional regulation of *PKCα* and endothelin-1 was further confirmed by the recovery of *PKCα* and endothelin-1 mRNA levels in $Wnt5a^{-/-}$ ES cells transfected with *Wnt5a* (Figure 3D, upper gels). The protein levels of β-catenin and PKCα were also increased by transfection of *Wnt5a* in $Wnt5a^{-/-}$ ES cells (Figure 3D, lower gels).

To identify the role of PKCα in the Wnt5a-induced endothelial differentiation of ES cells, we examined the effects of both Gö6976, a PKCα-specific inhibitor,[28] and PKCα siRNA. Treatment of differentiated $Wnt5a^{+/-}$ ES cells with either Gö6976 or PKCα siRNA decreased the levels of *Flk-1*, *Flt-1*, and endothelin-1 mRNA (Figure 4A and 4B). The reduction of Flk-1 level by PKCα siRNA was confirmed in immunoblotting analyses (Figure 4C). Moreover, Gö6976 or PKCα siRNA treatment abolished the increase of *Flk-1*, *Flt-1*, and endothelin-1 mRNA levels in $Wnt5a^{-/-}$ ES cells transfected with *Wnt5a* (supplemental Figure VI, A and B). The role of β-catenin in endothelial differentiation was also directly investigated by measuring the effects of β-catenin siRNA (Figure 4D). The mRNA levels of *Flk-1*, *Flt-1*, and endothelin-1 were concomitantly reduced by β-catenin siRNA in $Wnt5a^{+/-}$ ES cells differentiated for 4 days. Interestingly, the mRNA level of *PKCα* was also reduced by β-catenin siRNA (Figure 4D). Similarly, protein levels of Flk-1 and PKCα were reduced by β-catenin siRNA (Figure 4E).

To determine the importance of PKCα and β-catenin signaling in Wnt5a-mediated endothelial differentiation, we tested whether endothelial differentiation of $Wnt5a^{-/-}$ ES cells could be recovered by transfection with *PKCα* and/or β-catenin. Neither *Flk*-1 nor *Flt-1* mRNAs were induced during the differentiation process of $Wnt5a^{-/-}$ ES cells by PKCα or β-catenin alone (Figure 5A and 5B). However, cotransfection of $Wnt5a^{-/-}$ ES cells with both *PKCα* and β-catenin during the 4 day of differentiation process induced an increase in *Flk-1*, *Flt-1*, and endothelin-1 mRNA levels (Figure 5C). The induction of Flk-1 by *PKCα* and β-catenin was also confirmed by immunocytochemical analyses (Figure 5D), as well as by fluorescence-activated cell sorting analyses showing significant increment of $Flk\text{-}1^{+}$ cells (from 8.73% to 22.79%) by transfection of both *PKCα* and β-catenin into $Wnt5a^{-/-}$ ES cells (supplemental Figure VII). Overall, our results indicate that both Wnt/β-catenin and PKCα signaling are required for endothelial differentiation involving Wnt5a.

## Wnt5a Is Involved in Differentiation of ES Cells Into Endothelial Progenitor Cells

Several studies have identified a role of endothelial progenitor cells (EPCs) in repair of damaged endothelium.[29,30]

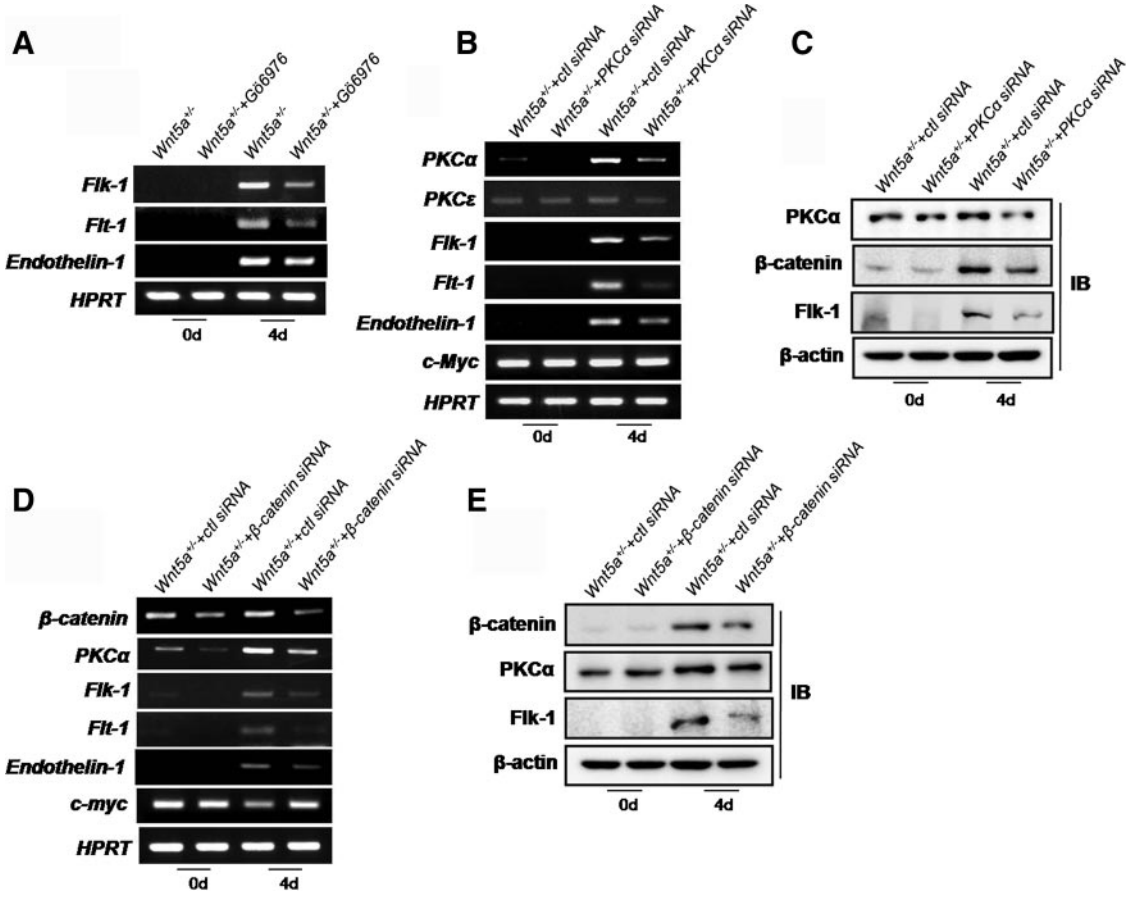


**Figure 4.** Effects of siRNA and inhibitor for PKC$\alpha$ and $\beta$-catenin in endothelial differentiation of *Wnt5a*$^{+/-}$ ES cells. A through E, For transient transfection or drug treatment, *Wnt5a*$^{+/-}$ ES cells were cultured for 1 day in the undifferentiation or differentiation medium and then transfected with a combination of 100 nmol/L PKC$\alpha$ and $\beta$-catenin siRNAs as indicated. In required cases, cells were treated with 1 $\mu$mol/L Gö6976 every day. *Wnt5a*$^{+/-}$ ES cells were further grown for 3 days in the differentiation medium and harvested. Cells were subjected to RT-PCR analyses (A, B, and D) to detect *Flk-1*, *Flt-1*, $\beta$-catenin, *PKC*$\alpha$, *PKC*$\varepsilon$, endothelin-1, c-Myc, and *HPRT* mRNAs and were subjected to immunoblotting analyses (C and E) to detect Flk-1, PKC$\alpha$, $\beta$-catenin, and $\beta$-actin proteins.

Therefore, we examined the involvement of Wnt5a in endothelial differentiation of ES cells into EPCs to identify any potential role of Wnt5a in the recovery of damaged endothelium. The Sca-1–positive EPCs from *Wnt5a*$^{+/-}$ cells (W-Sca-1$^{+}$) significantly induced *Flk-1* mRNA to levels equivalent to those of 4-day differentiated *Wnt5a*$^{+/-}$ ES cells and human umbilical vein endothelial cells (HUVECs) (Figure 6A). The Sca-1–positive EPCs from *Wnt5a*$^{-/-}$ cells transduced with *Wnt5a* (K-Sca-1$^{+}$) also induced *Flk-1*, *Flt-1*, and *Wnt5a* mRNAs (Figure 6A). However, no Sca-1–positive cells could be isolated from untransduced *Wnt5a*$^{-/-}$ ES cells (data not shown). The Sca-1–positive EPCs (W-Sca-1$^{+}$ and K-Sca-1$^{+}$) exhibited rapid labeling by acetylated low-density lipoprotein (Dil-Ac-LDL [1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–labeled acetylated-low density lipoprotein]), in accord with characteristics of endothelial cells (Figure 6B). These endothelial characteristics were confirmed by positive staining of Flk-1 or von Willebrand factor, an alternative endothelial marker, in Sca-1–positive EPCs by immunocytochemistry (Figure 6C and supplemental Figure VIII). The Sca-1–positive EPCs formed capillary-like tube structures, which were similar to those formed by HUVECs on Matrigel (Figure 6D). Overall, these findings indicate that endothelial differentiation of ES cells into EPCs, which are involved in the recovery of damaged endothelium and vascular tube formation, requires Wnt5a.

## Wnt5a Is Required for Vascular Development During Mouse Embryogenesis

To determine the in vivo role of Wnt5a, we examined vascular formation at E14.5 in *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ mice.[11] *Wnt5a*$^{-/-}$ but not *Wnt5a*$^{+/-}$ mouse embryo displayed edema, an indicator of a vascular defect,[31,32] at the upper dorsal area after basic morphological evaluation (Figure 7A). We first observed the formation of edema in the spinal cord of *Wnt5a*$^{-/-}$ but not in *Wnt5a*$^{+/-}$ embryos stained with hematoxylin/eosin (Figure 7B). An antibody against platelet-endothelial cell adhesion molecule (PECAM)-1, an endothelial cell surface marker, labeled the marginal layer of the spinal cord (Figure 7C, upper images) and cells in the dorsal root ganglia (Figure 7C, lower images), a site of vascular sprouting in the nervous system,[33,34] in *Wnt5a*$^{+/-}$ but not in *Wnt5a*$^{-/-}$ embryos. To identify whether the role of Wnt5a in vascular formation is restricted to the nervous system, we also checked the gut system, which has endothelial cell lineage in its formation.[35] However, the staining of PECAM-1 showed no difference between

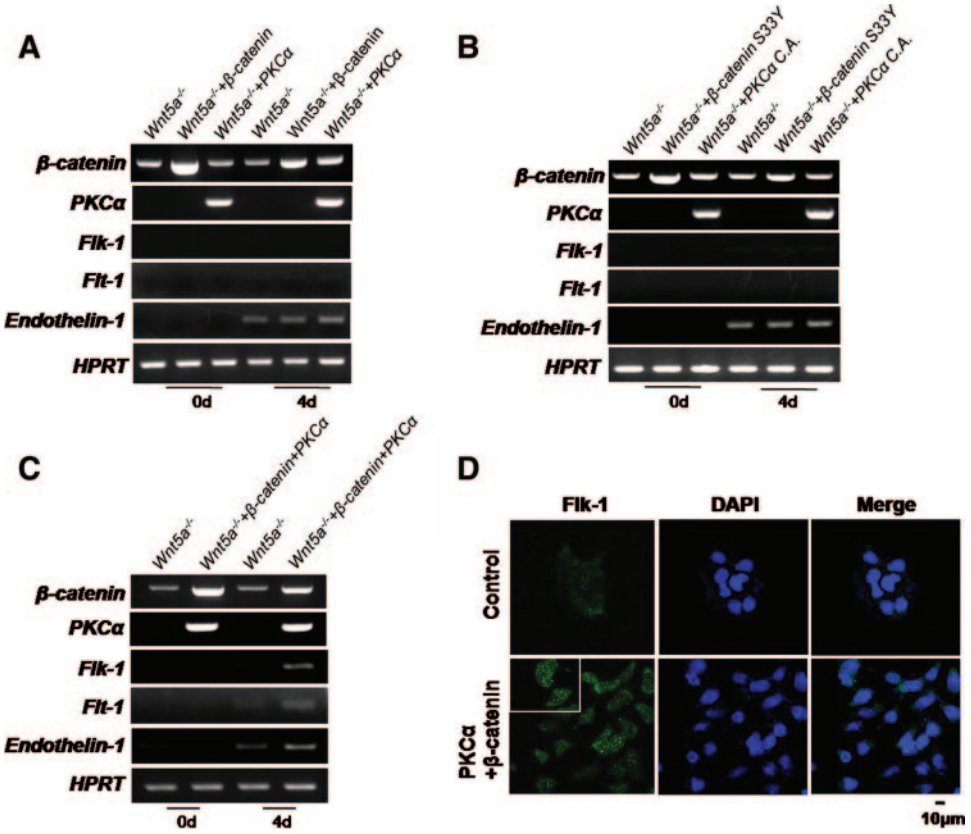


**Figure 5.** Roles of PKC$\alpha$ and $\beta$-catenin in endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells. A through D, For transient transfection, *Wnt5a*$^{-/-}$ ES cells were cultured as described in Figure 4 and then transfected with a combination of 1.0 $\mu$g of empty vector, PKC$\alpha$-pHACE, caPKC$\alpha$-pHACE, Flag–$\beta$-catenin–pcDNA3.0, and/or Flag–$\beta$-catenin S337-pcDNA3.0 as indicated. *Wnt5a*$^{-/-}$ ES cells were further grown for 3 days in the differentiation medium and harvested. Cells were subjected to RT-PCR analyses (A through C) to detect mRNA levels of the *Flk-1*, *Flt-1*, *h*$\beta$-catenin, *hPKC*$\alpha$, endothelin-1, and *HPRT* and were subjected to immunocytochemical analyses (D) to detect Flk-1 level. The boxed area is presented as a higher-magnification image in supplemental Figure XII. Scale bar=10 $\mu$m.

*Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ embryonic intestines (supplemental Figure IX), revealing the specificity of Wnt5a effect on the differentiation of endothelial cells during embryogenesis.

## Discussion

Wnt5a controls multiple physiological processes, including chondrocyte[13] and neural[11] development, by regulating cellular functions such as proliferation and differentiation. The finding of high expression of Wnt2, Wnt5a, and Wnt11 in Flk-1–positive cells has suggested that these Wnt proteins are potentially involved in endothelial commitment from ES cells.[18,36] However, the role of Wnt5a in vascular development and the mechanism by which Wnt5a regulates endothelial differentiation of ES cells have remained largely unknown.

The requirement for Wnt5a in endothelial differentiation of ES cells was clearly shown by the defects in differentiation of *Wnt5a*$^{-/-}$ ES cells. Moreover, the rescue of endothelial differentiation in *Wnt5a*$^{-/-}$ ES cells by infection with retroviruses encoding *Wnt5a*, or by treatment with recombinant Wnt5a, confirmed the essential role of Wnt5a in endothelial differentiation of ES cells. We also found that *Wnt2* and *Wnt7a* mRNAs are highly expressed in both *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ ES cells (supplemental Figure X). The failure of endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells without the supplementation of Wnt5a excludes the possibility of the compensatory roles of other Wnt proteins in the endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells.

Although different Wnts are known to preferentially activate canonical or noncanonical pathways, this distinction is not absolute, and it is likely to be dependent on the presence of specific signaling components, putative receptors, and other cofactors at the cell surface.[1] Wnt5a functions in the noncanonical pathways through signaling components such as PKC and JNK,[37,38] as well as in the canonical pathway involving $\beta$-catenin.[1,6] Our results showing specific activation of PKC and $\beta$-catenin during endothelial differentiation of *Wnt5a*$^{+/-}$ ES cells indicate that both the PKC and Wnt/$\beta$-catenin pathways are involved in the endothelial differentiation of ES cells by Wnt5a. A role for PKC$\alpha$ in vascular tube formation of endothelial cells was previously identified.[39–41] However, our results are the first to indicate that PKC$\alpha$ is specifically involved in the Wnt5a-induced endothelial differentiation of ES cells. We found that both the enzymatic activation and the transcriptional/translational upregulation of PKC$\alpha$ contribute to differentiation of ES cells. Similarly, the activation of the Wnt/$\beta$-catenin pathway by Wnt5a and the increase of endothelin-1 mRNA, a transcriptional target of $\beta$-catenin,[27] indicated that the Wnt/$\beta$-catenin pathway plays a prominent role in endothelial differentiation of ES cells. It has been previously reported that $\beta$-catenin signaling promotes angiogenesis of primary endothelial HUVECs.[42] We also demonstrated here the role of the Wnt/$\beta$-catenin pathway in endothelial differentiation of ES cells, as seen in reductions of *Flk-1*, *Flt-1*, and endothelin-1 mRNA levels and Flk-1 protein level in $\beta$-catenin siRNA-transfected *Wnt5a*$^{+/-}$ ES cells. We suggest that the endothelial differentiation of ES cells by Wnt5a via the Wnt/$\beta$-catenin signaling may be acquired by highly selective expression of target genes such as endothelin-1, because other transcriptional targets of Wnt/$\beta$-catenin including c-*Myc* and

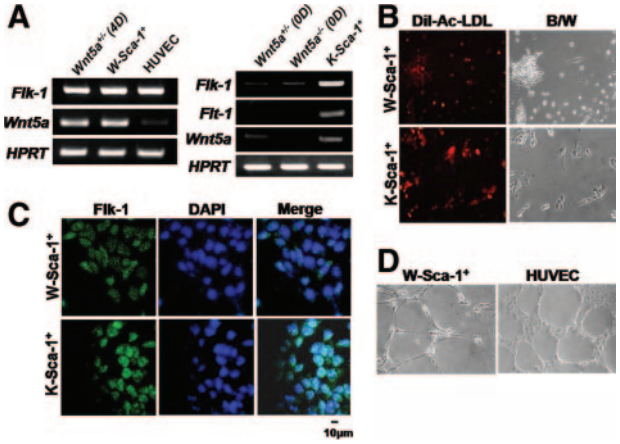


**Figure 6.** Generation and characterization of Sca-1$^+$ EPCs derived from *Wnt5a*$^{+/-}$ or *Wnt5a*$^{-/-}$ ES cells supplemented with Wnt5a. A, Differentiated *Wnt5a*$^{+/-}$ ES cells (*Wnt5a*$^{+/-}$ [4D]), undifferentiated *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ ES cells (*Wnt5a*$^{+/-}$ [0D] and *Wnt5a*$^{-/-}$ [0D], respectively), and HUVECs were grown as described in Materials and Methods. W-Sca-1$^+$ were sorted from the 4-day differentiated *Wnt5a*$^{+/-}$ ES cells. K-Sca-1$^+$ cells were sorted from *Wnt5a*$^{-/-}$ ES cells infected with *Wnt5a-LacZ* retroviruses. The mRNA levels of *Flk-1*, *Wnt5a*, and *HPRT* were measured by RT-PCR analyses. B, The images of phase contrast (black and white) and Dil-Ac-LDL uptake of W-Sca-1$^+$ and K-Sca-1$^+$ cells were captured by light and fluorescence microscopes. All of the images are ×200 magnifications. C, W-Sca-1$^+$ and K-Sca-1$^+$ cells were stained for immunocytochemical analyses as described in Figure 1C. Scale bar=10 μm. D, W-Sca-1$^+$ cells were transferred on Matrigel-coated plates, and rearrangement of cells and the formation of tube structure were captured by a light microscope equipped with digital charge-coupled device camera after 48 hours. HUVECs were used as positive control cells to verify tube-forming activity. All of the images are ×200 magnifications.

*cyclinD1* were not upregulated during endothelial differentiation.[43,44] However, we observed a weak induction of endothelin-1 mRNA during the endothelial differentiation of *Wnt5a*$^{-/-}$ ES cells, indicating that small portion of endothelin-1 mRNA is inducible independently of Wnt5a during the differentiation process. The expression of PKCα was reduced by β-catenin knockdown during the endothelial differentiation of *Wnt5a*$^{+/-}$ ES cells. Moreover, the lack of endothelial differentiation in *Wnt5a*$^{-/-}$ ES cells was rescued only when both *PKCα* and β-catenin were cotransfected. These results indicate that both the PKCα and β-catenin pathways are required for endothelial differentiation by Wnt5a. Furthermore, our data suggest that the canonical and noncanonical Wnt pathways function reciprocally in the process of endothelial differentiation by Wnt5a.

Involvement of Wnt5a in vascular development was shown by a defect of the vascular system at E14.5 in *Wnt5a*$^{-/-}$ mice. Similar phenotypes have been observed in the mutant embryos deficient in vascular endothelial growth factor-C.[45] The requirement for Wnt5a in normal vascular development of nervous system was further shown by a strong expression of PECAM-1 in the marginal layer and dorsal root ganglia of the spinal cord of *Wnt5a*$^{+/-}$ but not the *Wnt5a*$^{-/-}$ embryos. The marginal layers of the spinal cord are involved in the formation of the neural tube and express endothelial-specific proteins, such as neuropilin-1, vascular endothelial growth factor, hypoxia-inducible factor-1, and PECAM-1.[33,46] The endothelial cells participate in formation of tubes that extend from the gut plexus to the surface of the gut.[35] We observed no significant difference in the PECAM-1 staining of *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ embryonic intestines. These results suggest that vascular defect by *Wnt5a* knock out is not a general phenotype but a phenotype limited to specific systems such as nervous systems. Finally, our data provide first-time evidence that Wnt5a plays an important role in endothelial differentiation of ES cells and embryonic vascular development.

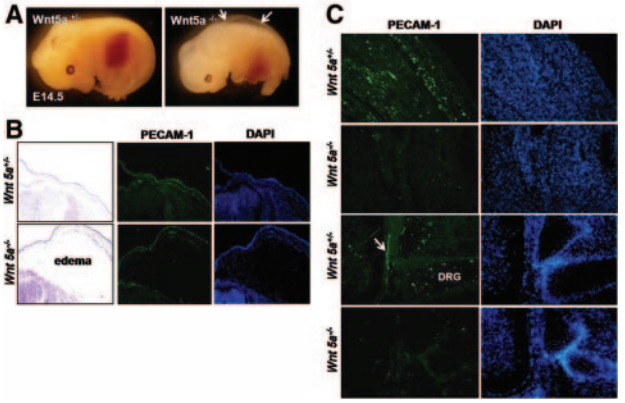


**Figure 7.** Effects of *Wnt5a* knockout on the vascular development of mouse embryos. A, Severe edema (arrows) was formed in whole embryo (E14.5) of *Wnt5a*$^{-/-}$ mouse. B, Transverse sections of the spinal cord for whole embryos (E14.5) of *Wnt5a*$^{+/-}$ and *Wnt5a*$^{-/-}$ mice were subjected to staining with hematoxylin/eosin (left images) or immunohistochemical staining with anti–PECAM-1 antibody (green; middle images). Nuclear counterstaining was performed with DAPI (blue, right images). All of the images are ×100 magnifications. C, The sagittal (upper images) sections of the marginal layer of the spinal cord and the transversal (lower images) sections of the spinal cord were visualized by immunohistochemical analyses as described above. Nuclear counterstaining was performed with DAPI (blue). An arrow indicates the marginal layer of the spinal cord. DRG indicates the dorsal root ganglia. All of the images are ×200 magnifications.

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## Disclosures

None.

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