Hematopoiesis

**Involvement of Runx1 in the downregulation of fetal liver kinase-1 (Flk-1) expression during transition of endothelial cells to hematopoietic cells.**

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Abstract

During early mouse embryogenesis, fetal liver kinase (Flk)-1, a receptor for vascular endothelial growth factor, and Runx1, a runt domain transcription factor, have prerequisite roles in the generation of hematopoietic lineages. Flk-1 expression is maintained in successive stages from mesoderm to endothelial cells and is downregulated in nascent hematopoietic cells, whereas Runx1 is expressed in embryonic sites of hematopoietic cells de novo generation as well as in practically all hematopoietic organs. Here we show that Runx1 represses Flk-1 during the development of hemogenic endothelial cells into hematopoietic cells. We established embryonic stem cell clones carrying the Venus gene, a modified version of yellow fluorescent protein, in the Runx1 locus and cultured them on OP9 cells without leukemia inhibitory factor. Flk-1+ cells appeared on day 3.5 and Runx1+ cells first appeared from the Flk-1+ fraction at day 4.5. The Flk-1+Runx1+ cells rapidly stopped expressing Flk-1 with further incubation, and eventually gave rise to CD45+ or TER119+ cells. Runx1 repressed the Flk-1 promoter transcriptional activity in an endothelial cell line and this repression required intact DNA-binding and transactivating domains of Runx1 protein. The repressor activity of Runx1 on endogenous Flk-1 was also confirmed by overexpressing Runx1 in embryonic stem cell differentiation cultures. These results provide novel insight into the role of Runx1 during the development of hematopoietic cell lineages.
Introduction

During mammalian embryogenesis, two waves of hematopoiesis develop in close association with the development of endothelial cells (ECs). The first wave, known as primitive hematopoiesis, occurs transiently in association with the formation of extraembryonic tissue structures called blood islands that consist of blood cells and surrounding angioblasts. The second wave is known as definitive hematopoiesis, during which the hematopoietic stem cells that function throughout life are generated. Accumulating evidence suggests that at least a part of the definitive hematopoietic cells (HPCs) are generated from a special subset of ECs designated hemogenic ECs. This notion was first suggested by histologic observations in many species of clusters of blood cells attached to the luminal wall of the dorsal aorta at the onset of definitive hematopoiesis that appeared to be budding from the ECs. More recently, the existence of hemogenic ECs was demonstrated in experiments in which multilineage HPCs including lymphocytes were induced from cells that were sorted from embryos according to their expression of EC markers.

Fetal liver kinase-1 (Flk-1), also known as vascular endothelial growth factor (VEGF) receptor-2, is a receptor tyrosine kinase that is indispensable for differentiation of HPCs and ECs. Its expression is detected during successive stages from early lateral mesoderm cells to ECs. Previously, we and others demonstrated that early Flk-1-expressing cells are the diverging point between HPCs (both the primitive and definitive) and ECs. Flk-1+ vascular-endothelial (VE)-cadherin− cells representing lateral mesoderm cells are induced first from embryonic stem (ES) cells and, as
mentioned above, the definitive HPC lineage diverge from the Flk-1\(^+\) cells after induction of the expression of EC markers\(^{18}\). During mesodermal specification, early Flk-1 expression persists mainly in the EC lineage; other lineages, including hemogenic ECs and HPCs quickly downregulate Flk-1, and this downregulation serves as one of the markers of hematopoietic commitment\(^{16,17}\).

The Runx1 gene encodes the DNA binding subunit (alpha-subunit) of a polyomavirus enhancer binding protein 2 (PEBP2) transcription factor complex and has a critical role in the generation of definitive hematopoiesis\(^{19}\). At mid-gestation in mouse, Runx1 is expressed by cell clusters on the ventral wall of dorsal aorta and cell-sorting experiments demonstrated that the presence of hematopoietic progenitors were exclusively within the Runx1\(^+\) population of the dorsal aorta, suggesting that Runx1 is expressed by hemogenic ECs\(^{20}\). In addition, Runx1 is implicated to have a role in the EC-to-HPC transition by observations that the budding cells are absent in Runx1 knockout mice\(^{21}\). Because Runx1 has a role in the differentiation of HPCs from ECs, it is likely involved, either directly or indirectly, in the process of downregulation of EC-specific molecules such as Flk-1. Indeed, while endogenous Flk-1 is expressed in both hemogenic and non-hemogenic ECs, a Flk-1 regulatory unit, which consists of a combination of a 5’-flanking region and a 3’ portion of the first intron of the Flk-1 gene, is suppressed in hemogenic ECs that express Runx1, whereas it is active in fully committed ECs that do not express Runx1\(^{22}\). These observations prompted us to investigate whether there is cross-talk between Runx1 and Flk-1 during the development of definitive hematopoiesis. Our results suggest that Runx1 serves as a
repressor of Flk-1 in mesoderm and ECs.

**Materials and Methods**

*Construction of the Runx -Venus knock-in targeting vector, and isolation of the knock-in clones*

A 12-kb genomic fragment containing the proximal promoter P2 and two exons of the *Runx1* gene was isolated from a mouse 129/Sv lambda genomic library and subcloned into pBluescript (Stratagene, La Jolla, CA). A floxed gene reporter cassette was inserted into the *Xho* site in the P2 – 5’ untranslated region (P2-5’UTR). The insert consists of a 1.7-kb splice acceptor sequence from the mouse *Engrailed–2* gene, *lacZ* reporter gene, transcription termination signals, and a floxed HSV-TK*neo* gene cassette (pL2-neo vector, a gift from Dr. H. Gu, NIAID/NIH, Rockville, MD). The Venus coding sequence, lacking polyA signals, was juxtaposed downstream of the floxed insert (Figure 1A). The targeting vector was linearized at a unique *Not*I site prior to electroporation of ES cells. CCE ES (10^7) cells were electroporated with 40 µg of linearized *Runx1* targeting construct and stably transfected clones were selected with 400 µg/ml G418. Correctly targeted clones were identified by Southern analysis of genomic DNA following digestion with *Bam*HI and hybridization with the 5’ external probe (Figure 1B). Single-copy integration of the vector DNA to the clones and the integrity of the targeted *Runx1* locus were confirmed by Southern blot analysis with an internal genomic probe spanning *Runx1* P2 – 5’UTR and the adjacent coding region. The floxed insert cassette was removed from correctly-targeted ES clones by transient
expression of Cre recombinase using pIC-Cre (a gift from Dr. H. Gu) (Figure 1C).

**Plasmids and transient transfections**

The pFlk-1prom/GL3 vector was generated by insertion of the –640bp/+299bp promoter fragment of the pGLacZ-Flk-1 promoter/enhancer\textsuperscript{25} into the K\textit{pn}I and \textit{Hind}III restriction sites of the pGL3 vector (Promega, Madison, WI). The pcDEFBOS/GL3 was generated by insertion of the \textit{Kpn}I-\textit{Xba}I fragment of the pGL3 vector into the \textit{Kpn}I and \textit{Xba}I restriction sites in the pcDEF3 vector. Deletion mutants of Flk-1 promoter, -474/299, -332/299, -240/299 and -475/-240 were made by cutting out the fragments between \textit{Kpn}I and \textit{Bai}I, \textit{Kpn}I and \textit{EcoT}14I, \textit{Kpn}I and \textit{Bst}XI or \textit{Bai}I and \textit{Bst}XI of pFlk-1prom/GL3 vector, respectively, and by self-ligation of the remnant vectors. Point mutations which disrupt the putative Runx1 binding sites at –201, +22 and +39 of the promoter were generated by PCR-based site-directed mutagenesis. The mutations at –201 changed the sequence from ACCA to CTTA, +22 from ACCC to CTTC, +39 from GTGTT to CTAAG. The introduced mutations and the integrity of non-mutated regions were confirmed by sequencing of cloned PCR products. Expression vectors for Runx1, Runx1 mutants, and PEBP2\textbeta\textsuperscript{26,27} were kind gifts from Dr. Motomi Osato (Institute of Molecular and Cell Biology, National University of Singapore).

Bovine aortic endothelial cells (BAEC; 2x10\textsuperscript{4}) were seeded in 12-well plates 24 hours prior to transfection. Cells were transfected using FuGENE6 (Roche Diagnostics, Mannheim, Germany) with 200 ng of reporter plasmid and 200 ng of expression vectors. Luciferase activity was normalized for transfection efficiency with the co-transfected 100 ng pRL-null (Promega) as previously described\textsuperscript{28}. Luciferase assays were
performed 48 hours after transfection using the dual luciferase reporter assay system (Promega) according to the manufacturer’s protocols. All trans-activation experiments were repeated at least three times.

ES lines with conditional Runx1 expression

The modified version of the tetracycline (Tet) regulatory system was used to induce Runx1 expression in ES cell clones essentially as described previously. The E14tg2a ES cells were stably transfected with the tetracycline transactivator (tTA). A tet-regulatable Runx1 construct was generated by inserting the mouse Runx1 cDNA into the EcoRI site of pUHD10-3IRESEGFP (a kind gift from Dr. Takumi Era, Center for Developmental Biology, Riken, Kobe). Tetracycline-inducible Runx1-expressing cell lines were established by stable transfection of pUHD10-3Runx1IRESGFP into the parental cells, and the ES cell lines with integrated pUHD10-3IRESGFP constructs were used as controls.

Cell culture and in vitro differentiation of ES cells

Culture of ES cells and OP9 stromal cells was performed as previously described. Induction of ES cell differentiation was also performed as described previously. Briefly, 3x10⁴ undifferentiated ES cells were transferred to each well of a type IV collagen-coated 6-well plate (BIOCOAT; Beckton Dickinson Labware, Bedford, MA) and incubated in alpha minimum essential medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 50 μmol/L 2-mercaptoethanol in the absence of leukemia inhibitory factor (LIF). Alternatively, cells were cultured on confluent OP9 cell layers in six-well plates (Becton Dickinson
Labware) at a density of 1x10^4 cells per well to induce differentiation. For hematopoietic differentiation, the total ES cells – OP9 culture was split 1:100 after 5 days of differentiation and reseeded on fresh OP9 cell layers in the presence of the following cytokines: mouse granulocyte colony stimulating factor (G-CSF) (100 ng/ml), murine stem cell factor (SCF) (100 ng/ml), mouse interleukin(IL)-3 (200 U/ml), human erythropoietin (Epo) (2 U/ml) and human VEGF (10 ng/ml). For B cell lineage differentiation the medium for ES cells cultured on OP9 stroma was supplemented with mIL-7 (20 ng/ml) and mFlt-3L (20 ng/ml). In both cases, the cytokine-supplemented cultures were maintained for 14 days before fluorescence activated cell sorter (FACS) analysis. Recombinant IL-3, SCF, and IL-7 were purchased from Pepro Tech Inc., Rocky Hill, NJ; recombinant G-CSF, Epo, VEGF, and Flt-3L was purchased from R&D Systems (Minneapolis, MN). Cultured cells were harvested with cell dissociation buffer (GIBCO BRL) and analyzed.

Monoclonal antibodies (MoAbs), cell staining, and sorting

The MoAb AVAS12 (anti Flk-1) was purified from hybridoma culture supernatants using protein G-sepharose columns (Pharmacia, Uppsala, Sweden) and labeled with allophycocyanin (APC) by standard methods. Fluorescein isothiocyanate-anti-CD45 (common leukocyte antigen) and fluorescein isothiocyanate-anti-Ter119 (erythroid marker), APC-anti-e-Kit, APC-anti-CD45, APC-anti-CD11b (Mac-1, monocyte/macrophage marker), APC-anti-Ly6G/C (Gr-1, granulocyte marker), and APC-anti-CD45R (B220, B-cell marker) were purchased from Pharmingen (Pharmingen, San Diego, CA). Nonspecific antibody binding was blocked using normal
mouse serum and cells were labeled with combinations of the above MoAbs. Stained cells were resuspended in Hank’s balanced salt solution (GIBCO BRL) containing 1% bovine serum albumin (Sigma-Aldrich Chemical Co., St Louis, MO) and 5µg/ml propidium iodide (PI; Sigma-Aldrich) to exclude dead cells. Cells were analyzed and sorted by FACS Vantage or Aria (Beckton Dickinson Immunocytometry Systems, San Jose, CA) using the CellQuest software (Beckton Dickinson Immunocytometry Systems).

**Immunoblotting**

Runx1 proteins were detected with rabbit polyclonal serum against Runx1 (Oncogene, Parmastadt, Germany) followed by treatment with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was prepared from sorted cell populations or cultured cells using ISOGEN (Nippon Gene, Toyama, Japan). RNA was reverse-transcribed with Superscript II reverse transcriptase (GIBCO BRL) and oligo (dT)12-18 primer (GIBCO BRL) according to the manufacturer’s instructions. PCR assays were performed in the reaction mixture containing 1x ExTaq Buffer (Takara Shuzo, Osaka, Japan), 200 µmol/L dNTPs (Pharmacia), 25 U/ml ExTaq DNA polymerase (Takara Shuzo), several dilutions of cDNA, and 2 µmol/L of specific primers. Sequences of primers for Flk-1 were as follows: forward 5’-GTGGATCTGAAAAGACGC-3’, reverse 5’-CATTCTTCTCCGATAGG-3’. Runx1 semi-quantitative RT-PCR was performed using Cell-to-cDNA™ II Kit (Ambion, Austin, TX). Sequences of primers for Runx1
and Ribosomal Protein 13 Large subunit were as follows: Runx1 forward 5'-CAATCGGCTTGTTGTGATGC-3', reverse 5’-TTCATCGTTGCCTGCCATGAC-3’, Ribosomal Protein 13 Large subunit forward 5’-GCTCAAGCTCATTGTGTGTT-3’, reverse 5’-GGAGACTGGCAAAGCCTTA-3’. PCR products were electrophoresed through a 1% agarose gel and stained with ethidium bromide.

Results

Establishment of Runx1-Venus knock-in ES cell lines.

First, we investigated the relation between Runx1 and Flk-1 expression during HPC development. Whereas Flk-1 expression is detectable by a combination of fluorescence-labeled specific MoAbs and flow cytometry, monitoring Runx1 expression requires markers that indicate its expression. For this purpose, we introduced the Venus gene reporter into one of the Runx1 alleles. The Venus gene was placed in the P2-5’UTR under the control of the Runx1 proximal promoter P2, which is active before the Runx1 distal P1 promoter during ES cell differentiation in vitro. Venus is a modified version of yellow fluorescent protein with an improved efficiency and speed of maturation that substantially increases the sensitivity of gene reporter analysis. The HSV-TK neoR-positive selection cassette was deleted to prevent possible interference with the proximal promoter (Figure 1A, C). No polyadenylation signals were inserted downstream of the Venus coding sequence, which allows the knock-in transcripts to utilize functionally important Runx1 3’-UTR, and to use an endogenous internal ribosome entry sequence (IRES) in the P2-5’UTR. To minimize the
interference with both IRES and proximal promoter functions, we introduced Venus gene into the UTR about 1200 bp upstream from the translation initiation codon and about 400bp downstream from the proximal promoter transcription start. This makes the recombinant transcripts effectively bicistronic and thereby reduces possible Runx1 gene dosage effects. The kinetics of early differentiation of the recombinant Runx1\textsuperscript{Venus/+} ES clones in terms of the changes in expression of Flk-1 were identical to the kinetics observed in the experiments using wild type ES cells (data not shown), whereas the diagnostic feature of Runx1 haploinsufficiency is significantly earlier emergence of Flk-1 mesoderm marker during ES cell differentiation \textit{in vitro}\textsuperscript{33}.

We measured the mRNA levels of endogenous Runx1 during \textit{in vitro} differentiation of the ES clones (Figure 2A). Runx1 expression was detected at day 3 at substantially higher level than at day 0 and this preceded the appearance of Venus\textsuperscript{+} population at day 4. The lag may be explained by rapid expansion of differentiating ES cells at day 0 to day 3, which severely decreases the amount of Venus protein in a single cell to the level of fluorescence not detectable by flow cytometry as Venus-positive. After day 4, the mRNA levels of Runx1 and the frequency of Venus\textsuperscript{+} cells in Flk-1\textsuperscript{+} population correlated well. These data suggest that the expression of Venus reflects the endogenous levels of Runx1. In addition, these Runx1\textsuperscript{Venus/+} ES lines were tested for specificity of the expression of the introduced reporter. Cultured on OP9 stromal cell layers in the presence of hematopoietic cytokines and VEGF, the recombinant ES cells efficiently differentiated into myeloid and lymphoid lineages. Venus fluorescence was detected in almost all CD45\textsuperscript{+} hematopoietic cells (Figure 2B). c-Kit\textsuperscript{+} immature hematopoietic cells
and a majority of B220+ lymphocytes expressed a high level of the Venus, whereas differentiated Mac-1 and Gr-1-positive cells expressed the reporter gene at lower levels (Figure 2B, C). These data suggest that the expression pattern of the Venus reporter gene faithfully reflects the known pattern of Runx1 expression in the hematopoietic lineages34-36.

### Runx1 expression during differentiation of Flk-1+ cells.

We next investigated Runx1 expression during the differentiation of mesoderm cells and ECs. ES cell differentiation was induced on OP9 stromal cells, which support preferential ES cell differentiation to mesodermal lineages. Like other ES cell lines16,17, Flk-1+ cells appeared around day 3.5 and the number of the Flk-1+ cells increased within the next 24 hours in the Runx1Venus+/+ ES cell (Figure 3A). Runx1-Venus+ cells appeared about 1 day later than that of the Flk-1+ fraction and the number of Runx1-Venus+ cells increased in the subsequent 24 hours (from 1.6% to 9.1%). Nearly all Runx1+ cells induced under these conditions were Flk-1+ at this stage. This observation is consistent with the results of a previous study by Lacaud et al37. In our study, Runx1 expression was investigated also at later stages of differentiation, and from day 6.5 to 7.5, the double-positive cells disappeared quickly and the Flk-1− population became dominant within the Runx1-Venus+ cells (11.1% versus 3.5%, respectively). Considering that Runx1 is a requisite molecule for the differentiation of ECs to HPCs, these data suggest a sequence of events in which Runx1 begins to be expressed in a portion of Flk-1− cells, which in turn triggers downregulation of Flk-1 and initiates transition to hematopoietic specification.
To confirm this possibility, we sorted the Flk-1^+Venus^+ cells on day 5.5 of differentiation and cultured them again on OP9 feeder layers to induce further differentiation (Figure 3B). Upon induction of differentiation, the double-positive cells quickly disappeared and eventually differentiated into HPCs (Figure 3B, C). In contrast, when we cultured the Flk-1^+Venus^− cells sorted from the same culture and incubated with OP9, 30% to 70% of the cells maintained Flk-1 expression and no HPCs were generated (Figure 3D), however, day 4 – 4.5 Flk-1^+Venus^− cells can form HPCs because some cells at this stage of differentiation still have the potential to upregulate Runx1/Venus (Figure 3A), but later Flk-1^+Venus^− fraction is completely devoid of such cells. These findings indicate that a subpopulation of Flk-1^+ cells that expresses Runx1 is the sole source of hematopoietic progenitors and Flk-1 is downregulated during HPC generation.

**Runx1 represses flk-1 promoter transcriptional activity in ECs**

During HPC differentiation, Runx1 might trigger multiple events and the rapid downregulation of Flk-1 in the Flk-1^+Runx1^+ cells can be either a direct or indirect outcome of Runx1 expression. To investigate whether Runx1 acts directly on Flk-1 transcription, we first examined Flk-1 promoter activity in the presence or absence of exogenous Runx1. We used bovine aortic endothelial cells (BAEC) in this assay, because the Flk-1 promoter is active specifically in ECs^{25,38}.

The -640/+299 fragment of the Flk-1 gene was ligated to firefly luciferase cDNA and transiently transfected into BAEC with or without Runx1-expressing plasmid vectors. Flk-1 promoter activity was reduced by 5- to 10-fold in the presence of Runx1 in a dose
dependent manner (Figure 4A). In contrast, Runx1 had no effect on the promoter of elongation factor-1 (EF-1). Runx1-dependent repression of the Flk-1 promoter is EC-specific, as we could not detect the negative regulatory effects of Runx1 on the Flk-1 promoter in other cell types, including NIH3T3, COS, or HEK293 cells (data not shown).

Runx1 is a member of the PEBP2 transcription complex and its DNA binding and transcriptional regulation activities are increased by binding with PEBP2β, the heterodimer partner. We evaluated the effects of PEBP2β on the repression of Flk-1 promoter transcription by Runx1. PEBP2β significantly augmented the effect of Runx1 (p<0.005) whereas the effect of Runx1 on the activity of the EF-1 promoter was not affected by the addition of PEBP2β (Figure 4B). This further confirms that the Flk-1 gene promoter is a specific target of gene silencing induced by the Runx1-mediated transcription complex.

There are seven putative consensus-binding sites for Runx1 within the Flk-1 promoter, although not all of them are complete match. We introduced deletion and point mutations in the sequence of the Flk-1 promoter to disrupt each putative Runx1-binding site and measured the promoter activity of these mutants (Figure 4C and D). Surprisingly none of the mutants reversed the Runx1-mediated transcriptional repression, suggesting the repressive effects are indirect.

To determine which Runx1 domain is required for this repression, we examined Runx1 deletion mutants in our assay (Figure 5A). The amino acids 242-371 were important for this inhibitory effect of Runx1 (Figure 5B). When we transfected BAEC
with the Runx1 mutant K83E, which maintains the ability to heterodimerize with the β-subunit but loses the DNA-binding ability, the Flk-1 promoter activity was not suppressed. Taken together, these data strongly suggest that Runx1 has negative regulatory effects on Flk-1 promoter activity in ECs and this repression requires intact DNA-binding and transactivating domains of Runx1 protein.

**Overexpression of Runx1 inhibits Flk-1 expression during in vitro differentiation of ES cells**

We next investigated whether Runx1-dependent repression of Flk-1 is observed not only in EC lines, but also in the ES cell differentiation culture. For this purpose, we used a Tet-off inducible system in combination with *in vitro* differentiation of ES cells (Figure 6A). We generated ES cell lines in which the tetracycline transactivator gene is driven by the CAG promoter and the Runx1 and/or green fluorescent protein (GFP) expression is under the control of the Tet operator sequence. Expression of Runx1 and/or GFP expression was induced in the absence of tetracycline, while neither Runx1 nor GFP expression was detected in the presence of tetracycline (Figure 6B, C).

When we cultured the control cell lines with inducible GFP for 4 days without LIF, Flk-1 expression was detected at equivalent levels in the presence or absence of tetracycline (Figure 6D). In contrast, when we cultured cell lines carrying inducible Runx1 transgene with or without tetracycline, the number of Flk-1-expressing cells was significantly reduced by Runx1 overexpression (3.7% versus 23.2%, p<0.001, n=3). The cell recovery and gross morphology did not differ with or without tetracycline treatment in either clone. This repression of Flk-1 expression was accompanied by a
reduction in Flk-1 transcripts (Figure 6E). The same results were obtained in 10 independent ES cell clones. These results suggest that Runx1 has the potential to downregulate the expression of endogenous Flk-1 gene during differentiation of ES cells \textit{in vitro}.

**Discussion**

During the EC-to-HPC transition, molecules determining the essential features of ECs as an integral part of the endothelial sheet are downregulated while at the same time HPC features are acquired. The budding of HPCs from the dorsal aorta, which regularly occurs in normal embryos is not detectable in Runx1 null-mutant mice\textsuperscript{21} and no hemogenic ECs are detected in the embryonic vessels of the mutant mice\textsuperscript{11}. These studies strongly suggest that Runx1 is directly involved in the EC-to-HPC transition. The aim of this study was to dissect this process, with particular focus on the relation between Runx1 and Flk-1.

Under OP9 stromal cell culture conditions, which preferentially support the differentiation of ES cells to a mesodermal lineage, Runx1 is induced mostly within the Flk-1\textsuperscript{+} population. This finding is largely consistent with the results of a previous study by Lacaud et al.\textsuperscript{37}, except that the time course of Runx1 expression was delayed 24 hours in the present experiment. While a part of this delay reflects the inherent difference between the two methods of ES cell differentiation, it is likely that the enhancement of HPC differentiation by haploinsufficiency of Runx1 accounts for this difference in the time course. Indeed, recent work by the same group indicates that
Runx1 haploinsufficiency profoundly affects the time course of ES cell differentiation into HPC\textsuperscript{33}. In contrast, our results indicated that \textit{Runx1}\textsuperscript{Venus/+} ES cells differentiated identically to \textit{Runx1}\textsuperscript{+/+} ES cells in terms of expression of Flk-1 mesodermal marker, suggesting Runx1 is at least partially intact in the recombinant allele. Currently we are intensively investigating the biological function of the knock-in allele \textit{in vivo}.

Runx1 is induced within 24 hours after the appearance of Flk-1\textsuperscript{+} cells, which is almost simultaneous with the expression of EC markers such as vascular endothelial cadherin. It is not clear which mechanism is responsible for the divergence of Runx1\textsuperscript{+} and Runx1\textsuperscript{−} ECs during EC commitment. It has been suggested that the EC-to-HPC transition occurs in a restricted region of the vascular system\textsuperscript{12,20,22}, therefore it is likely that an as yet unknown extrinsic signal is involved in this divergence. Nonetheless, our study clearly demonstrates that HPCs are generated only from the Flk-1\textsuperscript{+}Runx1\textsuperscript{+} population. Consistent with our results, generation of HPC in midgestation embryos was present exclusively within the Runx1\textsuperscript{+} population with or without EC features\textsuperscript{20}, indicating that the ability to undergo the EC-to-HPC transition is determined at a relatively early stage of mesoderm differentiation. Such fine tuning of the timing of Runx1 expression in relation to Flk-1 must be essential for supporting the process wherein the HPC progenitor cells are first integrated into the vascular system and subsequently differentiate to HPC.

In this study, we investigated the fate of Flk-1\textsuperscript{+}Runx1\textsuperscript{+} cells. Under our culture conditions, the majority of the double-positive cells differentiated into HPC, though a small population that downregulated Runx1 expression maintained EC characteristics.
There are ECs expressing Runx1 in vivo in the yolk sac, the vitelline and umbilical arteries, and in the ventral wall of the dorsal aorta in the aorta/genital ridge/mesonephros region where the definitive HPCs arise as cell clusters attached to the luminal wall. This supports the view that the program leading to Runx1 expression in Flk-1+ cells is likely to trigger the irreversible process toward HPC differentiation. This is consistent with our previous report that a subset of ECs in which a promoter/enhancer combination of the Flk-1 gene was active, but Runx1 expression was absent, could not give rise to HPCs.

Flk-1 is a VEGF receptor that has a central role in endothelial development. VEGF-Flk-1 signaling mediates proliferation, migration, and other EC-specific properties. Disruption of Flk-1 resulted in embryonic lethality with no ECs nor HPCs in vivo; however, Flk-1–/– ES cells can differentiate into both lineages in vitro, indicating that Flk-1 is required especially for the migration of progenitors into the proper microenvironment during embryogenesis. Heterozygous inactivation of the VEGF gene results in impaired development of the vascular and hematopoietic system. In chicken, a higher concentration of VEGF inhibits the differentiation of HPCs from VEGF-R2+ cells. These data indicate that precise regulation of VEGF signaling is necessary not only for EC development, but also for proper HPC development. In this study, we demonstrated that the Runx1+Flk-1+ sorted population quickly downregulates Flk-1 expression upon reseeding on a fresh OP9 stromal cell layer. These data suggest an inhibitory role of Runx1 in the regulation of Flk-1 expression. Indeed, Lacaud et al. reported that heterozygous inactivation of Runx1 accelerated mesodermal
differentiation using an ES in vitro system and a higher proportion of mesodermal cells express Flk-1 than normal ES cells. Conversely, we demonstrated that Runx1 specifically represses the promoter activity in a cell context-dependent manner. This repression was also observed during early mesoderm differentiation through which Flk-1 is induced. Taken together, these data provide evidence that Runx1, possibly among others, is a negative regulator of Flk-1 expression during mesoderm differentiation. It is possible that the tuning of HPC differentiation by the dose of Runx1 is mediated through the regulation of the Flk-1 expression level. On one hand, Runx1 downregulates the signal mediated by VEGF/Flk-1 and thereby inhibits the differentiation towards ECs at the diverging point of cellular specification, while Eichmann et al. demonstrated that strong VEGF-Flk-1 signaling inhibits HPC differentiation.

The Runx family of transcription factors acts as both transcription activators and a transcriptional repressors or silencers, which depends both on the cellular context and target molecules. The mechanisms of repression by the Runx family have been studied in detail. The recruitment of co-repressors, including mSin3A, TLE, SUV39H1, and histone deacetylase, is also involved in Runx1-mediated repression. In our study, disruption of the VWRPY motif had little impact on the repression, suggesting that TLE is not involved in this effect (αB1 (1-371) deletion mutant, Figure 5). Although our results with the Runx1 K83E mutant clearly showed involvement of DNA binding in the inhibitory function of Runx1, we could not determine the elements in Flk-1 promoter responsible for the repression in a reporter assay, suggesting Runx1 transcription factor
targets another gene in the chain of events leading to downregulation of Flk-1. Hypoxia inducible factor (HIF)-1 and HIF-2 positively regulate the Flk-1 promoter, and the binding sites for the SCL, GATAs, and Ets transcription factors have important roles in the proper expression of Flk-1 during development \textit{in vivo}\textsuperscript{48,49}. Whether these factors are directly silenced by Runx1 in the described inhibition of Flk-1 expression remains to be determined.

Our results showed that the amino acids 242-371, known as a transactivating domain, are important for the repression. These findings prompted us to study the role of bone morphogenetic protein (BMP)-Smad signaling pathway, because BMP4 is also involved in the differentiation of hematopoietic and endothelial lineage\textsuperscript{50}, and Smads family member molecules are known to interact with Runx1 at its transactivating domain\textsuperscript{51}. When Runx1 expression vector was co-transfected with expression constructs for Smad1, Smad5 or Smad8 together with a vector for constitutively active form of BMP receptor, the repressive effects of Runx1 were slightly attenuated, suggesting that Smads were not involved in this repression by Runx1 but rather sequestered Runx1 from the repressive effects (data not shown).

In summary, we demonstrated that Runx1 represses Flk-1 expression during the development of definitive hematopoeisis. These findings provide new insights into the understanding of diversification between hematopoietic and endothelial lineages.

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providing Runx1 expression vectors, vectors for the tet-regulatable system and pGLacZ-Flk-1 promoter/enhancer, respectively. We also thank Dr. Gang Huang, Dr. Jun Yamashita and Dr. Osam Mazda for valuable discussions and Natalia I. Samokhvalova for technical assistance.
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Figure Legends

Figure 1

Generation of VenusYFP (Venus) knock-in ES cells. (A) Targeting strategy used to insert the Venus gene reporter under P2 proximal promoter of Runx1 locus. Exons are presented as boxes; P2-5′UTR is grey, coding regions are light. Black triangles are loxP sites; the 5′- external and internal genomic probes used for screening are indicated, as well as the P2 proximal promoter of Runx1. SA-LacZ-stop, mouse Engrailed2 splice acceptor – LacZ – transcription stop cassette; neo, HSV-TK – neoR – polyA cassette;
pBlue, pBluescript II SK+; B, BamHI; X, XbaI; Xh, XhoI. (B) Southern blot analysis of the targeted allele. ES genomic DNA was digested by BamHI, blotted and hybridized with the 5’- external genomic probe. The properly targeted allele generates a 2.4-kb DNA band. (C) Southern hybridization analysis of Runx1fl/+ ES cells subjected to Cre recombinase–dependent excision of the floxed (loxP flanked) cassettes. Internal genomic probe hybridizes with 1.8 kb, 2.4 kb, and 8.8 kb XbaI genomic fragment derived from wild-type (+), Venus (V) knock-in, and the floxed (fl) Runx1 alleles, respectively.
Figure 2

Venus YFP expression during ES cell differentiation on OP9 stroma. (A) RT-PCR analysis of endogenous Runx1 mRNA transcribed from proximal promoter P2, and
corresponding Venus expression in undifferentiated ES cells and in Flk-1\(^+\) fraction of differentiated ES cells. Above triangles are the numbers of PCR cycles; days of ES cell differentiation on OP9 cell layer are indicated. RPL13, Ribosomal Protein 13 Large subunit. (B) Venus expression in ES cell – derived myeloid hematopoietic cells. (C) Venus expression in ES cell – derived B cells.

Figure 3
**FACS analysis of Venus knock-in ES cells differentiating on OP9 stromal cells.** (A) 
Runx1$^{Venus^+/+}$ ES cells were cultured on OP9 cells and analyzed for the expression of Flk-1 and Venus by FACS on the indicated days. (B) Flk-1$^+$Venus$^+$ cells were sorted on
day 5.5 of differentiation and recultured on OP9 stroma. Flk-1, CD45, and TER119 expression on Venus⁺ cells was then analyzed by FACS on the indicated days. (C) Giemsa staining of Venus⁺ cells harvested on day 2.5 of differentiation of sorted Flk-1⁺Venus⁺ cells (x1000). All cells looked like immature hematopoietic cells of definitive hematopoietic origin. (D) Flk-1⁺Venus⁻ cells were sorted on day 5.5 of differentiation and cultured again on OP9 stroma. On day 2.5 after sorting cells were harvested and stained for Flk-1, CD45, TER119 and analyzed by flow cytometry. The results shown are representative of three independent experiments.
Runx1 downregulates the activities of the *Flk-1* promoter in BAEC. (A) The activity of luciferase reporter gene under the control of the −640bp/+299bp promoter fragment
of mouse Flk-1 was measured in the presence of the indicated amounts (ng) of Runx1 expression vector (left panel). Note that luciferase activity was downregulated in the presence of Runx1 in a dose-dependent manner, while luciferase activity driven by elongation factor (EF-1) promoter was not affected (right panel). (B) The repressive effect of Runx1 on the Flk-1 promoter was augmented in the presence of PEBP2β (left panel), while EF-1 promoter activity was not affected (right panel). (C) Putative Runx1 binding sites in the murine Flk-1 promoter and functional analysis of the promoter. Open circle represent the putative Runx1 binding sites and closed circles are the binding sites mutated by PCR. (D) The activity of luciferase reporter under control of mutant Flk-1 promoters in bovine aortic endothelial cells. The results shown are representative of three independent experiments.
Figure 5

A

![Diagram showing the structures of full-length Runx1 (αB1), its deletion derivatives, and K83E mutants. K83E mutant has an A>G substitution in exon 3 resulting in a missense mutation.]

B

![Graph showing the effects of Runx1 and its mutants on Flk-1 promoter activity in BAEC. The results shown are representative of three independent experiments.]

Analysis of Runx1 functional domains involved in the Flk-1 promoter repression.

(A) Schematic illustration of the structures of full-length Runx1 (αB1), its deletion derivatives, and K83E mutants. K83E mutant has an A>G substitution in exon 3 resulting in a missense mutation. (B) Effects of Runx1 and its mutants on Flk-1 promoter activity in BAEC. The results shown are representative of three independent experiments.
Figure 6

A. tetracycline

\( \text{prom} \) Runx1 IRES GFP

Runx1 expression

(-) (+) tTA

tet-regulatable promoter

B. Cell number

\( \text{GFP} \)

- tet (-)
- tet (+)

C. tet-GFP tet-Runx1

Runx1

D. tet (+) tet (-)

\( \text{Flk-1} \)

\( \text{tet-GFP} \)

\( \text{tet-Runx1} \)

E. tet (+) tet (-)

\( \text{Flk-1} \)

\( \beta\text{-actin} \)
Tet-induced overexpression of Runx1 downregulates Flk-1 in differentiating ES cells. (A) The construct and strategy of Tet-regulated expression of Runx1 in ES cells. The expression of Runx1 and IRES-linked GFP is driven by a Tet-regulated promoter which is suppressed by the addition of tetracycline (Tet-off system). (B) and (C) Inducible expression of GFP and Runx1 by the removal of Tet in undifferentiated ES cells. GFP expression was analyzed by FACS (B) and Runx1 expression was analyzed by Western blotting (C) 48 hours after Tet removal. (D) and (E) Inducible expression of Runx1 downregulates Flk-1 expression during in vitro differentiation of ES cells. ES cells with Tet-inducible GFP constructs (upper lane) or Tet-inducible Runx1-IRES-GFP constructs (lower lane) were cultured on collagen type IV coated dishes in the presence or absence of tetracycline and analyzed for Flk-1 and GFP expression by flow cytometry (D) and for Flk-1 expression by semi-quantitative RT-PCR (E) on day 4.5 of differentiation. The results shown are representative of three independent experiments.
Involvement of Runx1 in the downregulation of fetal liver kinase-1 (Flk-1) expression during transition of endothelial cells to hematopoietic cells

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