

Hes1 immortalizes committed progenitors and plays a role in blast crisis transition in chronic myelogenous leukemia

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Hairy enhancer of split 1 (Hes1) is a basic helix-loop-helix transcriptional repressor that affects differentiation and often helps maintain cells in an immature state in various tissues. Here we show that retroviral expression of Hes1 immortalizes common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) in the presence of interleukin-3, conferring permanent replating capability on these cells. Whereas these cells did not develop myeloproliferative neoplasms

when intravenously administered to irradiated mice, the combination of Hes1 and BCR-ABL in CMPs and GMPs caused acute leukemia resembling blast crisis of chronic myelogenous leukemia (CML), resulting in rapid death of the recipient mice. On the other hand, BCR-ABL alone caused CML-like disease when expressed in c-Kit-positive, Sca-1-positive, and lineage-negative hematopoietic stem cells (KSLs), but not committed progenitors CMPs or GMPs, as previously reported.

Leukemic cells derived from Hes1 and BCR-ABL-expressing CMPs and GMPs were more immature than those derived from BCR-ABL-expressing KSLs. Intriguingly, Hes1 was highly expressed in 8 of 20 patients with CML in blast crisis, but not in the chronic phase, and dominant negative Hes1 retarded the growth of some CML cell lines expressing Hes1. These results suggest that Hes1 is a key molecule in blast crisis transition in CML. (*Blood*. 2010;115(14):2872-2881)

Introduction

The balance between activator and repressor basic helix-loop-helix transcription factors is crucial for the proper timing of cellular differentiation and normal morphogenesis of various tissues.¹ During embryogenesis, the basic helix-loop-helix protein hairy enhancer of split 1 (Hes1), functioning downstream of the Notch receptor,^{2,3} blocks differentiation of neural stem cells by antagonizing Mash1⁴ and affects the cell-fate decision of pancreatic epithelial progenitors.⁵ In the adult hematopoietic system, Hes1 blocks granulocyte colony-stimulating factor-induced granulocytic differentiation of the 32D cell line,⁶ preserving the long-term reconstituting ability of hematopoietic stem cells (HSCs) in vitro as well as in vivo.⁷ Hes1 also plays a significant role in the development of perinatal T cells,^{8,9} and knocking out Hes1 leads to lack of thymus.⁹

Recently, activating mutations of the *Notch1* and *Notch2* genes have been identified in more than 50% of human T-cell acute lymphoblastic leukemias¹⁰ and in a subset of non-Hodgkin lymphomas,¹¹ respectively, implicating Notch signal deregulation based on a genetic abnormality in human cancers. The effect of Notch signal aberration, however, has been largely confined to lymphoid lineages in the hematopoietic compartment. Indeed, enhanced Notch signaling provides the bone-marrow-to-thymus transition stage of early progenitors, with strong selective pressure toward thymic

T-cell precursors at the expense of B-cell and myeloid precursors.¹²⁻¹⁴ We recently found that up-regulation of Hes1 represents only a part of Notch signaling during the decision between mast cell and granulocyte lineage differentiation. Notch signaling does promote mast-cell development at the expense of granulocyte differentiation through up-regulation of both Hes1 and GATA-3 in common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs). However, up-regulation of Hes1 alone causes expansion of cells with myeloid progenitor phenotypes, rather than mast cell development, mediated through down-regulation of a transcription factor, C-enhancer binding protein α (C/EBP- α).¹⁵

A growing volume of evidence shows that down-regulation of C/EBP- α represents major events in human acute myelogenous leukemia (AML), through either genetic or epigenetic abnormalities. Therefore, it is postulated that Hes1 up-regulation may be involved in a subset of myeloid leukemias.

Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm that originates in an abnormal pluripotent bone marrow stem cell and is consistently associated with the *BCR-ABL* fusion gene. The disease is biphasic or triphasic; an initial indolent chronic phase is followed by one or both of the aggressive stages, the accelerated phase and blast crisis, resulting in expansion of immature leukemic cells. The mainstay of chronic phase to blast

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crisis transition is the differentiation block by additional genetic events in progenitor stages of CML cells¹⁶ that could otherwise differentiate during the chronic phase. Thus, the transformation of BCR-ABL-induced myeloproliferative neoplasm to full-blown blast crisis has been drawing tremendous attention from investigators.

Here we show that retroviral expression of Hes1 immortalizes CMPs and GMPs *in vitro*. Hes1 introduction together with BCR-ABL into CMPs and GMPs, the postulated origin of blast crisis transition in CML, induced CML blast crisis-like disease when intravenously administered to sublethally irradiated mice. Considering as well the study of Hes1 expression in CML patients, we propose that Hes1 is a unique experimental tool for studying the mechanisms of chronic phase to blast crisis transformation in CML.

Methods

Mice

C57BL/6 (Ly5.1) donor mice were purchased from Sankyo Labo Service Corporation. C57BL/6 (Ly5.2) recipient mice were purchased from SLC. Mice were kept at the Animal Center for Biomedical Research, University of Tokyo, according to institutional guidelines.

Bone marrow progenitor sort

Bone marrow cells were isolated from the femurs and tibias of C57BL/6 (Ly5.1) donor mice (8-10 weeks of age) and were incubated with biotinylated antibodies for lineage markers, including anti-CD3, anti-CD4, anti-CD8, anti-B220, anti-Ter119, and anti-Gr-1 antibodies (BD Biosciences PharMingen) followed by incubation with streptavidin Micro Beads (Miltenyi Biotec). The lineage marker-negative (Lin⁻) fraction was separated with an autoMACS separator or LS Columns (Miltenyi Biotec) and incubated with anti-CD34–fluorescein isothiocyanate, anti-CD116/32 (FcγRIII/II receptor)–phycoerythrin (PE), anti-c-Kit–allophycocyanin, streptavidin peridinin chlorophyll protein (BD Biosciences PharMingen), and anti-Sca-1–PE/Cy7 (eBioscience). Lin⁻c-Kit⁺Sca-1⁺, Lin⁻c-Kit⁺Sca-1⁻FcγR^{lo}CD34⁺, and Lin⁻c-Kit⁺Sca-1⁻FcγR^{hi}CD34⁺ cells (KSLs, CMPs, and GMPs, respectively)¹⁷ were sorted with a FACSAria cell sorter (BD Biosciences).

Transfection and retrovirus production for murine cells

Rat Hes1 cDNA, a gift from R. Kageyama (Kyoto University, Kyoto, Japan), was subcloned into a retrovirus vector, GCDNsam/internal ribosome entry site (IRES)–nerve growth factor receptor (NGFR), a gift from H. Nakauchi (University of Tokyo) and M. Onodera (National Center for Child Health and Development, Tokyo, Japan). BCR-ABL (p210) cDNA¹⁸ was subcloned into a retrovirus vector, GCDNsam/IRES-GFP.¹⁹ Mouse C/EBP-α cDNA, a gift from K. Akashi (Kyushu University, Fukuoka, Japan) and S. Mizuno (Dana-Farber Cancer Institute, Boston, MA), was subcloned into a retrovirus vector, pMYs-IRES-GFP.¹⁹ Plat-E²⁰ packaging cells maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum were transfected with retroviral constructs using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. The medium was changed a day after transfection, and retroviruses were harvested 48 hours after transfection, as previously described.^{19,20}

Transfection and retrovirus production for human cell lines

We generated a dominant-negative Hes1 (dnHes1) lacking a C-terminal WRPW (Trp-Arg-Pro-Trp) domain as described.²¹ The fragment of dnHes1 was subcloned into pMYs-IRES-GFP.¹⁹ Retrovirus packaging was done as described. Briefly, retroviruses were generated by transient transfection of Plat-A²⁰ packaging cells with FuGENE 6 (Roche Diagnostics).

Infection to progenitors

The retrovirus medium was placed in 24-well nontissue culture dishes for 4 hours at 37°C, precoated with 40 μg/mL of RetroNectin (Takara Bio) overnight at 4°C. After washing the wells with phosphate-buffered saline, sorted KSLs, CMPs, or GMPs were plated for infection for 48 to 60 hours with the coated retroviruses harboring GCDNsam/IRES-GFP-BCR-ABL (p210) or GCDNsam/IRES-NGFR-Hes1 or an empty vector as a control. Infection was done in StemSpan SFEM medium (StemCell Technologies) containing 100 ng/mL mouse stem cell factor (SCF), 100 ng/mL mouse thrombopoietin (TPO), and 100 ng/mL human FLT3 ligand (FL) for KSLs, or in Iscove modified Dulbecco medium (Sigma-Aldrich) containing 20% fetal calf serum, 50 ng/mL mouse SCF, 20 ng/mL mouse TPO, and 20 ng/mL mouse interleukin-3 (IL-3), 20 ng/mL human IL-6 (R&D Systems) for CMPs or GMPs.

Colony-forming assay

Retrovirus-infected cells were sorted at 48 to 60 hours from the initiation of infection with a FACSAria cell sorter (BD Biosciences) and used for colony-forming assay using Methocult 3231 (StemCell Technologies), supplemented with 50 ng/mL mouse SCF, 20 ng/mL mouse TPO, and 20 ng/mL mouse IL-3, 20 ng/mL human IL-6. A total of 1000 cells were cultured in each 2.5-cm dish in duplicate. The colony-forming cells were harvested and replated every 7 to 9 days and scored for colony formation. We defined a colony as “a group of cells, grown from a single parent cell, which is composed of more than 40 live cells.”

Mouse bone marrow transplantation

Bone marrow cells prepared from C57BL/6-Ly5.1 mice were infected with retrovirus containing Hes1 or BCR-ABL, and 0.1 to 2.6 × 10⁵ of Hes1/NGFR-sorted or BCR-ABL/GFP-sorted cells were injected through tail veins into C57BL/6-Ly5.2-recipient mice (8-12 weeks of age) after sublethal (5.25 Gy) or lethal (9.5 Gy) total body γ-irradiation (¹³⁵Cs). For the lethally irradiated mice, 2 × 10⁵ of C57BL/6-Ly5.2 mice-derived bone marrow cells were simultaneously injected for radioprotection. Probabilities of overall survival of the mice that received transplantations were estimated using the Kaplan-Meier method. Statistical differences were determined by the Wilcoxon test. All animal studies were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo.

Analysis of mice receiving transplantation

After transplantation, mice were monitored for signs of disease, such as cachexia, hyperpnea, or loss of gloss in fur. Autopsies were performed on moribund recipient mice. Peripheral blood count was analyzed by KX-21 Auto Analyzer (Sysmex). Morphology of the peripheral blood was evaluated by staining of air-dried smears with Hemacolor (Merck). Tissues including bone marrow, spleen, and liver were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Cytospin preparations of bone marrow and spleen cells were also stained with Hemacolor. Percentage of blasts, myelocytes, neutrophils, monocytes, lymphocytes, and erythroblasts was estimated by examination of at least 200 cells. To assess whether the leukemic cells were transplantable to secondary recipients, 0.1 to 5 × 10⁶ total bone marrow cells were injected into the tail veins of sublethally irradiated mice. Two recipient mice were used for each serial transplantation.

Flow cytometric analysis

Red blood cells were lysed using Red Blood Cell Lysing Buffer (Sigma-Aldrich) in peripheral blood or single-cell suspensions of bone marrow and spleen. After washing with phosphate-buffered saline, Fc receptor was blocked by incubating cells with 2.4G2 antibody (eBioscience) for 15 minutes at 4°C and then staining them with the following PE-conjugated monoclonal antibodies for 20 minutes at 4°C: Ly-5.1, Gr-1, CD11b, B220, CD19, CD3, CD4, CD8, c-Kit, Sca-1, CD34, and Ter119. Flow-cytometric analysis of the stained cells was performed with FACSCalibur

(BD Biosciences) equipped with CellQuest software (BD Biosciences) and FlowJo software (TreeStar).

Patients

CML patients were diagnosed at Hiroshima University Hospital and its affiliated hospitals. Diagnosis was based on morphologic, immunophenotypic, and, in some cases, real-time reverse transcription-polymerase chain reaction (RT-PCR) studies according to the French-American-British classification or World Health Organization classification. Patient samples were prepared after the research plan was approved by the Institutional Review Board at Hiroshima University, and written informed consent was obtained in accordance with the Declaration of Helsinki. Investigations were carried out in accordance with ethical standards authorized by the ethics committee of Hiroshima University and the ethics committee of the University of Tokyo (approval no. 20-10-0620).

Real-time RT-PCR

Total RNA was extracted from human bone marrow or peripheral blood cells using a TRIzol Kit (Invitrogen) according to the manufacturer's instructions, and converted to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Total RNA of mouse progenitors was extracted with RNeasy (QIAGEN) according to the manufacturer's instructions, and converted to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time RT-PCR was performed using a LightCycler Workflow System (Roche Diagnostics). cDNA was amplified using a SYBR Premix EX Taq (Takara). Reaction was subjected to 1 cycle of 95°C for 30 seconds, 45 cycles of PCR at 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 10 seconds. All samples were independently analyzed at least 3 times. The following primer pairs were used: 5'-CCAGTTTGCTTTCCTCATTCC-3' (forward) and 5'-TCTTCTCC-CCAGTATTCAAGTTC-3' (reverse) for human Hes1²²; 5'-GAGCTGAACGGGAAGCTCACTGG-3' (forward) and 5'-CAACTGTGAGGAGGGGAGATTCAG-3' (reverse) for human GAPDH²²; 5'-GAACAGCAACGAGTACCGGGTA-3' (forward) and 5'-CCCATGGCCTTGACCAAGGAG-3' (reverse) for mouse C/EBP- α ²³; 5'-CACAGGACTAGAACACCTGC-3' (forward) and 5'-GCTGGTGAAAAGGACCTCT-3' (reverse) for mouse hypoxanthine phosphoribosyltransferase (HPRT).²³ Relative gene expression levels were calculated using standard curves generated by serial dilutions of cDNA. Product quality was checked by melting curve analysis via LightCycler software (Roche Diagnostics). Expression levels were normalized by a control, the expression level of GAPDH mRNA for human samples, and HPRT mRNA for mouse samples.

Western blot analysis

To detect the expression of Hes1 or BCR-ABL (p210) proteins, equal numbers of cells from spleen or cell line were lysed, and Western blotting was performed as described with minor modifications.²⁴ Polyclonal rabbit anti-Hes1 antibody (H-140; Santa Cruz Biotechnology) and polyclonal rabbit anti-c-ABL antibody (K-12; Santa Cruz Biotechnology) were used for Hes1 or BCR-ABL detection, respectively.

Results

Retroviral transduction of Hes1 immortalizes CMPs and GMPs

NGFR-sorted Hes1-transduced KSLs, CMPs, and GMPs similarly generated compact and relatively large colonies, whereas empty vector-transduced KSLs generated a similar number of less large colonies. Empty vector-transduced CMPs and GMPs did not generate colonies (Figure 1A). Cytospin preparations of Hes1-transduced progenitors, stained with Hemacolor (Merck), showed blast-like morphologies, whereas those of empty vector-transduced KSLs contained bands, macrophages, and blasts (Figure 1B). Most

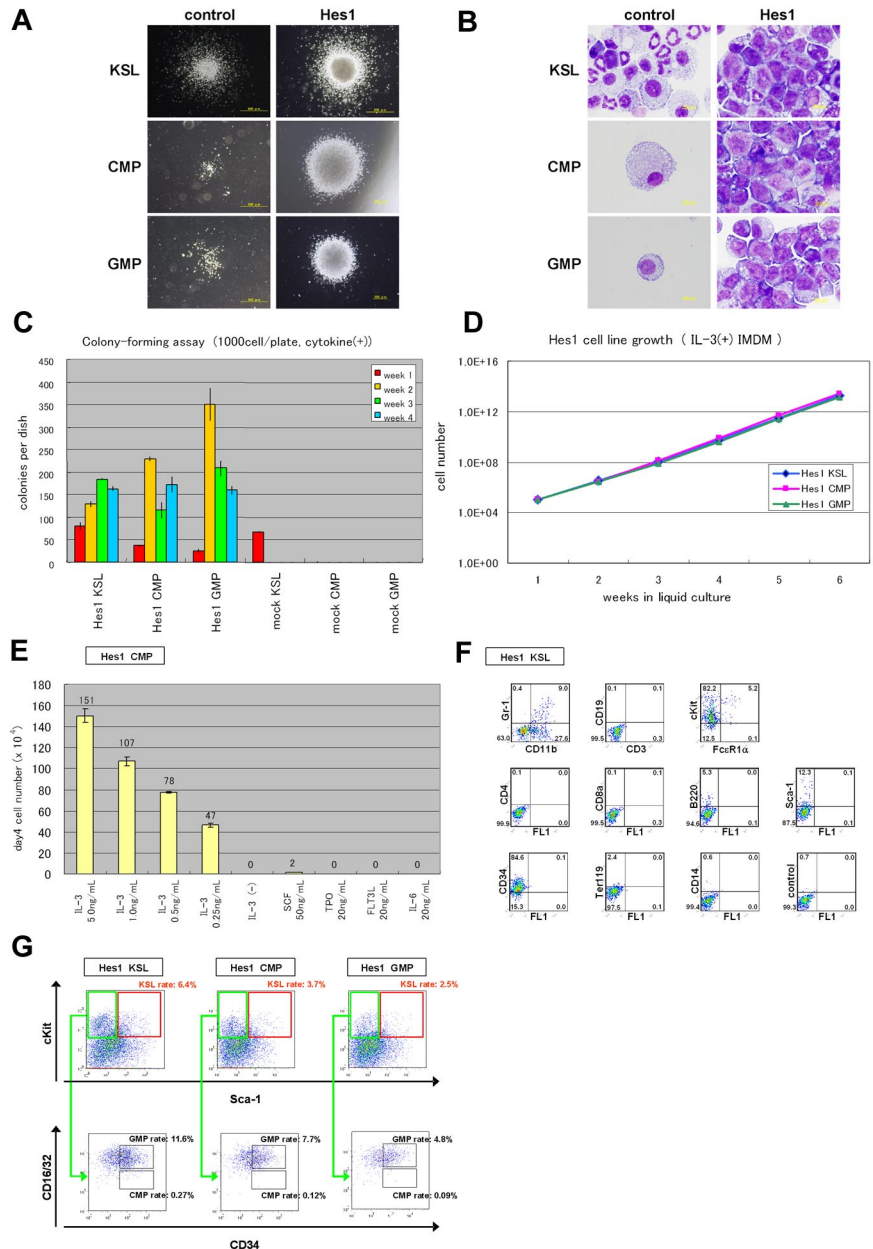
of the empty vector-transduced CMPs and GMPs died and few cells remained (Figure 1B). In serial colony-forming assays, both CMPs and GMPs transduced with Hes1 formed colonies after at least 4 rounds of replating, with the plating efficiency more than 15% at the fourth round (Figure 1C). Replating could be reproducibly maintained for more than half a year, implying immortalizing activity of Hes1 (Figure 1D). The Hes1-transduced KSLs, CMPs, and GMPs were dependent on the presence of IL-3, requiring concentrations more than 1 ng/mL (Figure 1E; supplemental Figure 1A-B, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). There was no significant difference between these cells in the dependency on IL-3. The majority of Hes1-transduced cells expressed c-Kit and CD34 at high levels, Sca-1 and CD11b at intermediate levels (Figure 1F, supplemental Figure 2A-B), irrespective of whether they were derived from KSLs, CMPs, or GMPs (supplemental Figure 2E).

The Lin⁻ cells were further analyzed by adopting 5-color flow cytometry that is used to identify bone marrow KSLs, CMPs, and GMPs. The expression levels of c-Kit, Sca-1, and CD34 were distributed over wide ranges. Approximately 2.5% to 6.4% of all nucleated cells showed a phenotype similar to KSLs, and another 4.8% to 11.6% showed a phenotype similar to GMPs. There were few cells that resembled CMPs (Figure 1G). We sorted the KSL-like cells, CMP-like cells, and GMP-like cells from each Hes1-transduced cell (Hes1-KSLs, Hes1-CMPs, and Hes1-GMPs) and cultured them for a week in methylcellulose. The same analysis by 5-color flow cytometry showed accumulation of GMP-like cells (~45.3%-83.5% of all nucleated cells) and moderate accumulation of KSL-like cells (~4.3%-23.4% of all nucleated cells) in the cultured cells (supplemental Figure 3A-C).

BCR-ABL replaces IL-3 in Hes1-immortalized cell lines

Because the Hes1-immortalized cell lines were IL-3 dependent for their growth in vitro, we examined whether additional signaling could replace IL-3. IL-3 signaling takes place mainly via Stat-, Ras-MAPK-, and PI3K-Akt-dependent pathways. It is also known that CML-specific BCR-ABL (p210) can replace IL-3 signaling in several experimental designs. Thus, we retrovirally expressed BCR-ABL together with Hes1. The combination of Hes1 and BCR-ABL enabled KSLs, CMPs, and GMPs to form colonies after repeated replating, not only in the presence of cytokines (Figure 2A left panel) but also in the condition free from cytokines (Figure 2A right panel). In contrast, KSLs, but not CMPs or GMPs, formed colonies by BCR-ABL transduction alone only when supplemented with cytokines (Figure 2A left panel), and they did not form any colonies without cytokines (Figure 2A right panel) or after replating with/without cytokines (Figure 2A both panels). In the liquid culture, it was shown that KSLs, CMPs, and GMPs transduced with both Hes1 and BCR-ABL were immortalized without cytokine supplementation (Figure 2B). The colonies made from Hes1- and BCR-ABL-transduced cells showed similar morphology with those from Hes1-transduced cells in the presence of a cytokine cocktail (Figure 2C). Importantly, the morphology of colony-forming cells derived from BCR-ABL-transduced KSLs was much more mature compared with those derived from Hes1- and BCR-ABL-transduced KSLs, CMPs, and GMPs, even in the same cytokine cocktail (Figure 2D). The majority of Hes1⁺BCR-ABL⁺ KSLs as well as Hes1⁺BCR-ABL⁺ CMPs and GMPs expressed CD34 at high levels, whereas they expressed c-Kit, Sca-1, and CD11b at intermediate levels (Figure 2E; supplemental Figure 2C-D), irrespective of whether they were derived from

Figure 1. Hes1-transduced KSLs, CMPs, or GMPs were immortalized in the presence of IL-3. (A) Typical colonies derived from Hes1- and empty vector-transduced KSLs, CMPs, and GMPs in the presence of SCF, TPO, IL-3, and IL-6. Images were obtained with an IX70 microscope and a DP70 camera (Olympus); an objective lens, UPlanFI (Olympus); original magnification $\times 40$ (bottom 2 in the right panels) and original magnification $\times 100$ (remaining 4 panels). (B) Giemsa staining of Hes1- and control vector-transduced KSLs, CMPs, and GMPs. Images were obtained with a BX51 microscope and a DP12 camera (Olympus); an objective lens, UPlanFI (Olympus); original magnification $\times 1000$. (C) Colony-forming assay from KSLs, CMPs, and GMPs transduced with Hes1 or empty vector. Hes1-transduced cells were replatable more than 4 times in vitro. Bars represent the number of colonies obtained per 10^3 cells after each round of plating in methylcellulose supplemented with SCF, TPO, IL-3, and IL-6. A representative result from 3 independent and reproducible experiments is shown. Error bars represent the SD from duplicate cultures. (D) Sustained growth of Hes1-transduced cells in liquid culture supplemented with 1 ng/mL IL-3. The number of cells was determined every 7 days by trypan blue staining, and 10^5 cells per well were seeded into a 6-well plate. Liquid culture was reproducibly continued for more than 6 months. (E) Cytokine requirement of Hes1-transduced CMPs. The cells were cultured in Iscove modified Dulbecco medium supplemented with indicated cytokines in duplicate. The numbers of cells were counted after 4 days of culture. A representative result from 2 independent and reproducible experiments is shown. Error bars represent the SD from duplicate cultures. Hes1-transduced KSLs and GMPs showed similar results (supplemental Figure 1A-B). (F) Flow-cytometric analysis of Hes1-transduced KSLs cultured in methylcellulose supplemented with SCF, TPO, IL-3, and IL-6. The dot plots represent Gr-1, CD19, c-Kit, CD4, CD8a, B220, Sca-1, CD34, Ter119, and CD14 labeled with a corresponding PE-conjugated monoclonal antibody versus CD11b, CD3, and Fc ϵ R1 α labeled with a corresponding fluorescein isothiocyanate-conjugated monoclonal antibody or FL1 with no monoclonal antibody. Hes1-transduced CMPs and GMPs showed similar expression patterns (supplemental Figure 2A-B). (G) Flow-cytometric analysis of Lin⁻-gated Hes1-transduced cells. Five-color analyses are used to identify KSL-like (top panels) and CMP-like and GMP-like cells (bottom panels) in the Hes1-transduced KSLs, CMPs, and GMPs. The number shows the percentage of cells in all nucleated cells. The analyzed cells were NGFR sorted at 48 to 60 hours from the initiation of Hes1- or control vector-transduction and cultured for the following lengths of time before the analysis: (A) 1 week, (B) 1 week, (C) 0 days, (D) 4 weeks, (E) 2 weeks, (F) 1 week, and (E) 2 weeks.



KSLs, CMPs, or GMPs (supplemental Figure 2F). Hes1⁺BCR-ABL⁺ KSLs, CMPs, and GMPs showed lower expressions of c-Kit and CD34 than KSLs, CMPs, and GMPs transduced with Hes1 alone (supplemental Figure 2E-F) when cultured in the presence of the same cytokine cocktail (SCF, TPO, IL-3, and IL-6). Expression of Hes1 or BCR-ABL in the Hes1 \pm BCR-ABL transduced CMP or GMP cell lines was confirmed by Western blot analysis (supplemental Figure 4A).

Hes1⁺BCR-ABL⁺ CMPs and GMPs rapidly induce AML/CML blast crisis-like disease in recipient mice

To examine the effect of Hes1 on leukemogenesis, Hes1-transduced KSLs, CMPs, and GMPs were injected through tail veins into C57BL/6-Ly5.2 recipient mice (8-12 weeks of age) via a sublethal (5.25 Gy) or a lethal (9.5 Gy) dose of total-body γ -irradiation (¹³⁵Cs). For the lethally irradiated mice, 2×10^5 bone marrow cells from C57BL/6-Ly5.2 mice were simultaneously injected for radioprotection. All the mice that received transplanta-

tions of Hes1-transduced KSLs, CMPs, and GMPs were kept healthy, and no recipients developed myeloproliferative neoplasms (MPNs) or leukemias for up to 250 days after the transplantation (Figure 3A). Regarding the nonleukemogenic nature of the stem/progenitor cells transduced with Hes1 alone, we⁷ and others²⁵ previously reported similar results, although the cell populations and/or experimental designs were not identical.

In agreement with the previous reports,²⁶ recipient mice injected with BCR-ABL-transduced KSLs developed fatal MPN within 30 days after the transplantation, whereas those injected with BCR-ABL-transduced CMPs and GMPs were kept healthy for more than 130 days. We did not find any signs of MPN or leukemias when mice were killed between 130 and 200 days after the transplantation (Figure 3B).

Because we found that the combination of Hes1 and BCR-ABL transduction conferred cytokine-independent immortalization on CMPs and GMPs, we injected Hes1⁺BCR-ABL⁺ KSLs, CMPs, and GMPs through tail veins into C57BL/6-Ly5.2 recipient mice

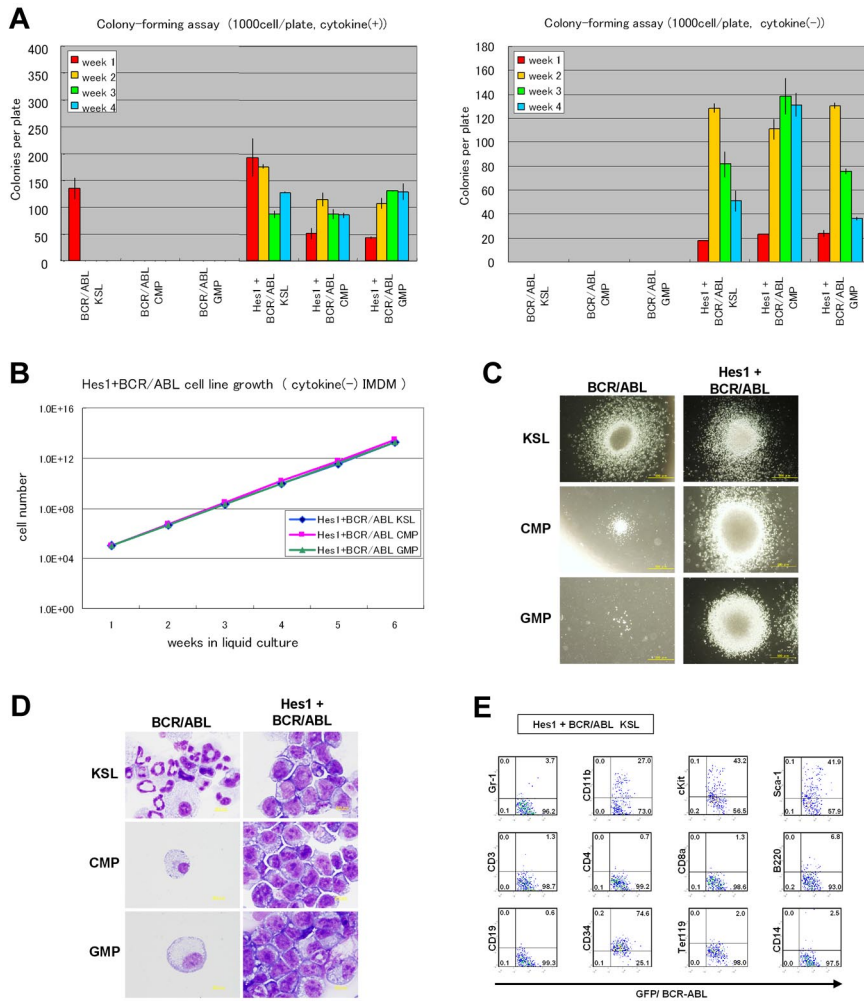


Figure 2. Hes1- and BCR-ABL-transduced KSLs, CMPs, or GMPs were immortalized independently of IL-3.

(A) Colony-forming assay of KSLs, CMPs, and GMPs transduced with BCR-ABL alone or Hes1 and BCR-ABL, cultured in methylcellulose with or without cytokine cocktail containing SCF, TPO, IL-3, and IL-6. Hes1⁺BCR-ABL⁺ cells could be serially replated more than 4 times both with or without cytokines. In contrast, whereas KSLs, but not CMPs or GMPs, transduced with BCR-ABL alone, formed colonies in the presence of cytokines, neither KSLs, nor CMPs, nor GMPs formed colonies without cytokine supplementation. Bars represent the number of colonies obtained per 10³ cells after each round of plating in methylcellulose. A representative result from 3 independent and reproducible experiments is shown. Error bars represent the SD from duplicate cultures. (B) Sustained growth of Hes1⁺BCR-ABL⁺ cells in liquid culture without cytokine supplementation. The numbers of cells were determined every 7 days by trypan blue staining, and 10⁵ cells per well were seeded into a 6-well plate. Liquid culture was reproducibly continued for more than 6 months. (C) Typical colonies derived from KSLs, CMPs, and GMPs transduced with BCR-ABL alone (left panels) or BCR-ABL and Hes1 (right panels) in the presence of SCF, TPO, IL-3, and IL-6. Images were obtained with an IX70 microscope and a DP70 camera (Olympus); an objective lens, UPlanFI (Olympus); original magnification ×100. (D) Giemsa staining of Hes1⁺BCR-ABL⁺ KSLs, CMPs, and GMPs. Images were obtained with a BX51 microscope and a DP12 camera (Olympus); an objective lens, UPlanFI (Olympus); original magnification ×1000. (E) Flow-cytometric analysis of Hes1⁺BCR-ABL⁺ KSLs cultured in methylcellulose supplemented with SCF, TPO, IL-3, and IL-6. The dot plots represent Gr-1, CD11b, c-Kit, Sca-1, CD3, CD4, CD8a, B220, CD19, CD34, Ter119, and CD14 labeled with a corresponding PE-conjugated monoclonal antibody versus expression of GFP/BCR-ABL. Hes1⁺BCR-ABL⁺ CMPs and GMPs showed a similar expression pattern (supplemental Figure 2C-D). The analyzed cells were GFP and NGFR sorted at 48 to 60 hours from the initiation of BCR-ABL- or Hes1+BCR-ABL transduction and cultured for the following lengths of time before the analysis: (A) 0 days, (B) 4 weeks, (C) 1 week, (D) 1 week, and (E) 1 week.

after sublethal irradiation. The numbers of cells injected varied among experiments, ranging from 17×10^2 to 15×10^4 , because of the difference in sorting efficiencies. All the mice receiving transplantations rapidly developed fatal AML/CML in blast crisis-like disease with no significant difference in latency, ranging between 18 and 39 days after the transplantation ($P < .867$) (Figure 4A). The tissue distribution of the disease was virtually the same among mice receiving KSLs, CMPs, and GMPs; they invariably demonstrated marked hepatosplenomegaly and lung hemorrhage resulting from infiltration of leukemic cells (Figure 4B). Expression of Hes1 and BCR-ABL in the spleen cells of recipient mice was confirmed by Western blot analysis (supplemental Figure 4B).

The morphology of bone marrow demonstrated increased myeloid blasts (Figure 4C), and the histology of spleen, liver, and lungs demonstrated extensive infiltration of leukemic cells (Figure 4D). The percentages of the blasts ranged between 28% and 55% of all nucleated bone marrow cells (mean, 36.5%) of the mice receiving Hes1- and BCR-ABL-transduced KSLs, CMPs, and GMPs. In contrast, the percentages of bone marrow blasts in the recipient mice receiving BCR-ABL-transduced KSLs were only 6% to 7% (Figure 5A). White blood cell counts in the peripheral blood of recipients with Hes1⁺BCR-ABL⁺ KSLs, CMPs, and GMPs were $2.4 \times 10^4/\mu\text{L}$ to $67.9 \times 10^4/\mu\text{L}$ (mean, $17.8 \times 10^4/\mu\text{L}$), whereas those with BCR-ABL-transduced KSLs showed moderate leukocytosis ranging between $2.9 \times 10^4/\mu\text{L}$ and $3.8 \times 10^4/\mu\text{L}$ (Figure 5B). The surface marker profiles of the bone marrow cells

from the recipients with Hes1⁺BCR-ABL⁺ cells expressed CD11b and Gr-1 at high levels, whereas they expressed c-Kit, Sca-1, and CD34 at intermediate levels (Figure 5C; supplemental Figure 5A-B), irrespective of whether they were derived from KSLs, CMPs, or GMPs (supplemental Figure 5C).

The long-term self-renewal properties of the leukemic cells derived from Hes1- and BCR-ABL-transduced CMPs or GMPs were tested by transplantation into secondary recipients; 0.1 to 5×10^6 total bone marrow cells were injected into the tail veins of sublethally irradiated mice. All recipient mice transplanted with more than 10^5 Hes1⁺ cells from bone marrow developed fatal AML/CML in blast crisis-like disease with latencies of between 18 and 75 days (supplemental Figure 4C). The disease was almost identical with the primary disease (data not shown).

Hes1 expression is elevated in a substantial subset of human CML blast crisis samples

The results presented from the mouse model experiments suggest a potential link between deregulated expression of Hes1 and human CML in blast crisis. We measured the Hes1 mRNA by real-time RT-PCR in 11 peripheral blood, 1 cerebrospinal fluid, and 8 bone marrow samples from CML in blast crisis patients; 19 bone marrow samples from CML in chronic phase patients; and 10 bone marrow samples from normal subjects. In 8 of 20 CML in blast crisis samples, we found that Hes1 mRNA levels were elevated by more than 4 times the average of normal bone marrow samples (Figure

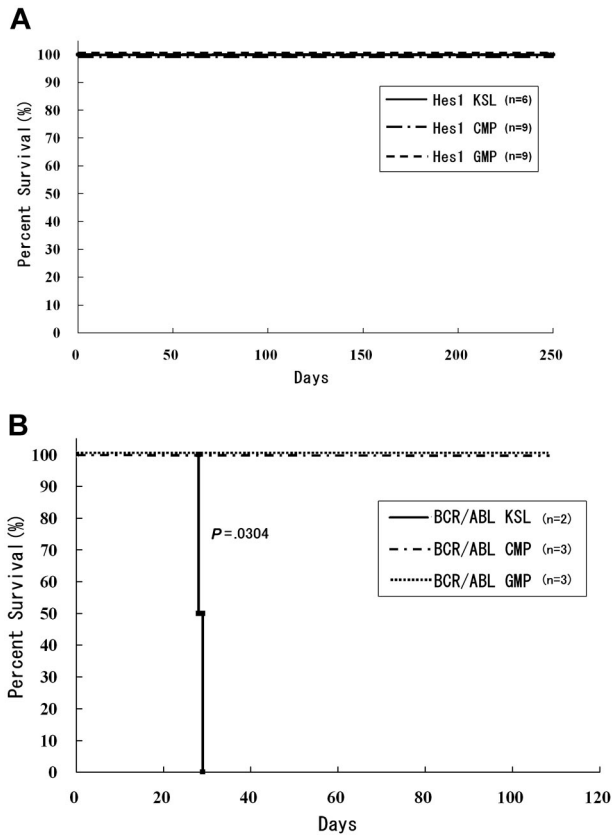


Figure 3. Mice transplanted with Hes1-transduced KSLs, CMPs, and GMPs were kept healthy. (A) Survival curves for mice injected with Hes1-transduced progenitors. No mice showed any signs of MPN for more than 250 days from transplantation. Data were analyzed by the Kaplan-Meier method. The numbers of transplanted mice are shown. Three independent experiments were performed. (B) Survival curves for mice injected with BCR-ABL-transduced progenitors. Mice transplanted with BCR-ABL-transduced KSLs developed fatal MPN within 30 days after transplantation, whereas mice transplanted with BCR-ABL-transduced CMPs or GMPs showed no evidence of disease when killed between 130 and 200 days after transplantation. Data were analyzed using the log-rank test. The 2 independent experiments were performed, and the total numbers of transplanted mice are shown.

6A). Interestingly, all but one of their phenotypes were myeloid, and 5 of 12 samples in which Hes1 mRNA levels were not elevated were derived from patients with B-cell lineage lymphoid crisis. On the other hand, the average of Hes1 mRNA levels in CML in chronic phase samples seemed to be lower than that of the normal bone marrow samples, with no sample exceeding twice the average. Clinical data of 20 patients with CML in blast crisis are shown in Table 1. The correlation coefficient between the blast percentage and the Hes1 mRNA level was -0.395 , indicating that the elevated Hes1 expression level was independent of the increase in the blast percentage.

To investigate the role of Hes1 in CML blast crisis, we measured the Hes1 mRNA by real-time RT-PCR in 5 human cell lines (K-562,²⁷ JK-1,²⁸ KCL-22,²⁹ TS9:22,³⁰ and JURL-MK1³¹), which were derived from CML in blast crisis. We found that, in 3 of 5 CML blast crisis cell lines, Hes1 mRNA levels were elevated compared with the normal bone marrow sample (Figure 6B). We transduced a dominant-negative Hes1 (dnHes1) lacking a C-terminal WRPW domain via retrovirus vector into the 3 cell lines (K-562, TS9:22, and JURL-MK1) in which Hes1 mRNA levels were elevated. Indeed, in 2 of these 3 cell lines, proliferation was significantly suppressed by transduction of dnHes1 (Figure 6C). The repression of C/EBP- α by Hes1 was also observed in Hes1-transduced KSLs, CMPs, and GMPs compared with control

vector-transduced KSLs, CMPs, and GMPs (Figure 6D). When C/EBP- α retrovirus vector was transduced to Hes1-transduced KSLs, CMPs, and GMPs, all of these cells differentiated to segmented neutrophils, suggesting that the expression of C/EBP- α reversed the function of Hes1 (supplemental Figure 6).

Discussion

In the present study, we demonstrated that retroviral transduction of Hes1 readily immortalizes myeloid progenitors at various stages.

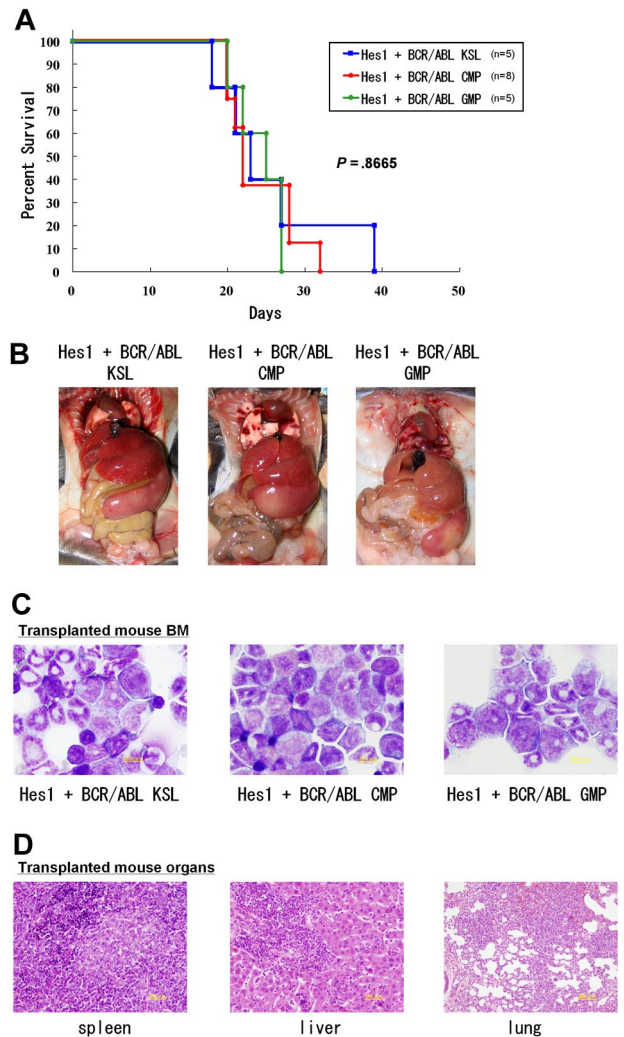


Figure 4. CMPs and GMPs transduced with the combination of Hes1 and BCR-ABL rapidly induced AML/blast crisis of CML. (A) Survival curves of mice. KSLs (n = 5), CMPs (n = 8), and GMPs (n = 5) transduced with the combination of Hes1 and BCR-ABL developed fatal AML/CML in blast crisis-like disease within 18 to 39 days, 20 to 32 days, and 20 to 27 days, respectively. Numbers of injected cells ranged 17×10^2 to 2.6×10^4 for KSLs, 5.5×10^4 to 15×10^4 for CMPs, and 4.0×10^4 to 13.8×10^4 for GMPs. There was no significant difference in latency of penetrance ($P < .867$). Statistical differences were determined using the log-rank test. Three independent experiments were performed, and the total numbers of transplanted mice are shown. (B) Tissue distribution of the leukemic cells. Mice transplanted with KSLs, CMPs, and GMPs transduced with the combination of Hes1 and BCR-ABL invariably demonstrated marked hepatosplenomegaly and lung hemorrhage, both resulting from infiltration of leukemic cells. (C) The morphology of bone marrow cells from representative recipient mice. Increased myeloid blasts were seen with no significant difference among KSLs, CMPs, and GMPs. (D) Histology of spleen, liver, and lungs from representative mice receiving Hes1⁺BCR-ABL⁺ GMPs. Vast infiltration of leukemic cells is seen. There were no differences in the histology among mice receiving Hes1⁺BCR-ABL⁺ KSLs, CMPs, and GMPs.

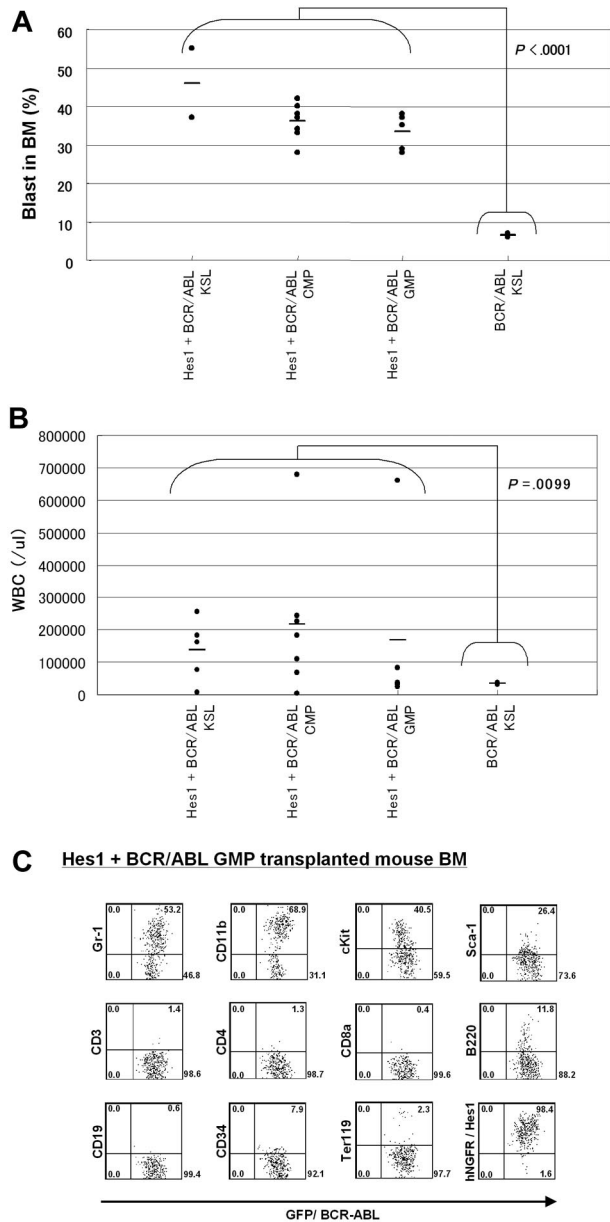


Figure 5. Comparisons of blast percentages in the bone marrow and peripheral blood leukocyte counts between mice receiving KSLs transduced with BCR-ABL alone and those receiving KSLs, CMPs, and GMPs transduced with the combination of Hes1 and BCR-ABL. (A) Blast ratios in the bone marrow. The mean blast ratios in all nucleated bone marrow cells were $6.5\% \pm 0.7\%$ and $36.5\% \pm 6.9\%$ in mice receiving KSLs transduced with BCR-ABL alone and in those receiving KSLs, CMPs, and GMPs transduced with the combination of Hes1 and BCR-ABL, respectively. The difference was statistically significant by the 2-sample *t* test with Welch correction ($P < .001$). (B) Peripheral white blood cell counts (WBCs). WBCs were $3.4 \pm 0.6 \times 10^4/\mu\text{L}$ and $17.8 \pm 20.3 \times 10^4/\mu\text{L}$ in mice receiving KSLs transduced with BCR-ABL alone and in those receiving KSLs, CMPs, and GMPs transduced with the combination of Hes1 and BCR-ABL, respectively. The difference was statistically significant by the 2-sample *t* test with Welch correction ($P < .001$). (C) Flow-cytometric analysis of bone marrow cells from mice receiving GMPs transduced with the combination of Hes1 and BCR-ABL. The dot plots represent GFP/BCR-ABL, NGFR is a marker of Hes1, and GFP is a marker of BCR-ABL transduction. The bone marrow cells derived from mice receiving KSLs or CMPs transduced with the combination of Hes1 and BCR-ABL showed essentially the same pattern (supplemental Figure 5A-B).

Moreover, when BCR-ABL is transduced together, Hes1 transforms differentiated myeloid progenitors, such as CMPs and GMPs, in addition to hematopoietic stem cell-rich population, such as KSLs,

to AML/CML in blast crisis-like cells, rapidly killing recipient mice. This result is in sharp contrast to the fact that a hematopoietic stem cell-containing population is required for BCR-ABL to cause MPN-like disease.

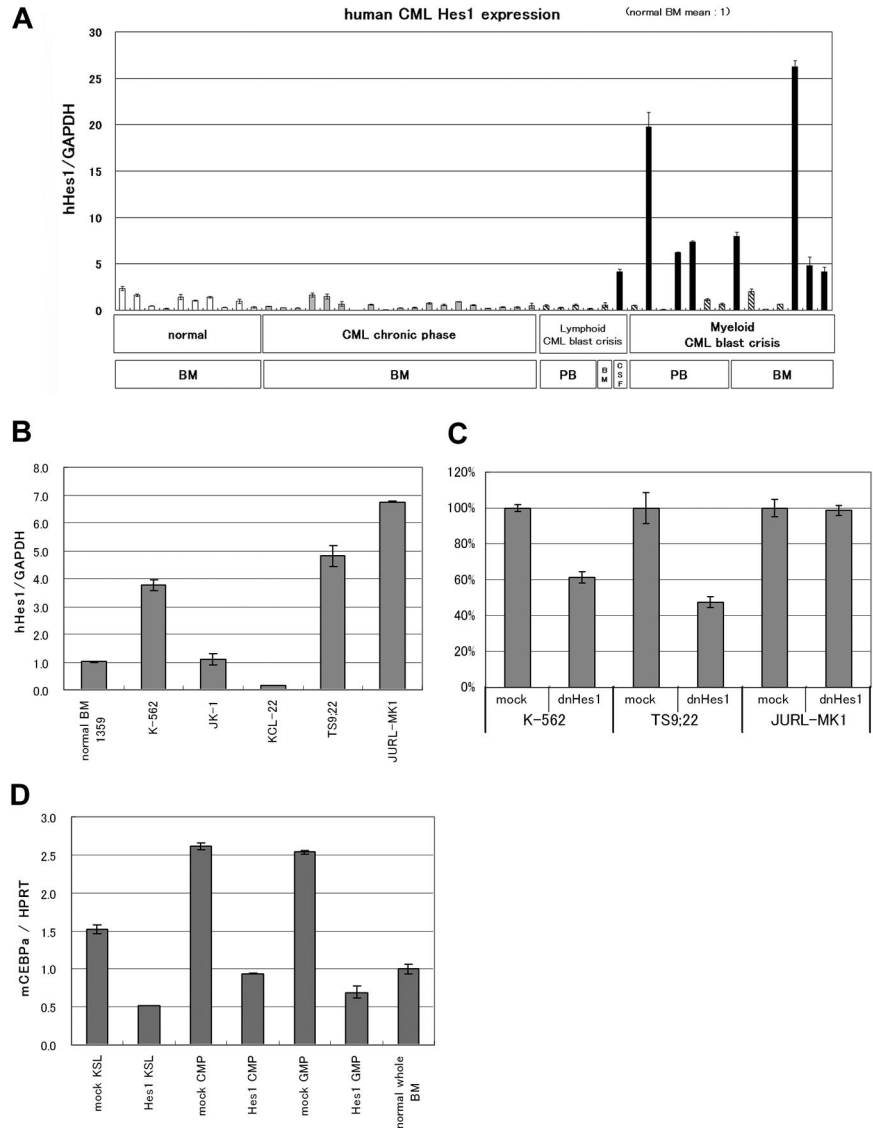
Hes1 is known as an effector molecule functioning downstream of Notch signaling. The activating mutations of the extracellular heterodimerization domain and/or the C-terminal PEST domain of Notch1 have been identified in approximately 50% of human T-cell acute lymphoblastic leukemias.^{10,32} We have recently identified gain-of-function mutations of *Notch2* in conjunction with increased copy numbers of the mutation-carrying *Notch2* allele in a subset of B-cell lymphomas.¹¹ A possible association between deregulated Notch signaling is also reported in Hodgkin lymphoma, anaplastic large cell lymphoma, small-cell lung cancer, and prostate adenocarcinoma, etc.³³ Regarding myeloid malignancies, however, only one paper reports the identification of the activating mutation of Notch1 in 1 of 12 human AML samples.³⁴ Given that Notch signaling is among the strongest inducers of T-cell lineage commitment^{12,13} and that increased Notch signaling could block myeloid lineage commitment,¹⁵ deregulated Notch signaling might antagonize, rather than promote, the development of myeloid malignancies. However, Hes1 does not necessarily represent Notch signaling. Indeed, other extracellular signaling, such as Sonic Hedgehog,³⁵ could affect Hes1 expression, and cross-talk between Hes family proteins and molecules in various cell signaling pathways, such as Stat3,³⁶ has been demonstrated.

We previously reported that Hes1 preserved highly purified hematopoietic stem cells in vitro and contributed to the expansion of transduced hematopoietic stem cells in the recipients' bone marrow,⁷ but the effect of Hes1 transduction on myeloid progenitors was not evaluated in detail. We have now found the myeloid progenitor-immortalizing activity of Hes1. In addition, accumulation of KSL- and GMP-like population in Hes1-transduced cells implicates a role for Hes1 in leukemic stem cells. On the other hand, we have also found that the in vitro growth of the Hes1-immortalized cells is dependent on cytokine signaling and that Hes1 alone is insufficient to be fully leukemogenic when overexpressed. The mainstay of the Hes1 effects on myeloid progenitors appears to be blockade of differentiation, although other functions, such as reversion from the quiescent state to the actively cycling state,²¹ may also be involved. In the present study, we confirmed that Hes1 expression represses C/EBP- α , a transcription factor having important roles in myeloid differentiation, in mouse KSLs and committed progenitors as we reported.¹⁵ Moreover, transduction of C/EBP- α reversed the differentiation block caused by Hes1 expression, which partially explains the mechanism of blocked myeloid differentiation by Hes1. C/EBP- α is frequently mutated in AML with the normal karyotype.³⁷⁻³⁹ In other human AML without C/EBP- α mutations, reduced C/EBP- α expression, possibly through deregulated epigenetic control, is not uncommon and is associated with poor prognosis.^{40,41} Furthermore, mice injected with mutated C/EBP- α -transduced bone marrow cells develop myelodysplastic syndrome and AML.⁴² Therefore, reduction of C/EBP- α function is highly relevant to the development and/or progression of myeloid malignancies. Hes1, therefore, might be involved in human myeloid malignancies through suppression of C/EBP- α .

Up-regulation of Hes1 is shown in a subset of human rhabdomyosarcomas²¹ and medulloblastomas.^{43,44} In the present study, we have detected elevated expression of Hes1 in 8 of the 20 samples from CML in blast crisis patients, but not those from CML

Figure 6. Hes1 expression was elevated in approximately 40% of patients with CML in blast crisis.

(A) Real-time RT-PCR for Hes1 in bone marrow or peripheral blood cells from healthy subjects, patients with CML in chronic phase, or patients with CML in blast crisis. Expression levels were normalized by GAPDH mRNA. RNA from normal bone marrow cells served as a control (mean of 10 RNA levels of normal bone marrow was defined as 1). Hes1 mRNA levels exceeded 4 (solid bar) in 8 of 20 samples from CML in blast crisis patients. The correlation coefficient determined by the Wilcoxon signed-rank test between blast ratio and Hes1-expression level was -0.395 . PB indicates peripheral blood; BM, bone marrow; CSF, cerebrospinal fluid. The solid bar represents CML in blast crisis exceeding 4; the hatched bar represents CML in blast crisis less than 4. (B) Hes1 expression in 5 human CML blast crisis cell lines. Expression levels of HES1 in K-562, JK-1, KCL-22, TS9:22, and JURL-MK1 were evaluated by real-time RT-PCR and were normalized by GAPDH mRNA. (C) Growth repression by transduction of dnHes1 (a dominant-negative Hes1) retrovirus vector into 3 human cell lines (K-562, TS9:22, and JURL-MK1). Six days after retrovirus transduction, cell numbers were counted. Growth is shown as a percentage of the control cells that were transduced with control vector. A representative result from 2 independent and reproducible experiments is shown. Error bars represent the SD from duplicate cultures. (D) Real-time RT-PCR for C/EBP- α in Hes1-transduced KSLs, CMPs, and GMPs compared with control vector-transduced KSLs, CMPs and GMPs. Total RNA was extracted at 60 hours from the initiation of Hes1-transduction. Error bars represent the SD from 2 independent experiments in (A-B,D).



in chronic phase patients. Although it is yet to be confirmed by a larger number of samples from CML as well as AML patients, this result indicates an interesting connection between the mouse model of AML/CML in blast crisis-like disease and human leukemia. In addition, we have demonstrated that transduction of dnHes1 represses the proliferation in 2 of 3 human cell lines of CML in blast crisis. These results suggest that Hes1 plays an important role in blast crisis of CML.

Although the origin of CML is considered to be a hematopoietic stem cell, blast crisis has been shown to be a result of transformation of myeloid progenitors.¹⁶ BCR-ABL can cause MPN-like disease when introduced into the hematopoietic stem cell population but cannot induce MPN or leukemia when introduced into differentiated myeloid progenitors.²⁶ Therefore, development of full-blown AML/CML in blast crisis-like disease in mice with differentiated progenitors only by cotransduction with Hes1 and BCR-ABL may represent a true model of blast crisis of CML. In this context, Hes1 is a possible crisis-promoting gene like other examples, such as activated β -catenin¹⁶ and BCL-2,⁴⁵ both of which caused CML in blast crisis-like disease in mice when transduced into GMPs together with BCR-ABL.

Several AML-associated fusion gene products, such as MLL-ENL,⁴⁶ MOZ-TIF2,²⁶ and MLL-AF9,⁴⁷ have been demonstrated to confer replating capacity on CMPs and GMPs, and eventually to transform these cells into leukemia-initiating cells. Unique to our findings is the fact that we transduced a wild-type transcription factor, Hes1, and found that such simple up-regulation of a transcription factor led to similar transformation phenotypes. A substantial number of examples have indicated that loss of function or altered function, rather than gain of function, of transcription factors, including MLL, MOZ, Runx1, RAR α , C/EBP- α , etc, is associated with leukemogenesis. If up-regulation of Hes1 is indeed involved in human leukemias, this represents a new mechanism of leukemogenesis.

In modeling CML in mice, the present model provides a powerful tool by which we can induce 2 distinct phases of CML from stem cells or progenitors using BCR-ABL gene: a chronic phase-like state by transduction of KSL with BCR-ABL alone and a blast crisis-like state by cotransduction of CMPs and GMPs with BCR-ABL and Hes1.

In conclusion, we have developed a useful mouse model for CML blast crisis and have indicated that Hes1 is a key molecule in blast crisis transition in CML. The present mouse model will aid

Table 1. Clinical data of 20 patients with CML in blast crisis

Sample name	Source	Blast ratio	Hes1/GAPDH	Phenotype	Chromosome aberration	Clinical features
228 CML BC	PB	57	0.49	B-ALL	46,XY,t(9;22)/46,XY,+der(1;14)(q10;q12),t(9;22)	—
428 CML BC	PB	100	0.27	B-ALL	t(9;22)	—
984 CML BC	PB	60	0.56	B-ALL	46,XX,t(9;22)(q34;q11.2)	—
3259 CML BC	PB	100	0.16	B-ALL	t(9;22)	—
1385 CML BC	BM	81	0.56	B-ALL	t(9;22)	—
1107 CML BC	CSF	100	4.16	B-ALL	t(9;22)	The blasts increased drastically in CNS.
1 CML BC	PB	90	0.51	Myeloid	t(9;22)	—
219 CML BC	PB	12	19.72	Myeloid	45,XX,-7,t(9;22)	BM was dry tap composed of 100% blasts.
393 CML BC	PB	20	0.08	Myeloid	46,XX,t(9;22),add(17)(p11)	—
1088 CML BC	PB	30	6.22	Myeloid	46,XX,t(9;22)(q34;q11.2)	BM was dry tap.
1299 CML BC	PB	20	7.35	Myeloid	t(9;22)	BM was dry tap composed of 23% blasts.
1824 CML BC	PB	51	1.15	Myeloid	t(9;22)	BM was dry tap.
3153 CML BC	PB	7	0.66	Myeloid	t(9;22)	BM was dry tap. The blasts in BM increased up to 42% after taking this sample.
232 CML BC	BM	11	7.96	Myeloid	47,XY,+8,t(9;22)	The blasts in BM increased drastically up to 44% after taking this sample.
452 CML BC	BM	54	2.01	Myeloid	46,dic(17)(q10),t(9;22)	—
916 CML BC	BM	24	0.11	Myeloid	t(9;22)	—
1091 CML BC	BM	28	0.67	Myeloid	t(9;22)	—
811 CML BC	BM	25	26.25	Myeloid	46,XX,t(1;9;22)(q44;q34;q11.2)	—
3332 CML BC	BM	22	4.17	Myeloid	t(9;22)	—
3847 CML BC	BM	62	4.79	Myeloid	t(9;22)	—

CML indicates chronic myelogenous leukemia; BC, blast crisis; PB, peripheral blood; B-ALL, B-cell acute lymphoblastic leukemia; BM, bone marrow; CSF, cerebrospinal fluid; —, not applicable; and CNS, central nervous system.

understanding of the molecular mechanisms underlying blast crisis of CML and might lead to a better therapeutic outcome for this difficult disease.

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Authorship

Contribution: F.N. did all the experiments and participated actively in writing the manuscript; M.S.-Y. and J.K. assisted with the experiments and actively participated in designing the experiments; Y.K., N.K., T.U., K.H., and K.K. assisted with the experiments; Y.H. and H.H. provided human samples; S.O. and M.K. participated in interpretation and designing the experiments; and T.K. and S.C. conceived the project, secured funding, and actively participated in manuscript writing.

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