

Human CD34⁺ stem cells express the *hiwi* gene, a human homologue of the *Drosophila* gene *piwi*

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Hematopoietic stem cells (HSCs) are characterized by their dual abilities to undergo differentiation into multiple hematopoietic cell lineages or to undergo self-renewal. The molecular basis of these properties remains poorly understood. Recently the *piwi* gene was found in the embryonic germline stem cells (GSCs) of *Drosophila melanogaster* and has been shown to be important in GSC self-renewal. This study demonstrated that *hiwi*, a novel human homologue of *piwi*, is also present in human CD34⁺ hematopoietic progenitor cells but not in more differ-

entiated cell populations. Placing CD34⁺ cells into culture conditions that supported differentiation and rapid exit from the stem cell compartment resulted in a loss of *hiwi* expression by day 5 of a 14-day culture period. Expression of the *hiwi* gene was detected in many developing fetal and adult tissues. By means of 5' RACE cloning methodology, a novel putative full-length *hiwi* complementary DNA was cloned from human CD34⁺ marrow cells. At the amino acid level, the human HIWI protein was 52% homologous to the *Drosophila* protein. The transient ex-

pression of *hiwi* in the human leukemia cell line KG1 resulted in a dramatic reduction in cellular proliferation. Overexpression of *hiwi* led to programmed cell death of KG1 cells as demonstrated by the Annexin V assay system. These studies suggest that *hiwi* maybe an important negative developmental regulator, which, in part, underlies the unique biologic properties associated with hematopoietic stem and progenitor cells. (Blood. 2001;97:426-434)

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Introduction

Hematopoietic stem cells (HSCs) have the unique ability to undergo self-renewal and to differentiate into cells belonging to multiple hematopoietic lineages.^{1,2} These properties allow stem cells to maintain hematopoiesis throughout the life span of an organism. The knowledge of the behavior of HSCs is limited due to their rarity, difficulty of efficient isolation, and sensitivity to manipulation.^{1,2} The self-renewal capacity of several classes of stem cells is thought to be controlled by external signals and intrinsic cellular processes.^{1,4} Over the last 2 decades, a variety of external stimuli (cytokines, matrix proteins) that alter HSC self-renewal have been the subject of intense investigation. Although a number of such external signals that interact with specific receptors on HSC have been identified, the signaling mechanisms that govern HSC self-renewal have eluded investigation. Intrinsic cellular mechanisms that regulate stem cell self-renewal have been explored in a variety of model systems including germline stem cells (GSCs) in several lower species. *Drosophila* has been a particularly useful model for studying biologic processes that are conserved in higher developmental systems.⁵⁻¹² Therefore, in an attempt to define candidate genes that are responsible for human HSC self-renewal, we have explored the expression of genes in humans that have recently been demonstrated to play a role in *Drosophila* GSC self-renewal.

The GSCs provide a continuous source of totipotent cells for the production of gametes needed for fertilization.⁸ They are similar to HSCs in their ability to not only self-renew but also to remain capable of generating large numbers of differentiated daughter

cells.^{8,9} The intracellular mechanisms that serve as the determinants of asymmetric-segregating cell fates of GSCs depend not only on the basic cell cycle machinery but also on a family of recently identified genes, some of which are evolutionarily conserved.^{7,9}

A group of somatic cells in *Drosophila*, termed terminal filament cells, which lie distal and immediately adjacent to the GSCs, have been shown to regulate GSC division.^{8,10,11} Laser ablation of the terminal filament increases the rate of oogenesis by 40%.¹² Loss of function mutations in a gene found in the terminal filament, termed *piwi*, leads to a failure of stem cell maintenance^{7,10}; *piwi* is expressed not only in the terminal filament but also in the germline. Loss of *piwi* function in the germline, however, is not known to affect GSC division. The protein encoded by *piwi* is extraordinarily well conserved along the evolutionary tree, being found in both *Caenorhabditis elegans* and primates.⁷ Our laboratory has attempted to determine if such genes were present in primitive hematopoietic cells and if they might play a role in HSC development. We report here the presence of a human homologue of the *piwi* gene, termed *hiwi*, in a variety of primitive hematopoietic cells. The *hiwi* gene represents a candidate gene that may play a role in determining or regulating HSC development.

Materials and methods

Isolation of human and baboon CD34⁺ cells

Adult human bone marrow (BM) samples (15-30 mL) were aspirated from the posterior iliac crests of healthy donors after informed consent was

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obtained according to guidelines established and approved by the University of Illinois at Chicago Institutional Review Board. Heparinized marrow aspirates were diluted with Dulbecco phosphate-buffered saline (DPBS) Ca⁺⁺- and Mg⁺⁺-free (Biowhittaker, Walkersville, MD). Diluted marrow was then underlaid with Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), and centrifuged at 800g for 30 minutes at 20°C. The mononuclear cell fraction was collected and the CD34⁺ cells were immunomagnetically enriched using the MACS CD34 Isolation Kit (Miltenyi Biotec, Auburn, CA). Briefly, cells were incubated with hapten-labeled anti-CD34 antibody (QBEND-10, Becton Dickinson) in the presence of blocking reagent, human IgG (Bayer, Elkhart, IN), and then with antihapten coupled to MACS microbeads. Labeled cells were filtered through a 30 μm nylon mesh and separated using a high-gradient magnetic separation column. Magnetically retained cells were eluted and stained with monoclonal antibodies (MoAb) and analyzed using flow cytometric methods. The flow-through population was identified as CD34⁻ cells. The purity of the CD34⁺ population was routinely more than 90% (data not shown).

The BM aspirates were obtained from the humeri and iliac crests of juvenile baboons (*Papio anubis*) after ketamine (10 mg/kg) and xylazine (1 mg/kg) anesthesia according to the guidelines established and approved by the University of Illinois at Chicago Animal Care Committee. Heparinized marrow was diluted 1:15 in phosphate-buffered saline (PBS) and the mononuclear cell fraction obtained by centrifugation over 60% Percoll (Pharmacia AB) at 500g for 30 minutes at 20°C. The antihuman CD34 MoAb K6.1 (a gift from the Naval Medical Research Institute, Bethesda, MD), a murine IgG_{2a}, which cross-reacts with baboon CD34 antigen, was used for the selection of the CD34⁺ fraction of marrow cells.¹³ The mononuclear cells were suspended in PBS containing 0.2% bovine serum albumin (BSA; Sigma Chemical, St Louis, MO) and stained first with biotin-conjugated K6.1 (20 μg/mL), washed, and labeled with Miltenyi streptavidin-conjugated iron microbeads (Miltenyi Biotec) and selected as described above according to manufacturer's instructions.

Flow cytometric analysis and sorting

Isolated human CD34⁺ cells were further fractionated based on the expression of CD38 antigen.¹⁴ Nonspecific staining was blocked using 0.1% heat-inactivated human gamma globulin (Bayer). Cells were stained with anti-CD34 MoAb conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson) and anti-CD38-phycoerythrin (PE) (Becton Dickinson). Control cells were incubated with fluorochrome-conjugated isotype-matched IgG1-FITC (Becton Dickinson) and IgG1-PE (Becton Dickinson). Immediately prior to sorting 1 μg/mL propidium iodide (PI) was added for the identification and exclusion of nonviable cells. Cells were sorted and analyzed on a FACSVantage cell sorter (Becton Dickinson). FITC, PE, and PI were excited at a wavelength of 488 nm using an argon ion laser. The CD34⁺ cells were sorted into CD38⁻, CD38^{lo}, and CD38^{hi} subpopulations. Positive fluorescence for each of the markers was established as fluorescence more than 99% of isotype-matched irrelevant murine IgG₁ controls. Cell aggregates or debris were excluded by forward and 90° light scatter. All staining for analysis and sorting was done in the presence of 0.2% BSA in PBS on ice.

Stroma-free expansion cultures

To promote differentiation of human CD34⁺ cells, a stroma-free suspension culture was established as previously described.¹⁵ Tissue culture dishes (35 mm; Corning, Corning, NY) were seeded with 1 × 10⁶ CD34⁺ cells/well in 3 mL Iscove modified Dulbecco medium (IMDM) (BioWhittaker) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cultures were placed at 37°C in 100% humidified atmosphere of 5% CO₂ in air. At initiation of cultures and at 72- to 96-hour intervals, cultures received a combination of recombinant cytokines: stem cell factor (SCF), interleukin (IL)-3, and granulocyte colony-stimulating factor (G-CSF) all at 100 ng/mL (R&D Systems, Minneapolis, MN). Cultures were maintained at a cell concentration of 5 × 10⁵ to 2 × 10⁶ viable cells/mL.

Leukemia cell lines

The TF-1 lymphoblast cell line; Jurkat, a T-lymphocyte cell line; CEM, an acute T lymphoblast cell line; BV-173, a B-cell precursor cell line; K-562, a chronic myeloid cell line; KG1 and KG1a, acute myeloid cell lines; and SUPB13, an acute B lymphoblastic cell line were obtained through the American Type Culture Collection (ATCC; Rockville, MD; SUPB13, a gift from Steve Smith at the University of Chicago, Chicago, IL). The lines were maintained in RPMI 1640 (Biowhittaker) supplemented with 10% heat-inactivated FBS (except for KG1 and KG1a, which required the presence of 20% FBS), 2 mM L-glutamine, 100 U/mL penicillin, 1 mg/mL streptomycin (Biowhittaker). Granulocyte/macrophage CSF (GM-CSF; 5 ng/mL; Peprotech, Rocky Hill, NJ) was added to TF-1 culture. Cell density was maintained at 1 × 10⁵ to 1 × 10⁶ viable cells/mL.

Mesenchymal stem cells

Mesenchymal stem cells were isolated and expanded from normal human BM aspirates as described by Pittenger and coworkers.¹⁶ These purified cells were kindly provided by Osiris Therapeutics (Baltimore, MD).

Stromal cells

Bone marrow stroma was grown by seeding 1 × 10⁶ low-density BM cells/162 cm² (Corning) flask in low-glucose Dulbecco modified Eagle medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine, 100 U/mL penicillin, 1 mg/mL streptomycin.^{17,18} Adherent cells were split at confluency and the nonadherent cells were discarded. Adherent cells were passaged 4 times and were then termed marrow stroma.^{17,18}

RNA isolation

Total RNA was isolated from hematopoietic, stromal, and mesenchymal cells using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Cells were pelleted and then resuspended in 1 mL Trizol per 5 × 10⁶ cells by repeated pipetting. The cell lysate was then incubated for 5 minutes at room temperature and extracted with 0.2 volumes chloroform by vortexing for 1 minute. The sample was then centrifuged for 30 minutes at 13 000 rpm (12 000g), 4°C in a microcentrifuge. The RNA was precipitated using 2 volumes isopropanol, mixed and allowed to sit at room temperature for 10 minutes. The RNA was centrifuged for 45 minutes at 13 000 rpm (12 000 g). The RNA was washed with 75% ethanol, briefly dried, and resuspended in RNase-free water or diethyl pyrocarbonate-treated (DEPC; Sigma Chemical) water (0.1%). RNA was then quantitated using a DU 650 spectrophotometer (Beckman Instruments, Palo Alto, CA). RNA was DNase treated using DNase I enzyme (Life Technologies) according to the manufacturer's instructions.

Cloning of *hiwi* complementary DNA from human testis

Polymerase chain reaction (PCR) amplification was performed on RNA samples using either a RNA PCR Core Kit (PerkinElmer, Foster City, CA), according to the manufacturer's instruction, except that High Fidelity Platinum *Taq* DNA Polymerase (Life Technologies) was substituted for AmpliTaq or a Stratagene ProSTAR First-Strand RT-PCR Kit (Stratagene, La Jolla, CA). One microgram of total RNA was used for complementary DNA (cDNA) synthesis using random hexamers to prime first-strand synthesis. The synthesized CD34⁺ cell cDNA was divided and used for PCR amplification. As a control, duplicate cDNA synthesis reactions were performed for each experiment without the addition of reverse transcriptase. Control PCR amplification reactions were performed using primers for glyceraldehyde phosphate dehydrogenase cDNA (GAPD; forward, 5'-ggctgagaacgggaagctgtcat-3'; reverse, 5'-cagccttccatgggtgaaga-3') for 1 cycle at 94°C/2 minutes; 5 cycles at 94°C/10 seconds, 70°C/2 minutes; 5 cycles at 94°C/10 seconds, 68°C/2 minutes; 25 cycles at 94°C/10 seconds, 66°C/2 minutes, and 1 cycle at 72°C/10 minutes, which produced a 142-base pair (bp) product. β₂-Microglobulin primers were also used as an

internal control (β_2 -microglobulin: forward, 5'-ctcgcgctactctctcttc-3'; reverse, 5'-catgtctgcattccactaac-3') producing a 329-bp product. PCR amplification primers were designed based on the partial published *hiwi* DNA sequence found in the Genbank database (accession number AF104260). Detection of *hiwi* in CD34⁺ DNase treated RNA was performed using the primer pair hiwiF269 5'-gaagcagcctgtctgtcagc-3' and hiwiR269 5'-gaatcaaagctcaaacccagctctc-3' producing a 269-bp product. Two primer pairs were initially designed to be used in 5' Rapid Amplification of cDNA Ends (5' RACE) PCR strategy cloning¹⁹ AKSrev1 (reverse primer #1; 5'-cgctgtatgtgtctgctcagc-3') and AKSrev2 (reverse primer #2; 5'-gggagaacactaccactctcagcctg-3'). AKSrev2 is 32 nucleotides upstream from AKSrev1 and serves as a nested internal control for secondary PCR amplification. Marathon Ready Human Testis cDNA Kit (Clontech Laboratories, Palo Alto, CA) was used for primary amplification, according to manufacturer's instructions. First round PCR amplification consisted of AKSrev1 and AP-1 (5'-ccatctaatacgaactactagggc-3'), supplied within the cDNA Kit, under the following conditions: 1 cycle at 94°C/2 minutes; 5 cycles at 94°C/10 seconds, 71°C/2 minutes; 5 cycles at 94°C/10 seconds, 69°C/2 minutes; 25 cycles at 94°C/10 seconds, 67°C/2 minutes, and 1 cycle at 72°C /10 minutes. A single PCR product was separated on a 1% SeaPlaque Agarose (FMC, Rockland, ME) 1 × TAE gel stained with ethidium bromide and purified from the agarose using a Wizard PCR Prep Kit (Promega, Madison, WI). The isolated PCR fragment was then used in a second round of PCR amplification that used the same PCR amplification conditions listed above except that AKSrev2 was substituted for AKSrev1 and AP-2 (5'-actcactataggctcagcggc-3') replaced AP-1. All PCR reactions were performed in a PerkinElmer Thermal Cycler 9700 (PerkinElmer) or Stratagene Robocycler Gradient 96 Thermal Cycler.

Cloning of *hiwi* cDNA from human CD34⁺ cells

Human CD34⁺ cells were purified and total RNA isolated as previously described. Based on a sequence obtained from the human testis clone, primers were designed to amplify a putative full-length cDNA clone, FLhiwifor1 (forward, 5'-atgatcttgggtgacacagaaggcagaa-3') and FLhiwirev1 (reverse 5'-gaggtagtaaggcgggttgacagtacaga-3'). PCR amplification conditions were as follows: 1 cycle at 94°C/2 minutes; 5 cycles at 94°C/10 seconds, 72°C/2 minutes; 5 cycles at 94°C/10 seconds, 70°C/2 minutes; 25 cycles at 94°C/10 seconds, 68°C/2 minutes, and 1 cycle at 72°C/10 minutes.

DNA sequencing

Direct DNA sequencing of the PCR product was performed using an ABI Prism Dye Terminator Cycle Sequencing Reaction Kit (PerkinElmer) according to the manufacturer's instructions. Additional primers (Integrated DNA Technologies, Coralville, IA) were synthesized based on analyzed sequence to obtain the complete cDNA sequence.

Tissue distribution analysis

The PCR amplification was performed on 3 different multiple tissue cDNA panels: human I, human II, and human fetal (Clontech Laboratories). The following PCR conditions were used for amplification with primer pair GSP2F4 (forward, 5'-ccttgcagctacgccacaagctg-3') and GSP1R1966 (reverse, 5'-cccacatgtgttagtgagcatcc-3'): 1 cycle at 94°C/2 minutes; 35 cycles at 94°C/10 seconds, 70°C/15 seconds, 72°C/45 seconds; and 1 cycle at 72°C/10 minutes, which produced a 557-bp product. Positive samples were separated on a 1% SeaPlaque Agarose (FMC) 1 × TAE gel stained with ethidium bromide and purified from the agarose using the Wizard PCR Prep Kit according to the manufacturer's instructions. Asymmetric restriction endonuclease digestion was performed to determine the identity of the PCR products (data not shown).

Subcloning of *hiwi* into expression vector

After obtaining the putative full-length *hiwi* cDNA, the fragment was then subcloned into the XhoI/NotI sites of the pCIneo Mammalian Expression Vector (Promega) and used for expression studies.

Transfection and G418 selection of transfected KG1 cells

Transfection of KG1 cells was accomplished using electroporation. Electroporation of plasmid DNA into KG1 cells was performed using a Bio-Rad Gene Pulser II System (Bio-rad Laboratories, Hercules, CA). Fifty micrograms of pCIneo or pCIneo-*hiwi* was electroporated per 1×10^5 KG1 cells accompanied with 2 μ g linearized vector for each respective condition as well as a mock control that consisted of KG1 cells alone. All cells were washed with and then resuspended in DPBS prior to electroporation. The electroporation conditions were as follows: 300 V, 950 μ F, using a 0.4-cm gapped cuvette (Bio-rad Laboratories) for each condition. Cells were then centrifuged and washed twice with DPBS and resuspended in IMDM supplemented with 20% heat inactivated FBS, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2 mM L-glutamine and incubated overnight at 37°C in a 100% humidified incubator containing 5% CO₂ in air. Cells were then counted and stained for viability using a 0.4% trypan blue solution (Sigma) on the following day. Cells were then plated at a density of 2×10^4 viable cells per well in a flat-bottom 96-well plate (Corning) in IMDM supplemented with 5% heat inactivated FBS, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2 mM L-glutamine. Cells exposed to each experimental condition were plated in 6 separate cultures for 7 days. Several sets of transfected cells were plated under similar conditions for detection of the *hiwi* gene by PCR and for antibiotic selection. Similar populations of cells (pCIneo vector alone, the pCIneo-*hiwi*, or cells alone) were then incubated in IMDM supplemented with 20% FBS, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2 mM L-glutamine containing 1 mg/mL Geneticin Selective Antibiotic (G418 sulfate; Life Technologies) for 3 weeks.

Cell proliferation assay

Cell proliferation and survival were measured by cellular uptake of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich), which measures living cells.¹⁵ Fifty microliters of a 1 mg/mL solution of filter sterilized MTT in DPBS was added to each culture volume of 200 μ L containing 2×10^4 viable cells per well and then incubated for 4 hours at 37°C in a 100% humidified incubator, 5% CO₂ in air. Approximately half of the volume was then carefully removed (without disrupting the solubilized complex) and replaced with developing reagent that consisted of 40 mM hydrochloric acid in isopropanol. After thoroughly homogenizing the solubilized complex with the developing reagent, the plate was then read on a ELX-800 ELISA plate reader (Bio-tek Instruments, Winooski, VT) at wavelength of 570 nm with a reference wavelength of 630 nm as previously described.¹⁵ This procedure was performed on days 1 to 6 and then on day 9.

PCR detection of *hiwi* in transfected KG1 cells

The KG1 cells (5×10^5) that were transfected with either the pCIneo vector alone, the pCIneo-*hiwi*, or cells alone were then harvested. Total RNA was isolated and cDNA was synthesized as previously described. PCR amplification conditions were similar to those described previously.

Apoptosis assay

The KG1 cells were transfected under the 3 separate conditions as previously described. Condition 1 consisted of a mock transfection, condition 2 consisted of an empty vector control transfection, and condition 3 consisted of the vector containing *hiwi*. An additional sample of KG1 cells was also serum-starved for 24 hours in IMDM, 2 mM L-glutamine, 100 U/mL penicillin, and 1 mg/mL streptomycin (Biowhittaker). Incubation conditions are similar to those described above. Transfected cells were maintained in culture for up to 32 hours and then assayed for programmed cell death by the Annexin V assay system (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were washed with DPBS, collected, and then resuspended in binding buffer containing Annexin V-FITC and PI at room temperature. Acquisition and analysis of data were performed on a FACSCalibur (Becton Dickinson) using the CellQuest Analysis Software (Becton Dickinson).

Chromosome localization of *hiwi* gene by computer-based mapping

The *hiwi* chromosome localization was achieved by comparing expressed sequence tags (EST) and the sequence tagged site (STS) to the partial published sequence of *hiwi* and verifying position through computer-based radiation hybrid homology searches.^{20,21}

Fluorescence in situ hybridization (FISH)

Metaphase spreads were prepared from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes of a healthy human male, according to standard protocols. Cells were treated with 5-bromodeoxyuridine (BrdU) at an early replicating phase to induce banding pattern.²² Slides were stained with Hoechst 33258 (1 mg/mL) for 10 minutes and exposed to UV light (302 nm) for 30 minutes. Before hybridization, metaphase slides were pretreated with RNase (100 mg/mL) and pepsin (20 mg/mL) to avoid nonspecific hybridization. An approximate 80 kilobase (kb) genomic DNA fragment was cloned into the pBEObac 11 vector after screening a human BAC library (Human Genome Systems, St. Louis, MO) with the original *hiwi* cDNA clone and was labeled with biotin 11-dUTP (Sigma Chemical) by nick translation according to standard protocols. The FISH procedure was carried out in 50% formamide and 10% dextran sulfate in 2 × standard sodium citrate (SSC) as described earlier.^{23,24} Repetitive sequences were suppressed with a 10-fold excess of human Cot-1 DNA (Life Technologies). After overnight incubation, nonspecific hybridization and background signals were eliminated by washing the slides 3 times with 50% formamide/2 × SSC and 3 times with 2 × SSC at 42°C. Specific hybridization signals were visualized using FITC-conjugated avidin (Vector Laboratories) and counterstained with DAPI (4'-6'-diamino-2-phenylindole; Sigma; 0.025 mg/mL).²⁵ A multicolor image analysis system was used for acquisition and display of hybridization signals of metaphase chromosomes. The system consists of a Zeiss Axioplan Imaging fluorescence microscope (Zeiss, Stuttgart, Germany) attached to a PC workstation.

Results

hiwi is expressed in CD34⁺ hematopoietic cells but not in CD34⁻ cells

Semiquantitative reverse transcriptase PCR (RT-PCR) was performed using RNA isolated from immunomagnetically separated CD34⁺ and CD34⁻ cells from nonhuman primates (*Papio anubis*), and from humans. A 269-bp band correlating to the same size as seen in the human testis positive control was viewed in the CD34⁺ population and not in the CD34⁻ population of both human and baboon. Sequence analysis confirmed that the PCR product obtained was the *hiwi* cDNA (Figure 1). To examine if *hiwi* expression was restricted to the most primitive subpopulation of human CD34⁺ cells, CD34⁺ cells were sorted according to CD38 expression into 3 subpopulations: CD34⁺CD38⁻, CD34⁺CD38^{lo}, CD34⁺CD38^{hi} (Figure 2A). Semiquantitative RT-PCR on DNase-treated RNA isolated from each population showed that each of the 3 subpopulations expressed *hiwi*. These data indicate the *hiwi*

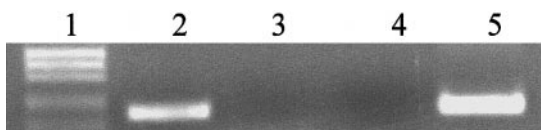


Figure 1. *hiwi* is expressed in human CD34⁺ cells but not in CD34⁻ hematopoietic cells. Electrophoretic gel shows positive signal for *hiwi* in CD34⁺ sample and no signal in CD34⁻ sample by semiquantitative RT-PCR. Lane 1, 123-bp DNA ladder; lane 2, CD34⁺ sample; lane 3, CD34⁻ sample; lane 4, negative control (water) PCR reactions; lane 5, positive control (human testis, 269 bp).

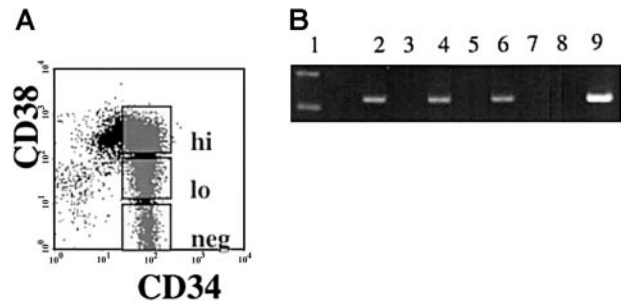


Figure 2. Expression of *hiwi* in subpopulations of CD34⁺ cells. Immunomagnetically selected CD34⁺ human adult BM cells were sorted based on the CD38 expression. (A) Gates were established for the flow cytometric isolation of CD34⁺CD38^{hi}, CD34⁺CD38^{lo}, and CD34⁺CD38^{neg}. (B) Semiquantitative RT-PCR of *hiwi* in CD34⁺ subpopulations. Lane 1, 123-bp DNA ladder; lane 2, CD34⁺CD38^{lo} sample; lane 3, CD34⁺CD38^{neg} (-RT) sample; lane 4, CD34⁺CD38^{hi} sample; lane 5, CD34⁺CD38^{hi} (-RT) sample; lane 6, CD34⁺CD38^{hi} sample; lane 7, CD34⁺CD38^{hi} (-RT) sample; lane 8, negative control (water); lane 9, positive control (human testis, 269 bp).

expression is not limited to the most primitive progenitor cell population (Figure 2B).

Expression of *hiwi* diminishes as CD34⁺ cells differentiate

The CD34⁺ cell differentiation was promoted in a suspension culture system to which SCF, IL-3, and G-CSF were added each at 100 ng/mL every 3 to 4 days. Bazil and colleagues¹⁵ have previously shown these conditions allow for rapid proliferation and differentiation causing the cells to leave the CD34⁺ compartment. Aliquots of cells were harvested on days 0, 1, 3, 5, 7, 10, and 14 and analyzed by flow cytometry for CD34⁺ expression and by semiquantitative RT-PCR for the *hiwi* messenger RNA (mRNA) (Figure 3A). On day 0, the starting cell population was composed of 99% CD34⁺ cells. Day 5 CD34⁺ content diminished to 20% of the expanded cell population; by day 7, fewer than 0.1% of the cells were CD34⁺, and by day 10 there were no detectable CD34⁺ cells present. By day 3, *hiwi* expression was markedly reduced and no longer detectable by semiquantitative RT-PCR by day 5. Concurrently β_2 -microglobulin gene expression was used as an internal

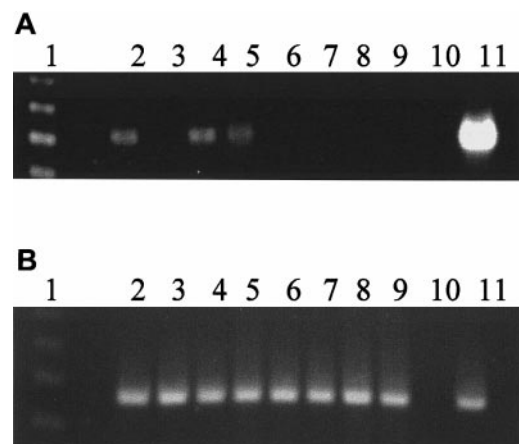


Figure 3. Loss of *hiwi* expression during differentiation of CD34⁺ marrow cells. CD34⁺ cells were placed in suspension culture with G-CSF, IL-3, and SCF at 100 ng/mL added every 3 to 4 days. (A) Expression of *hiwi* as detected by semiquantitative RT-PCR. Lane 1, 123-bp DNA ladder; lane 2, day 0 CD34⁺ cells; lane 3, day 0 CD34⁻ cells; lane 4, day 1 culture sample; lane 5, day 3 culture sample; lane 6, day 5 culture sample; lane 7, day 7 culture sample; lane 8, day 10 culture sample; lane 9, day 14 culture sample; lane 10, negative control (water); lane 11, positive control (human testis, 269 bp). (B) Internal control of β_2 -microglobulin gene expression throughout 14 days of culture (330 bp). Lane numberings correspond to the same samples as in panel A.

control. The levels of β_2 -microglobulin remained constant throughout the 14 days of culture (Figure 3B).

Expression of *hiwi* in leukemia cell lines

To examine whether the *hiwi* gene was expressed in leukemia cells, we analyzed a variety of immortalized human leukemia cell lines belonging to various lineages (Figure 4). Analysis of 8 immortalized leukemia cell lines showed that the *hiwi* mRNA transcript was not detectable by RT-PCR.

Expression analysis of *hiwi*

To determine the expression pattern of *hiwi* in various adult and fetal tissues other than CD34⁺ marrow cells, PCR amplification was performed using *hiwi*-specific primers on cDNA samples (Clontech Laboratories). Fetal cDNA samples ranged from 18 to 36 weeks of gestation. The expression level was determined through semiquantitative PCR amplification and revealed a wide distribution of *hiwi* through most fetal and adult tissues (Figure 5). The highest level of expression in fetal tissues was found in the kidney. Analysis of adult samples showed that *hiwi* was also expressed in a wide range of tissues such as the prostate, ovary, small intestine, heart, brain, liver, skeletal muscle, kidney, and pancreas. The highest level of expression was seen in the testis followed by the kidney. Expression of *hiwi* was not detected in mesenchymal stem cells or marrow stroma.

Cloning of *hiwi* from human marrow CD34⁺ cells

The presence of *hiwi* in CD34⁺ hematopoietic cells was determined through PCR amplification by designing a primer pair that spanned from amino acid 364 to 524 of the published partial coding sequence generating a 480-bp fragment that corresponds to the C-terminal end of the protein. After positively identifying the PCR product to be that of *hiwi* through dye terminator cycle sequencing, primers were then designed to amplify the potential full-length gene from human testis cDNA by using a 5'RACE cloning methodology that allows the PCR amplification of a given gene of interest by using a small region of a known sequence. AKSrev1 and AKSrev2 were also based on the published partial *hiwi* coding sequence and acted as a nested primer pair for primary and secondary PCR amplification reactions and correspond to base pairs 1391 to 1415 and 1330 to 1359, respectively. After using the 5' RACE methodology on the human testis cDNA sequence and 2

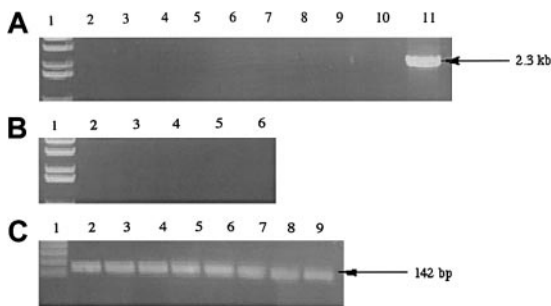


Figure 4. Expression of *hiwi* in human leukemia cell lines. (A) Lane 1, 1-kb ladder; lane 2, TF-1; lane 3, Jurkat; lane 4, KG1a; lane 5, KG1; lane 6, K562; lane 7, CEM; lane 8, BV173; lane 9, SUPB13; lane 10, negative control (water); lane 11, human testis positive control (2.3 kb). (B) Samples without reverse transcriptase; same setup as panel A, excluding lanes 7 through 11 (samples 7-9 were donated cDNAs and total RNA samples were not available to perform the -RT control). (C) GAPD internal control (142 bp); lane numberings correspond to the same samples as in panel A, excluding lanes 10 and 11.

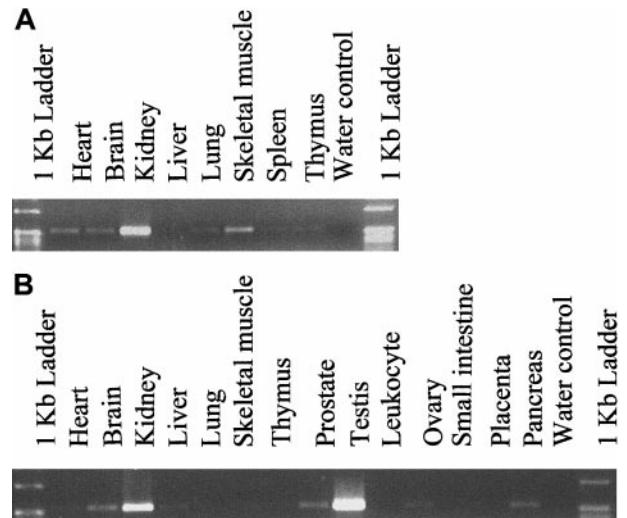


Figure 5. Tissue distribution of *hiwi* mRNA. (A) Human fetal tissues. (B) Human adult tissues (557 bp for both panels).

rounds of PCR amplification (the second round consisting of a nested PCR amplification), a putative 2.3-kb full-length coding sequence was cloned. After several rounds of sequencing through primer walking, an open reading frame was determined. When compared to the Genbank database of nonredundant clones, the

PIWI	MADDQGRGRRRPLNEDDSSTSRGSGDGPVKVFRGSSGDPADFPRIEASRRRALLAEAPR	61
HIWI	M-----IP-----G-----R-----L-----VNTNRQLNDHV--	
PIWI	REGGPPERKPGWDQYDYLNTRPVELVSKKGDGVPVLMQTNPFRLKTKPEWRIVHYHVEFE	122
HIWI	-----K-----E--SRKSGGIIVRLSTNHFLTRSPQWALVQYHIDYN	
PIWI	PSIENFVRMGVLSNHANLLGSGVLPDGLQFPTRKPEQBITVLVSGSKLDIEYKISIKFV	183
HIWI	PLMEARRLSALLFOEDLIGKCHAFDGTILFLPKRKLQKQVTEVFSKTRNGEDVRITITLT	
PIWI	GFISCAEPRFLQVNLILRRSMKGLNELVGRNLPDPRAKIBIREPFKMEIWPFCYETSIRQH	244
HIWI	NELPPTSPTCLQFYNIIPRLKIMNLLQIGRYNYNENPIDIPISHERLVIWPGFTTSIIQY	
PIWI	EKDILLGTEITHKVMRTETIYDIMRRCSHNFARH--QDEVVNVLDLVLDTYNNRTYRIN	305
HIWI	ENSIMLCTDVSHKVLRSFVLDLDFMNFYHQTEHKKPQKQVSKELIGLVLLTRYNKTYRVD	
PIWI	DVDFGQTPKSTF--SCKGRDISFVEYLLTKYIRIRDRHNPQLLISK--NRDKALKTNASELVV	366
HIWI	D+D+ Q PKSTF G ++SF+EYY +YN I D QP+L+S+ R ++ +HPG+ TSI Q-	
PIWI	DIDWDQPKSTFKKADGSEVSPLEYYRKYQYNEITDLKQFVLVSPKRRRPGGTLPGPAM	427
HIWI	LPELCLRVTLGNAEMRSNFQLRAMRSYTRMNPQR---TDRLRAFNRHLQNTPEVVKVLR	
PIWI	LPELCLRVTLGNAEMRSNFQLRAMRSYTRMNPQR---TDRLRAFNRHLQNTPEVVKVLR	488
HIWI	LPELCLRVTLGNAEMRSNFQLRAMRSYTRMNPQR---TDRLRAFNRHLQNTPEVVKVLR	
PIWI	DWMELDKNVEVQGRIGQONIVFHNKGFVAGEN---ADWRHFRDRQMLTTPSGDLGR	549
HIWI	DW+ D N+ GRI+ + I H G N ADW + R +++ LD W	
PIWI	DVIAEQRNSHELRTLLDSLYRAASGMLRIRSPQEFIIYDRTGTVYRAMDDCVRSDPKLI	610
HIWI	LLIYTRRNVEAANSILQNLFKVTPAMGQMQR--AIMIEVDRTATYLRVLRQKVTADTQIV	
PIWI	LCIVPNDNAERYSSIKRKYVDRVPTQVVLTKTKPKYSLSMIAKIAIQLNCKLGYTPW	671
HIWI	CL+ ++ ++Y+IKK D P+Q V +T K ++M+IATKIA+Q+NCK+G W	
PIWI	VCLSSNRKDKYDAIKKYLCTDCTPSCVAVARTLGRQQTVMATATKIALQMNCKMGGELW	732
HIWI	MIEPLSGMGTIGFDIAKSTRDRKRAYGALLASMDLQNSYTFSTVECSAFDVLANTLWP	793
PIWI	+++PL +M +G D +R+ +AS++ + +FS L+ L	
HIWI	RVDIPLKLVMIYVIGIDCYHDMTAGRRSIAGFVASIN--EGMTRWFSRICIPDRQQLVDGLKV	
PIWI	MIKALRQVQHEHRKLPRIYVYRDRGVSSGSLKQLFVFEVVDIIEKLTKEYARVQLSPPQL	854
HIWI	+ ALR + + +PSRI+ YRDGV G LK L +BV ++ LK+ P+L	
PIWI	CLQAALRAWNSCNEYMPSRIIYVRDGVGDQLKLVNVEVYVQPLDCLKISGRGYN---PRL	
PIWI	AIYVIVTRSMNTRFPLNG----QNPFGTIVDDVITLPERYDFYLVSQVROGCTVSPSTYNY	859
HIWI	IYV + +TRTF QNP PGT+D +T PE YDF+VSQ VR G+VSPT YNY	
PIWI	LYIVVTRSMNTRFPLNG----QNPFGTIVDDVITLPERYDFYLVSQVROGCTVSPSTYNY	
HIWI	LYIVVTRSMNTRFPLNG----QNPFGTIVDDVITLPERYDFYLVSQVROGCTVSPSTYNY	
PIWI	LYSSMGLSPKMKQLTYKMLYNYNSGTRVPAVQCQYAKLALVGTNLHLSIPQNALBK	
HIWI	+Y + GL P+ +Q+LYK+CH+YNYN G RVPA CQYA KLA LVG +H P +L	
PIWI	IYDNSGLKPDHIQRLTYKMLYNYNSGTRVPAVQCQYAKLALVGTNLHLSIPQNALBK	
HIWI	+ YL 859	
PIWI	KFYLL	
HIWI	RLYYL	

Figure 6. HIWI protein alignment. Amino acid alignment of the HIWI protein (Genbank accession number AF264004) to the *D. melanogaster* PIWI protein (Genbank accession number AF104355). Amino acid identities are indicated by identical amino acid matches, whereas mismatches are indicated by a blank space. The + signs refer to positions in which the nature of the residue is conserved as reported by BLASTP homology alignment. Numbering begins at the initiation codon of the PIWI sequence.

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human HIWI protein showed a 52% homology to the *Drosophila* PIWI protein at the amino acid level (Figure 6). Primer pairs were then designed to amplify the full-length coding sequence from CD34⁺ hematopoietic cell cDNA through PCR amplification. A 2.3-kb band was detected in the CD34⁺ cDNA sample but not in the no-template control. No bands were detectable in the absence of reverse transcriptase (–RT) during the cDNA synthesis step. Control PCR amplification with primers to GAPD confirmed that the quantity and integrity of the RNA could be PCR amplified. The PCR product was sequenced and the identity was confirmed to be that of the *hiwi* gene by comparing it to the Genbank database.

Overexpression of *hiwi* leads to a diminished proliferative capacity of KG1 cells as measured by MTT

The KG1 cells were chosen for these studies due to their lack of expression of the *hiwi* mRNA as demonstrated by RT-PCR (Figure 4). Figure 7 shows that the proliferative capacity of the mock and pCneo vector-treated cells was similar, whereas treatment with the pCneo-*hiwi* construct led to a greatly diminished proliferation of KG1 cells. To determine whether or not the *hiwi* gene was actually expressed in the transfected cells, PCR was performed. Figure 8 shows the presence of the *hiwi* transcript from day 1 (24 hours after transfection) until cultures were terminated (day 9). The declining levels of the expressed gene were likely due to the fact that the cells were not subjected to G418 antibiotic selection. To further assess the function and integrity of each construct, populations of cells that were simultaneously transfected were placed in the presence of 1 mg/mL active G418 for 3 weeks. After this period, the pCneo-*hiwi* construct and the pCneo empty vector contained similar numbers of viable cells. The pCneo-*hiwi* construct further continued to express the *hiwi* gene (as determined by PCR, data not shown). The mock transfected cells were, however, characterized by a high degree of cell death at day 6 (> 96%, data not shown). These studies demonstrate that both the cytomegalovirus (CMV) promoter and the SV40 promoter (which was driving neomycin phosphotransferase gene) were actively driving transcription of their respective genes and that *hiwi* gene expression was present, but to an unknown level. This study indicates that *hiwi* inhibits

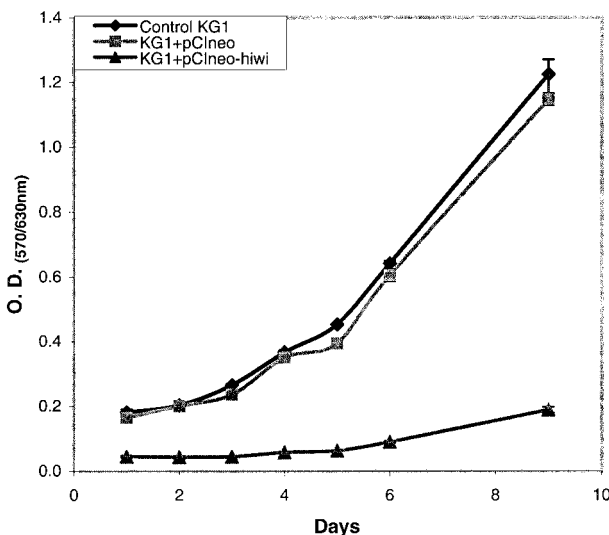


Figure 7. MTT assay of KG1 cells with or without *hiwi*. KG1 cells were subjected to transfection under 3 different conditions (control KG1, KG1 + pCneo, KG1 + pCneo-*hiwi*) and were cultured for a 9-day period. An MTT assay was performed on each day to assess cell proliferation from days 1 through 6 and then on day 9.

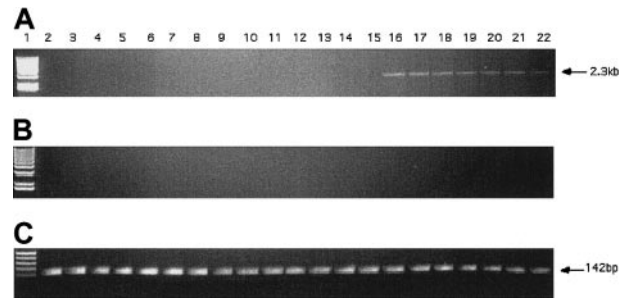


Figure 8. PCR detection of *hiwi* DNA in transfected KG1 cells. (A) PCR amplification of *hiwi* on mock transfected KG1 cells (d1-6, d9; lanes 2-8), empty vector transfected KG1 cells (d1-6, d9; lanes 9-15), and *hiwi*-containing vector-transfected KG1 cells (d1-6, d9; lanes 16-22). Lane 1 is a 1-kb DNA ladder. (B) Samples without reverse transcriptase; same setup as panel A. (C) GAPD internal control (142 bp), same setup as panel A.

KG1 cell proliferation and therefore suggests that this gene may play a role in the negative regulation of hematopoietic cells.

Apoptosis of KG1 cells induced by *hiwi*

To determine a potential mechanism for the reduced proliferative capacity of KG1 cells that overexpress the *hiwi* gene product, apoptosis of these cells was evaluated using Annexin V as an indicator of programmed cell death and PI as a measure of cell viability. Figure 9D-F shows such an analysis after an 8 hours of incubation demonstrating an approximate 4.4-fold increase (9.6%) in the percentage of cells undergoing apoptosis, (positive for Annexin V but negative for PI) when compared to the mock (1.8%) and the empty vector control (2.4%). The degree of apoptosis after 12 hours of incubation is also greater in the *hiwi* overexpressing cells as compared to the control cells. Flow cytometric analysis at 32 hours of culture (Figure 9J-L) exhibited an increase in the number of cells that were PI and annexin positive, suggesting that the cells had proceeded to a necrotic state. These data indicate that most of the cell population containing the *hiwi* gene underwent apoptosis and eventually entered a terminal state of cell death.

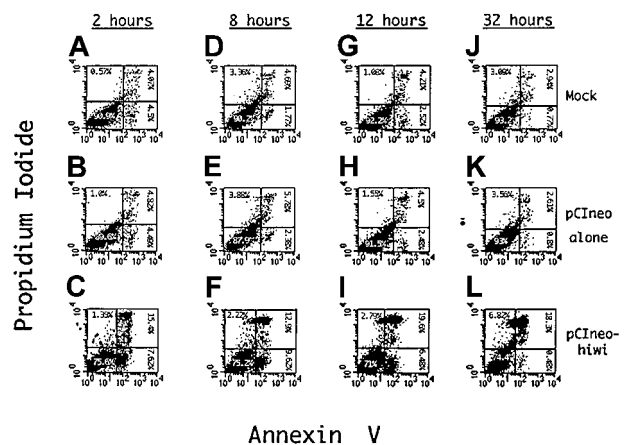


Figure 9. Induction of apoptosis in KG1 cells overexpressing the *hiwi* gene. KG1 cells were transfected under mock, pCneo empty vector, or pCneo-*hiwi* conditions. Cells were harvested at 2, 8, 12, and 32 hours, washed with PBS, and resuspended in binding buffer containing Annexin V-FITC and PI. The X-axis represents log fluorescence intensity for Annexin V staining; the Y-axis represents log fluorescence intensity for PI staining. Panels A-C show flow cytometric analysis at a 2-hour time point that consists of a mock, a pCneo empty vector, and the pCneo-*hiwi* transfected KG1 cell populations. Panels D-F, G-I, and J-L show the same analysis but at 8, 12, and 32 hours (respectively) after transfection.

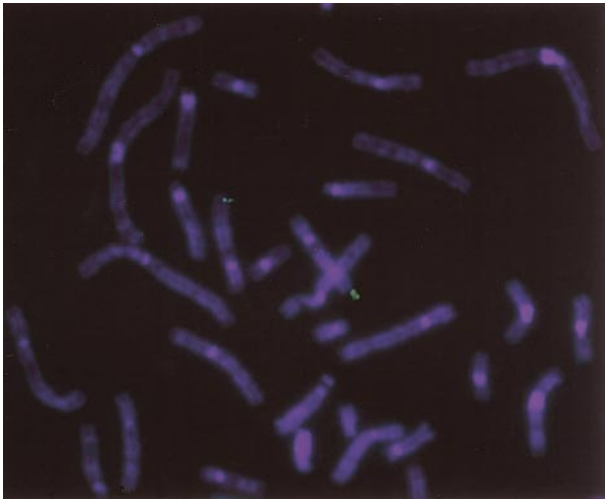


Figure 10. Chromosomal localization by FISH analysis. Chromosomal localization of *hiwi* using a *hiwi* genomic clone shows hybridization of the genomic probe to the q arm of chromosome 12, specifically between 12q24.2 through 12q24.32.

Chromosomal localization of *hiwi*

The chromosomal location of *hiwi* was determined by STS computer-based mapping.^{20,21} Four different STS clones (Genbank accession numbers AA639672, AA904973, AA969660, and AI25224) were found to have significant homology to the *hiwi* gene. These 4 clones along with the partial published *hiwi* sequence were then mapped to the reference interval of D12S340-D12S97 (147.5-160.9 cM) by radiation hybridization (Unigene cross-reference Hs. 128673). The physical position of *hiwi* was located at 489.71 cR3000 (P1.43, stSG53541) on the q arm of chromosome 12, specifically between 12q24.2 through 12q24.32 as represented by the cytogenetic ideogram. The physical locale of *hiwi* does not currently show an association with any hematologic disorders (as demonstrated by STS/EST homology comparisons).

Chromosomal localization of the human *hiwi* gene by FISH analysis

To directly determine the chromosomal location of *hiwi*, a *hiwi* genomic clone was hybridized on metaphase chromosomes derived from a human peripheral blood cell culture. Identification of the human chromosomes was based on their DAPI-banding pattern that resembles G-bands achieved by conventional trypsin-Giemsa treatment. Thirty-two metaphase spreads were analyzed showing specific localization to chromosome 12 as follows: specific hybridization signals were seen 9 times (28.1%) in 2 chromatids, 10 times (31.3%) in 3 chromatids, and 11 times (34.4%) in 4 chromatids; no hybridization signals were seen in 2 metaphases (6.2%). A very small number of nonspecific hybridization sites were seen (Figure 10). Traditional FISH analysis confirmed the computer-based chromosomal localization of *hiwi*.

Discussion

A stem cell can undergo self-renewal as well as generate differentiated progeny. The capacity of an HSC to remain undifferentiated and be capable of reconstituting a myeloablated host as well as its ability to generate multiple differentiated cell types is central to its pivotal role in normal hematopoiesis. Despite the improved ability of various laboratories to isolate and manipulate pure populations

of murine and human HSCs,^{26,27} our understanding of mechanisms by which a stem cell divides and retains its unique biologic properties has eluded the efforts of a large number of investigators.²⁸⁻³³ Understanding the basis of these properties is required if progress in stem cell transplantation and gene therapy are to occur. Stem cell division is likely controlled by both intracellular mechanisms as well as cell to cell interactions. The intracellular mechanisms of a stem cell would include basic cell cycle machinery as well as machinery responsible for cell division.

Elucidation of the genetic program that underlies the unique biologic properties of HSCs has been the focus of a growing number of laboratory groups.^{33,34} These laboratories have attempted to approach this daunting objective by using a variety of approaches. Array technology, for instance, now permits one to monitor simultaneously the expression patterns of thousands of genes during cellular differentiation and response.^{35,36} The key to the successful implementation of such technology to the study of stem cell biology is the development of the means to assign priority to such genes and to determine their function.

Recently our laboratory and several others have taken a different approach to analyze the genetic organization of human HSCs.³⁷⁻³⁹ Genes that were originally shown to affect stem cell development in lower species have been shown subsequently to be expressed by human hematopoietic cells and to have profound regulatory effect on human hematopoiesis. Lower organisms such as *Drosophila*, *C. elegans* and *Danio rerio* (zebra fish) have been used as effective models for studying mechanisms that are conserved among diverse developmental systems.^{5,39,40} Studies from *Xenopus*, for instance, have revealed a multitude of genes involved in mesoderm induction including members of the transforming growth factor β superfamily, fibroblast growth factor, and at least 19 members of the *Wnt* gene family in diverse species ranging from roundworm and insects to humans.^{41,42} The *Wnt* gene family members have subsequently been shown to have profound effects on murine and human hematopoiesis. With this experience in mind, we attempted to extend this approach to identify genes of interest that might serve as candidate regulators of HSC self-renewal and development.

In *Drosophila*, stem cells exist in the germline at the apical tip of each ovariole, the germarium, which is the functional portion of the ovary.^{12,43} Each ovary consists of 10 to 17 ovarioles. Each germarium contains 2 to 3 GSCs that are in direct contact with specialized somatic cells, the basal terminal filament cells.^{8,11,43} GSCs undergo asymmetric divisions to produce daughter stem cells and a differentiated daughter cell, a cystoblast. Recently a number of genes including *dpp*, *piwi*, *pumilio*, and *fs¹Yb* have been identified and shown to be essential for GSC maintenance.^{7,10,44} Among these genes, *piwi* has been of special interest. It has recently been demonstrated to be an essential stem cell gene in *Drosophila* and *C. elegans* and to be expressed in tissues belonging to many species including human. The *Drosophila piwi* gene is required for asymmetric division of GSCs but is not required for differentiation of committed daughter cells. Expression of *piwi* in adjacent somatic cells, terminal filament cells, regulates GSC division.⁷ We have therefore, embarked on studies of *piwi* homologue gene expression during human hematopoiesis with the hope of determining if this gene might regulate human HSC function.

Our laboratory has demonstrated that the human equivalent of *piwi*, termed *hiwi*, is expressed in a variety of human tissues including primitive hematopoietic cells. The gene was cloned from human marrow CD34⁺ cells and the HIWI protein was shown to have 52% homology with the *Drosophila* PIWI protein at the

amino acid level. In addition, the gene was localized to the q arm of chromosome 12 between 12q24.2 through 12q24.32.

By using 2 different approaches, we have shown that *hiwi* expression within the hematopoietic compartment is unique to the most primitive hematopoietic progenitors and is diminished or absent in more differentiated cells. CD34⁺ marrow cells contain hematopoietic stem and early progenitor cells, which express *hiwi*, in contrast to CD34⁻ cells, which are predominantly composed of more differentiated precursor cells. Although studies in the mouse have recently demonstrated the presence of quiescent stem cells within the CD34⁻ subpopulation, the presence of such a CD34⁻ stem cell in humans remains uncertain.^{27,45} Nevertheless, this CD34⁻ stem cell population would potentially represent an extremely small fraction of the total HSC population. Furthermore, when CD34⁺ cells were placed in conditions that favored differentiation in vitro, *hiwi* gene expression declined synchronously with cellular differentiation. Both groups of studies indicate that *hiwi* expression is unique to the more primitive cellular compartment and that its expression might serve as a genetic marker of progenitor and stem cells.

Because leukemia is frequently accompanied by expansion of the hematopoietic compartment, we anticipated that leukemia cells would express *hiwi*, a gene that is associated with self-replication. To our surprise, when 8 immortalized leukemia cell lines were analyzed, *hiwi* mRNA transcripts were undetectable. These studies suggest that *hiwi* expression may not be a component of the genetic program that accompanies leukemogenesis. By contrast, *hiwi* expression appears to be limited within the hematopoietic compartment to normal CD34⁺ cells. These data indicate that the lack of *hiwi* expression may be a consequence of the leukemic transformation event. Further testing of this potentially important finding will be pursued with primary leukemia cells.

To determine if *hiwi* plays a regulatory role in human hematopoiesis, we attempted to overexpress the *hiwi* gene in an immortalized acute myeloid leukemia cell line, KG1. The biologic activity of *hiwi* was assessed using a standard cell proliferation assay (MTT), which demonstrated that overexpression of the *hiwi* gene product actually caused a decrease in cell proliferation when compared with 2 separate controls. The magnitude of *hiwi* activity was evaluated by direct comparison of cells exposed to the empty vector control and the mock transfection control. Under both of these conditions, KG1 cells were capable of proliferating normally without the presence of the *hiwi* gene or interference from the empty vector control. Comparing the optical density of both controls with the *hiwi*-expressing culture shows a dramatic functional decline in the metabolic machinery of the cells in this culture condition. The overexpression of the *hiwi* gene product promoted apoptosis in KG1 cells. The rapid progression of apoptosis can be seen at the 8-hour time point where a majority of the cells in the *hiwi*-containing population have undergone apoptosis and have started to proceed into the necrotic state. These studies suggest that *hiwi* overexpression causes programmed cell death. Lack of apoptosis in the cells transfected with the *hiwi* gene, which were maintained in selective media, may be due to the SV40 promoter (which drives the transcription of the neomycin phosphotransferase

gene), outcompeting the CMV promoter (which directs the transcription of the *hiwi* gene) because of the selective pressure placed on the SV40 promoter by the G418 selection. This may account for diminished expression of the *hiwi* gene product while the transcription of the neomycin phosphotransferase gene continues. Emerman and coworkers have reported a state of gene suppression in cells genetically modified with a retrovirus in which one gene is shutdown while the second gene undergoes normal transcription due to promoter competition.⁴⁶ Because the KG1 cells were electroporated and put into media that was lacking G418 for the apoptosis experiments, there would be no need for the activation of the SV40 promoter allowing the CMV to transcribe the *hiwi* gene unperturbed, thus allowing for the rapid induction of apoptosis resulting in eventual cell death.

During steady-state hematopoiesis, most stem cells are quiescent or cycling extremely slowly.⁴⁷⁻⁵² Stem cell quiescence may be a passive process involving the absence of proliferation or an active process that occurs as a consequence of a variety of negative inhibitors of hematopoiesis.⁵³⁻⁵⁵ The observation that *hiwi* expression is associated with diminished proliferation of an immortalized leukemia cell line suggests that the expression of this gene might play a role in maintenance of stem cell quiescence or down-regulation of stem cell or progenitor cell cycling. This is somewhat surprising because it has been reported that *piwi* causes cellular division within a *Drosophila*-based model.⁵⁶ Further studies to address the issue of *hiwi* function will be conducted in primitive primary HSCs.

In *Drosophila*, *piwi* is expressed both in the terminal filament cells and the germline. The *piwi* gene in the terminal filament functions to affect stem cell self-replication, whereas *piwi* expression in the germline does not appear to be required for GSC self-replication.⁷ We attempted to determine if a similar relationship existed between marrow stroma cells and HSCs. Many of the regulatory signals that control stem cell development are dependent on cellular interactions between marrow stroma and HSCs.^{17,18} Although *hiwi* was expressed in marrow CD34⁺ cells, it was not expressed by marrow stroma or marrow mesenchymal stem cells, which are capable of differentiating into not only marrow stroma but also other components of the hematopoietic niche such as adipocytes, osteoblasts, tenoblasts and cartilage forming cells.¹⁶ These data indicate that the potential role of *hiwi* in human stem cell development is quite different from that which occurs in the *Drosophila* model. It remains possible that the *hiwi* present in CD34⁺ cells may play a role as an intrinsic regulator of stem cell self-replication. Testing of this hypothesis remains an objective of our ongoing investigations.

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