

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Jamieson CHM, Ailles LE, Dylla SJ, et al. Granulocyte–macrophage progenitors as candidate leukemic stem cells in blast-crisis chronic myelogenous leukemia. *N Engl J Med* 2004;351:657-67.

Supplementary Appendix

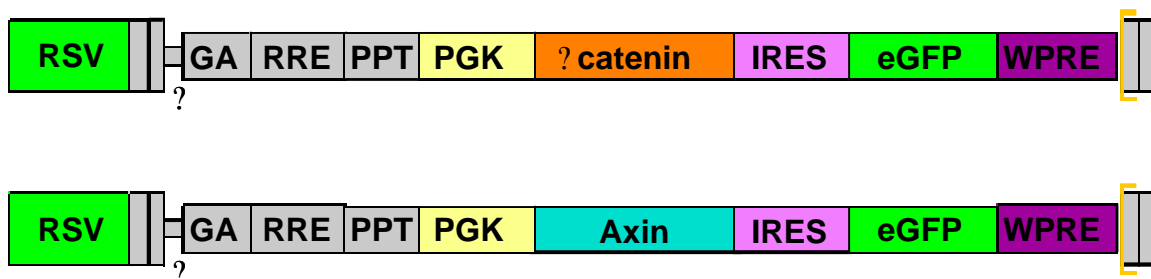
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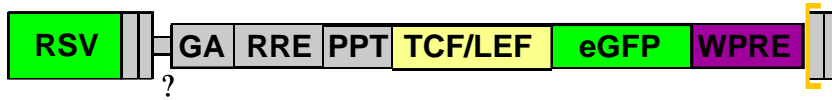
Supplement to:

Supplemental Method 1. Lentiviral Transduction with LEF/TCF-GFP, PGK- β -catenin-IRES-GFP or PGK-Axin-IRES-GFP

Individual colonies were plucked into 50 microliters of myelocult media (H5100, Stem Cell Technologies), gently vortexed and resuspended in methylcellulose (150 microliters) supplemented with human cytokines including stem cell factor (SCF, 10 ng/ml); Flt3 ligand (FL, 10 ng/ml), IL-3 (20 ng/ml), IL-6 (10 ng/ml), IL-11 (10 ng/ml), thrombopoietin (TPO, 50 ng/ml); and erythropoietin (EPO, 4 units/ml) as previously described with or without lentiviral vectors containing LEF/TCF-GFP, phosphoglycerate kinase- β -catenin-IRES-GFP, phosphoglycerate kinase-Axin-IRES-GFP or a phosphoglycerate kinase-IRES-GFP control vector as previously described and cultured in 96 well plates (Falcon) for 14 days in a humidified 37°C 5%CO₂ incubator.⁴¹ Colonies were scored on day 14 and GFP fluorescence was analyzed using a Zeiss inverted fluorescence microscope and photomicrographs were obtained at 40x magnification with the aid of SPOT software.

Lentiviral Vector Constructs:





Supplemental Method 2. Quantitative RT-PCR for *BCR-ABL*, *β-catenin* and *LEF-1*

RNA was isolated from 40 to 300 normal or CML hematopoietic stem cells, common myeloid progenitors, granulocyte/macrophage progenitors, or megakaryocyte/erythroid progenitors.⁴⁸ The *BCR-ABL* TaqMan quantitative RT-PCR reaction was performed with P210 *BCR-ABL* specific primers and a P210 BCR-ABL probe as previously described.⁴⁹

The quantitative RT-PCR assay for *β-catenin*, *LEF-1* and *HPRT* (as a control) was performed using SYBR Green core PCR reagents (Applied Biosystems) and sequence-specific primers (see below) with specific primers including:

	Forward	Reverse
<i>β-catenin</i>	5'AATCAGCTGGCCTGGTTTGA3'	5'GGCCAATCACAATGCAAGTTC3'
<i>LEF-1</i>	5'CCAGAGCATCTTGCATCCAAA3'	5'TTGCGCATGACAGGCAAAT3'
<i>HPRT</i>	5'CGTCTTGCTCGAGATGTGATG3'	5'TTTATAGCCCCCTTGAGCAC3'

on total RNA, isolated using the RNeasy Mini kit (Qiagen), that was reverse transcribed for 50 min at 42°C using Oligo(dT) primers and Superscript™ II reverse transcriptase (Invitrogen) . Amplification was performed with 50 cycles of two-step PCR (15s at 95°C and 60s at 60°C) after initial denaturation (95°C for 10 min) using an ABI Prism 7700 Sequence Detector System (Applied Biosystems). Amplification of HPRT mRNA as an endogenous control was used to standardize reactions across samples. To compare relative target gene expression in the different samples, we designated one of the normal (NL) bone marrow samples as a reference and expressed the averaged sample value as percentage of the reference value.

Supplemental Method 3. Confocal Fluorescence Microscopy

Normal (n=5) or CML (chronic phase; n=2, accelerated phase; n=3, blast crisis; n=4, post-imatinib; n=2, imatinib resistant; n=2) hematopoietic stem cells or granulocyte/macrophage progenitors were FACS sorted onto glass slides. Slides were washed for 5 min in PBS, stained with anti-human CD45-FITC antibody (Anti-Hle-1; BD) for 1 hr, washed in PBS, fixed in 4% paraformaldehyde, for 10 min, and washed for 5 min in PBS/0.1% Tween-20 (PBS-T). Non-specific antibody binding was blocked with 5%

goat serum, 1% BSA, 1:100 Fcγ receptor antibody in PBS-T and PBS for 1 hr, followed by staining for 1 hr with a mouse monoclonal antibody to nuclear-β-catenin as described previously.⁵⁰ Slides were then washed with PBS-T, stained with an Alexa 594-conjugated goat anti-mouse antibody, washed, incubated for 10 min with Hoechst 33342 (Molecular Probes), rinsed with PBS-T, covered with Prolong antifade (Molecular Probes) and a coverslip. Confocal images were obtained with a dual photon Zeiss LSM510 confocal fluorescence microscope at 100x magnification. Excitation and emission spectra were 543 nm and 565–615 nm, respectively for β-catenin-Alexa 594. To visualize Hoechst 33342, a dual photon laser system with a 776 nm excitation wavelength, a beam splitter and a band pass of 435-485 nm was used while the excitation wavelength for CD45-FITC was 488 nm. Three dimensional renderings of confocal images were made with Volocity™ software.

Supplemental Table 1.**Table 1. A) Characteristics of Pre-Imatinib CML Patient Samples**

Pt.	Age	Sex	Type	Phase at Time of Sample Analysis	Prior Therapy
1	76	M	BM	CP, 100% Ph+	IFN-a
2	27	M	BM	CP, 100% Ph+	IFN-a
3	40	F	BM	CP, 100% Ph+	IFN-a/ara-C
4	49	M	BM	CP, 100% Ph+	IFN-a /Hydrea
5	46	M	BM	AP, 81% Ph+/clonal evolution trisomy 8	IFN-a
6	66	M	BM	AP, 100% Ph+/clonal evolution	IFN-a /ara-C/ homoharringtonine
					+
					ara-C/Hydrea
7	53	M	BM	AP, 100% Ph+/clonal evolution trisomy 8	IFN-a /Hydrea/6-M

8 71 M PB AP, 100% Ph+

IFN-a /Hydrea

Table 1. A) Characteristics of Pre-Imatinib CML Samples Analyzed for BCR-ABL Expression

9	63	F	BM	AP, 100% Ph+	Hydrea + ATRA
10	32	M	PB	AP, 100% Ph+/clonal evolution	Hydrea
11	41	M	PB	AP	Hydrea
12	62	M	BM	Myeloid BC, 100% Ph+ /clonal evolution	IFN-a /Hydrea
13	81	F	PB	Myeloid BC, 100% Ph+	none
14	67	F	PB	Lymphoid BC, 100% Ph+	Hydrea/ara-C/ Vincristine/ prednisone

Table 1. B)**Characteristics of Post-imatinib CML Samples**

Pt.	Age	Sex	Type	Phase at Time of Sample Analysis	Prior Therapy
1	76	M	BM	CP CHR, 100% Ph+ after imatinib	IFN-a /Hydrea imatinib (6 months)
2	65	M	BM	CP + CHR, 95% Ph+ after imatinib	homoharringtonine ara-C/ IFN-a imatinib (15 months)
3	27	M	BM	CP CHR and CCR after imatinib	IFN-a /Hydrea/ alloBMT imatinib (6 months)
4	54	M	PB	AP CHR and 80% Ph+ after imatinib	IFN-a /Hydrea imatinib (6 months)

Table 1. C) Characteristics of Imatinib resistant CML Samples

Pt.	Age	Sex	Type	Phase at Time of Sample Analysis	Prior Therapy
1.	65	F	PB	AP CHR, 100% Ph+	IFN-a /Hydrea imatinib+/-FTI (2 months)
2	74	F	PB	AP Hematologic Relapse, 100% Ph+	IFN-a /Hydrea/ imatinib + ara-C+ Hydrea (30 months)
3	58	M	PB	AP Hematologic Relapse, 100% Ph+	Hydrea/alloBMT/ imatinib (21 months)

4	43	M	BM	AP	IFN-a /Hydrea/ alloBMT imatinib + ara-C imatinib + FTI (10 months)
				CHR, 100% Ph+	
				clonal evolution, trisomy 8	

Table 1. C) Characteristics of Imatinib resistant CML Samples

Pt.	Age	Sex	Type	Phase at Time of Sample Analysis	Prior Therapy
5	62	M	BM	a) AP Hematologic Relapse, 100% Ph+	IFN-a /Hydrea imatinib + arsenic (1 year)
			PB	b) BC Persistent Hematologic Relapse 100% Ph+ and Clonal Evolution trisomy 8	imatinib + FTI (2 months)

BM=bone marrow; PB=peripheral blood; CP = chronic phase; AP=accelerated phase;

BC=blast crisis; Ph+=Philadelphia chromosome positive; CHR=complete hematologic

remission; FTI=farnesyl transferase inhibitor; alloBMT=allogeneic bone marrow
transplant; IFN-a=interferon alpha; ara-C=cytosine arabinoside

Supplemental Figure Legends

Supplemental Figure 1. A) The average percentage of CD34⁺ cells in the lineage negative fraction (\pm S.E.M.) from normal bone marrow (n=11) versus chronic phase CML (n=5), accelerated phase CML (n=6, P<0.05), blast crisis CML (n=4, P<0.05), post-imatinib CML (n=6, P<0.05) and imatinib resistant CML (n=4) samples. B) Typical myeloid progenitor profiles, gated on CD34⁺CD38⁺Lin⁻ cells, from normal bone marrow, chronic phase CML, accelerated phase CML and blast crisis CML. C) Myeloid progenitor profiles, gated on CD34⁺CD38⁺Lin⁻ cells, from pre- and post-imatinib CML samples

Supplemental Figure 2. Model of the canonical Wnt signaling pathway. In the absence of dickkopf 1 (Dkk)/Kremen (K) inhibition, LDL-receptor related protein 6 (LRP6) binds to the Wnt/Frizzled (Fz) complex and transduces a signal through dishevelled (Dvl) resulting in dephosphorylation of cytoplasmic β -catenin protein and dissociation from the axin/APC/GSK complex. Non-phosphorylated (activated or stabilized) β -catenin then translocates to the nucleus and binds to the LEF/TCF transcription factor complex where it mediates a change in gene expression and cell fate.

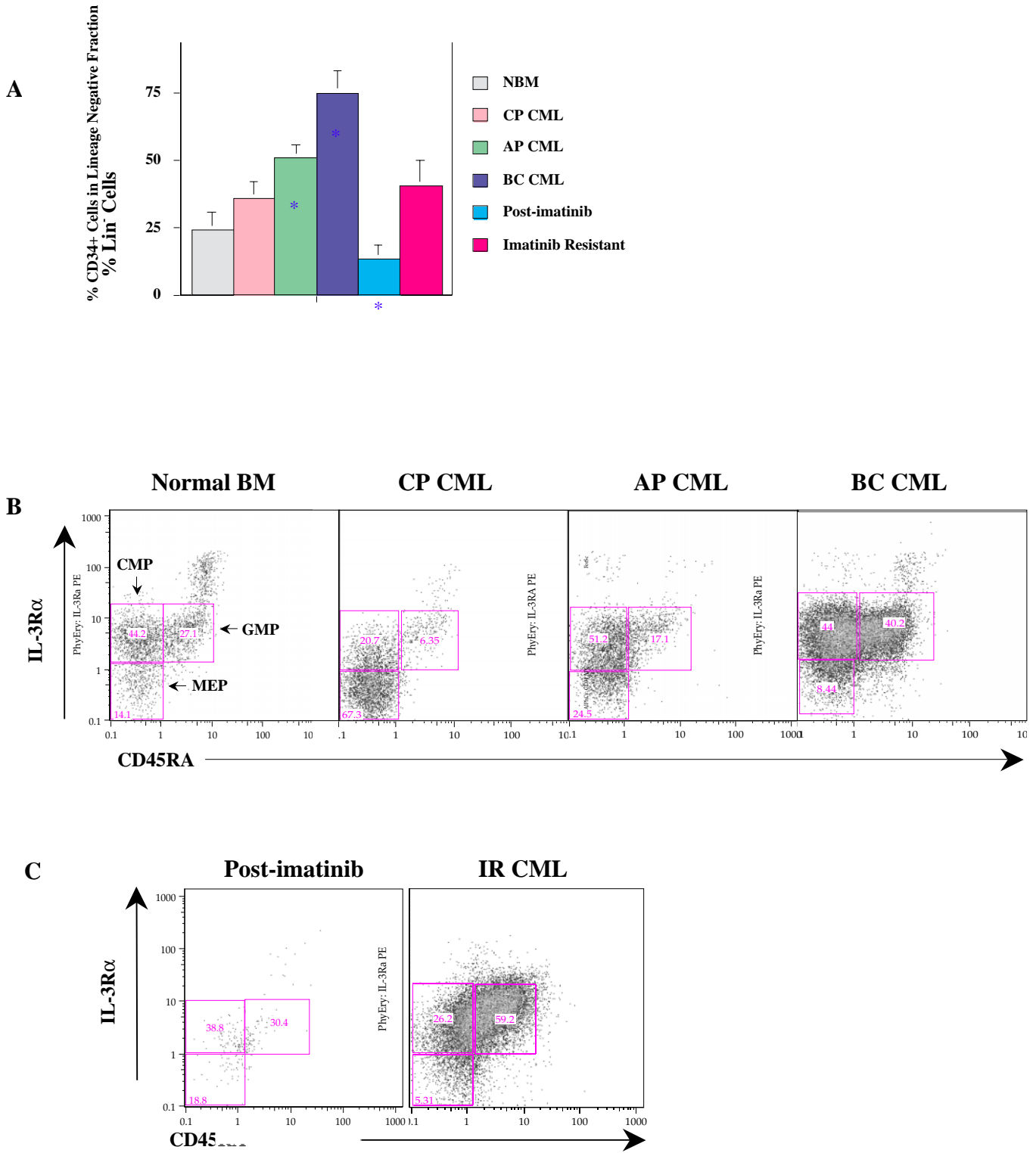
Supplemental Figure 3. LEF/TCF-GFP reporter assay of activated β -catenin in colonies derived from normal (left) or blast crisis CML (right) CD34⁺Lin⁻ cells.

Supplemental Figure 4. β -catenin (upper panel) and LEF-1 (lower panel) transcript levels in hematopoietic stem cells or granulocyte/macrophage progenitors from normal bone marrow, or chronic phase, blast crisis or imatinib resistant CML patients. Transcript levels were internally controlled with HPRT, and shown relative to normal bone marrow hematopoietic stem cells.

Supplemental Figure 5. Colony area (μm^2) of control accelerated phase CML colonies (left), compared with β -catenin (center) and axin (right) transduced colonies.

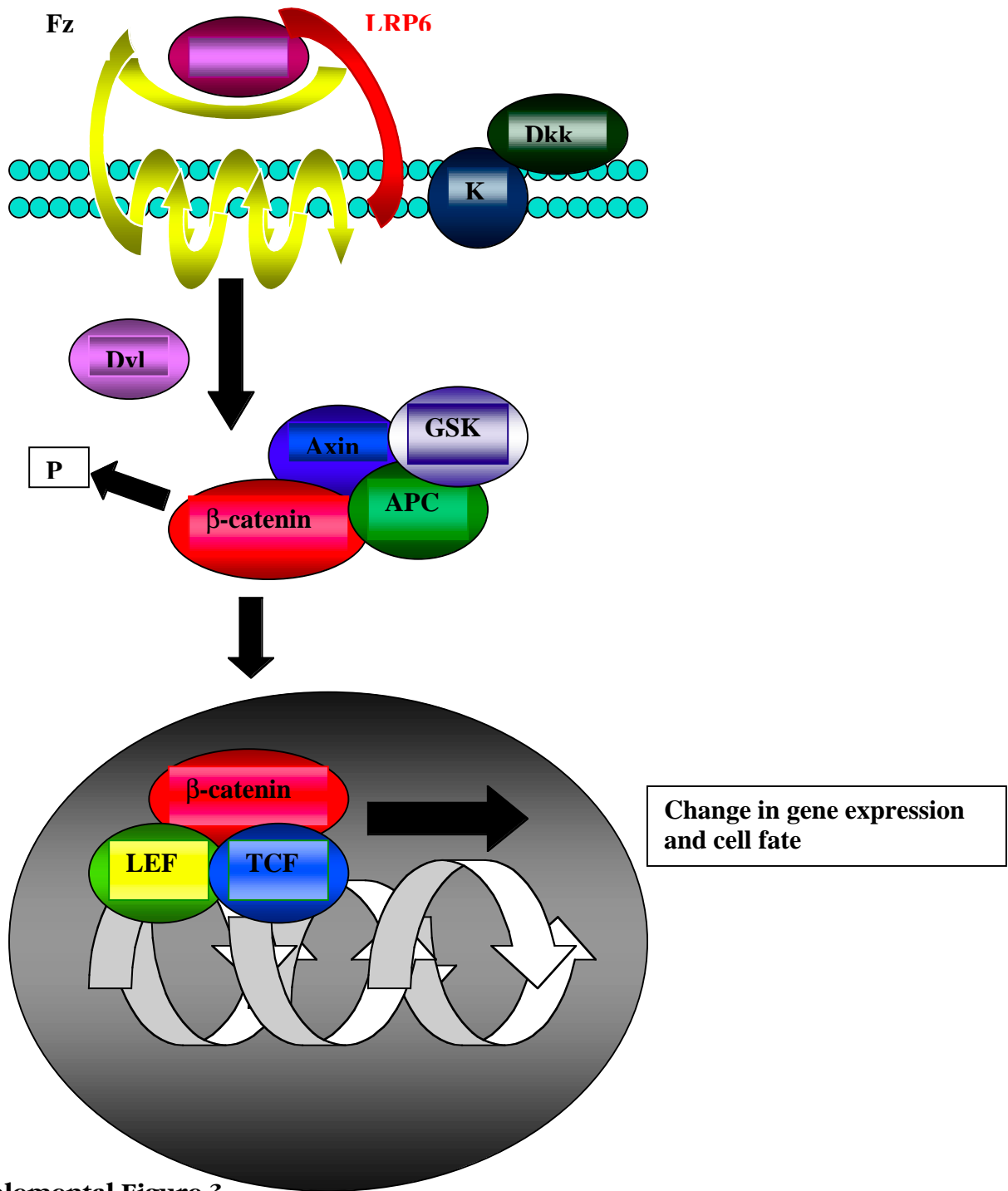
Photomicrographs were obtained at 40x magnification with the aid of a Zeiss inverted microscope and Spot software which was also used to measure colony area.

Supplemental Figure 1.



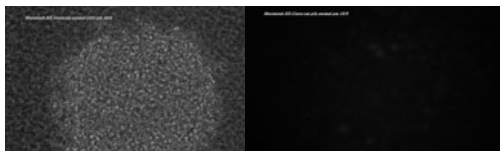
Supplemental Figure 2.

Fz=Frizzled Receptor
LRP6=LDL-receptor related protein 6

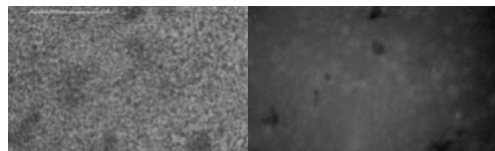


Supplemental Figure 3.

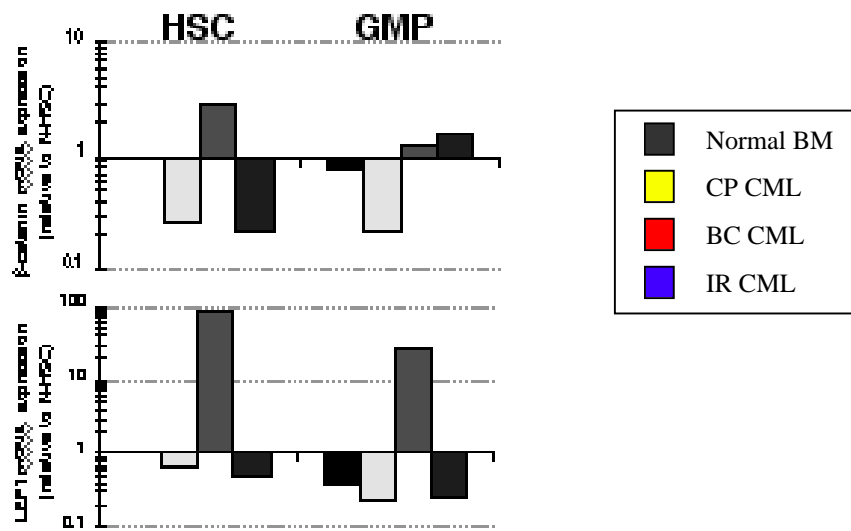
Normal CD34+Lin- CFC GFP



CML BC CD34+Lin- CFC GFP



Supplemental Figure 4.



Supplemental Figure 5.

