The fetal liver lymphoid-primed multipotent progenitor provides the prerequisites for the initiation of t(4;11) *MLL-AF4* infant leukemia

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Supplementary Material and Methods

Mice

The animal work was done under regulation of the UK Home Office. Males and females from the Mll-AF4[1] and the VEC-Cre[2] line were mated to obtain Mll-AF4-expressing embryos. The day of the plug was counted as day 0 of embryonic development.

Cell sorting of HSC/MPP, LMPP and LK/CLP populations

The fetal liver was dissected and dissociated in Flow Cytometry Staining Buffer (ThermoFisher Cat# 00-4222-26) using a 21Gx15mm needle attached to a syringe (BD Microlance Cat# 10472204-X, BD Cat# 3000185). Cells were stained using the following antibody mix in Flow Cytometry Staining Buffer: APC anti-mouse CD3ε antibody (clone I45-2C11, Biolegend Cat# 100312), APC anti-mouse TER-119 antibody (clone TER119, Biolegend Cat# 116212), APC anti-mouse F4/80 antibody (clone BM8, Biolegend Cat# 123116), APC anti-mouse Nk1.1 antibody (clone PK136, Biolegend Cat# 108709), APC anti-mouse Ly-6G/Ly-6C (Gr-1) antibody (clone RB6-8C5, Biolegend Cat# 108412), PE/Cy7 anti-mouse/human CD45R/B220 antibody (clone RA3-6B2, Biolegend Cat# 103222), PE/Cy7 anti-mouse CD19 antibody (clone 6D5, Biolegend Cat# 115520), APC-eFluor 780 CD117 (ckit) antibody (clone 2B8, ThermoFisher Cat# 47-1171-80), Alexa Fluor® 700 anti-mouse CD45 antibody (clone 30-F11, Biolegend Cat# 103128), Pacific Blue™ anti-mouse Ly-6A/E (Sca-1) antibody (Clone E13-161.7, Biolegend Cat# 122519), PE anti-mouse CD127 antibody (clone A7T34, ThermoFisher Cat# 12-1271-82), biotin anti-mouse CD135 (Flt3) antibody (clone A2F10, ThermoFisher Cat# 13-1351-81). Cells were stained for 20 minutes on ice and washed once with Flow Cytometry Staining Buffer. Cells were then resuspended in diluted Qdot 655 Streptavidin Conjugate (ThermoFisher Cat# Q10123MP) and incubated for 20 minutes on ice. Cells were washed once and resuspended in diluted SYTOX™ Green Nucleic Acid Stain (ThermoFisher Cat# S7020) to exclude dead cells. Sorting was done on a BD FACSAria™ II (BD Biosciences).

Transplantation of CD45.1/2 mice with HSC/MPP, LMPP and LK/CLP

On the day of transplant, recipient mice (CD45.1/2) received two doses of 4.6 Gy at a 3 hours interval. Donor cells (CD45.2/2) were injected through the tail vein along with bone marrow helper cells (CD45.1/1). For HSC/MPP transplants, we used 100 000 helper cells and for LMPP and LK/CLP, we used 20 000 helper cells. Mice were administered antibiotics after transplantation through their drinking water (0.1 mg/mL enrofloxacin, 10% Baytril solution from Bayer). For secondary transplants, the number of bone marrow cells transplanted was adjusted according to the repopulation in primary recipient (85% repopulation in primary recipient, 2 x 10^6 total bone marrow cells injected). Mice were bled on a monthly basis, and blood counts were measured on a Celltac MEK-6500K (Nihon Kohden). Red blood cell lysis was achieved with BD Pharm Lyse™ lysing solution according to the manufacturer's instructions (BD Biosciences Cat# 555899). Cells were stained in Flow Cytometry Staining Buffer using the following antibodies: FITC CD45.2 antibody, (clone 104, ThermoFisher Cat# 11-0454-81), APC-eFluor 780 CD45.1 monoclonal antibody (clone A20, ThermoFisher Cat# 47-0453-80), eFluor450 CD11b monoclonal antibody (clone M1/70, ThermoFisher Cat# 48-0112-80), Alexa Fluor® 700 Ly-6G/Ly-6C (Gr-1) antibody (clone RB6-8C5, Biolegend Cat# 108422), PE/Cy7 CD45R/B220 antibody (clone RA3-6B2, Biolegend, Cat# 103222), Brilliant Violet 605™ CD19 antibody (clone 6D5, Biolegend Cat# 115539), APC mouse IgM monoclonal antibody (clone II/41, ThermoFisher Cat# 17-5790-82), PE CD3e (clone 145-2C11, Biolegend Cat# 100308). For sorting/analysis of hematopoietic stem and progenitor cells in organs and analysis of B cell populations in primary and secondary recipients, we used the following antibodies: the APC lineage cocktail from the sorted E14 FL cells, FITC CD45.2 antibody, (clone 104, ThermoFisher Cat# 11-0454-81), APC-eFluor 780 CD45.1 monoclonal antibody (clone A20, ThermoFisher Cat# 47-0453-80), Brilliant Violet 421™ CD117 (c-Kit) antibody (clone 2B8, Biolegend Cat# 105827), APC-eFluor 780 CD117 antibody (clone 2B8, ThermoFisher 47-1171-82), PE/Cy7 Ly6A/E (Sca-1) antibody (clone E13-161.7, Biolegend Cat#122513), PB Sca1 antibody (clone E13-161.7, Biolegend, Cat# 47-1171-82), PerCP/Cy5.5 CD34 antibody (clone HM34, Biolegend, Cat# 128607), PE CD135 antibody (clone A2F1, Biolegend, Cat# 135306), biotin anti-mouse CD135 (Flt3) antibody (clone A2F10, ThermoFisher Cat# 13-1351-81), Alexa Fluor® 700 CD48 antibody (clone HM48-1, Biolegend, Cat# 103425), PE/Cy7 CD150 antibody (clone TC15-12F12.2, Biolegend, Cat# 115914), Alexa

Fluor® 700 CD45R/B220 antibody (clone RA3-6B2, Biolegend, Cat# 103232), PE/Cy7 CD19 antibody (clone 6D5, Biolegend, Cat# 115520), PerCP CD43 antibody (clone 1B11, Biolegend, Cat# 121222), Brilliant Violet 421™ CD24 antibody (clone M1/69, Biolegend, Cat# 101825), APC CD127 antibody (clone A7R34, Biolegend, Cat# 135011). Cells were incubated on ice for 20 minutes, washed twice with Flow Cytometry Staining Buffer and resuspended in diluted SYTOX AADvanced (ThermoFisher, Cat# S10274) to exclude dead cells. Data was acquired on a BD LSRFortessa™ (BD Biosciences). For end of study analysis, cell types were identified as follows: HSCs (LSK CD34+/- FLT3- CD150+ CD48-), LMPPs (LSK CD34+/- FLT3+), CLP (Lin- ckitlow Sca1low IL7R+ FLT3+), LK (Lin- ckit+ Sca1-), pre-pro-B (CD45.2+ ckit- CD43+ CD24low B220+ CD19-) and pro-B (CD45.2+ ckit+ CD43+ CD24+ B220+ CD19+).

Cell Cycle

Sorted HSC/MPP, LMPP and LK/CLP cells were collected in Flow Cytometry Staining Buffer and an equivalent volume of 5 µg/mL DAPI 1% IGEPAL (Sigma-Aldrich D9542 and CA-630) solution was added. Cells were incubated at room temperature for 1 minute, in the dark. Data was acquired on a BD LSRFortessa™ (BD Biosciences).

RNA extraction, reverse transcription and quantitative PCR

RNA extraction and reverse transcription were performed using the RNeasy Micro Kit (QIAGEN Cat# 74004) and iScript Ready-to-Use cDNA Supermix (Bio-Rad Laboratories Ltd Cat# 1708841) according to the manufacturer's instructions. Primer sequences are: *Flt3*.F/R (gccagttcagcccgccta/agattccctcggactggtgc), *Meis1*.F/R (attcacactgctggagacgc/cgtcgtacctttgcgccatc), *Cdk6*.F/R (ccaacgtggtcaggttgttt/gccgggctctggaactttat), *Mcl1*.F/R (agaggctgggatgggtttgt/ccctattgcactcacaaggc), *Twist1*.F/R (gccggagacctagatgtcatt/ccacgccctgattcttgtga), *Runx1*.F/R (ttcgcagagcggtgaaagaa/tgctgtctgaagccatcgtt), *Hoxa9*.F/R (ccacgcttgacactcacact/cagccgggttattgggatcg), *Hmga2*.F/R (gcctgctcaggagactgaag/ggaagtagaaagaccgtggca), *Bcl2*.F/R (ggaggctgggatctttgt/acttgtggcaggtatgc), *Lmo2*.F/R (ctggacccgtctgaggaac/gacacccacagaggtcacag), *Ikaros*.F/R (ccctgaggacctgtccactac/acgcccattctcttcatcac), *Il7r*.F/R

(cgaaactccagaacccaaga/aatggtgacacttggcaagac), *E2a*.F/R (aagaggacaagaaggacctgaa/ttattggccatacgcctctc), *Pax5*.F/R (accatcaggacaggacatgg/gcggactacatctgggagtg), Mll-AF4 F/R (agtgggcatgtagagggatc/atggctcagctgtactaggc) and beta-actin F/R (tcctgtcctcactgtcca/gtccgcctagaagcacttgc). Quantitative PCR was carried out with Brilliant III Ultra-Fast SYBR QPCR (Agilent Technologies Cat#600883) according to the manufacturer's instructions. Data was acquired on a QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher).

Data analysis, statistics and graphs

Analysis of flow cytometry data was performed with FlowJo (version 10) and graphs were generated with GraphPad Prism (version 6). Statistical analysis was performed with GraphPad using a non-parametric t test (Mann-Whitney) with a bi-lateral p-value. Data are presented as Mean ± SEM.

References for the supplementary

1. Metzler M, Forster A, Pannell R, et al. A conditional model of MLL-AF4 B-cell tumourigenesis using invertor technology. Oncogene. 2006;25(22):3093-3103.
2. Chen MJ, Yokomizo T, Zeigler B, Dzierzak E, Speck NA. Runx1 is required for the endothelial to hematopoietic cell transition but not thereafter. Nature. 2009;457(7231):887-891.

Supplementary Figure 1

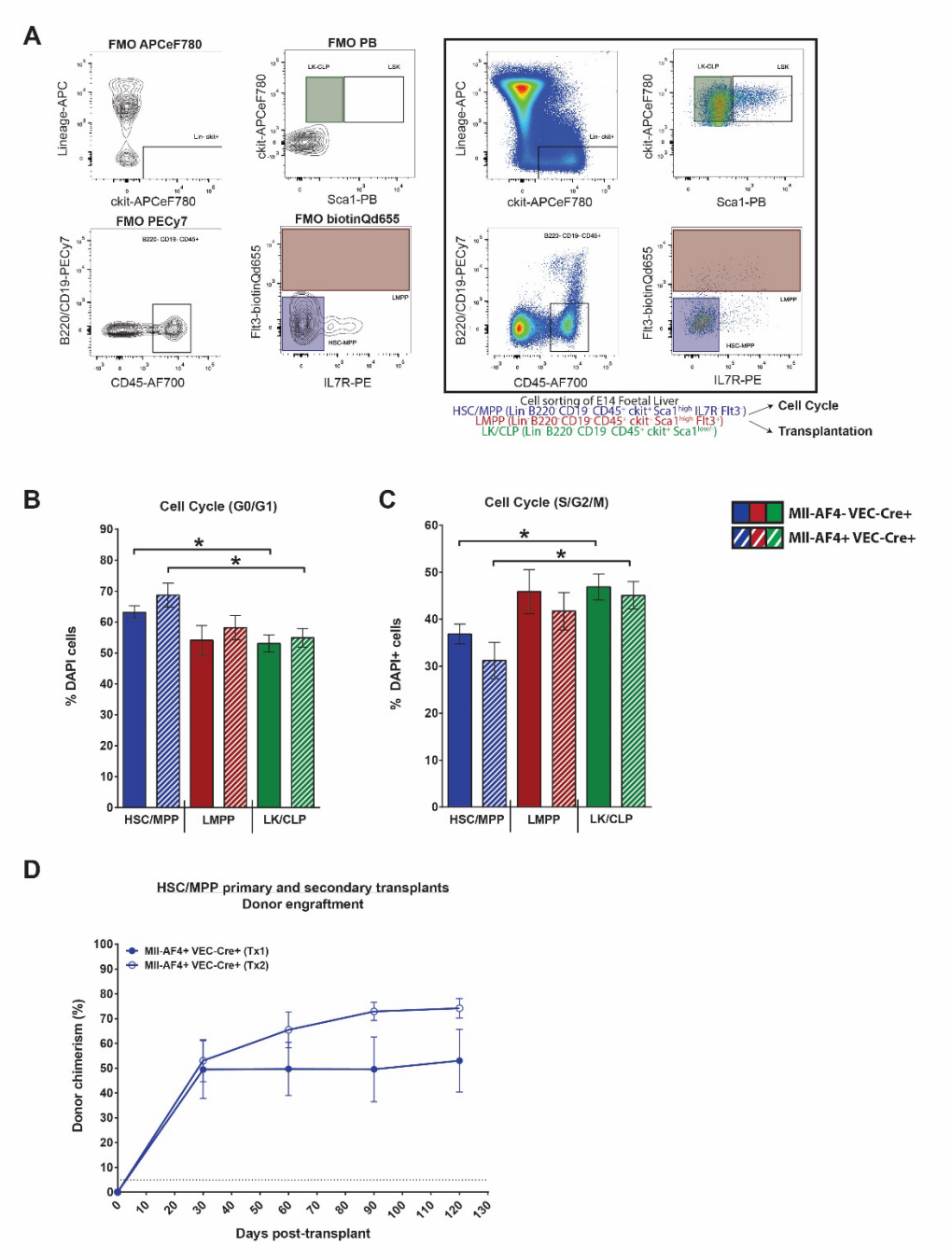

Figure S1. Sorting strategy and cell cycle analysis of E14FL HSC/MPP, LMPP and LK/CLP cells (A) Sorting strategy of E14 FL HSC/MPP (Lin- B220- CD19- CD45+ ckit+ Sca1high IL7R- Flt3-), LMPP (Lin- B220- CD19- CD45+ ckit+ Sca1high Flt3+) and LK/CLP (Lin- B220- CD19- CD45+ ckit+ Sca1low/-). (B,C) Cell cycle analysis (n = 5-7). HSC – hematopoietic stem cell, MPP – multipotent progenitor, LMPP - lymphoid-primed multipotent progenitor, LK – Lin- ckit+, CLP – common lymphoid progenitor. (D) Repopulation of Mll-AF4+ VEC-Cre+ E14 FL HSC/MPPs in primary and secondary recipients.