Transgenic mouse production

To generate the TRE-surv transgene construct, mouse survivin cDNA was excised with BamHI and HindIII from the previously described K14-survivin vector (a generous gift from Dr. Dario Altieri).[1] The pBI-G vector (Invitrogen) was cut with SalI and the survivin cDNA was blunted and cloned into the linearized pBI-G vector. For additional transgenic mRNA stability, a 2.1 kb fragment of the 3′ end of the human-globin gene (including the 3′ intron and a poly(A) signal; base pairs 71022-72711 from the translation start of the clone, described in GenBank accession number NT_000007) was cloned downstream of the survivin cDNA using the HindIII and BstEII cut sites. Vector sequences were removed by digest with BstEII and AseI and a 7.8 kb linearized sequence was gel purified and cleaned for injection using a Geneclean Turbo kit (Q-Biogene, Inc.). Transgenic founders were generated by the Boston University Trangenic Core via pronuclear injection using FVB strain mice from Jackson Laboratories, Bar Harbor, ME. Positive transgenic founders were identified through Southern blot analysis of genomic tail DNA using a radiolabeled survivin cDNA probe. Genotyping was also confirmed via PCR of genomic tail DNA using transgene specific primers; sense, 5′-gaggctggcttcatccactg-3′ and antisense, 5′-ctttgcaattttgttcttggctc-3′, amplifying a 300 bp survivin PCR product. The PF4-tTA activator mouse was established as described by Nguyen et al[2] and genotyped via Southern blot using a radiolabeled tTA-VP16 probe.[2,3] PF4-tTA transgenic mice were also identified by PCR. The primers for genotyping PF4-tTA transgenic mice were; sense, 5′-gcttaatgaggtcggaatcg-3′ and antisense, 5′-gcgacttgatgctcttgatc-3′, amplifying a 400 bp tTA-VP16 PCR product.[2] Heterozygous TRE-survivin and PF4-tTA mice were crossed and resulting litters were genotyped via PCR as described above. Double transgenic mice, PF4-tTA+/TRE-survivin+ (PF4-surv) were used for analyses. Wild type littermates were used as controls.

To generate the GATA1-survivin transgene, the human survivin cDNA was inserted within an engineered unique Not I site within exon 2 of GATA1, which lies between 7 kb of GATA-1 upstream regulatory sequences and approximately 3.0 kb of sequences downstream of exon 2, including exons 3, 4, and 5. The rabbit globin poly (A) region and SV40 sequence were inserted at the 3′ end of the survivin insert to provide polyadenylation signals and a specific transgene detection element.[4] After confirming the sequence of the survivin gene and the regions at the junction between the GATA-1 regulatory elements and the insert, we prepared linear DNA by removing vector sequences by digestion with Age I and Kpn I and purifying the DNA by gel electrophoresis, electroporation and purification through an elutip column (Schleicher-Schuell). DNA was injected into oocytes of the CD1 strain were performed by the University of Chicago Transgenic Core Facility. From these injections, we obtained 7 transgenic founders that harbor the transgene, as determined by PCR of tail DNA. Primers used to genotype these mice include: sense, 5′-ctgtgtctcacaacccttc-3′ and antisense, 5′-ccatcgatggtcaatccatggcagccagc-3′. Wild type littermates were used as controls in all studies.

Megakaryocyte enrichment by MACS® magnetic bead purification system

This method was used to purify MKs for analysis by western blot. BM from two mice in each group was cultured for 3 days in the presence of TPO. Cells were spun down and washed twice with staining buffer (0.5% BSA, 2 mM EDTA, and PBS, pH 7.2). BM was then labeled with MK specific antibody, anti-CD41-FITC (BD Pharmingen, cat# 553848) using a 1:200 dilution in staining buffer and incubated on ice for 20 minutes. Following two washes in staining buffer, BM cells were resuspended in 270 µl of buffer and 30 µl of anti-FITC labeled microbeads (Miltenyi Biotech, cat# 37-048-701). After a 15 minute incubation on ice, cells were washed,

spun down, and resuspended in 500 µl of buffer, before loading onto an equilibrated large cell separation column (Miltenyi Biotech, cat# 130-042-202) fitted with a 25 gauge needle for flow resistance. The column was washed three times with buffer and unlabeled BM cells were collected. The column was then removed from the magnetic stand and bound CD41-FITC labeled MKs were eluted with 1 mL of buffer. Cells were immediately spun down and pellets were snap frozen in liquid nitrogen. Pellets were stored at −80°C until use via western blot analysis.

Flow cytometry

Single cell suspensions from bone marrow or spleen were treated with hypotonic KCl to lyse red cells. The remaining cells were washed and stained in PBS+1% BSA + 5% NGS on ice for 1 hour with either anti-CD41-FITC, anti-Ter119-PE/anti-CD71-FITC, anti-Mac1-FITC/anti-Gr1-PE, or anti-c-kit-APC (BD Pharmingen), washed, and analyzed on a BD FacsCanto or LSRII. Data were analyzed using FlowJo software (Treestar). For the analysis of survivin expression in erythroid cells in transgenic animals, single cell suspensions of bone marrow from GATA-1-surv mice and wild type littermates were treated with hypotonic KCl to lyse red cells, washed and stained in PBS+1% BSA + 5% NGS on ice for 1 hour with anti-Ter119-PE, and then sorted by FACS using a MoFlo (Cytomation).

Megakaryocyte Colony Assays

Single cell suspensions were obtained from the bone marrow of 6–8 week old transgenic and non-transgenic littermates by flushing the femurs with cold PBS+1% BSA. Red cells were lysed in hypotonic KCl and nucleated cells were washed and plated (30,000 per ml) in MegaCult-C for CFU-MK (Stem Cell Technologies, Inc, Vancouver, Canada). Cells were cultured for 7 days at 37 degrees with 5% CO2. Following incubation, CFU-MK slides were then fixed and stained with acetylcholiniodide according to the manufacturer's specification and acetylcholinesterase positive colonies were enumerated.

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