Contractile Forces Sustain and Polarize Hematopoiesis from Stem and Progenitor Cells

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SUMMARY

Self-renewal and differentiation of stem cells depend on asymmetric division and polarized motility processes that in other cell types are modulated by nonmuscle myosin-II (MII) forces and matrix mechanics. Here, mass spectrometry-calibrated intracellular flow cytometry of human hematopoiesis reveals MIIB to be a major isoform that is strongly polarized in hematopoietic stem cells and progenitors (HSC/Ps) and thereby downregulated in differentiated cells via asymmetric division. MIIA is constitutive and activated by dephosphorylation during cytokine-triggered differentiation of cells grown on stiff, endosteum-like matrix, but not soft, marrow-like matrix. In vivo, MIIB is required for generation of blood, while MIIA is required for sustained HSC/P engraftment. Reversible inhibition of both isoforms in culture with blebbistatin enriches for long-term hematopoietic multilineage reconstituting cells by 5-fold or more as assessed in vivo. Megakaryocytes also become more polyploid, producing 4-fold more platelets. MII is thus a multifunctional node in polarized division and niche sensing.

INTRODUCTION

Stem cells must be able to self-renew and also give rise to diverse cell types by asymmetric division in appropriate microenvironments (Knoblich, 2010). This differential segregation of cell fate determinants produces progenitors that expand symmetrically to generate tissue. Hematopoietic stem cells (HSCs, as a subset of $CD34^+$ cells) exemplify these key properties of stem cells in that they are often quiescent in niches of the bone marrow (BM), but they and/or their daughter cells polarize and divide asymmetrically in suitable niches to generate progenitors that further divide and specialize to terminally differentiated erythroid, megakaryocyte, and white cell lineages. A number of models for marrow and soluble signal regulation of HSC maintenance and differentiation have been described (Trumpp et al., 2010), but many physical aspects of hematopoiesis remain unclear. Many cell types apply forces to the matrix that they adhere to, and the flexibility of extracellular matrix is already known to modulate differentiation of marrow-derived mesenchymal stem cells (MSCs) (Engler et al., 2006) as well as the expansion of adult HSCs and progenitors (HSC/Ps) (Holst et al., 2010). In both of these latter studies, myosin-II (MII) inhibition revealed a key role for actomyosin forces in adhesion and sensing of matrix. However, cell contractile forces contribute to many processes in stem cell and progenitor maintenance and division with likely relationships to differentiation.

Cytokinesis is driven by nonmuscle MII in a cell's cortex, and the asymmetry of stem cell division in *C. elegans* is also established by MII (Ou et al., 2010). Differentiation in embryogenesis indeed requires active MII (Conti et al., 2004), and while inhibition of MII in adherent embryonic stem cells (ESCs) increases survival in culture by preserving intercellular contacts (Chen et al., 2010), inhibition can also lead to multinucleated cells (Canman et al., 2003). Actomyosin forces generally stabilize the plasma membrane with an active cortical tension or rigidity (Merkel et al., 2000), but these forces also drive cell rounding in cytokinesis (Sedzinski et al., 2011) and can change dramatically in differentiation (of MSCs) (Engler et al., 2006). Indeed, while it has been known for many years that as granulocytes differentiate they become soft to better traffic from marrow through the endothelial barrier and into the circulation (Lichtman, 1970), any MII changes in such cells leaving the marrow or in other hematopoietic cells is currently unknown.

Mammals express three isoforms of MII: A (*MYH9*), B (*MYH10*), and C (*MYH14*), and each is regulated transcriptionally as well as posttranslationally. MIIA is found in most tissues (Ma et al., 2010) including blood (Maupin et al., 1994) and is essential to embryonic differentiation (Conti et al., 2004). MIIB is particularly enriched in brain and cardiac tissues, and it is often polarized to the rear of migrating cells (Vicente-Manzanares et al., 2008; Raab et al., 2012). Recent studies have revealed roles for MIIB in hematopoiesis, specifically in megakaryocyte (MK) differentiation (Lordier et al., 2012) and in the asymmetric process of erythroid enucleation (Ubukawa et al., 2012). MIIB myofilaments are known to attach more strongly to and detach

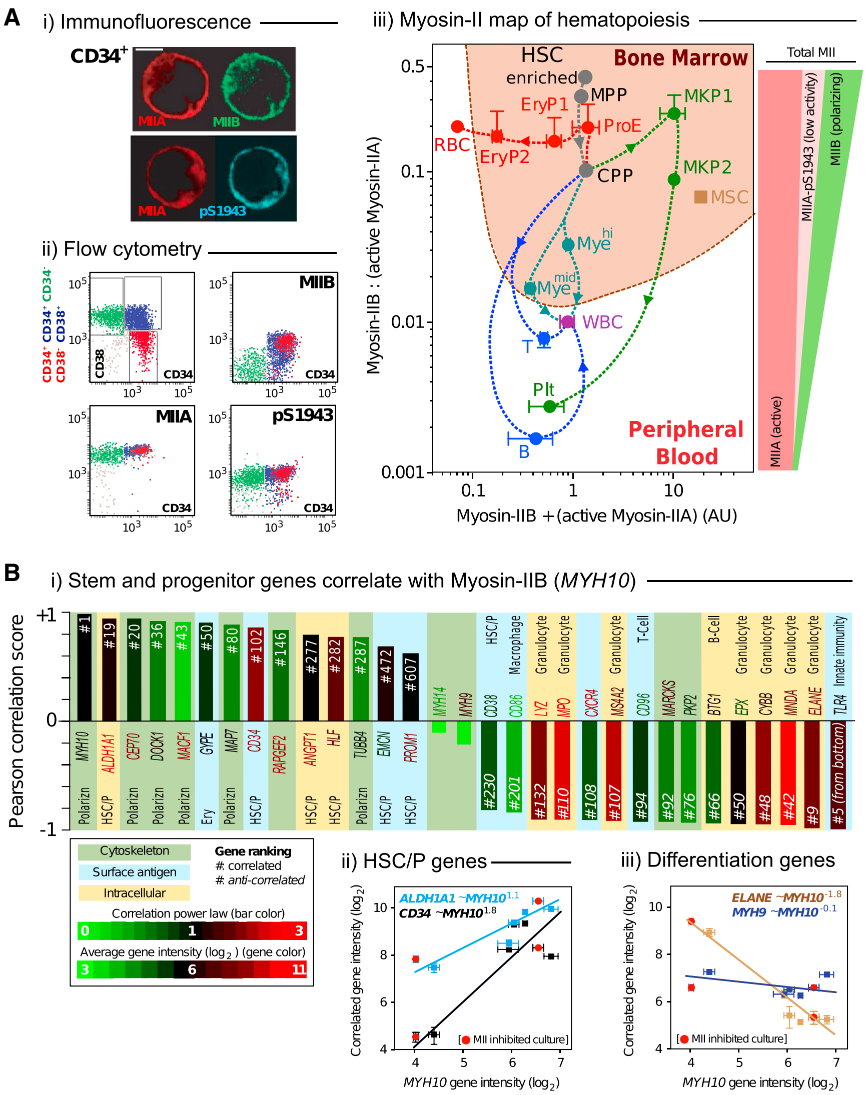

Figure 1. Two-Component Lineage Trajectories of MII Isoform States in Hematopoiesis

(A) MIIB relative to active fraction of MIIA (non-phosphorylated MIIA), transformed to a measurable B:A ratio versus sum total intensity (a.u.). (Ai) Images of coimmunostained MIIA and MIIB (bars = 5 μm). (Aii) Representative intracellular FACS dot plots show expression of MIIA, pS1943, and MIIB (y axis) across subpopulations (markers indicated in x axis). (Aiii) Mean fluorescent intensity of MIIs for each subpopulation from flow cytometry was normalized to an internal fluorescence control (A549), and B:A was calibrated to an absolute ratio from mass spectrometry analyses of MSCs (B:A = 6:94). The perforated endothelium schematically illustrates the permeable barrier between bone marrow and circulating cells. MKP, MK Progenitor 1 (CD34$^+$CD41$^+$), 2 (CD34$^-$CD41$^+$); ProE, Proerythroblast (CD44$^+$GPA$^-$); EryP, Erythroid Progenitor 1 (CD44$^+$GPA$^+$), 2 (CD44$^-$GPA$^+$); Plt, Platelet; T, B, Lymphoid; Mye^{mid}, Mye^{hi}, Bone marrow CD33$^+$ myeloid. WBC: Mean result for PB. Mean ± SEM of n $\geq$ 3, with errors bars omitted if <5% of mean.

(B) Key genes correlated with *MYH10* and ranked by |Pearson correlation| > 0.75 or fit with a power-law. (Bi) Data sets were derived from RMA summarized microarray analyses of fresh populations of HSC-enriched, MPP, CPP, and cultured CD34$^+$-derived cells control or treated with Blebb (see Supplemental Experimental Procedures). Colors in bar graphs and gene symbols respectively represent power law exponents or gene intensities, and they are normalized by minimum levels (green: 0 or $\log_2 3$) and maximum levels (red: 3 or $\log_2 11$) of correlated genes using *MYH10* as a reference (black: 1 or $\log_2 6$). Representative correlation plots between *MYH10* and HSC/P (Bii) or differentiation (Biii) gene markers are shown (mean ± SEM of n $\geq$ 2).

See also Figure S1, Table S1, and Table S2.

more slowly from F-actin than MIIA, resulting in higher force generation per MIIB (Wang et al., 2003). However, MIIB in human ESCs or stem cells in general has unknown functions. MIIC appears restricted to epithelial cells (Ma et al., 2010) and serves here as a useful negative control in expression analyses. Here, we reveal critical roles of MIIA and MIIB in adult hematopoiesis and use that understanding to enrich highly heterogeneous HSC/Ps for long-term hematopoietic stem cells.

RESULTS

MII Isoforms Switch from B-and-A to Only A in Human Adult Hematopoiesis

Immunofluorescence of human CD34$^+$ cells reveals cortical MIIB as well as MIIA (Figure 1Ai), but flow cytometry and immunoblots show that myosin levels vary with surface markers and also across differentiated lineages (Figure 1Aii, Figures S1A and S1B, available online). Mass spectrometry-calibrated intracellular flow (MS-IF) cytometry (Figure S1C, Supplemental Experimental Procedures) was developed to quantify absolute isoform stoichiometry, which is not possible by antibody methods alone due to differential sensitivities of antibodies to isoforms. MS-IF cytometry of diverse hematopoietic cell types reveals that MIIB is no more than ∼30% of total MII across cell types and has a large dynamic range of ∼5,000-fold compared to ∼80-fold for MIIA (Table S1). However, MS also revealed MIIA phosphorylation at S1943 (pS1943), which deactivates MIIA through myofilament disassembly (Dulyaninova et al., 2007), and so a phosphospecific antibody was used to estimate the pS1943 stoichiometry of MIIA through a calibration scheme using mutant GFP-MIIA (see Supplemental Experimental Procedures). Based on this, ∼50%–60% of MIIA is phosphorylated as pS1943 in the three key HSC/P subpopulations of CD34$^+$ cells (Figure S1D) per standard surface markers (Majeti et al., 2007; Novershtern et al., 2011):

{“HSC enriched” cells: CD34$^+$, CD38$^-$, CD90$^+$, CD45-RA$^-$, CD133$^+$}

{Multipotent progenitor (“MPP”): CD34$^+$, CD38$^-$, CD90$^-$, CD45-RA$^-$, CD133$^+$}, {“Common potent progenitor (“CPP”): CD34$^+$, CD38$^+$, CD90$^-$, CD45-RA$^+$, CD133$^-$}.

In differentiation, pS1943 generally decreases as MIIA is activated (Figure S1D), except for MKs in which pS1943 is similarly high as in HSC/Ps.

Phosphorylation at S1943 is equivalent to inhibiting ~50% of MIIA activity (Raab et al., 2012), so that for any cell type, *active*-MIIA = (50% of total MIIA) × (1 + MIIA fraction of *non*-pS1943). The result for *active*-MIIA proves to be nearly constant throughout hematopoietic differentiation and makes a useful common denominator. As such, a myosin map of hematopoiesis shows the highest stoichiometric ratio of (MIIB: *active*-MIIA) ≈ 1:2 (50%) in “HSC-enriched” (Figure 1Aiii) and a ratio of (MIIB: *active*-MIIA) ≥ 1:10 for the other marrow-restricted cells that include nucleated erythroid progenitors, MKs, and nonhematopoietic MSCs. For cell types that predominantly exit the marrow, the ratio (MIIB: *active*-MIIA) generally decreases as MIIB is specifically repressed—except for red blood cells (RBCs), which repress both myosins in similar proportion. Hematopoietic differentiation thus involves a major switch from MIIB + MIIA to active MIIA alone.

The programmatic nature of MII changes in hematopoiesis is evident in the fact that all nine of the marrow-resident cell types measured here cluster together in the MII map (Figure 1Aiii). Based on the “marrow” space in such a map being roughly half as large as that spanned by all measured cell types, we estimate a clustering probability $p \approx (^1/_2)^{11}(^1/_2)^5 = 0.000015$. This high significance p provides a metric of the systematic consistency of our MII measurements in addition to revealing a broad MII program.

Since MIIB was highest at the protein level in CD34$^+$ subpopulations, microarray profiling of the different stem/progenitor/differentiated cells allowed us to identify genes that correlate with expression of *MYH10* (Figure 1Bi). *CD34* correlated strongly with *MYH10* in showing a power law exponent of 1.8 (Figure 1Bii), whereas the differentiation gene *ELANE* is strongly anticorrelated with a power law of −1.8 exponent (Figure 1Biii). *MYH9* shows no correlation with *MYH10*. Transcripts that fit well were further assessed with a second, novel algorithm for a robust list (Table S2). A key subset is ranked on the correlation with *MYH10* and color-coded for the power law. Consistent with protein-level analyses, both *MYH9* and *MYH10* transcripts are of similar (midrange) intensity. About 1% of *MYH10*-correlated genes are known HSC/P markers, while >10% of the most anticorrelated genes are specific lineage markers. The profiling indeed shows maintenance of HSC/P markers (e.g., *CD34* and *ALDH1A1*) and suppression of differentiation genes (e.g., *ELANE*) (Figures 1Bii and 1Biii). Transcriptome dynamics thus align well with protein dynamics along with the differentiation trajectory.

MIIB Polarization in Marrow-Derived CD34$^+$ Cells

Some of the *MYH10*-correlated genes (*CEP70*, *DOCK1*, *MACF1*, *MAP7*, and *TUBB4*) not only polarize but also have roles in motility via the microtubule system. MIIB might be more cortical than MIIA in round, uncultured CD34$^+$ cells (Figure 2A), and in the ~50% of CD34$^+$ cells that are polarized in culture, MIIB is clearly enriched in the uropod by 75%, while MIIA is diffuse and uniform. The microtubule organizing center (MTOC) was also in the uropod as expected (Giebel et al., 2004). However, because cytoskeletal polarization in MSCs is suppressed by soft microenvironments (Raab et al., 2012), it became important to measure the elasticity of intact BM. Therefore we sectioned fresh bone lengthwise to expose the marrow for probing by atomic force microscopy. We determined a microscale elasticity of ~0.3 kilopascal (kPa) (Figure 2B), which is >10^6-fold softer than rigid bone and plastic. HSC/Ps have also been seen to localize at or near the osteoblastic endosteum of bone, which we had previously measured to have an elasticity >30 kPa (Engler et al., 2006). The softness of marrow is likely a product of both high cellularity and low extracellular matrix density: fibronectin (FN) is ubiquitous in marrow but is denser near the endosteum where collagen-I is also high (Nilsson et al., 1998). CD34$^+$ cells clearly anchor specifically to FN versus collagen-I (Figures S2A–S2C), and so we FN-functionalized gels of polyacrylamide to test matrix elasticity effects on MIIB polarization. Similar to our findings for marrow-derived MSCs (Raab et al., 2012), MIIB polarization in CD34$^+$ cells is suppressed in cultures on marrow-mimetic soft matrix but promoted in cultures on endosteum-mimetic stiff matrix (Figure 2C). Blebb rapidly abolishes MIIB polarization on stiff matrix without affecting membrane intensity of MIIB. MII activity and stiff matrix (including rigid plastic if cells adhere) thus drive polarization in CD34$^+$ cells.

Local stresses independent of adhesion can polarize MII in *Dictyostelium* cells that are pulled into micropipettes by aspiration (Ren et al., 2009). Hematopoietic cells were likewise aspirated at low stress (<1 kPa) after transfection of GFP-MIIA or MIIB, and within just 20 min, MIIB polarized by more than 10-fold into the stressed projection (Figure 2D), while MIIA polarized much less. Importantly, receptors such as integrins do not engage the micropipette wall, and so polarization can indeed be independent of adhesion. Partial knockdown of MIIB in CD34$^+$ cells followed by aspiration also showed greater distension of the membrane as well as membrane fragmentation (Figure 2E), and knockdown cells also showed an ~20% decrease in migration through a 3 μm pore filter (Figure S2D). Functionally, MIIB polarization in CD34$^+$ cells is thus protective of membrane shape changes produced by cell forces.

Asymmetric Division Is Biophysically Regulated by MIIB

Large cortical tensions are generated in cells as they round up and divide during asymmetric division (Sedzinski et al., 2011). Because *MYH10* correlates with a half-dozen genes involved in asymmetric division of hematopoietic cells (Ting et al., 2012) (Table S3), confocal imaging and partial knockdown (Figure 3Ai) were used to assess MIIB in asymmetric division of CD34$^+$ cells (Figure 3Aii), which occurs in ~30% of cells (consistent with Lordier et al., 2012). MIIB enriches toward the CD34^{hi} daughter cell, concentrating near the cleavage furrow by ~3-fold (Figure 3B), whereas CD34 appears segregated between cells but otherwise locally homogenous, consistent with lateral mobility of this membrane protein. The results suggest that high cortical tensions in the cleavage furrow have a similar effect on receptor-independent localization of MIIB as that of local stressing by a micropipette.

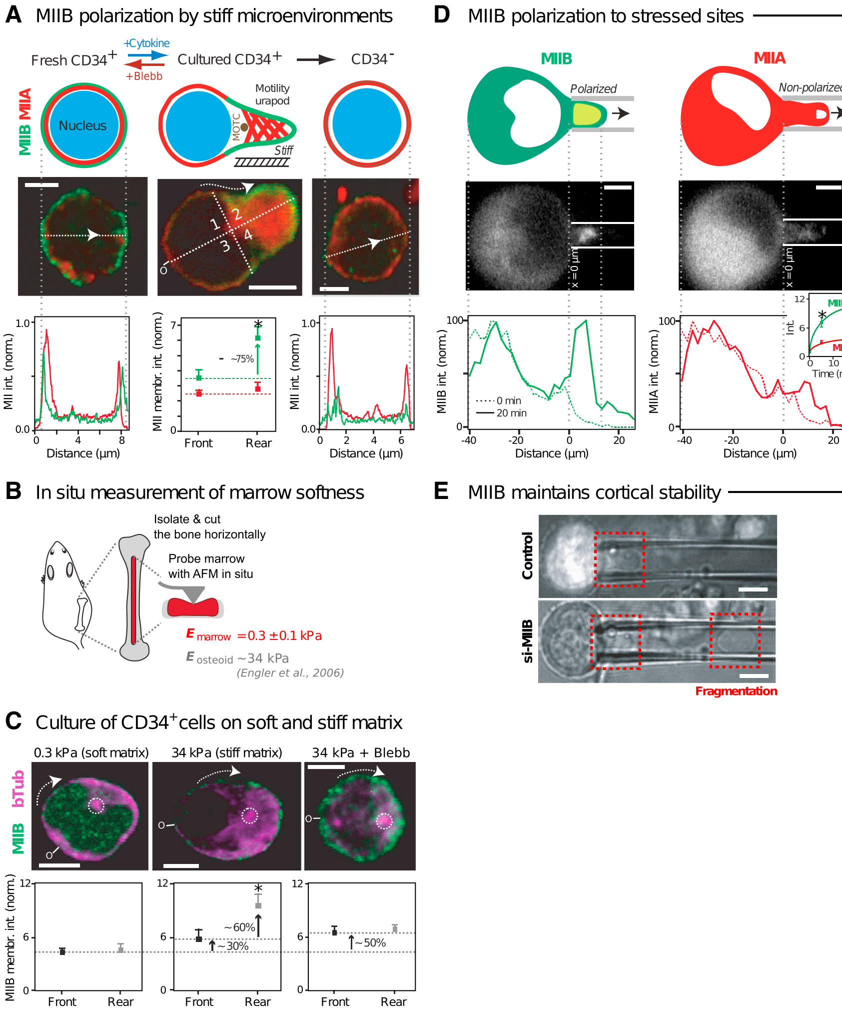

Figure 2. MIIB Is Polarized in Motile CD34⁺ Cells and Localizes to Where Cells Are Stressed

(A) MIIB is more membrane-localized than MIIA in CD34⁺ cells before and after serum-free culture, but not in CD34⁻ cells. Bar = 5 μm. For the left and right panels, protein intensity was measured across the cell diameter (representative images and diameters are shown). For the middle panel, cortical intensity was measured clockwise (arrow) from the front of the cell origin ("0"). "Front" is the summed intensity over contour in the first and fourth quadrants (~50% of the total contour), while "Rear" is the summed intensity over the remaining contour.

(B) Measurement of in situ marrow elasticity by atomic force microscopy (AFM). Young's modulus E_{marrow} is obtained from indentation measurements performed at different locations across the exposed marrow samples. The force versus indentation curves are fit by $F = E\,\delta^2\,(2/\pi)\,(\tan\alpha)/(1-\nu^2)$, where δ is the indentation, ν is the Poisson ration (assumed to be 0.5), and α is the half opening angle of the AFM tip (Sneddon, 1965). From 88 measurements done on four mouse tibia or femur samples, E_{marrow} = 0.32 ± 0.07 kPa.

(C) Arrows indicate direction of intensity measurement as described for Figure 2A middle panel.

(D) Stress-induced localization of mCherry-MIIB and less so GFP-MIIA are demonstrated in micropipette aspiration of MEG01 cells. Yellow region in cartoon represents MII accumulation and white regions represent MII^{lo} areas. Representative images and intensity measurements across cell midline are shown with the inset kinetics of MIIA and MIIB intensities normalized by the intensity at t = 0 min. (Pressure Δp = 1 kPa; bar = 10 μm).

(E) MIIB maintains cortical stability of CD34⁺ cells, based on aspiration of cells with or without knockdown with si-MIIB. (Δp = 1 kPa; bar = 5 μm).

For all image analyses, n ≥ 10 each sample, two donors, mean ± SEM, and *p < 0.05. See also Figure S2.

Partial knockdown of MIIB abolishes the asymmetry and also the segregation of CD34 (Figure 3B, bottom). Whereas asymmetric division of CD34⁺ cells results in 6-fold higher MIIB in the CD34⁺ daughter than in the CD34⁻ daughter, knockdown decreases the MIIB level in CD34⁺ to that in CD34⁻ and suppresses asymmetric division (Figure 3C). Prolonged cultures of MIIB knockdown cells increase the relative number of CD34⁺ progenitors with more colony forming unit-granulocyte and macrophage (CFU-GM) (Figure 3D), consistent with MIIB regulating asymmetric division when late CD34⁺ progenitor cells transition to CD34⁻ cells and when CD34 molecularly segregates between daughter cells. Tracking of division using carboxyfluorescein diacetate succinimidyl ester (CFSE) (Hawkins et al., 2007) shows that partial knockdown of MIIB increases the number of CD34⁺CD38⁻ cells by 2-fold (Figure 3Ei) or for CD34⁺CD38⁺ cells by 1.5-fold (Figure 3Eii), whereas CD34⁻ numbers remain unaltered (Figure 3Eiii). On the other hand, partial MIIA knockdown (~30%) did not alter numbers of any subpopulations (Figure S2E). The data reveal MIIB as a major factor in asymmetric division and differentiation of CD34⁺ cells to CD34⁻ cells.

MIIA Dephosphorylates in Differentiation to a Mechanically Active State

MIIA is often the dominant MII isoform and can influence MIIB (Raab et al., 2012), which implies that phosphoregulation of MIIA can in principle influence hematopoiesis (Figure 4A). We therefore examined regulation by niche factors of pS1943 in CD34⁺ cells and found the highest levels of pS1943 in uncultured CD34⁺ cells (Figure 4Bi; Figure S1D), with levels systematically decreased upon differentiation with Thrombopoietin (Tpo) and Granulocyte-Colony Stimulating Factor (G-CSF), but not Stem Cell Factor (SCF) alone (Figure 4Bii). Transforming Growth Factor-β (TGF-β) promotes HSC hibernation (Yamazaki et al., 2011) and blocks the decrease in pS1943 with cytokines (Figure 4B). Remarkably, Blebb mimics TGF-β (Figure 4B). In contrast, very low pS1943 in myeloid CD33⁺ cells (Figure S1D) and in the human monocytic cell line (THP-1) are consistent with rapid proliferation of THP-1 in suspension (which can be blocked by Blebb). Both CD34⁺ and CD34⁻ cell numbers anticorrelate with the level of pS1943, and the half-maximal effect was observed at ~35% pS1943-MIIA (Figure 4B). MIIA is thus modulated by cytokines critical to precirculation differentiation.

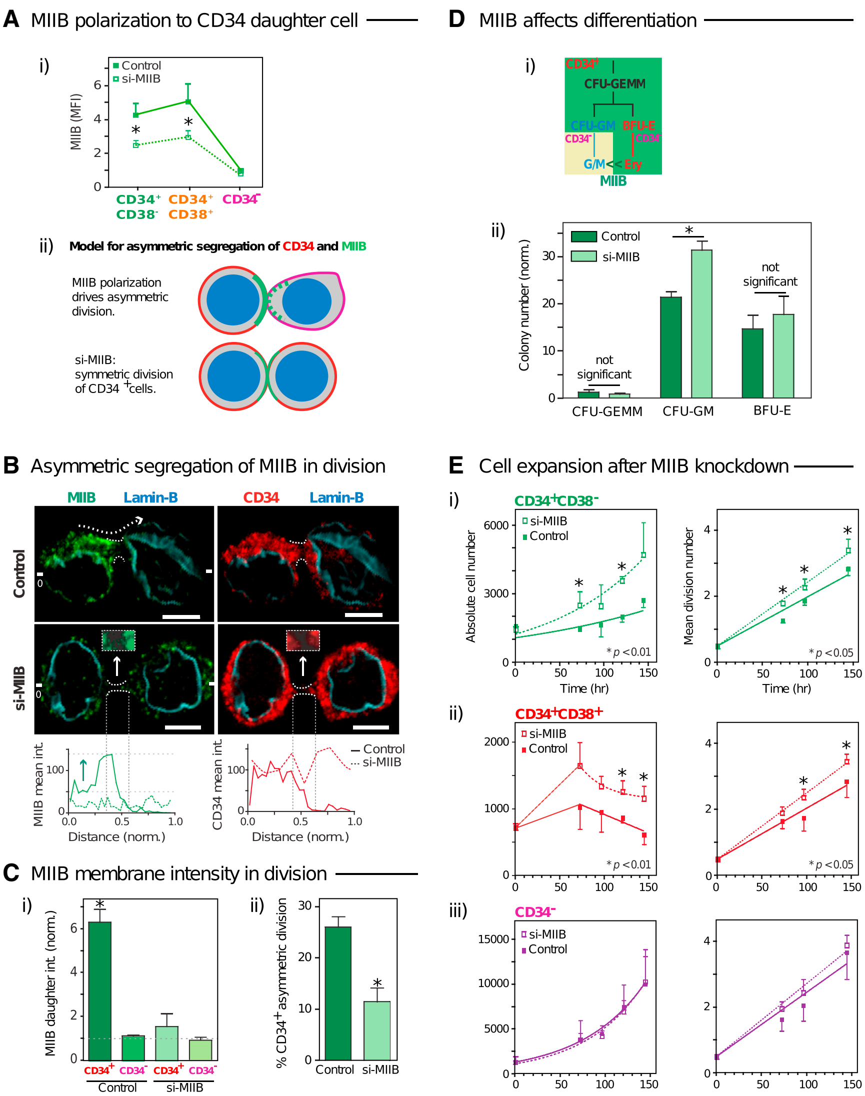

Figure 3. MIIB Polarizes in and Promotes Asymmetric Division of CD34$^+$ to Differentiated Cells

(A) Partial knockdown of MIIB decreases protein by ~40% in CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$. (Ai) Mean Fluorescence Intensity (MFI) of MIIB protein was measured by flow cytometry. (Aii) Hypothetical model for asymmetric segregation of CD34 and MIIB in division shown for WT versus MIIB knockdown.

(B) MIIB segregates asymmetrically in dividing CD34$^+$-derived cells unless MIIB is knocked down (top). Bar = 5 μm. Intensities of MIIB and CD34 were measured (bottom) along the membrane contour of dividing cells from "0" through the cleavage furrow to the antipole, with distance normalized by total length. A green arrow indicates the difference in MIIB intensity between periphery and cleavage furrow.

(C) MIIB membrane intensity bifurcates to CD34^{hi} and CD34^{lo} daughters in dividing cell pairs (Ci), unless MIIB is knocked down. Percentage of asymmetrically dividing CD34$^+$ cells is suppressed with MIIB siRNA versus control (Cii). Greater than forty cells per group.

(D) Colony forming assays after 3 days in methylcellulose medium supplemented with cytokines. (Di) A relationship among different progenitors in terms of CD34 and MIIB expression. (Dii) CFU-GM increases with MIIB knockdown.

(E) Absolute CD34$^+$ cell numbers expand after MIIB knockdown, with normalization to an initial total of 10,000 cells (left), and CFSE tracking shows an increase in mean division number per time (right). Slopes for si-MIIB are as follows: control, (0.02: 0.015) for CD34$^+$CD38$^-$ (Ei) and CD34$^+$CD38$^+$ (Eii), and (0.02: 0.02) for CD34$^-$ (Eiii). For all graphs, *$p < 0.05$ between Control (= Scrambled) versus MIIB siRNA for each data point, mean ± SEM, and n ≥ 3 donors.

See also Figure S2 and Table S3.

Fresh CD34$^+$CD38$^-$ are softer than CD34$^+$CD38$^+$ (Figure 4Ci), and consistent with the high pS1943-MIIA in CD34$^+$ cells, cells transfected with a site-specific MIIA phosphomimetic mutant (S1943D) fragment more often (from a weak cortex) and also divide more slowly compared to wild-type controls (Figures 4Cii and 4Ciii). These functional results all indicate that high pS1943-MIIA impacts cell mechanics and limits cell division, and hence, differentiation. Transcriptional profiles reveal perturbation of pathways that regulate MIIA phosphorylation in CD34$^+$ cells by Blebb (Table S4). Matrix mechanics therefore have an understandable effect on pS1943-MIIA as well (Figure 4Di): soft FN-coated gels (20 μg/ml) maximize pS1943 in CD34$^+$ cells treated with cytokines compared to stiff matrix, while CD34$^-$ cells appear unaffected (Figure 4Dii). This response to stiff matrix is blocked with the phosphomimetic, deactivating S1943D-MIIA (Figure 4Diii). Increased cell spreading as part of matrix engagement on stiff substrate thus requires MIIB in CD34$^+$ cells (Figure 2C), whereas differentiated cells use nonphosphorylated MIIA. At the same FN density as above, the number of CD34$^+$CD38$^-$ is 4-fold higher on soft matrix relative to stiff matrix (but Blebb eliminates the difference), whereas the number of CD34$^+$CD38$^+$ remains constant (Figure 4E). CD34$^+$CD38$^-$ cells are thus sensitive to matrix elasticity, with sensitivity modulated by MII.

In Vivo Roles in HSC/Ps: MIIB Contributes to Differentiation, whereas MIIA Confers Survival

Based on our in vitro results, a major knockdown of MIIB in human cells grafted into BM should repress asymmetric division and lead to (1) an accumulation of human cells in marrow and (2) a suppression of circulating human blood cells. To test this hypothesis, fresh human CD34$^+$ BM cells were transduced with shRNA-carrying lentivirus to knock down MIIB, which was followed by puromycin selection of transduced cells. Cells were injected directly into bone of NOD/SCID/IL-2R$\gamma^{-/-}$ (NSG) mice to study BM retention both at 16 hr and at 20 weeks (Figure 5Ai); this same duration has been described by others (Notta et al., 2011) as providing "a stringent test of long-term

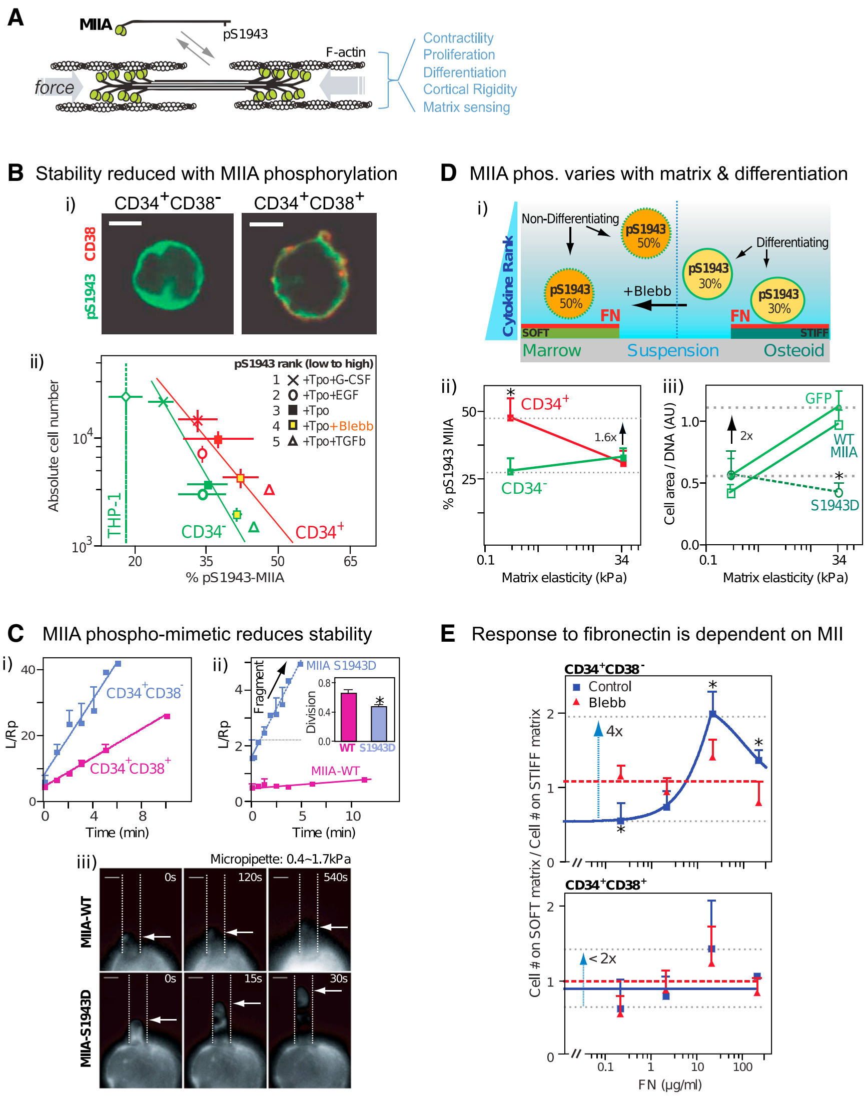

Figure 4. Phosphorylation of MIIA Regulates the Biophysics of CD34^{+} Differentiation

(A) Dephosphorylation of MIIA at S1943 promotes assembly and function.

(B) CD34^{+} differentiation with soluble factors decreases pS1943. (Bi) Representative images showing pS1943 expression in fresh CD34^{+} cells. Bar = 5 μm. (Bii) pS1943 (normalized to MIIA) was measured by flow cytometry [n = 3 donors, ± SEM; fit to Log Y = aX + b (a,b): (CD34^{+}: −0.05, 5.74; in red), (CD34^{-}: −0.10, 6.74; in green)]. Minimum pS1943 per MIIA was measured for THP-1 cells (0.07 ± 0.01). All cells were treated with SCF and indicated cytokines. pS1943% values were normalized as described in Figure S1D (n = 3, ± SEM). pS1943 percentage for SCF only = ~40%.

(C) MIIA S1943D phosphomimetic decreases both cortical stiffness and cytoskeletal stability. Aspiration length L, normalized by pipette radius, Rp (L/Rp), versus time for various cells with (*slope, intercept, effective viscosity η*) were as follows: (Ci) CD34^{+}CD38^{-} (5.7/min, 8.3, 3.2 Pa/s) and CD34^{+}CD38^{+} (2.2/min, 4.7, 8.5 Pa/s); (Cii) MIIA-WT: (0.02/min, 0.5, 1,400 Pa/s), MIIA-S1943D (0.70/min, 1.6, 40 Pa/s). n = 5, ±SEM. The inset bar graph in (Cii) shows the fraction of transfected COS-1 cells after MIIB knockdown that undergo cell division (2n and 4n cells) as calculated by subtracting the fraction of polyploid cells (n = 3, ±SEM, *p < 0.05). (Ciii) Representative images of aspiration of transfected COS-1 cells (bar = 10 μm).

(D) CD34^{+}CD38^{-} cells sense matrix elasticity with changes in pS1943-MIIA similar to cytokines (Di). (Dii) Soft matrix maintains high pS1943 in CD34^{+}. *p < 0.05 (three donors, ±SEM). (Diii) pS1943 limits matrix sensing: cell area was normalized to DNA to correct for ploidy of COS-1. *p < 0.05 for GFP-S1943D 34 kPa versus GFP or GFP-MIIA 34 kPa (n ≥ 20, ±SEM).

(E) CD34^{+} numbers on soft matrix (0.3 kPa) scaled by stiff matrix (34 kPa) increase with fibronectin (FN) density unless MII is inhibited. For CD34^{+}CD38^{-}, EC_{50} ~22.4 μg/ml, *p < 0.05 (n ≥ 3, ±SEM).

See also Table S4.

repopulation'' of human xenografts injected into the femurs of NSG mice for which ''HSCs were operationally defined by lymphomyeloid engraftment that persisted for at least 20 weeks after transplant.'' Standards for mouse HSCs differ from those of human (Doulatov et al., 2012), but 12–16 weeks is currently considered as ''long-term engraftment'' (Oguro et al., 2013). We injected directly into marrow rather than into blood to avoid any potential effect of knockdown on trafficking from blood to marrow. Human cells in mice were identified by dual immunostaining for hCD45 and hCD47 (Figure S3A), since human RBC and platelets do not express hCD45 while hCD47 confers immunocompatability to all human cells within NSG mice (Rodriguez et al., 2013; Takenaka et al., 2007). Partial permanent MIIB knockdown (by ~40%) (Figure 5Aii) leads to 3-fold greater retention of cells in marrow when they are assayed just 16 hr after marrow injection (Figure 5Bi); this could reflect the fact that knockdown impairs migration through constraining micropores by 20% ($p < 0.05$; Figure S2D). Despite this initial retention advantage, the percentage of human peripheral blood (PB) cells in circulation at 6 weeks after transplantation is 7-fold lower for the MIIB knockdown cells (Figure 5Bii), and the difference is maintained after 20 weeks (Figures 5Ci and 5Cii). Sustained engraftment is evident in control mice with significant human cell numbers in marrow and four of five mice showing human cells in PB. In contrast, MIIB knockdown cells were 6-fold more abundant in marrow, but only one of five mice had human cells in circulation. MIIB is thus required to generate PB cells.

Because MIIA is the dominant isoform in hematopoietic cells and is phosphoregulated distinctly in marrow cells versus PB cells, we characterized MIIA contributions to hematopoiesis by performing competitive transplants of BM from tamoxifen-inducible cre-*Myh9* knockout mice. These conditional knockout cells (with surface marker CD45.2) were mixed 1:1 with cells from

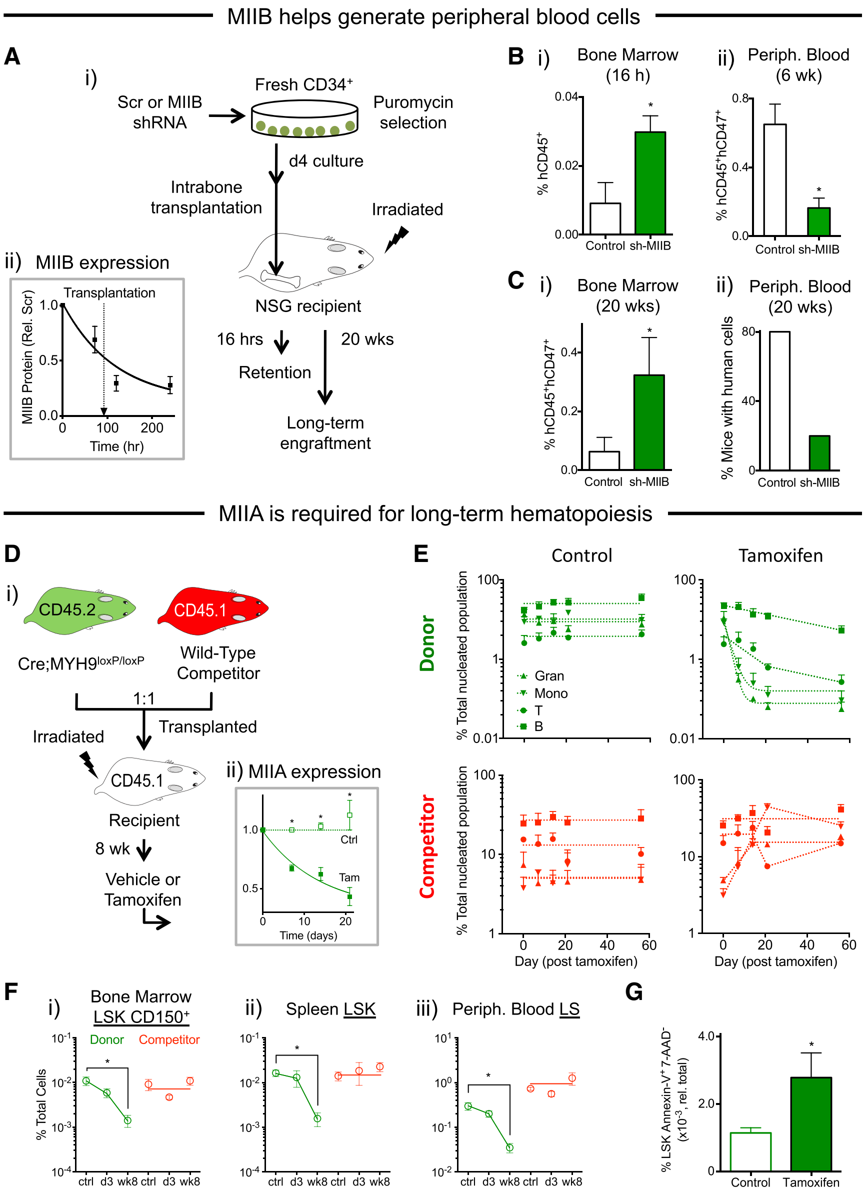

Figure 5. MII Isoforms Regulate Hematopoiesis In Vivo

(A) Scheme for in vivo experiments to test MIIB functions (Ai). (Aii) MIIB expression kinetics with shRNA knockdown relative to control. $t_{1/2}$ = 81 hr. n = 5 mice for each group (±SEM for all graphs), transplanted via an intratibial route with 5 × 10^3 BM $CD34^+$ cells per sublethally irradiated mouse. Transplantation occurred 4 days after lentiviral transduction, with 2 days puromycin selection.

(B) MIIB knockdown increases short-term (16 hr) retention in bone marrow (BM) (Bi), but decreases short-term (6 week) generation of peripheral blood (PB) (Bii). *$p < 0.01$ control (scrambled) versus MIIB shRNA.

(C) MIIB knockdown increases long-term (20 weeks) BM engraftment (Ci), but suppresses PB generation (Cii). For PB generation, the number of positively engrafted mice is shown (≥0.1% total nucleated cells).

(D) Scheme for in vivo experiment to test MIIA functions (Di). BM cells from Cre:$Myh9^{loxP/loxP}$ (CD45.2) and from WT competitor (CD45.1) were transplanted at a 1:1 ratio into lethally irradiated WT recipients. (Dii) Eight weeks after reconstitution, mice were treated with tamoxifen to delete *Myh9* as assayed by protein expression (*$p < 0.01$, control versus tamoxifen, n ≥ 3, ±SEM for all graphs). Deletion occurred with $t_{1/2}$ = 9.6 days. n ≥ 8 mice for each group from two independent experiments (±SEM for all graphs).

(E) PB lineages with deleted *Myh9* are lost from circulation with kinetics similar to clearance of WT cells. The donor (top) and competitor (bottom)-derived granulocyte (Gran, Gr-1^+Mac-1^+, larger side scatter), monocyte (Mono, Gr-1^+Mac-1^+, smaller side scatter), T cell (T, $CD3^+$), and B cell (B, $B220^+$) lineages in PB were quantified at the indicated time points after vehicle (left) or tamoxifen (right) treatment. Decay half-lives for tamoxifen-treated donor Gran, Mono, T, and B are 1.3, 1.7, 24.2, and 18.8 days, respectively

(F) *Myh9* deletion decreases HSC/P subpopulations across different hematopoietic organs in the long term (8 weeks), but not in the short term (3 days). Donor and competitor HSC/P cells were quantified in BM (LSKCD150^+, Fi), spleen (LSK, Fii), and PB (LS, Fiii) (control versus treated, *$p < 0.01$).

(G) *Myh9* deletion increases apoptosis of LSK. Treatment was for 3 days (*$p < 0.01$).

wild-type mice (CD45.1) and injected into sublethally irradiated recipient mice (CD45.1) (Figure 5Di). This knockout strategy with mouse cells instead of human cells proved necessary for understanding MIIA because our in vitro results for proliferation indicated no effect with partial knockdown of MIIA in contrast to major defects with MIIB partial knockdown (Figure 3A). At 8 weeks after transplantation of the mixed cells, the total percentage of donor and competitor blood cells was ~50% each, and upon tamoxifen treatment, MIIA decreased as expected only in CD45.2 donor cells (Figure 5Dii). In PB, donor myeloid cells decreased rapidly compared to lymphoid cells ($t_{1/2}$ = ~30–40 hr versus ~20–25 days) (Figure 5E), but these half-lives are within 2-fold of those reported for both myeloid (Basu et al., 2002; van Furth and Cohn, 1968) and lymphoid (Fulcher and Basten, 1997; Sprent and Basten, 1973) lineages in mouse blood.

MIIA loss therefore does not greatly affect viability of terminally differentiated lymphoid cells, while blood cell production from progenitors is clearly suppressed. Consistent with this, we find in BM that Lin^- Sca-1^+ c-Kit^+ (LSK) $CD150^+$ cells (which include progenitors or HSC/Ps; Kiel et al., 2005) are reduced 10-fold at just 8 weeks after *Myh9* deletion (16 weeks since transplant), with similar results for LSK in spleen and LS in blood (Figures 5Fi, 5Fii, and 5Fiii, respectively). MIIA is thus required for sustained engraftment in vivo and hematopoiesis. An early apoptotic fraction (Annexin-V^+ and 7-AAD^-) of the LSK population also increased just 3 days after *Myh9* deletion (Figure 5G), although the total LSK number remained unchanged at this time point (Figure 5F). Irreversible loss of MIIA therefore suppresses differentiated cell numbers in the long term as defective HSC/Ps progressively apoptose.

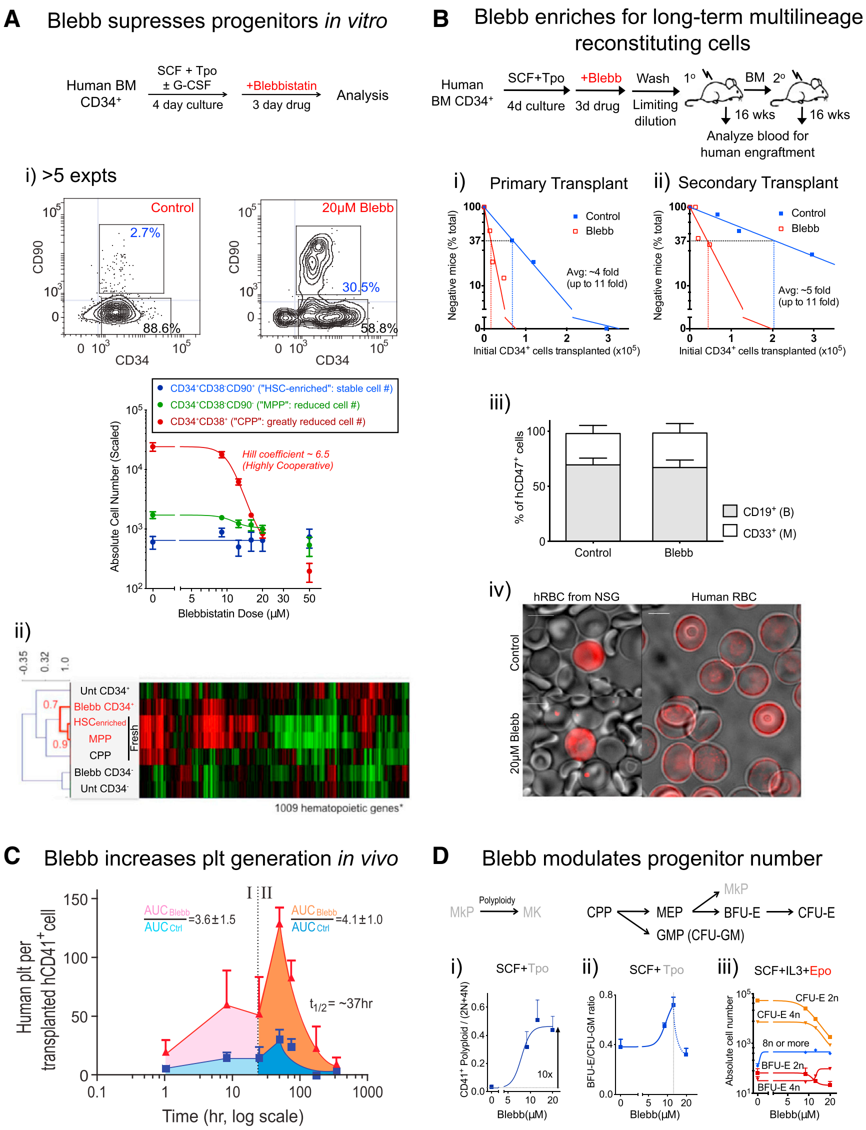

Figure 6. MII Inhibition Maintains HSC-Enriched Population with Long-Term Multilineage Reconstitution Potential

(A) Scheme for in vitro experiments (top). (Ai) Representative flow cytometry contour plots for $CD34^+$ subpopulations, with dose-dependence of 2n cells showing 15.6- ± 4.1-fold enrichment at 20 μM Blebb. Absolute cell numbers were scaled to 10^4 initial cells and fit to dose-response curves: CPP and MPP IC_{50} = 10.5 μM; HSC-enriched numbers = 646 ± 77 (n ≥ 5 donors, ±SEM). These IC_{50} values are within ~2-fold of the inhibition constant K_i for pure MII (Kovács et al., 2004). (Aii) Blebb-treated $CD34^+$ cells show a gene expression profile similar to fresh $CD34^+CD38^-$ for hematopoietic genes (Table S4 and Table S5). Values are derived from two experiments.

(B) Limiting dilution serial transplant analyses show functional HSCs after myosin inhibition after 16 weeks (long-term). (Top) Scheme for in vivo experiments. (Bi) Limiting dilution primary transplant. The number of transplanted $CD34^+$ cells versus the percentage of unsuccessful engraftment determines the frequency of repopulating cells (n = 26 recipients per group from three independent experiments; $p < 0.0005$). (Bii) Secondary transplantation of BM from primary transplant demonstrates the maintenance of higher HSC frequency with Blebb compared to control (n ≥ 13 recipients per group, $p < 0.01$) (See Figure S3B). Transplantation with Blebb-exposed $CD34^+$-derived cells shows similar multilineage engraftment in the NSG mice compared to control cells, including myeloid ($CD33^+$), lymphoid ($CD19^+$) (Biii) (±SEM), and erythroid (GPA^+) (Biv). Bar = 5 μm.

(C) Kinetics of human-$CD41^+$ platelets in circulation were measured after transplantation of human $CD34^+$-derived cells and normalized by the initial number of $CD41^+$ cells transplanted. Areas under curves show significant differences between drug-treated and control. $p < 0.05$ in both phase I and phase II from at least nine recipients in three experiments (±SEM).

(D) Effects of Blebb on progenitors. (Di) Enrichment of polyploid MKs by Blebb (n = 4). y axis represents the ratios between polyploid MKs and 2n + 4n MKs. EC_{50} = 7.5 μM; Hill coefficient = 7.0. (Dii) Enrichment of BFU-E relative to CFU-GM in the absence of Epo, evaluated by colony forming assays. The maximum ratio was observed at 12.5 μM. IC_{50} = 10 μM, Hillslope = 5.0 (n = 3, ±SEM). (Diii) Sensitivity of erythroid progenitors to Blebb in the presence of Epo. BFU-E = $CD34^+IL\text{-}3R^+CD36^-$; CFU-E = $CD34^-IL\text{-}3R^-CD36^+$. Absolute values were normalized to 10^4 initial cell input and fit to dose-response curves. IC_{50}, Hill coefficient for CFU-E, 2n: 8.7 μM, −4.4 and 4n: 12.9 μM, −6.3; BFU-E, 2n: 10.9 μM, −9.7, 4n: 13 μM, 28, and Poly ≥ 8n: 0.2 μM, 2.0. (n = 2, ±SEM).

See also Figure S3, Table S4, and Table S5.

Transient Inhibition of MII with Blebbistatin Spares Only Long-Term Multilineage Reconstituting Cells

Blebbistatin is a reversible inhibitor of all MII isoforms, and dose-response studies of $CD34^+$ cultures show that it has a surprising but understandable effect: the diploid "HSC-enriched" population (as phenotypically defined per Majeti et al., 2007 and Novershtern et al., 2011) proves relatively stable to a 3-day treatment, which is long relative to the cell cycle, while the Blebb-treated MPP and CPP are depleted by 1.8-fold (±0.5) and 31-fold (±16), respectively. By suppressing only the progenitors and sparing the HSC-enriched population, the net effect is an enrichment of the latter among total $CD34^+$ cells by up to 16-fold (Figure 6Ai). Whole-genome transcript profiles indeed show that Blebb cultures correlate well with fresh HSC-enriched cells and MPP, but not CPP (Figure 6Aii, Table S4, and Table S5), whereas control $CD34^+$ cultures correlate with fresh CPPs. Blebb treatment beyond 3 days showed a progressive decrease in the HSC-enriched population, consistent with the conditional knockout studies above that suggest that MIIA is essential for hematopoiesis in vivo (Figures 5D–5F).

Functional tests of HSC enrichment by Blebb were conducted after washing out the drug and involved measuring the frequency of human cells in NSG mice after limiting dilution serial transplantations into multiple primary and secondary recipients (Figure 6B). A total duration of 32 weeks in primary plus secondary xenografts was chosen as sufficient to assess long-term multilineage engraftment of human HSCs in NSG mice (Notta et al., 2011). Our blood analyses 16 weeks after primary transplantation showed that positive engraftment required fewer $CD34^+$ cells (~1 in 10,000) from Blebb-treated cultures compared to control cultures (Figure 6Bi; Figure S3B). If long-term multilineage engraftment were due solely to progenitors (such as MPPs), then the fact that Blebb-treated cultures have relatively fewer progenitors (Figure 6A) would have required that more (not fewer) Blebb-treated $CD34^+$ cells be injected for reconstitution. Both treated and control cultures also showed a similar percentage of human $CD34^+CD38^-$ and $CD34^+CD38^+$ populations in BM after transplantation (Figure S3D), indicative of engraftment, and Blebb results also compare well to uncultured $CD34^+$ cells in previous studies (Nishino et al., 2011). Sustained secondary engraftment provides an assay for cells with appropriate stem cell properties (Doulatov et al., 2012; Notta et al., 2011; Oguro et al., 2013) and our secondary transplantation results show that Blebb maintains a higher fraction of the HSC-enriched population compared to untreated cultures (~5-fold once again). Both treated and control human $CD34^+$ transplants produced a similar percentage of multilineage myeloid and lymphoid cells (Figure 6Biii) and a minor fraction of enucleated human RBCs in the NSG mice (Figure 6Biv). The latter were enriched by flowing blood through a microfluidic channel coated with anti-hCD47 and then staining for the erythroid-specific marker hGPA (Figure S3C).

MKs are unique among blood cells in being naturally polyploid and become more so in vitro with Blebb treatment, which also increases in vitro proplatelet formation (Shin et al., 2011). In the NSG mice, human platelets ($CD41^+$) showed two phases of circulation up to 2 weeks after transplantation that also seemed to benefit from Blebb treatments (Figure 6C). In phase I, human platelets are released into the circulation almost immediately and reach an initial peak between ~1–20 hr, consistent with intravenously infused MKs (Figure S3E) (Fuentes et al., 2010). Phase II peaks at ~20–90 hr and reflects a successful lodging of MKs in the marrow. Importantly, human cells treated with Blebb generate more human platelets per transplanted $CD41^+$ cell by about 4-fold in both phases, and shear forces appear to be important in regulating the size of human platelets derived from MKs (Figure S3F). Blebb indeed enriches for mature polyploid MKs in culture by ~10 fold (Figure 6Di). For other lineages, the sensitivity of individual progenitor lineages to Blebb proves cytokine dependent (Figure 5A; Figure S3G). For SCF and Tpo $CD34^+$-derived cells, the IC_{50} for CFU-GM is lower than that of BFU-E, with Blebb producing up to a 2-fold higher ratio of BFU-E to CFU-GM (Figure 6Dii). Erythroid lineages are thus preserved under non-Epo and submaximal MII inhibition. In contrast, when cells are cultured with Epo, both CFU-E and BFU-E numbers are reduced (Figure 6Diii). Functional studies thus reveal that short-term reversible MII inhibition in combination with specific cytokines enriches for HSCs, mature MKs, and even erythroid progenitors.

Sustained Blebbistatin Induces Apoptosis of Dividing $CD34^+$ Cells via Aryl Hydrocarbon Receptors and p53 Pathways

Control cultures show that CPP and MPP progenitors undergo 2- to 3-fold more divisions as expected compared to the phenotypically marked HSC-enriched population based on CFSE tracking (Figure 7A), whereas Blebb-treated cells (20 μM) do not divide. Decay rates of CFSE for $CD34^+$ subpopulations in either the presence or the absence of G-CSF (Figures S4A and S4B) indicate that Blebb accelerates decay, consistent with enhancing cell death. With the phenotypical HSC-enriched population, this is likely due to inhibition of cytokinesis of 4n cells trying to divide at the point drug was added. Apoptosis was measured by cleaved caspase-3 and increased 2-fold with Blebb (Figure 7B; Figures S4C–S4F), although G-CSF modulates survival (Figure 7B; Figure S4B). Suppression of MPP and CPP cell numbers with Blebb is therefore explained by cytokinesis-associated death, which seems consistent with similar roles for MII in apoptosis in *C. elegans* (Ou et al., 2010).

Transcription profiles of viable fractions from drug-treated and control cells implicate intersecting pathways that involve the aryl hydrocarbon receptor (AHR) and p53 (Table S4). AHR antagonists counteract apoptosis (Vaziri and Faller, 1997) and expand human HSC/Ps in culture (Boitano et al., 2010) (Figure S5A). The antagonist CH-223191 improves viability of both control $CD34^+$ and $CD34^-$ cells, specifically the cycling cells (4n) (Figure 7B). The number of apoptotic cells with Blebb treatment is also systematically decreased by the more potent StemRegenin-1 (SR1) (Figure 7C). Both CH-223191 and SR1 rescue $CD34^+$ cells from cell death by Blebb, since cell numbers approximate control conditions (Figure 7D; Figure S5B).

Total p53 protein was then assayed in the presence of Blebb and/or SR1. Total p53 protein increased ~2 fold with Blebb but reversed by SR1 (Figure 7E). Cyclic-pifithrin-α tests whether induction of apoptosis is dependent on p53-mediated transcription activity (Zuco and Zunino, 2008). The phenotypic "HSC-enriched" population is increased ~2-fold with cyclic-pifithrin-α in both control and Blebb-treated cells (Figure 7F) with an EC_{50} close to previous reports (30 nM) (Pietrancosta et al., 2005). AHR and p53 are thus implicated in Blebb-induced apoptosis.

DISCUSSION

Asymmetric division provides a means to maintain stemness while generating the many differentiated cells required for a tissue with high turnover such as blood (10^5 nucleated cells/s). However, it has been unclear as to how two interconnected daughter cells physically sort components to become distinct. While asymmetry of stem cell division in *C. elegans* is driven by its one isoform of MII (Ou et al., 2010), the mammalian homolog, MIIA, is expressed in many cells other than stem cells and unlike MIIB, MIIA polarizes very weakly if at all (Vicente-Manzanares et al., 2008; Raab et al., 2012). Compared to any other hematopoietic lineage, $CD34^+$ cells express the most MIIB relative to MIIA. MIIB polarizes strongly to regions of high cell tension or curvature where it can physically break the symmetry of cytokinesis (Sedzinski et al., 2011). It is therefore almost predictable that MIIB in $CD34^+$ cells will polarize near a cleavage furrow and define the $MIIB^{hi}$ daughter cell in asymmetric division (Figure 3). Since MIIB is localized near the membrane and is known to link to membrane proteins (Clark et al., 2006), $MIIB^{hi}$ could also help sort cell surface proteins such as CD34 and thereby correlate with $CD34^{hi}$ as seen. Depletion of MIIB from the $CD34^{lo}$ daughter cell is also propagated as a

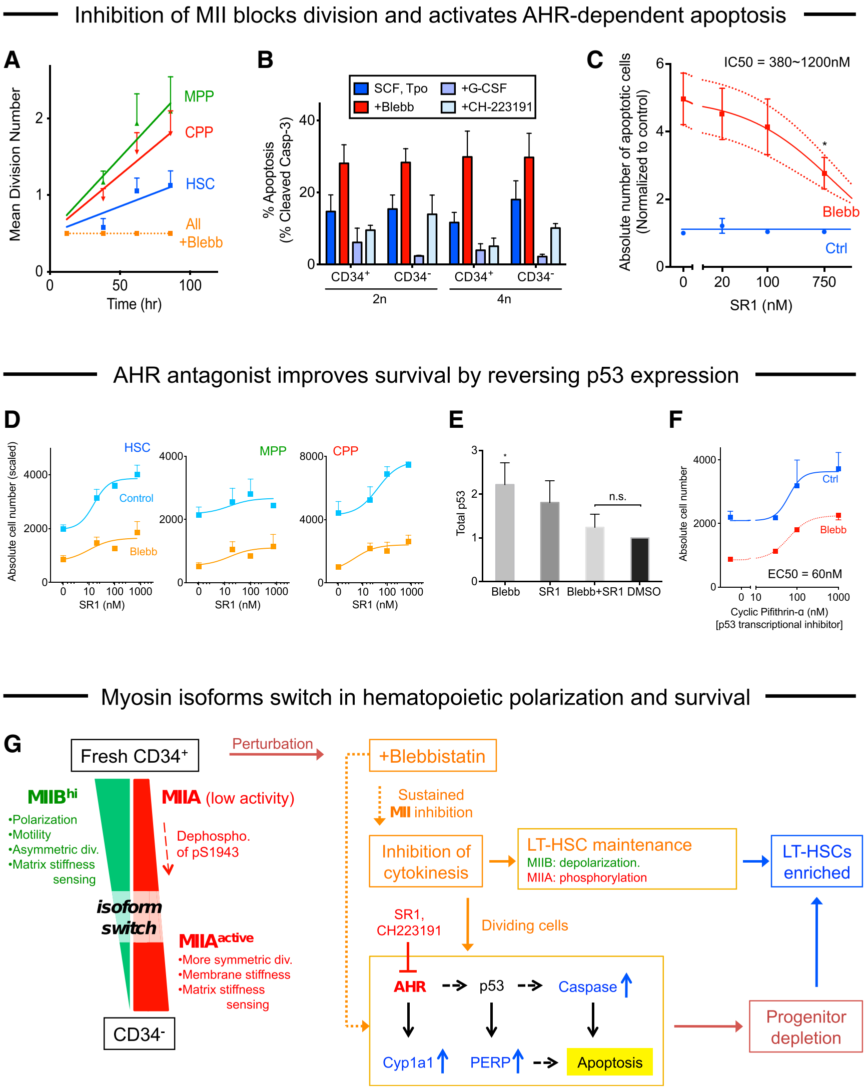

Figure 7. Inhibition of MII Blocks Division and Activates AHR-Dependent Apoptosis

(A) The mean division numbers for each HSPC subpopulation were calculated by fitting Gaussians to CFSE data, and Blebb blocks division ($n \geq 3$ donors, ±SEM).

(B) Increased apoptosis by sustained MII inhibition. CD34$^+$-derived cells cultured in SCF and Tpo were treated with Blebb for 3 days and fixed, followed by intracellular flow cytometry with the anti-(cleaved caspase-3) and 7-AAD for DNA ($n = 3$, ±SEM, $p < 0.05$ for all except CD34$^-$ 4n).

(C) SR1 decreases the absolute number of apoptotic cells generated by Blebb treatment. Nucleated (Hoechst$^+$) Annexin-V$^+$ 7-AAD$^-$ cells were quantified by flow cytometry calibrated by APC-beads. Absolute values were normalized to control (vehicle-treated cells). IC_{50} = ~380–1,200 nM, Hill coefficient = 0.62. *$p < 0.05$ Blebb +0 nM versus +750 nM SR1 ($n = 3$, ±SEM).

(D) Phenotypic HSC-enriched population is maximized by synergy between myosin inhibition and AHR antagonism. CD34$^+$-derived cells in SCF and Tpo were treated with different doses of the selective AHR antagonist SR1, with or without 20 μM Blebb for 3 days. Absolute values were normalized to 10^4 initial cell input and fit to dose-response curves. EC_{50} and maximum cell number for HSC-enriched, Control: 15.5 nM, 4000, and Blebb: 10 nM, 1800; MPP, Control: 15.5 nM, 2200, and Blebb: 15.5nM, 1,100; CPP, Control: 41.5 nM, 7,500, and Blebb: 4.7 nM, 2,400. Hill coefficient for all the graphs is ~1–2. $n \geq 3$ (±SEM).

(E) SR1 reverses upregulation of p53 protein by Blebb. Total p53 protein was quantified by intracellular flow cytometry. *ANOVA $p < 0.05$, Tukey's HSD Test $p < 0.05$ for DMSO versus Blebb ($n = 3$, ±SEM).

(F) Pharmacological inhibition of p53 transcription by cyclic-pifithrin-α increases the absolute cell number of phenotypic HSC-enriched. EC_{50} = 63.1 nM for both control and Blebb, Hill coefficient = 2.6, 1.8 for control and Blebb, respectively ($n = 3$, ±SEM).

(G) Summary of results for biological functions of MII in adult hematopoiesis with perturbations of MII pathways.

See also Figure S4, Figure S5, and Table S4.

key aspect of the MII isoform switch that defines and delineates hematopoiesis (Figure 7G).

How *MYH10* is ultimately repressed in differentiation requires further study: RUNX1 downregulates *MYH10* during MK differentiation (Lordier et al., 2012), but RUNX1 does not anticorrelate in general with *MYH10* and is not required for normal functions once HSCs are formed from vascular endothelial cells during embryonic development (Chen et al., 2009). Nevertheless, asymmetric processes are hinted at by a number of polarizable proteins in our early CD34$^+$ cells, including Cdc42, which polarizes in correlation with HSC aging (Florian et al., 2012). Generic polarization of a protein in hematopoietic cells seems predictive of a role in asymmetric division (Beckmann et al., 2007), but MIIB's role in physically breaking the symmetry of cytokinesis seems unique and motivates deeper study of biophysical factors that feed back into transcription programs and perhaps even regulate cancer stem cell differentiation (Cicalese et al., 2009).

Human HSC/Ps expand when cultured on flexible tropoelastin matrices plus serum and cytokines (Holst et al., 2010) and also expand when cultured serum-free on endothelial cells that secrete cytokines (Butler et al., 2012). However, endothelial cells and secreted matrix on plastic could be locally soft or regionally rigid. On FN-coated hydrogels that are marrow-mimetic soft or else endosteal-like stiff, minimal cytokines in serum-free media can for some conditions enrich for early CD34$^+$ cells, consistent with our finding that both soft gels and blebbistatin suppress MIIB polarization and enhance pS1943-MIIA in early CD34$^+$ cells.

Blood Cells that Lack Myosin-II Become Polyploid or Die Trying

Motile cells that are sufficiently adherent can generate enough traction forces to pull themselves apart even in the absence of MII, whereas cells in suspension or daughter cells that cannot crawl away with sufficient force (to break the intercellular bridge) tend to become polyploid (Zang et al., 1997). HSC/Ps grow well as suspension cells that do not adhere and spread strongly on substrates compared to other solid tissue cell types, and they only possess a thin cortical cytoskeleton; cytokinesis defects are thus likely to favor polyploidy in these cell types. In a blood cancer line that only expresses MIIA, partial knockdown of MIIA indeed increases polyploidy in vitro as does blebbistatin and the cancer cells survive (Shin et al., 2011). In healthy human and mouse primary cells, however, such a process of endomitosis is usually seen only for MKs (among blood cells at least), which implies that other cell types are either never tetraploid or apoptose if they become so. Irreversible ablation in vivo of MIIA in primary blood cells indeed enhances apoptosis and depletes most dividing blood cell types (Figures 5D–5G). This seems consistent with cell death in blebbistatin treatments being downstream of MII inhibition. Although the specificity of this drug has been questioned (Shu et al., 2005), the reversible 3-day treatment here with blebbistatin of primary CD34+ cells in vitro increases ploidy of viable MKs and enhances apoptosis of progenitors with slower dividing stem/progenitor cells dying only with more sustained drug treatments. Our pharmacological results with AHR inhibitors provide some functional evidence of a transcriptome-implicated link between a failure of these normal primary, nonadherent cells to divide and AHR upstream of p53 in apoptosis (Figure 7G, right), but since AHR is primarily nuclear, any interaction with MII is likely to be indirect.

Translation

Mouse knockouts of MIIB are embryonic lethal (Ma et al., 2010), and since MIIA is at least weakly polarizable (Figure 2D), any deficiencies or mutations in MIIB might be partially compensated by MIIA. Inhibiting both isoforms transiently, as shown here, might be exploited to further maintain and perhaps expand HSCs and maybe other stem cells in suitably designed microenvironments. Importantly, given the successful long-term engraftment of drug treated cells, genes that are truly essential for hematopoiesis (profile in Figure 5Aii) might be clearly identified. Enrichment of highly adherent stem cells by the methods here might require optimization of adhesion to both suppress motility and provide sufficient anchorage signals for viability. Our findings ultimately reveal not only a biophysical hierarchy of actomyosin forces in adult hematopoiesis but also some utility in controlling those forces to enrich for stem cells.

EXPERIMENTAL PROCEDURES

MS-IF Cytometry

For intracellular flow cytometry, cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, and resuspended in 0.1% saponin in HBSS. The samples were then stained with antibodies against MIIA and MIIB for 30 min at room temperature, along with hematopoietic surface markers and Hoechst 33342, followed by secondary antibody staining conjugated with Alexa 488 and 647 (Invitrogen). The samples were analyzed on an LSR II (BD) to obtain the mean fluorescent intensity (MFI) values of MIIB and MIIA across different samples and subpopulations. Each value was normalized by a standard cell line (MFI from COS cells) to correct for differences in fluorescence intensities caused by laser fluctuations. Normalized MFI values from flow cytometry were then calibrated by MS results for MSCs (Raab et al., 2012) that served as a standard to calculate the stoichiometric ratio between MIIB and MIIA of each sample.

Standard methods can be found in Supplemental Experimental Procedures for MS, microarray, gene correlation analysis, cell culture, confocal microscopy, micropipette analysis, construction of matrix-coated gels, and xenotransplantation, among other techniques.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 5. Unless otherwise noted, all statistical comparisons were made by unpaired two-tailed Student t test and were considered significant if $p < 0.05$. All dose-response data were fitted to sigmoidal dose-response with variable slope with x axis in a log scale.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.10.009.

AUTHOR CONTRIBUTIONS

J.W.S. and D.E.D. designed research; J.W.S., A.B., K.R.S., D.A.C., I.L.I., and F.R. performed research; J.S. and P.C.D. contributed to new analytic tools; C.L. and C.G. engineered, and C.A.H. supplied, the *MYH9* floxed mutant mice; and J.W.S., K.R.S., J.S., J.A.C., and D.E.D. wrote the paper.

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