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14. ABSTRACT

We have fully documented the function of angiogenin (ANG) in protecting and mitigating damages caused by radiation exposure. We have examined the effect of ANG deficiency on HSPC, LymPro, MyePro, and found that ANG deficiency the number of HSPC, LymPro, and MyePro. We have also found that ANG deficiency resulted in more active cycling of HSPC and LymPro but restricted cycling of MyePro. As the consequence of ANG deficiency on hematopoietic cells, we found that ANG deficiency reduced animal survival upon exposure to radiation. We have also assessed the therapeutic activity of recombinant ANG protein as a radio-protective and radio-mitigative agent, and found that treatment with ANG protein, either prior to or post γ-irradiation, significantly extended survival of animals, thus demonstrating the protective and mitigative functions of ANG against radiation-induced BM damage. We have also documented the beneficial effect of ANG in enhancing stem cell transplantation.

15. SUBJECT TERMS
Angiogenin, radiation damage, radioprotection, radio-mitigation, hematopoietic stem and progenitor cells, myeloid-restricted progenitors

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1. Introduction

The hypothesis to be tested in this project was that angiogenin (ANG) has a protective role against hematopoietic syndrome of the acute radiation syndrome (H-ARS) and is able to attenuate the effect of residual bone marrow damage (RBMD) after radiation exposure. The objective of this proposal was to obtain proof-of-principle data that supplemental therapy of ANG protein is a feasible strategy for developing medical countermeasure against H-ARS. We planned to examine the effect of ANG deficiency and overexpression on radio-sensitivity in terms of animal survival, severity of H-ARS, and RBMD. *Ang* knockout and transgenic mice were planned to be used for this purpose. They would be irradiated with different doses of γ-radiation and analyzed for complete blood count (CBC), bone marrow (BM) and peripheral blood (PB) profiles including hematopoietic stem cell (HSC), immature and mature myeloid, lymphoid, and erythroid. Post-irradiation survival would also be recorded and compared with that of mic. The protective and therapeutic activities of ANG against H-ARS would also be examined. For this purpose, recombinant mouse ANG protein would be administered into mice 24 h before, immediate or 24 hours after they receive γ-irradiation, and the effect on animal survival and functional recovery of hematopoiesis would be determined.

2. Keywords

Angiogenin, radio protection, radio mitigation, medical countermeasure for radiation exposure, strategic national stockpile

3. Accomplishments

What were the major goals and objectives of the project?

The major goal is to obtain proof-of-principle data that supplemental therapy of ANG protein is a feasible strategy for developing medical countermeasure against H-ARS. This goal is to be accomplished by 4 major tasks. 1) Determine the effect of ANG levels on post-irradiation survival; 2) Analyze the effect of ANG levels on hematopoietic recovery after radiation exposure; 3) Assess the effect of ANG protein supplemental therapy on post-irradiated animal survival; 4) Examine the effect of ANG protein supplemental therapy on function of hematopoietic system.

What was accomplished under these goals?

We have 1) determined the survival of WT and ANG KO mice after exposure to 7.25 Gy, 7.76 Gy, and 8.25 Gy radiation; 2) analyzed complete blood counts and peripheral blood profiles of WT and ANG KO mice after radiation exposure; 3) examined the beneficial effect of ANG protein supplemental therapy on post-irradiation animal survival; 4) examined the protective activity of ANG treatment prior to radiation exposure; 5) examined the effect of ANG protein supplemental therapy on function of hematopoietic system; 6) examined the effect of knocking out ANG receptor PlexinB2 in hematopoietic cells on radiation-induced hematopoietic damage and animal survival.

<u>Significant Results:</u>

1) *Ang*$^{-/-}$ mice displayed reduced survival (Fig. 1A) following exposure to various doses of γ-radiation, accompanied by decreased blood leukocyte recovery (Fig. 1B), reduced total BM cellularity (Fig. 1C), reduced hematopoietic stem and progenitor cells (HSPC) (Fig. 1D) and lymphoid-restricted progenitor (LymPro) number (Fig. 1F), and more active cycling (Fig. 1E and H). In contrast, myeloid-restricted progenitors (MyePro) in

Ang$^{-/-}$ mice showed reduced cell number (Fig. 1H), but restricted proliferation (Fig. 1I) following irradiation.

2) Upon γ-irradiation, *Ang*$^{-/-}$ mice also demonstrated increased apoptosis in all cell types (Fig. 1I), as well as reduced lymphoid and myeloid colony formation (Fig. 1J). Together, these data suggest that ANG deficiency leads to reduced animal survival, accompanied by diminished cell number, perturbed cell cycling, and elevated apoptotic activity in hematopoietic cells.

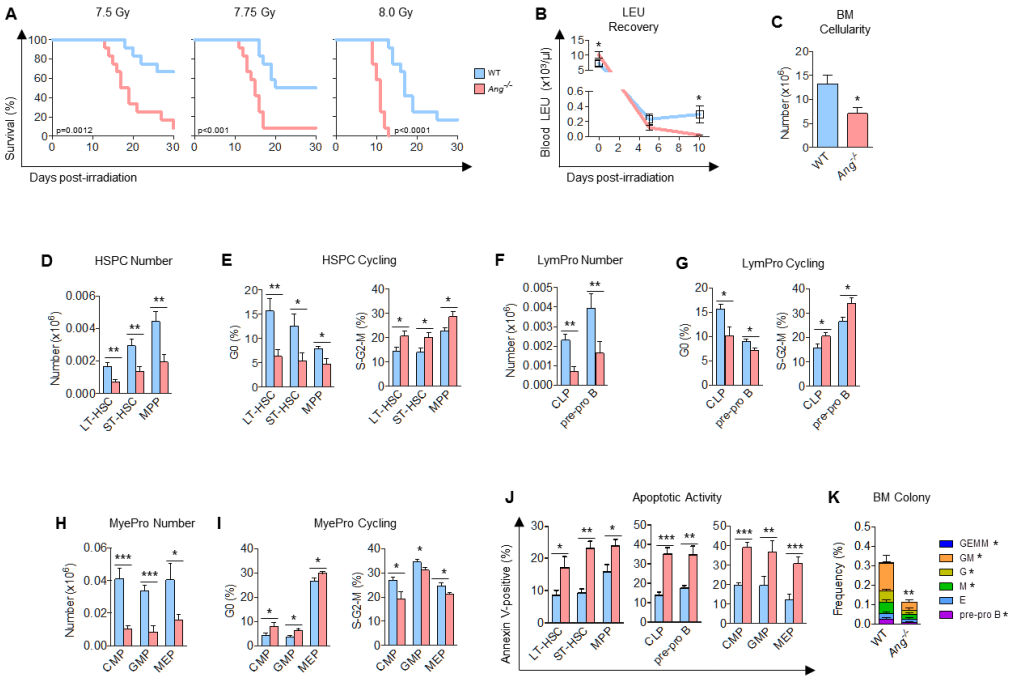


Fig. 1. Increased sensitivity of *Ang*$^{-/-}$ mice to γ-irradiation. (**A**) Kaplan-Meier survival curves of WT or *Ang*$^{-/-}$ mice subjected to 7.5 Gy (left), 7.75 Gy (middle), or 8.0 Gy (right) radiation (n=12). (**B**) Blood leukocyte recovery in WT or *Ang*$^{-/-}$ mice treated with 8.0 Gy (n=10). (**C-J**) BM cellularity (**C**), HSPC number per femur (**D**), HSPC cycling (**E**), LymPro number per femur (**F**), LymPro cycling (G), MyePro number per femur (**H**), MyePro cycling (**I**), apoptotic activity (**J**), and colony formation (**K**) of WT or *Ang*$^{-/-}$ mice treated with 4.0 Gy TBI (n=6). Animals were sacrificed and analyzed on day 7 post-irradiation.

3) Treatment with recombinant ANG protein enhances animal survival. We pretreated WT or *Ang*$^{-/-}$ mice with ANG daily for three successive days and irradiated mice with 8.0 Gy 24 hours following the final ANG treatment. Significantly, the 30-day survival rate increased from 20% to 90% after ANG treatment (Fig. 2A, solid lines), indicating that ANG is radioprotective. Importantly, 80% of *Ang*$^{-/-}$ mice also survived following ANG pretreatment whereas 100% of untreated *Ang*$^{-/-}$ mice died (Fig. 2A, dotted lines). Pre-treatment with ANG protected against radiation (4 Gy)-induced loss of bone marrow cellularity (Fig. 2B) and increase in cycling of HSPC (Fig. 2C). In contrast, ANG pre-treatment not only prevented the loss of MyePro but also promoted their proliferation (Fig. 2C). Moreover, ANG protected against radiation-induced apoptosis in all cell types (Fig. 2E), and led to enhanced colony formation (Fig. 2F) and post-transplant

reconstitution (Fig. 2G). Together, these data demonstrate the protective function of ANG against radiation-induced BM damage, likely through induction of HSPC quiescence and promotion of MyePro proliferation.

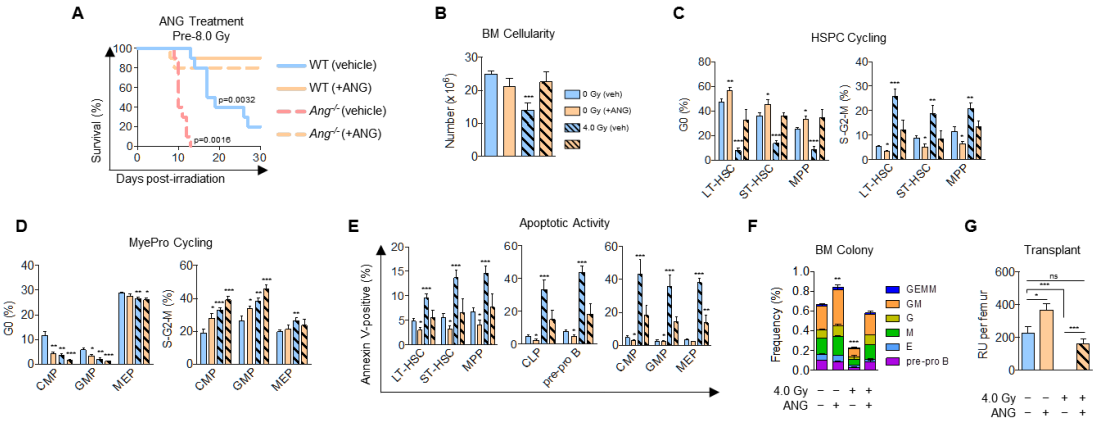


Fig. 2. Protective activity of ANG against radiation-induce damage. (**A**) Survival of WT or *Ang*$^{-/-}$ mice treated with ANG daily for three successive days 24 h pre-8.0 Gy (n=10). (**B-G**) Bone marrow cellularity (**B**), HSPC cycling (**C**), MyePro cycling (**D**), apoptotic activity (**E**), colony formation (**F**), and post transplant reconstitution (**G**) of WT mice pre-treated with ANG daily for three successive days 24 h before 4.0 Gy TBI (n=6). Animals were sacrificed and analyzed on day 7 post-irradiation.

4) ANG has therapeutic activity as a radio-mitigating agent. We irradiated mice with 8.0 Gy and began ANG treatment 24 hours later, and found that the majority of ANG-treated mice survived, including ANG-treated *Ang*$^{-/-}$ mice, suggesting that ANG has radio-mitigating capabilities (Fig. 3A). A similar enhancement of survival was observed when ANG treatment was begun immediately following irradiation (Fig.3B) Importantly, treatment with ANG 24 hour post-irradiation prevented TBI-induced reduction of overall BM cellularity (Fig. 3C), as well as HSPC cells and MyePro (Fig. 3D).

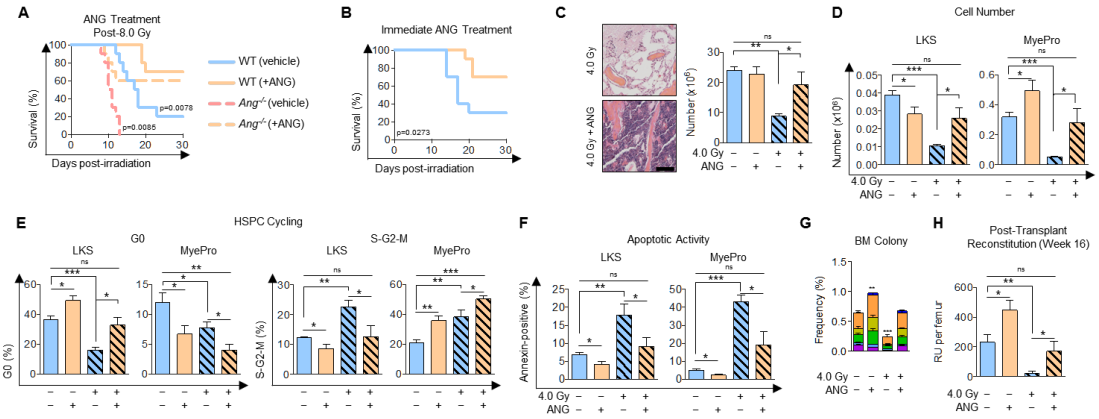


Fig. 3. Mitigative activity of ANG against radiation-induce damage. (**A**) Survival of WT or *Ang*$^{-/-}$ mice treated with ANG daily for three successive days 24 h post-8.0 Gy (n=10). (**B**) Survival of WT mice treated with ANG immediately following 8.0 Gy (n=10). (**C-H**) BM cellularity of femurs (**C**), LKS and MyePro cell number per femur (**D**), cell cycling (**E**), apoptotic activity (**F**), colony formation (G), and post-transplant reconstitution (H) of WT mice treated with ANG daily for three successive days 24 h post-4.0 Gy (n=6). Scale bar = 100 µm.

5) ANG has dichotomous role toward stem and progenitor cells in the hematopoietic system in hematopoietic regeneration after radiation damage. We found that post-irradiation treatment of ANG restricted proliferation of HSPC, and simultaneously enhanced proliferation of MyePro (Fig. 3E). Further, ANG prevented radiation-induced apoptosis in both HSPC cells and MyePro (Fig. 3F). Significantly, defects in colony formation (Fig. 3G) and post-transplant reconstitution (Fig. 3H) can be rescued by ANG treatment.

6) We also assessed the protective and mitigative effect of ANG in lethally-irradiated animals and found that ANG treatment either before or after lethal irradiation improved survival (Fig. 4A) and enhanced BM cellularity (Fig. 4B). Moreover, ANG significantly increased the LD50 when treatment was begun 24 hours post-irradiation (Fig. 4C). Further, treatment with ANG upregulated pro-self-renewal genes in HSPC cells and led to enhanced pro-survival transcript levels and reduced pro-apoptotic transcripts in both HSPC cells and MyePro (Fig. 4D). Importantly, ANG treatment enhanced rRNA transcription only in MyePro (Fig. 4D) and tiRNA production only in HSPC cells (Fig. 4E) following radiation, consistent with its dichotomous role in promoting and restricting cell proliferation in these two cell types.

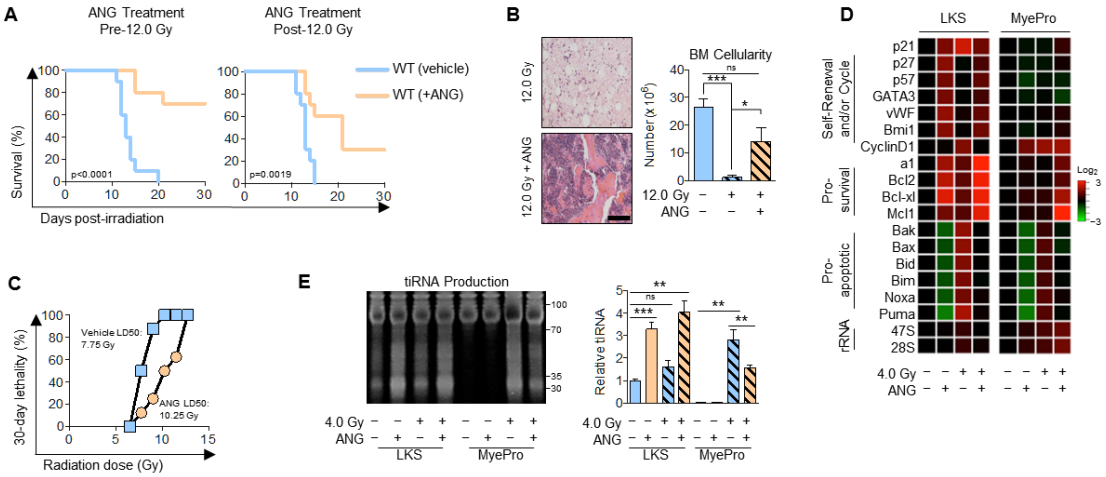


Figure 4. ANG enhances radioprotection and radioresistance. (**A**) Survival of WT mice treated with ANG daily for three successive days 24 h prior or post-12.0 Gy. (**B**) H&E and BM cellularity of femurs of WT mice treated with ANG daily for three successive days 24 h post-12.0 Gy (n=6). Scale bar = 100 μm. (**C**) LD50 of mice treated with ANG daily for three successive days beginning 24 h post-TBI (n=8). (**D**-**E**) qRT-PCR analysis of pro-self-renewal, pro-survival, pro-apoptotic, and rRNA transcripts (**D**, n=6), and tiRNA production (**E**, n=3) in LKS or MyePro sorted from irradiated mice (4.0 Gy) and treated with 300 ng/ml ANG.

7) We have deleted *PlxinB2* (*Plxnb2*) gene in hematopoietic cells by crossing Mx-1 Cre mice with *Plxnb2* floxed mice. We found that HSPC from Mx1-specific *Plxnb2*$^{-/-}$ mice cycle more actively, compared to those from Mx1 WT mice. Mx1-specific *Plxnb2*$^{-/-}$ mice showed increased LT-HSC number in BM, and elevated peripheral blood cell counts relative to Mx-1 WT mice. We treated Mx1 WT or Mx1-specific *Plxnb2*$^{-/-}$ mice with 7.75 Gy γ-irradiation and observed premature death of all Mx1-specific *Plxnb2*$^{-/-}$ animals (Fig.

5). These data suggest Plxnb2 in hematopoietic cells is the functional receptor for ANG to mediate the radiop-rotective and radio-mitigative activity of ANG.

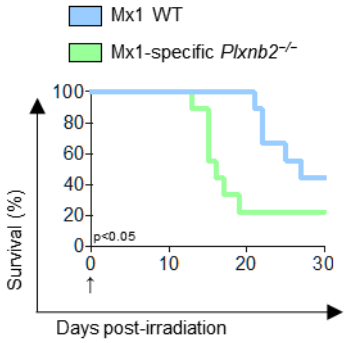


Fig. 5. Survival of Mx1-specific *Plxnb2*$^{-/-}$ mice following a single exposure to 7.75 Gy γ-irradiation (n=9).

Together, these results establish a model by which ANG interacts with PLXNB2 to simultaneously stimulates proliferation of rapidly-responding myeloid-restricted progenitors and preserves HPSC stemness, in association with enhanced hematopoietic regeneration and improved survival upon radiation exposure.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

The findings were reported in a paper published in Cell on Aug 11, 2016 (Appendix 1).

Goncalves, K. A., Silberstein, L., Li, S., Severe, N., Hu, M. G., Yang, H., Scadden, D. T., and Hu, G. F. (2016) Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells. Cell 166, 894-906.

Tufts Medical Center also issued a press release on publication of above paper that contains these finding (Appendix 2).

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Our findings provide a practical application of recombinant ANG for preventing and mitigating radiation exposure in a scenario of catastrophic accident or terrorism. ANG was found to be effective against radiation-induced hematopoietic syndrome and significantly enhanced survival when administered 24 hours either before or after radiation exposure. Currently, there are no FDA-approved drugs to treat severely irradiated individuals. An efficacy requirement mandated by The Radiation and Nuclear Countermeasures Program at the National Institute of Allergy and Infectious Diseases, and ANG apparently satisfies this requirement. Moreover, current standard-of-care approaches, including (G- Neuprogen (G-CSF) and its derivatives, which is on the list of Strategic National Stockpile, target a limited progenitor cell pool and require repeated doses to combat radiation-induced neutropenia. In this regard, ANG is a promising candidate as a medical countermeasure for radiation exposure.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

We have filed a patent application

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Nothing to report

6. Products

Publications

One paper has been published: Appendix I

Goncalves, K. A., Silberstein, L., Li, S., Severe, N., Hu, M. G., Yang, H., Scadden, D. T., and Hu, G. F. (2016) Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells. Cell 166, 894-906.

Patent applications

One patent application has been filed: Appendix III

David Scadden, Peter Kharchenko, Lev Silberstein, Guo-fu Hu, Kevin Goncalves. Treatment with angiogenin to enhance hematopoietic reconstitution. Serial no.: 62/315281.

7. Participants and Other Collaborating Organizations

Name:	Guo-fu Hu, PhD
Project Role:	PD/PI
Nearest Person month worked:	3 (27.5%)
Contribution to Project:	Conceived ideas, designed experiments, interpreted data, and wrote manuscript
Name:	Shuping Li, PhD
Project Role:	Postdoctoral Fellow
Nearest Person month worked:	6 (100%)
Contribution to Project:	Performed survival analysis, FACS analysis, and transplantation.
Name:	Nil Vanli
Project Role:	Graduate Student
Nearest Person month worked:	2
Contribution to Project:	Prepared ANG proteins, maintained animal clones.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to report

8. Special reporting requirements
None

9. Appendices
Appendix I: Cell – "Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells"

Appendix II: PUBLIC RELEASE: 11-AUG-2016 - Tufts Medical Center researchers find new functions of blood cell protein in transplant - TUFTS MEDICAL CENTER

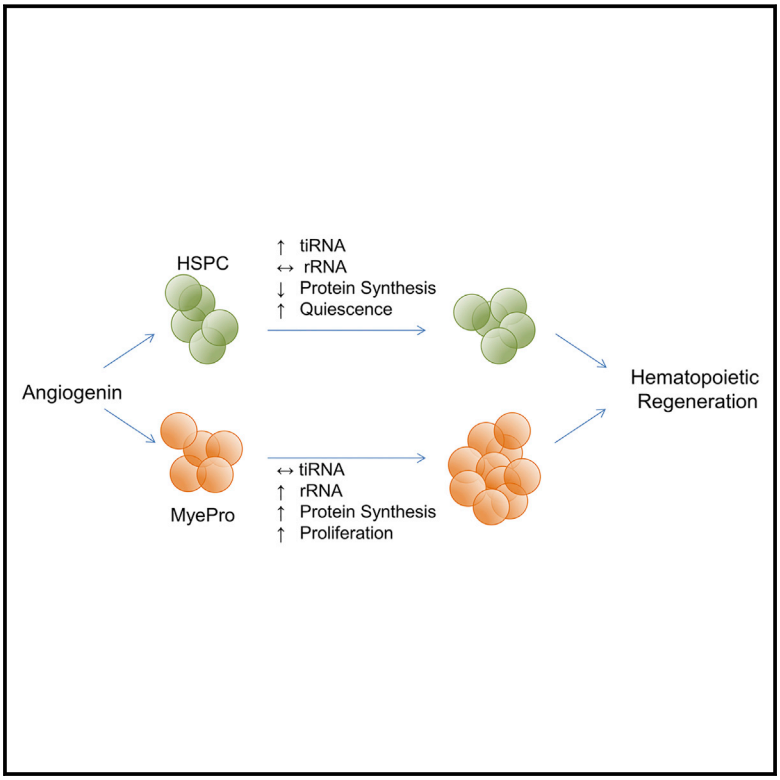
Appendix III: US Patent Application – Application Number: 62/315,281 3/30/16

Article

Cell

Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells

Graphical Abstract



Highlights

- HSPC quiescence and progenitor cell proliferation are simultaneously enhanced by ANG
- The dichotomous effect of ANG is related to differential RNA processing
- ANG prevents and mitigates radiation-induced bone marrow failure
- ANG dramatically improves transplantation efficiency of mouse and human HSPCs

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In Brief

A new niche-specific modulator maintains the stemness of hematopoietic stem/progenitor cells while promoting the proliferation of myeloid progenitor cells through the regulation of RNA metabolism.

Goncalves et al., 2016, Cell *166*, 894–906
August 11, 2016
http://dx.doi.org/10.1016/j.cell.2016.06.042

CellPress

Cell

Article

Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells

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http://dx.doi.org/10.1016/j.cell.2016.06.042

SUMMARY

Regulation of stem and progenitor cell populations is critical in the development, maintenance, and regeneration of tissues. Here, we define a novel mechanism by which a niche-secreted RNase, angiogenin (ANG), distinctively alters the functional characteristics of primitive hematopoietic stem/progenitor cells (HSPCs) compared with lineage-committed myeloid-restricted progenitor (MyePro) cells. Specifically, ANG reduces the proliferative capacity of HSPC while simultaneously increasing proliferation of MyePro cells. Mechanistically, ANG induces cell-type-specific RNA-processing events: tRNA-derived stress-induced small RNA (tiRNA) generation in HSPCs and rRNA induction in MyePro cells, leading to respective reduction and increase in protein synthesis. Recombinant ANG protein improves survival of irradiated animals and enhances hematopoietic regeneration of mouse and human HSPCs in transplantation. Thus, ANG plays a non-cell-autonomous role in regulation of hematopoiesis by simultaneously preserving HSPC stemness and promoting MyePro proliferation. These cell-type-specific functions of ANG suggest considerable therapeutic potential.

INTRODUCTION

A population of quiescent adult stem cells with self-renewal and differentiation capabilities is required for tissue homeostasis and regeneration (Orford and Scadden, 2008). Stem cell quiescence has been shown to protect cells from exhaustion, especially under stress, which is essential for both continuous cell output and prevention of malignant transformation (Nakamura-Ishizu et al., 2014). In the hematopoietic system, this is achieved by both cell-intrinsic and -extrinsic factors. Cell-cycle and epigenetic regulators—as well as pathways involved in growth control, including cyclin-dependent kinases and inhibitors, Rb, PI3K, and p53–have been demonstrated as cell-intrinsic regulators of hematopoietic stem/progenitor cells (HSPCs) proliferation (Ito and Suda, 2014; Nakamura-Ishizu et al., 2014). Various secreted and cell-surface factors that are produced by bone marrow (BM), including angiopoetin-1, thrombopoietin, SCF (stem cell factor), CXCL12, and TGF-β (transforming growth factor β) (Ito and Suda, 2014; Mendelson and Frenette, 2014; Morrison and Scadden, 2014), have been shown to extrinsically regulate HSPCs.

Recent strides have been made to therapeutically harness growth control properties of hematopoietic stem cells (HSCs) in an effort to improve hematopoietic regeneration in the clinic. In the context of HSC transplantation (SCT), in particular, low numbers of HSPCs result in low transplantation efficacy, which can markedly affect the survival of patients undergoing SCT (Smith and Wagner, 2009). Therefore, expanding transplantable cell numbers has been a long-standing goal (Boitano et al., 2010; Delaney et al., 2010; Fares et al., 2014; Himburg et al., 2010; North et al., 2007). Preserving HSC function can be at odds with expansion strategies, but advances in improved BM homing (Li et al., 2015) and maintained stemness through protection against extraphysiologic oxygen shock (Mantel et al., 2015) are being made. To our knowledge, however, no studies to date have accomplished preserving HSC regenerative capacity through quiescence while enabling progenitor expansion.

Angiogenin (ANG), also known as RNase5, is a member of the secreted vertebrate-specific RNase superfamily and has angiogenic (Fett et al., 1985), neurogenic (Subramanian and Feng, 2007), neuroprotective (Subramanian et al., 2008), and immune-regulatory (Hooper et al., 2003) functions. Under growth conditions, ANG promotes proliferation and enhances survival in a variety of cell types, including endothelial (Kishimoto et al., 2005), neuronal (Kieran et al., 2008), and cancer (Yoshioka et al., 2006) cells. The growth stimulatory function of ANG is mediated through rRNA transcription (Tsuji et al., 2005) and requires nuclear translocation of ANG (Xu et al., 2003). Under stress, ANG is translocated to stress granules (SGs) and mediates the production of tRNA-derived stress-induced small RNA (tiRNA); these small RNA species enhance cellular survival by simultaneously suppressing global protein translation, saving anabolic energy,

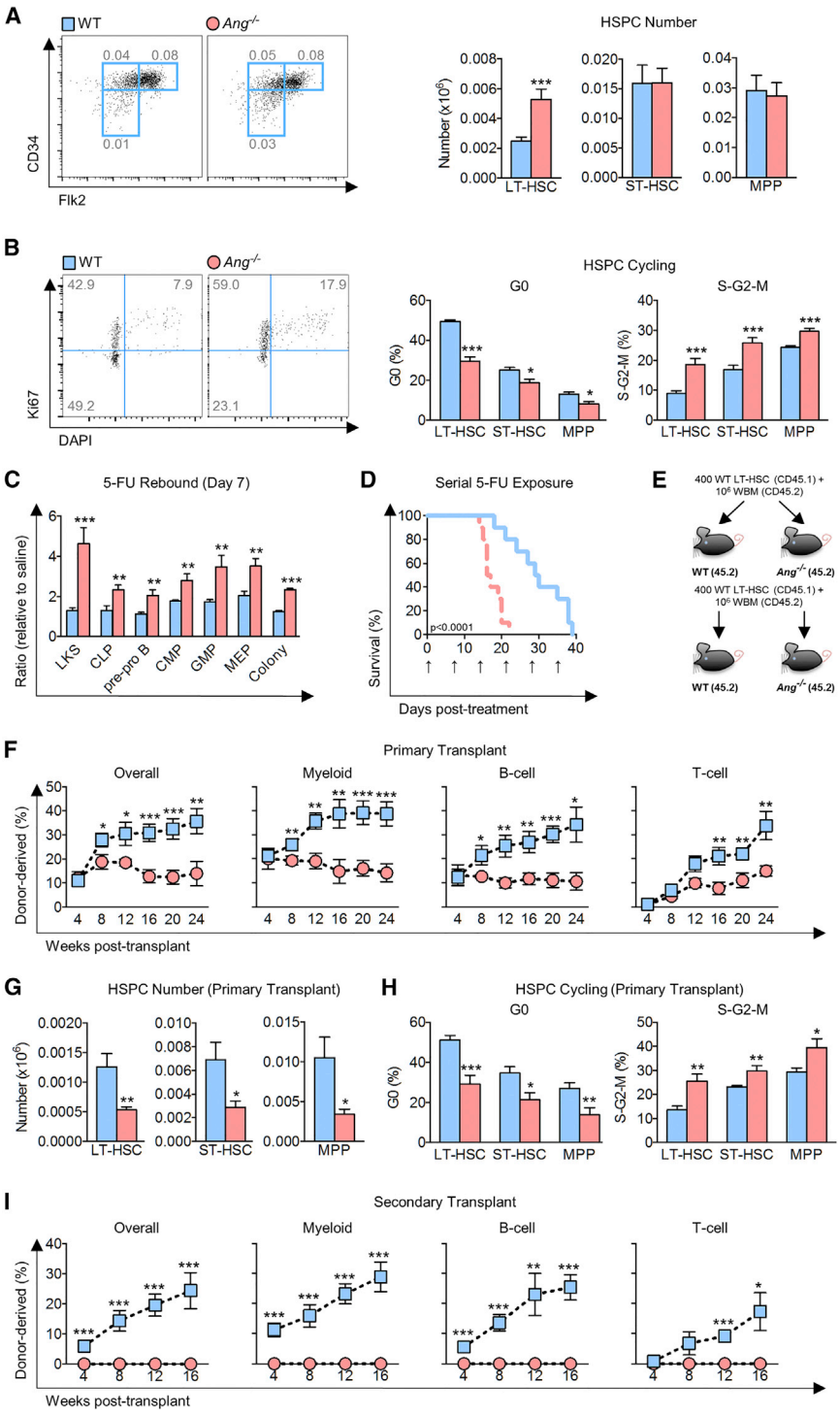

Figure 1. *Ang* Deficiency Results in Loss of HSPC Quiescence and Defective Transplantation

(A and B) Quantification of primitive hematopoietic cell number per femur (A; n = 12) and cell-cycle status (B; n = 8) in $Ang^{-/-}$ mice.

(C) Quantification of stem and progenitor cells in $Ang^{-/-}$ mice on day 7 post-exposure to 150 mg/kg 5-FU (n = 8).

(D) Survival of $Ang^{-/-}$ mice following weekly 5-FU (150 mg/kg) exposure (n = 10). Arrows indicate day of injection.

(E) Experimental schema of serial transplant using WT or $Ang^{-/-}$ hosts.

(F–H) Multi-lineage donor cell chimerism (F), HSPC number per femur (G), and HSPC cell-cycle status (H) after competitive primary transplantation of WT LT-HSCs into lethally irradiated WT or $Ang^{-/-}$ recipients (n = 8).

(I) Chimerism after secondary transplantation of sorted LT-HSCs from primary recipients into WT or $Ang^{-/-}$ secondary recipients (n = 8).

Bar graphs represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

See also Figure S1 and Tables S1 and S2.

and permitting internal ribosomal entry sequence (IRES)-mediated protein translation of anti-apoptotic genes (Emara et al., 2010; Ivanov et al., 2011; Yamasaki et al., 2009).

In this study, we demonstrate that ANG restricts proliferation of primitive HSPCs but stimulates proliferation of myeloid-restricted progenitor (MyePro) cells. We also demonstrate that ANG mediates tiRNA production in HSPCs but promotes rRNA transcription in MyePro cells. Importantly, these properties of ANG are reflected by enhanced hematopoietic regeneration and animal survival upon treatment with recombinant ANG protein following radiation-induced BM failure and a dramatic increase in the level of hematopoietic reconstitution by ANG-treated mouse LT-HSCs (long-term HSCs) and human $CD34^+$ cord blood (CB) cells. Therefore, ANG is a previously unrecognized regulator of HSPCs, with unique RNA processing function relevant to radiation-induced BM failure and clinical SCT.

RESULTS

ANG Plays a Non-Cell-Autonomous Role in Regulation of LT-HSC Quiescence and Self-Renewal

We sought to examine the functional role of ANG in hematopoiesis because it was originally found to be differentially expressed in BM osteolineage cells in close proximity to transplanted HSPCs (Silberstein et al., 2016). The presence of ANG mRNA in mesenchymal cells from BM was confirmed by qPCR of sorted subsets of cells (Figure S1A). We then profiled HSPCs (Figure S1B) in the BM of *Ang* knockout ($Ang^{-/-}$) mice and found a 2-fold increase in the number of LT-HSCs, but not ST-HSCs (short-term HSCs) or multi-potent progenitors (MPPs), in $Ang^{-/-}$ BM (Figure 1A).

Consistent with this finding, a reduction in G0 phase and a corresponding increase in S/G2/M phases of the cell cycle (Figure 1B), as well as enhanced bromodeoxyuridine (BrdU) incorporation (Figure S1C), were observed in *Ang*$^{-/-}$ LT-HSCs. *Ang*$^{-/-}$ ST-HSCs and MPPs also displayed increased cycling (Figures 1B and S1C) but to a less severe degree (for LT-HSCs, ST-HSCs, and MPPs, respectively, the percentage increases in S/G2/M phases were 107.7 ± 23.7, 52.2 ± 10.0, and 21.9 ± 4.6, and the percent decreases in G0 phase were 40.1 ± 4.3, 24.3 ± 7.0, and 37.6 ± 10.3), which, combined with elevated apoptosis across hematopoietic lineages in *Ang*$^{-/-}$ mice (Figure S1D), might partially explain why no difference in cell number was observed for ST-HSCs and MPPs (Figure 1A). These patterns were also observable by alternative cell-surface markers (SLAM/CD48) for HSPC subtypes (Cabezas-Wallscheid et al., 2014) (Figures S1E and S1F), confirming that *Ang*$^{-/-}$ LT-HSCs cycle more actively.

Despite the significant increase in LT-HSC number in *Ang*$^{-/-}$ BM (Figures 1A and S1E), only mild lymphocytosis was apparent in 8- to 12-week-old mice at baseline (Table S1). However, under conditions of stress, progenitor response to the genotoxic agent, 5-fluorouracil (5-FU), was markedly exaggerated in *Ang*$^{-/-}$ mice (Figure 1C). Further, exposure of these animals to serial proliferative stress, such as weekly injections of 5-FU, resulted in excess animal morality (Figure 1D). Consistent with the phenotype of stress-induced exhaustion (Orford and Scadden, 2008), aged 22-month-old *Ang*$^{-/-}$ mice developed leukopenia (Table S2) and showed a marked reduction in the number of primitive hematopoietic cells in the BM (Figure S1G), accompanied by more active HSPC cycling (Figure S1H). Aged *Ang*$^{-/-}$ mice also displayed reduced functional capabilities by in vitro methylcellulose assays (Figures S1I and S1J) and in vivo competitive transplantation (Figures S1K and S1L).

To further characterize the functional significance of elevated cycling in *Ang*$^{-/-}$ HSPCs, we performed transplant experiments by injecting either sorted LT-HSCs (Figure 1E) or total BM (Figure S1M) into lethally irradiated wild-type (WT) or *Ang*$^{-/-}$ hosts. In both experiments, impaired long-term multi-lineage reconstitution was observed in *Ang*$^{-/-}$ hosts (Figures 1F and S1N), with particularly pronounced impairment at later time points. Notably, WT HSPCs in the ANG-deficient microenvironment displayed a significantly reduced HSPC number, accompanied by more active cycling (Figures 1G and 1H). To rule out a homing defect as a cause of impaired reconstitution in *Ang*$^{-/-}$ hosts, CFSE-labeled CD45.1 Lin^{-} cells were injected into irradiated WT or *Ang*$^{-/-}$ recipients, and no difference in the percentage of LKS cells or MyePro cells in the BM of these animals was observed 16 hr after transplantation (Figure S1O).

In order to evaluate the effect of niche-derived ANG (Silberstein et al., 2016) on HSC self-renewal, we carried out serial transplantation experiments. While competitive transplantation demonstrated no detectable hematopoietic contribution by LT-HSCs that had been passaged through ANG-deficient primary recipients (Figure 1I), non-competitive transplantation of whole BM cells from primary *Ang*$^{-/-}$ recipients resulted in the death of all secondary *Ang*$^{-/-}$ recipients (Figure S1P). The marked inability to reconstitute in both transplant settings indicates severe loss of HSC self-renewal capacity in ANG-deficient hosts. Taken together, these data demonstrate that ANG plays a non-cell-autonomous role in the regulation of quiescence and self-renewal of primitive hematopoietic cells, particularly LT-HSCs.

ANG Enhances MyePro Proliferation

The finding that ANG restricts cell cycling of HSPCs is the first known evidence for a suppressive activity of ANG on cell proliferation, as all previous studies revealed that ANG promotes cell proliferation (Li and Hu, 2010). Therefore, we examined cell-type-specific effects of ANG in various cells of the hematopoietic lineage. We observed that, while *Ang*$^{-/-}$ LKS cells cycle more actively, *Ang*$^{-/-}$ MyePro cells showed reduced cycling by Ki67 (Figure 2A) and BrdU (Figure S2A) staining.

The cell-type specificity of ANG was further illustrated by analyzing lymphoid-restricted progenitor (LymPro) cells and MyePro cells, including common lymphoid progenitor (CLP), pre-pro B, common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), and megakaryocyte-erythroid progenitor (MEP) cell types. As with HSPCs, we found that *Ang*$^{-/-}$ CLPs and pre-pro B cells (Figure S2B) cycle more actively (Figure S2C) and incorporate more BrdU (Figure S2D), suggesting that ANG restricts LymPro proliferation. In contrast, *Ang*$^{-/-}$ MyePro cells, including CMPs, GMPs, and MEPs, displayed less active cycling (Figure S2F) and reduced BrdU incorporation (Figure S2G), accompanied by a reduction of CMP and GMP numbers (Figure S2E). Importantly, we observed restricted proliferation of myeloid-biased MPP3s and more active cycling of lymphoid-biased MPP4s (Cabezas-Wallscheid et al., 2014) in *Ang*$^{-/-}$ mice (Figure 2B). Together, these data indicate that the function of ANG is cell-type specific: while ANG restricts cell proliferation in primitive HSPCs and LymPro cells, it promotes proliferation of MyePro cells. This cell-type specificity is observable within the earliest phenotypically defined lineage-biased progenitor cell types: MPP3 and MPP4.

Cell-type-specific regulation of ANG was confirmed by the fact that *Ang* deletion resulted in decreased expression of cycle checkpoint or self-renewal genes, including *p21*, *p27*, *p57*, *GATA3*, *vWF*, and *Bmi1* (Cheng et al., 2000; Frelin et al., 2013; Kent et al., 2009; Matsumoto et al., 2011; Park et al., 2003) in LKS cells but not in MyePro cells (Figure S2H). In contrast, the cell-cycle-related gene, cyclin D1, was decreased in MyePro cells, but not in LKS cells, upon *Ang* deletion (Figure S2H). We then examined the effect of recombinant ANG protein on cultured HSPCs and MyePro cells. Remarkably, culture with ANG for 2 hr in PBS led to a dose-dependent increase in the expression of pro-self-renewal genes in LKS cells (Figure 2C). No such change was noted in MyePro cells. In contrast, cyclin D1 was enhanced by culture with ANG in MyePro cells but not in LKS cells (Figure 2C). A similar pattern was observed in LT-HSCs cultured with ANG for 2 hr in PBS (Figure S2I) or under longer culture conditions in cytokine-supplemented S-clone media (Figure S2J). Notably, addition of exogenous ANG led to elevated levels of pro-self-renewal genes in *Ang*$^{-/-}$ LT-HSCs, as was seen in WT cells (Figure S2K). Together, these data demonstrate that ANG differentially regulates gene expression in HSPCs and MyePro cells, including genes relevant for proliferation and self-renewal.

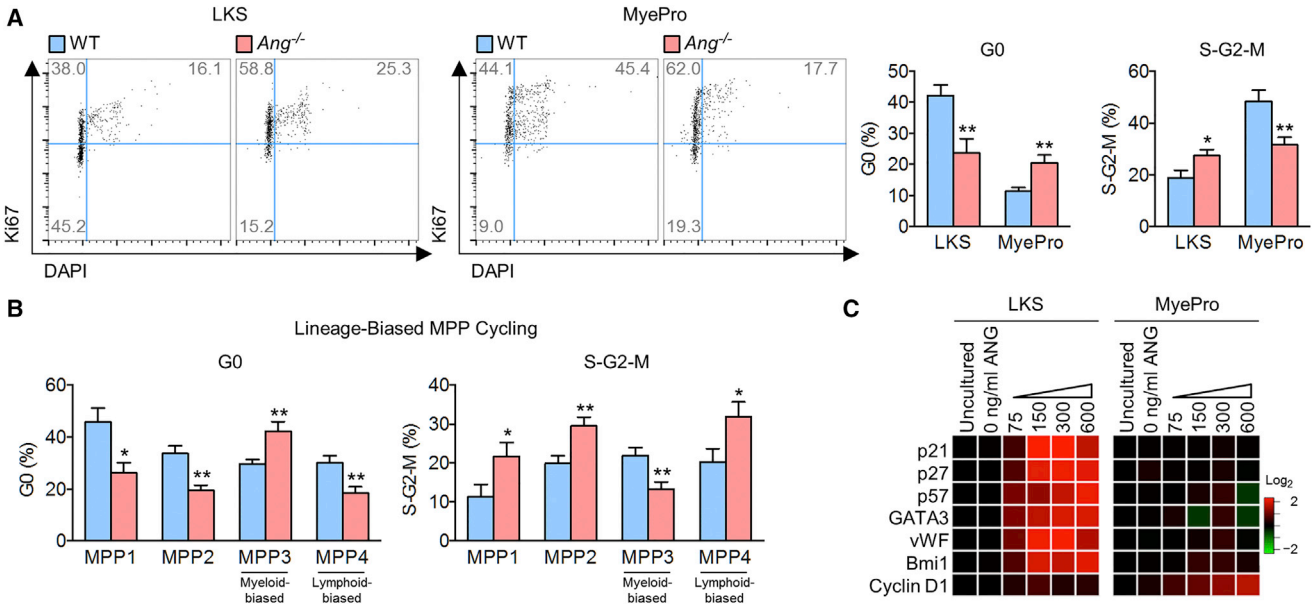

Figure 2. Dichotomous Effect of ANG in LKS and MyePro Cycling

(A and B) Cell-cycle status of LKS cells and MyePro cells (A; n = 8) and of MPP1-4 cells (B; n = 6) in *Ang*$^{-/-}$ mice.

(C) qRT-PCR analysis of pro-self-renewal transcripts from sorted LKS cells or MyePro cells treated with mouse ANG protein (0–600 ng/ml; n = 6).

Bar graphs represent mean ± SEM, and heatmaps represent mean. *p < 0.05; **p < 0.01.

See also Figure S2.

ANG Dichotomously Regulates Protein Synthesis in LKS and MyePro Cells

ANG has been shown in other cell types to regulate global protein synthesis, a housekeeping function recently shown to be tightly regulated in primitive HSCs (Signer et al., 2014). To determine whether ANG regulates protein synthesis in HSPCs, we assessed in vivo protein synthesis in *Ang*$^{-/-}$ mice by a fluorogenic assay using O-propargyl-puromycin (OP-Puro) (Signer et al., 2014). Consistent with their cell-cycle profile (Figures 2A and S2A), *Ang*$^{-/-}$ LKS cells showed a higher rate of protein synthesis, while *Ang*$^{-/-}$ MyePro cells demonstrated reduced protein synthesis (Figure 3A). This cell-type specificity was also evident when BM was analyzed with more specific hematopoietic cell markers (Figure S3A) or when assessing cells in G0/G1 phase (Figure S3B). In vivo administration of OP-Puro did not alter BM cellularity or LT-HSC frequency (Figures S3C and S3D). Significantly, in vitro culture of LKS cells with ANG led to reduced protein synthesis, while the addition of ANG to MyePro cells led to enhanced protein synthesis (Figure 3B). Together, these data demonstrate that the effect of ANG on protein synthesis is cell-type specific.

The Restrictive Function of ANG in HSPCs Is Mediated by tiRNA

To reveal the biochemical mechanism for this dichotomous effect of ANG on protein synthesis, we first assessed rRNA transcription, which is stimulated by ANG in other cell types (Kishimoto et al., 2005; Tsuji et al., 2005). The addition of ANG led to enhanced rRNA transcription in MyePro and whole BM cells, but not in LKS cells (Figure 3C), while *Ang* deletion resulted in reduced rRNA transcription in MyePro and whole BM cells but not in LT-HSCs (Figure S3E). These findings are consistent with increased proliferation and protein synthesis observed in MyePro cells following ANG treatment.

ANG has been shown to reprogram protein synthesis as a stress response to promote survival under adverse conditions. This function of ANG is mediated by tiRNA, a noncoding small RNA that specifically permits translation of anti-apoptosis genes while global protein translation is suppressed so that stressed cells have adequate time and energy to repair damage, collectively promoting cell survival (Emara et al., 2010; Ivanov et al., 2011; Yamasaki et al., 2009). To assess whether ANG-mediated regulation of protein synthesis is tiRNA dependent, we assessed bulk small RNA production by electrophoresis. LKS cells exhibited dramatically higher small RNA production than MyePro cells at baseline (Figure 4A). tiRNA level was normally low in Lin^{+}-differentiated cell types, although it became detectable when a higher amount (15 μg) of total RNA was assessed (Figure S3F). Importantly, the addition of ANG led to markedly elevated tiRNA levels in LKS cells (Figure 4A). Equal loading was affirmed by tRNA levels (indicated by arrows in Figure 4A). The addition of ANG to Lin^{+} cells did not result in an increase in tiRNA levels, in contrast to significantly elevated tiRNA levels following ANG treatment of HSPCs (Figure S3F; compare to Figure 4A). Furthermore, tiRNA levels in *Ang*$^{-/-}$ LKS cells were substantially reduced but not completely diminished (Figure S3G). As ANG is the only RNase that has been demonstrated to mediate tiRNA production (Yamasaki et al., 2009), this finding suggests that other unidentified RNases may be responsible for the remaining level (29%) of tiRNA. Moreover, we also observed

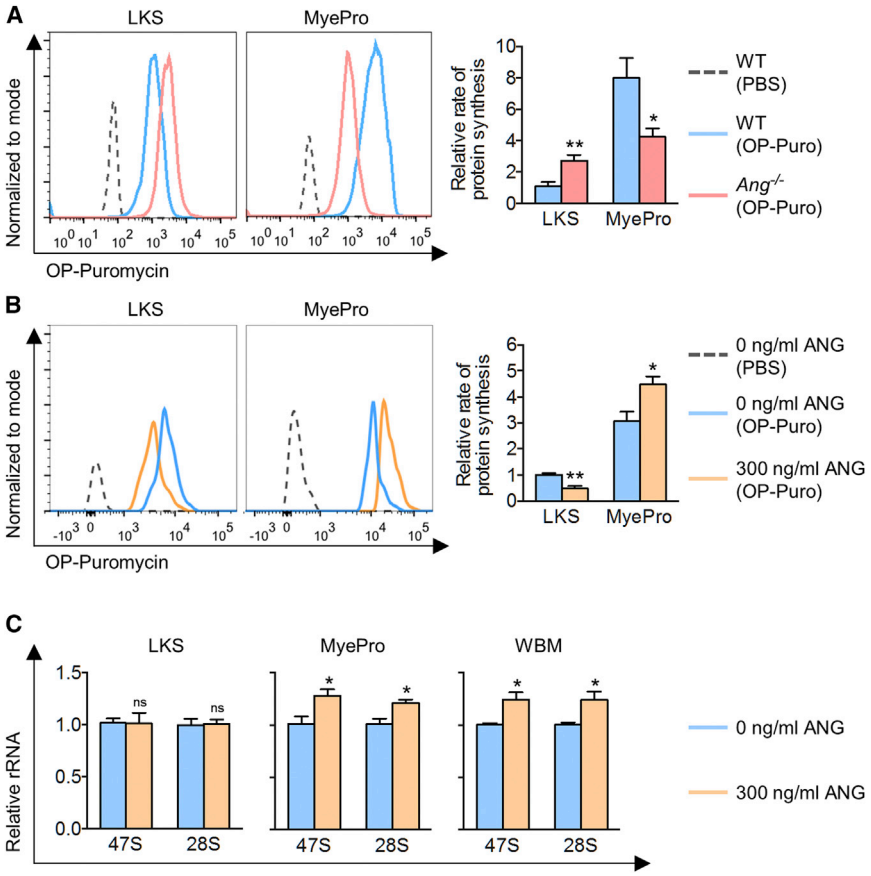

Figure 3. ANG-Mediated Regulation of Protein Synthesis Is Cell-Type Specific

(A) In vivo OP-Puro incorporation in WT or *Ang*$^{-/-}$ LKS cells and MyePro cells. Cells were analyzed 1 hr after OP-Puro administration. Bar graphs indicate relative values to WT LKS (n = 5).

(B) In vitro OP-Puro incorporation following 2-hr ANG treatment of LKS and MyePro cells. Bar graphs indicate relative values to untreated LKS (n = 6).

(C) qRT-PCR analysis of rRNA with primers targeting mature and precursor transcripts following 2-hr ANG treatment of LKS and MyePro cells, using various primer sets (n = 3). Bar graphs indicate relative values to untreated cells.

Error bars indicate mean ± SEM. *p < 0.05; **p < 0.01; ns, not significant.

See also Figures S3 and S4.

an increase in tiRNA production in MyePro cells by sodium arsenite (SA), which was suppressed by exogenous ANG protein (Figure S3H). In contrast, tiRNA production in LKS cells was not increased by SA but was enhanced by ANG both at steady-state and under SA-induced oxidative stress conditions (Figure S3H). These results demonstrate that ANG differentially regulates tiRNA in LKS and MyePro cells under both homeostatic and stress conditions.

To ensure that bulk small RNA reflect tiRNA, we analyzed the levels of a representative tiRNA, tiRNA-Gly-CCC, by northern blotting in ANG-treated LKS and MyePro cells. tiRNA-Gly-GCC was previously shown to be expressed in hematopoietic tissues, including BM and spleen, but was neither examined in primitive hematopoietic cells nor functionally validated (Dhahbi et al., 2013). Figure 4B shows that tiRNA-Gly-GCC was significantly elevated in LKS cells, relative to MyePro cells, and was further enhanced by exogenous ANG. Together, these data identify tiRNA as a distinct RNA species that is abundantly expressed in HSPCs and that is regulated by ANG.

To determine whether tiRNA was responsible for restricted protein synthesis in HSPCs, we transfected synthetic tiRNA-Gly-GCC in LKS and MyePro cells and assessed protein synthesis in vitro using OP-Puro. As tiRNA requires its 5′-phosphate to suppress protein synthesis (Ivanov et al., 2011), we used an inactive, dephosphorylated synthetic tiRNA-Gly-GCC, termed (d)5′-P-tiRNA, as a negative control. Expectedly, transfection of active 5′-P tiRNA, but not inactive (d) 5′-P-tiRNA, led to a significant reduction in the rate of protein synthesis in both LKS and MyePro cells (Figure 4C). Thus, tiRNA transfection phenocopies exogenous ANG on restriction of protein synthesis in LKS cells, which has been shown in Figure 3B. We also found that myeloid and lymphoid progenitor colony formation was restricted upon transfection of whole BM with active 5′-P tiRNA but not inactive (d)5′-P-tiRNA (Figure S3I). Moreover, transfection of active 5′-P-tiRNA led to upregulation of pro-self-renewal and pro-survival genes, and downregulation of pro-apoptotic genes, in both LKS and MyePro cells by lipofection (Figure 4D) and electroporation (Figure S3J).

The exact subcellular compartment where tiRNA is produced by ANG is currently unknown (Saikia et al., 2014), but the growth and survival function of ANG has been correlated to its SG localization in stressed cells (Pizzo et al., 2013). The finding that ANG produces tiRNA and restricts protein synthesis only in LKS cells prompted us to examine differential localization of ANG in SGs between LKS and MyePro cells. ANG is known to be internalized through receptor-mediated endocytosis and translocated to either the nucleus or SGs, depending on cell state, to mediate rRNA or tiRNA production, respectively (Pizzo et al., 2013). We found that ANG was colocalized with poly(A)-binding protein (PABP), an SG marker, in LKS cells but not in MyePro cells (Figure S4A). Furthermore, we found that RNase/ANG inhibitor 1 (RNH1)—an endogenous ANG inhibitor that has been shown to regulate the subcellular localization of ANG (Pizzo et al., 2013) and that is expressed in BM cell subsets under steady-state conditions (Figure S4B)—is localized in SGs in MyePro cells but not in LKS cells (Figure S4C). This opposing localization pattern of RNH1 and ANG was further examined by double immunofluorescence (Figure S4D) and fluorescence resonance energy transfer (FRET; Figure S4E), which showed that ANG and RNH1 colocalize and interact in the nucleus, but not in the

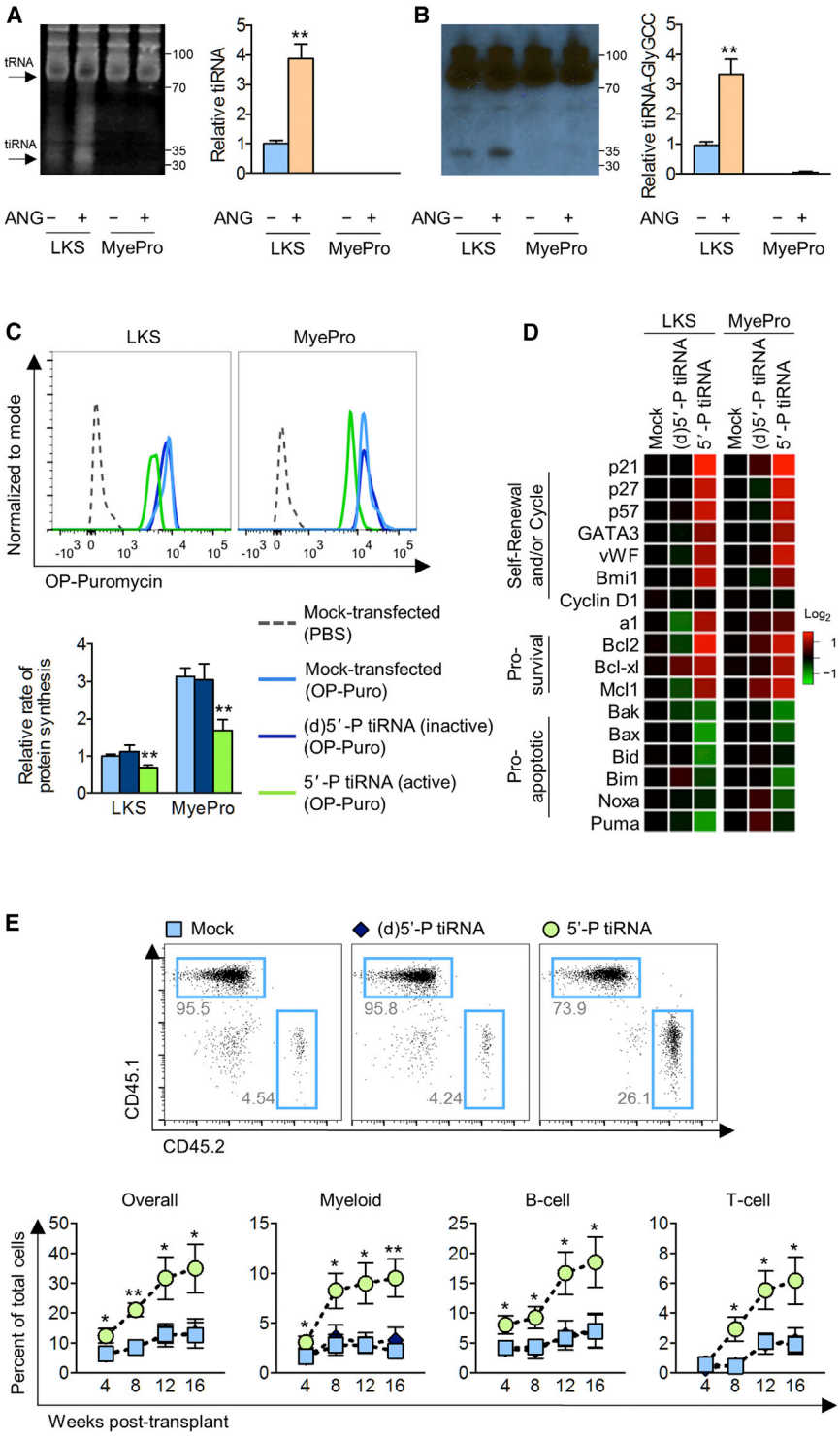

Figure 4. ANG-Mediated Regulation of Protein Synthesis Is Correlated with Cell-Type-Specific tiRNA Production

(A and B) Small RNA production (A; n = 3) and northern blot analysis of tiRNA-Gly-GCC (B; n = 3) following 2-hr treatment of LKS and MyePro cells with ANG. Bar graphs indicate relative values to untreated LKS.

(C and D) OP-Puro incorporation (C; n = 5), and qRT-PCR analysis of pro-self-renewal, pro-survival, and pro-apoptotic transcripts (D; n = 5) in LKS and MyePro cells transfected with inactive (d) 5′-P tiRNA or active 5′-P tiRNA.

(E) Post-transplant reconstitution of LKS cells transfected with inactive (d)5′-P tiRNA or active 5′-P tiRNA (n = 7).

Error bars indicate mean ± SEM, and heatmaps represent mean. *p < 0.05; **p < 0.01.

See also Figures S3 and S4.

cytoplasm, of LKS cells and in the cytoplasm, but not in the nucleus, of MyePro cells. Thus, RNH1, which is known to stoichiometrically inhibit ANG with a femtomolar dissociation constant, K_d (Lee et al., 1989), likely inhibits nuclear ANG but not cytoplasmic ANG in LKS cells, suppressing rRNA production but permitting tiRNA production, whereas it inhibits cytoplasmic ANG, but not nuclear ANG, in MyePro cells to allow rRNA transcription but not tiRNA production.

To assess whether tiRNA-mediated regulation of protein synthesis affects HSPC function, we transfected LKS cells with synthetic tiRNA and competitively transplanted those cells into WT hosts. We observed significantly enhanced long-term multi-lineage post-transplant reconstitution of cells transfected with active 5′-P-tiRNA, relative to untreated LKS cells or cells transfected with inactive (d)5′-P-tiRNA (Figure 4E). As ANG stimulates tiRNA production in LKS cells, these data strongly suggest that ANG may enhance the regenerative potential of HSPCs by tiRNA-mediated alterations of protein synthesis.

ANG Is a Pro-regenerative Factor after Radiation-Induced Damage

To assess the pro-regenerative role of ANG, we first examined the function of ANG in the context of radiation-induced cell damage. We found that *Ang* mRNA levels were elevated in mixed niche cells 1 day post-irradiation (Figure S5A), but not in whole BM cells (Figure S5B) or sorted LKS and MyePro cells (Figure S5C). Consistent with this finding, ANG serum levels were also significantly elevated (Figure S5D), suggesting that niche cells increase ANG synthesis as a response to irradiation. *Ang*$^{-/-}$ mice displayed reduced survival following exposure to various doses of γ radiation (Figure S5E), accompanied by reduced blood leukocyte recovery (Figure S5F). When animals were analyzed

at day 7 post-irradiation (Figure S5G), we observed reduced total BM cellularity (Figure S5H; Table S3), reduced HSPC and LymPro cell numbers (Figures S5I and S5K), and more active cycling (Figures S5J and S5L). These data are consistent with the quiescence-inducing effect of ANG on HSPCs and LymPro cells. In contrast, *Ang*$^{-/-}$ MyePro cells showed reduced cell number (Figure S5M) but restricted proliferation (Figure S5N) following γ irradiation. Importantly, this dichotomous regulation of proliferation was observed in lineage-biased MPP3 and MPP4 cells under radiation stress (Figures S5O and S5P), as observed at steady state (Figure 2B). *Ang*$^{-/-}$ mice also demonstrated increased apoptosis in all primitive hematopoietic cell types examined (Figure S5Q), as well as reduced lymphoid and myeloid colony formation in response to γ irradiation (Figure S5R).

To determine whether treatment with ANG enhances survival, we pretreated WT or *Ang*$^{-/-}$ mice with ANG daily for 3 successive days and irradiated mice with 8.0 Gy 24 hr following the final ANG treatment (Figure S6A). Significantly, the 30-day survival rate increased from 20% to 90% after ANG treatment, indicating that ANG is radioprotective (Figure 5A). Importantly, 80% of *Ang*$^{-/-}$ mice also survived following ANG pretreatment whereas 100% of untreated *Ang*$^{-/-}$ mice died. These results indicate that both endogenous and exogenous ANG are radioprotective. Pretreatment with ANG protected against radiation-induced loss of the total BM number (Figure S6B) and various BM cell subsets (Table S4) and restricted HSPC and LymPro cell cycling (Figures S6C and S6D). In contrast, ANG pre-treatment not only prevented radiation-induced loss of MyePro cell number (Table S4) but also further promoted their proliferation beyond those enhanced by γ irradiation (Figure S6E). Moreover, ANG protected against irradiation-induced apoptosis in all primitive hematopoietic cell types examined (Figure S6F) and led to enhanced colony formation (Figure S6G) and post-transplant reconstitution (Figure S6H). Together, these data demonstrate the protective function of ANG against radiation-induced BM damage, likely through induction of HSPC quiescence and promotion of MyePro cell proliferation.

To assess a potential therapeutic use of ANG as a radio-mitigating agent, we irradiated mice with 8.0 Gy and immediately began ANG treatment (Figure S6I), and we observed enhanced survival in ANG-treated mice (Figure S6J). We also irradiated mice with 8.0 Gy and began ANG treatment 24 hr later (Figure S6K). Significantly, the majority of ANG-treated mice survived, including ANG-treated *Ang*$^{-/-}$ mice, suggesting that ANG has radio-mitigating capabilities (Figure 5B). Importantly, treatment with ANG 24 hr post-irradiation prevented radiation-induced reduction of overall BM cellularity (Figure 5C), as well as that of LKS cells, MyePro cells (Figures 5C and 5D), and other BM cells (Table S4). Consistent with its dichotomous role in cell-cycle kinetics, ANG restricted the proliferation of LKS cells and simultaneously enhanced proliferation of MyePro cells under radiation stress (Figure 5E). Furthermore, ANG prevented radiation-induced apoptosis in both cell types (Figure 5F). These effects on cell number, cycling, and apoptosis were also apparent in further defined stem and progenitor cell populations (Figures S6L–S6O; Table S4). Significantly, defects in colony formation (Figure S6P) and post-transplant reconstitution (Figure 5G) could be rescued by ANG treatment.

We also assessed the protective and mitigative effects of ANG in lethally irradiated (12.0-Gy) animals and found that ANG treatment either before or after lethal irradiation improved survival (Figure 5H) and enhanced BM cellularity (Figure 5I) as well as peripheral blood content (Table S5). Consistent with these findings, ANG significantly increased the LD_{50} (lethal dose 50) when treatment was begun 24 hr post-irradiation (Figure 5J). Furthermore, treatment with ANG upregulated pro-self-renewal genes in LKS cells and led to enhanced pro-survival transcript levels and reduced pro-apoptotic transcripts in both LKS cells and MyePro cells under radiation stress (Figure 5K). ANG treatment enhanced rRNA transcription only in MyePro cells, both before and after irradiation (Figure 5K). In LKS cells, ANG did not promote rRNA transcription (Figure 5K) but enhanced tiRNA production under radiation stress (Figure 5L), as has been observed under steady-state (Figure 4A) and under oxidative stress conditions (Figure S3H). These results indicate that the dichotomous role of ANG in regulating proliferation of HSPCs and MyePro cells is preserved under stress conditions. Together, these results establish a model by which ANG simultaneously stimulates proliferation of rapidly responding MyePro cells and preserves HPSC stemness, in association with enhanced hematopoietic regeneration and improved survival.

Ex Vivo Treatment of LT-HSCs with Recombinant ANG Enhances Post-transplant Reconstitution

The in vitro (Figure 2C; Figures S2H–S2K) and in vivo (Figure 5; Figures S5 and S6) activity of ANG in preserving HSPC stemness and in enhancing regeneration prompted us to assess its capacity in improving SCT and its potential for clinical development. Treatment of LT-HSCs with ANG in culture for 7 days led to a dose-dependent decrease of cell proliferation in WT and *Ang*$^{-/-}$ cells (Figure 6A), consistent with its ability to restrict HSPC proliferation. Notably, LKS cells cultured in the absence of ANG resulted in a reduction of tiRNA expression relative to uncultured cells (Figure 6B). In contrast, cells cultured in the presence of ANG not only maintained baseline tiRNA levels but also their responsiveness to further ANG treatment (Figure 6B).

To test whether restriction of proliferation would enhance transplantation efficiency, we competitively transplanted LT-HSCs that were either freshly isolated or had been cultured for 2 hr with or without 300 ng/ml ANG (Figure S7A), the physiological concentration of both mouse (Figure S5D) and human (Yoshioka et al., 2006) ANG. Significantly, we observed that a 2-hr treatment with ANG led to a dramatic increase in multi-lineage post-transplant reconstitution over 24 weeks (Figure 6C). We also found that improved transplant efficiency was observed with LT-HSCs cultured with ANG for 7 days (Figure S7B). Enhanced regeneration was observed over 16 weeks upon secondary transplant without further ANG treatment (Figure 6D), and elevated peripheral blood counts were observed 1 year post-transplant without any indication of leukemia development (Table S6). Significantly, removal of ANG from the media after 7 days in culture did not induce proliferation (Figure S7C), and enhanced levels of pro-self-renewal transcripts were retained (Figure S7D). Treatment with ANG had no effect on homing (Figure S7E). Importantly, treatment of *Ang*$^{-/-}$ LT-HSCs with exogenous ANG ameliorated post-transplant reconstitution defect of

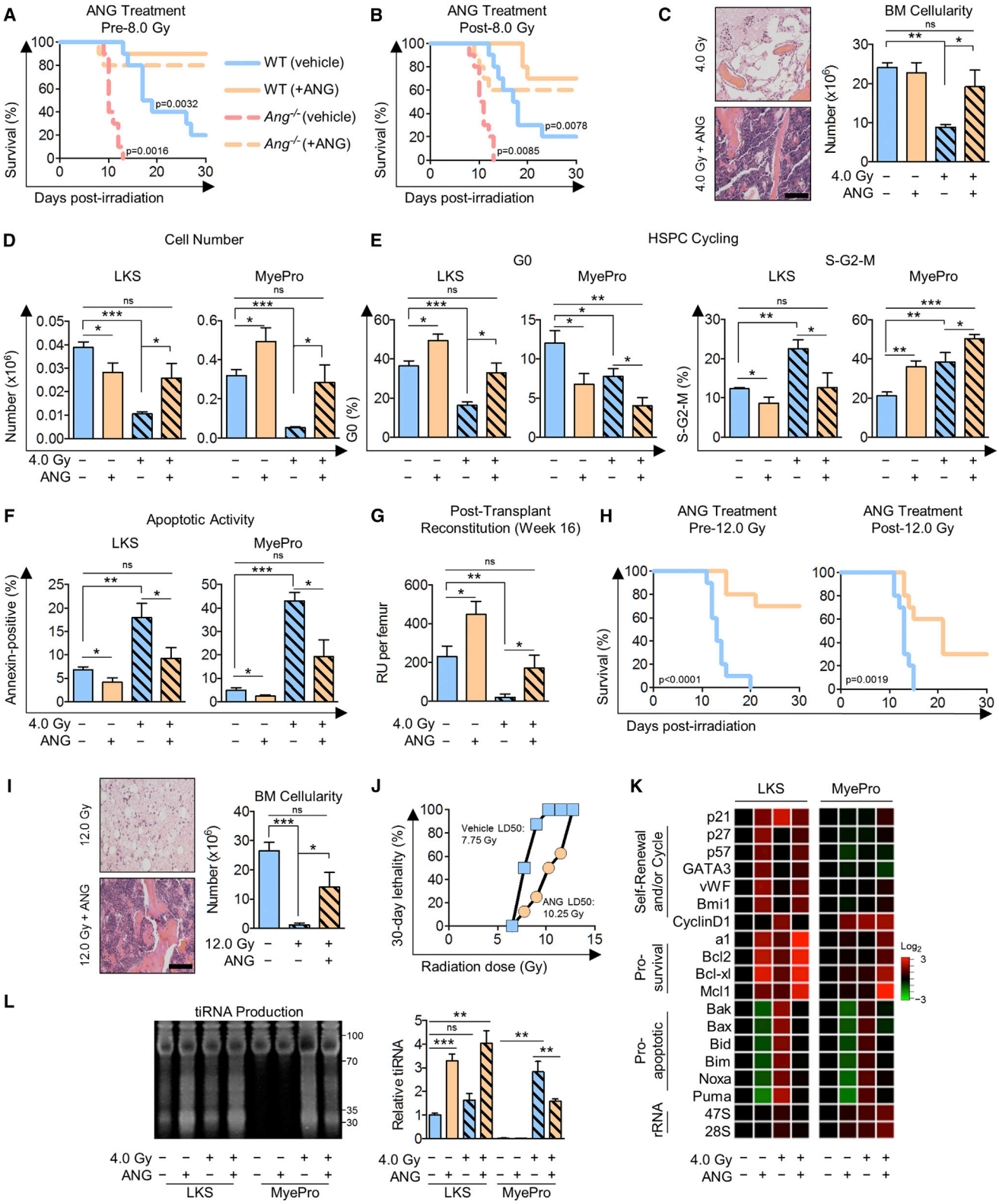

Figure 5. ANG Enhances Radioprotection and Radioresistance

(A) Survival of WT or *Ang*$^{-/-}$ mice treated with ANG daily for 3 successive days 24 hr pre-8.0 Gy (n = 10).

(B) Survival of WT or *Ang*$^{-/-}$ mice treated with ANG daily for 3 successive days 24 hr post-8.0 Gy (n = 10).

(legend continued on next page)

Ang$^{-/-}$ cells and led to enhanced reconstitution over WT cells by week 16 (Figure 6E). Together, these data demonstrate that treatment of LT-HSCs with exogenous ANG significantly enhances their regenerative capabilities upon relatively short exposure, and this effect is long lasting.

ANG Improves Regeneration of Human Cells

Given that ANG significantly improved transplantation efficiency of mouse LT-HSCs, we examined whether human ANG has similar pro-regenerative capabilities in human CD34$^+$ CB cells. Consistent with the anti-proliferative effect of ANG on mouse LT-HSCs, treatment with human ANG led to a dose-dependent reduction of CD34$^+$ CB-cell proliferation over 7 days (Figure 7A) and an elevated level of pro-self-renewal transcripts (Figure S7F), whereas ANG variants that are defective in its ribonucleolytic activity (K40Q) or in receptor binding (R70A) (Hallahan et al., 1991) were inactive (Figures 7A and S7F). Interestingly, R33A ANG, despite having a defective nuclear localization sequence (Moroianu and Riordan, 1994), recapitulated the effect of WT ANG in restricting proliferation (Figure 7A) and enhancing self-renewal signature (Figures 7B and S7F). It is significant to note that a 2-hr exposure to human ANG is adequate for CD34$^+$ human CB cells to upregulate pro-self-renewal genes (Figure 7B), which greatly enhances the translational capability of ANG in improving SCT. The fact that the R33A ANG variant is as active as WT ANG points to the dispensable role of nuclear ANG in HSPCs and reinforces the finding that cytoplasmic localization of ANG is important in preservation of HSPC stemness. Furthermore, ANG treatment of CB cells led to slightly elevated numbers of primitive colonies (Figure S7G). Together, these data indicate that in vitro properties of mouse ANG faithfully translate in a human setting and suggest that the cellular mechanisms underlying mouse HSC regeneration may also translate into human cells.

To assess whether ANG improves transplantation efficiency of human cells, we transplanted CD34$^+$ CB cells that had been cultured for 2 hr in the presence or absence of ANG into NSG (NOD scid gamma) mice at limit dilution and found that treatment with ANG led to elevated frequencies of human CD45$^+$ cells across all doses examined in BM at 16 weeks post-transplant (Figure 7C). Importantly, enhanced regeneration was multi-lineage, as confirmed by the presence of both CD19 B-lymphoid cells and CD33 myeloid cells in BM (Figures S7H and S7I). Remarkably, calculated LT-HSC frequency was 8.9-fold higher in ANG-treated human CD34$^+$ CB cells relative to untreated cells (Figure 7D). Further, enhanced reconstitution was observed upon secondary transplant without further ANG treatment (Figure 7E). These data highlight the translational capacity of ANG in improved transplantation efficacy of clinically relevant human cells.

DISCUSSION

Our study highlights several important findings. First, ANG has a cell-type-specific role in regulating proliferation of HSPCs versus MyePro cells: while promoting quiescence in the former, ANG stimulates proliferation in the latter. Second, we identified a novel RNA-based mechanism by which hematopoiesis is regulated. Importantly, ANG promotes tiRNA production in LKS cells, in association with enhanced stemness in vitro and in vivo. We also show that increased tiRNA production results in reduced levels of global protein synthesis in HSPCs. In contrast, ANG stimulates rRNA transcription in MyePro cells, but not in HSPCs, leading to increased protein synthesis and proliferation. To our knowledge, this is the first report of cell-type specificity in RNA processing that leads to, or originates from, a different cellular state. How cell differentiation state results in the very distinctive effects of ANG is intriguing and of practical consequence, as demonstrated by our studies. Defining the basis for this cell-type specificity is beyond the scope of this report but will be of particular interest for how exogenous signals and intrinsic properties may induce distinctive RNA processing in specific cell types in response to ANG.

Our findings are important, given recent reports demonstrating tight regulation of protein synthesis in HSPCs (Signer et al., 2014). Furthermore, a number of mutations or defects in ribosome function or protein synthesis have been shown to either promote or resist malignant hematopoiesis (Narla and Ebert, 2010). To our knowledge, however, no factors have been shown to link the regulation of HSPC quiescence at the level of protein synthesis. Moreover, a potential therapeutic benefit of using these properties to promote hematopoietic regeneration has not been explored. Modulating tiRNA to alter protein synthesis and cell fate is unique among prior reports of regulatory mechanisms and is of particular interest because of its ability to be affected by a cell-exogenous source. The notion that tiRNA can be cell-state specific in regulating hematopoiesis offers the possibility that similar distinct mechanisms may apply to other tissue types. Discerning whether this is the case and how they may induce altered cell characteristics will help define whether tiRNA represents a common regulatory lever in mammalian biology.

Third, we demonstrate two potential therapeutic uses for ANG. We found that recombinant ANG recapitulates the growth-suppressive properties in vitro and can remarkably improve post-transplant reconstitution of mouse LT-HSCs and human CD34$^+$ CB cells in vivo. Previous studies have identified numerous factors that expand stem cell number in vitro and in vivo (Boitano et al., 2010; Delaney et al., 2010; Fares et al., 2014; Himburg

(C–G) H&E and BM cellularity of femurs (C), LKS and MyePro cell number per femur (D), cell cycling (E), apoptotic activity (F), and post-transplant reconstitution (G) of WT mice treated with ANG daily for 3 successive days 24 hr post-4.0 Gy (n = 6). Scale bar, 100 μm.

(H) Survival of WT mice treated with ANG daily for three successive days 24 hr prior or post-12.0 Gy.

(I) H&E and BM cellularity of femurs of WT mice treated with ANG daily for 3 successive days 24 hr post-12.0 Gy total body irradiation (TBI) (n = 6). Scale bar, 100 μm.

(J) LD_{50} of mice treated with ANG daily for 3 successive days beginning 24 hr post-TBI (n = 8).

(K and L) qRT-PCR analysis of pro-self-renewal, pro-survival, pro-apoptotic, and rRNA transcripts (K; n = 6) and tiRNA production (L; n = 3) in LKS or MyePro cells sorted from irradiated mice (4.0 Gy) and treated with 300 ng/ml ANG.

*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

See also Figures S5 and S6 and Tables S3–S5.

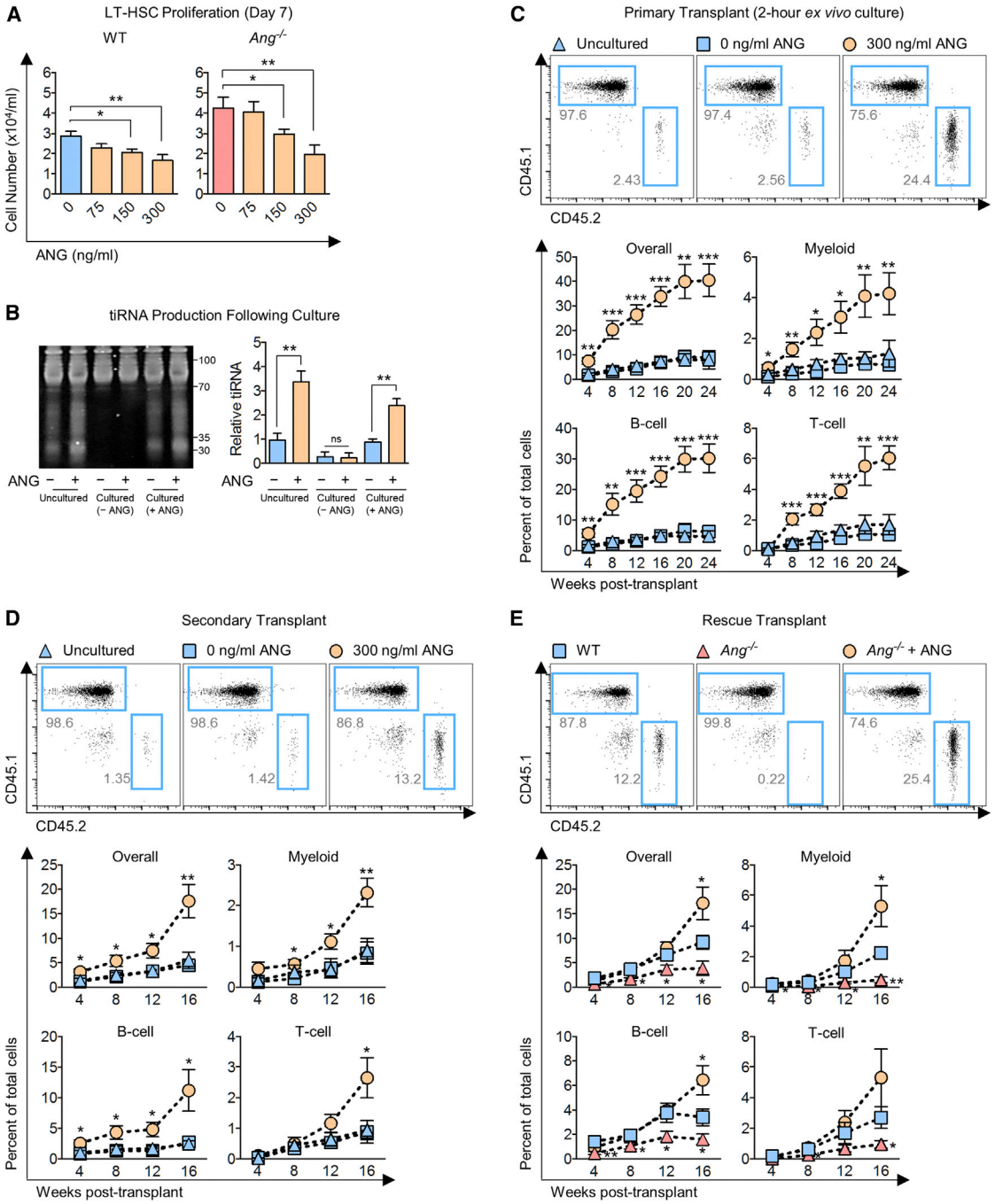

Figure 6. ANG Enhances Post-transplant Reconstitution

(A) Cell density on day 7 from sorted WT or *Ang*$^{-/-}$ LT-HSCs (1,875 cells per milliliter) cultured in the presence of various doses of ANG (n = 6).

(B) tiRNA levels following 7-day culture with 0 or 300 ng/ml ANG. After culture, cells were harvested and again treated with 0 or 300 ng/ml ANG (indicated by + or −) for 2 hr prior to analysis by electrophoresis (n = 3).

(C) Post-transplant reconstitution of WT LT-HSCs (CD45.2) after 2-hr ex vivo treatment with ANG (n = 8–9).

(D) Secondary transplant of WT LT-HSCs (CD45.2) without further ex vivo ANG treatment (n = 7–8).

(E) Post-transplant reconstitution of WT or *Ang*$^{-/-}$ LT-HSCs that were cultured in the presence or absence of 300 ng/ml ANG for 2 hr and competitively transplanted in WT hosts (n = 7).

Error bars indicate mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

See also Figure S7 and Table S6.

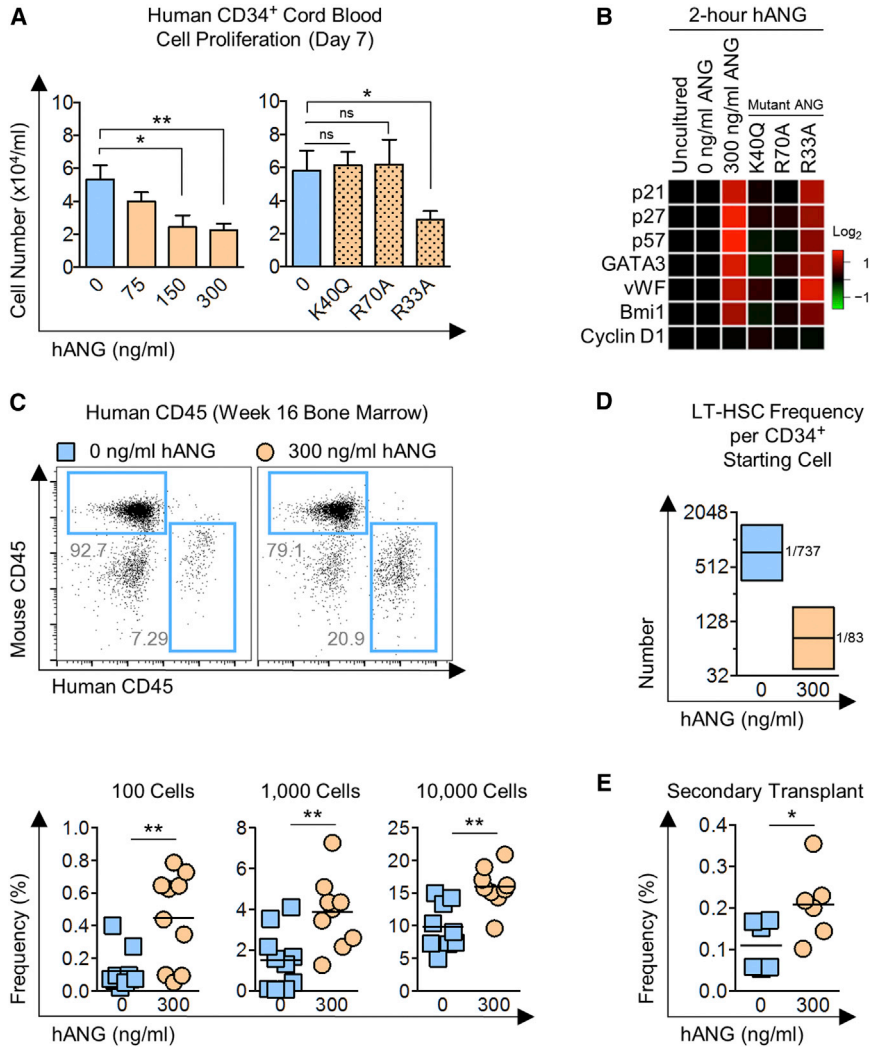

Figure 7. ANG Enhances Post-transplant Reconstitution of Human CD34⁺ CB Cells

(A) Cell number on day 7 from human CD34⁺ CB cells (2,500 cells per milliliter) cultured in the presence of various doses of ANG or ANG variants: K40Q (enzymatic variant), R70A (receptor-binding variant), or R33A (nuclear localization variant) at 300 ng/ml (n = 6). hANG, human ANG protein.

(B) qRT-PCR analysis of pro-self-renewal transcripts in human CD34⁺ CB cells following 2 hr culture with 300 ng/ml human ANG protein (n = 6).

(C) Human CD45 cells in BM of NSG mice transplanted with human CD34⁺ CB cells treated with or without human ANG (300 ng/ml) for 2 hr. BM was harvested 16 weeks post-transplant (n = 9–10).

(D) LT-HSC frequencies (black line) and 95% confidence intervals (shaded boxes) for each transplant condition from Figure 7C ($p = 8.28 \times 10^{-5}$).

(E) Secondary transplant of ANG-treated human CD34⁺ CB cells from primary recipient. BM was harvested 4 weeks post-transplant (n = 6).

Error bars indicate mean ± SEM, and heatmaps represent mean. *$p < 0.05$; **$p < 0.01$; ns, not significant.

See also Figure S7.

et al., 2010; North et al., 2007); however, it has been noted that cycling HSPCs engraft less well upon transplantation and undergo faster exhaustion (Nakamura-Ishizu et al., 2014; Passegué et al., 2005), likely as a consequence of differentiation and loss of stemness. Our finding that ANG improves HSPC stemness warrants further testing of ANG as a means of improving transplantation outcomes in the setting of limiting HSPC cell numbers. Further, the ability of ANG to serve as a radio-mitigant is also of considerable interest, particularly given its ability to rescue animals when administered 24 hr post-irradiation injury. Translation of this ability to humans is obviously complex, but the potential to reduce mortality following radiation exposure is of considerable significance. Functionally, we demonstrate that ANG simultaneously preserves stemness and promotes progenitor cell proliferation following radiation damage, in contrast to other reported approaches of HSPC regeneration or protection from genotoxic injury. The success of hematopoietic regeneration depends upon rapid reconstitution of mature blood cell pools, to avoid infections and bleeding complications, and long-term generation of mature cells from a durable cell source (Doulatov et al., 2012; Smith and Wagner, 2009). These two functions are provided by progenitor and stem cell populations, respectively.

Currently, there are no FDA (U.S. Food and Drug Administration)-approved drugs to treat severely irradiated individuals (Singh et al., 2015). A number of hematopoietic growth factors have been shown to mitigate hematopoietic syndrome of acute radiation syndrome; however, only a few candidates have been demonstrated to improve survival when administered 24 hr post-irradiation (Himburg et al., 2014), an efficacy requirement mandated by The Radiation and Nuclear Countermeasures Program at the National Institute of Allergy and Infectious Diseases. Moreover, current standard-of-care approaches, including granulocyte colony-stimulating factor (G-CSF) and its derivatives, target a limited progenitor cell pool and require repeated doses to combat radiation-induced neutropenia (Singh et al., 2015). In this regard, ANG is a promising candidate as a medical countermeasure for radiation exposure, as only three ANG treatments are needed for improved animal survival, even if started 24 hr after a lethal (12.0-Gy) dose. The long-term effect of ANG treatment in post-irradiated mice, however, is not clear and is the subject of future studies. It will, therefore, be important to assess whether ANG promotes the survival of genetically aberrant HSPCs and/or leads to development of leukemia.

Overall, we demonstrate that the unique growth and survival properties of ANG in primitive hematopoietic cells can be therapeutically harnessed for improvement of tissue regeneration.

EXPERIMENTAL PROCEDURES

Animal Studies

Ang$^{-/-}$ mice were generated in house. B6.SJL and NSG mice were purchased from The Jackson Laboratory. For aged-animal experiments, 22-month-old WT (NIH/NIA [National Institute on Aging]) and *Ang*$^{-/-}$ mice were used. For all other studies, age-matched 7- to 12-week-old mice were used. Littermates and gender-matched animals were used whenever possible. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Tufts University/Tufts Medical Center.

Cell-Surface Markers for Stem and Progenitor Subtypes

The following cell-surface markers were used: LKS (Lin$^-$c-Kit$^+$Sca1$^+$), MyePro (Lin$^-$c-Kit$^+$Sca1$^-$), LT-HSC (Flk2$^-$CD34$^-$ LKS or CD150$^+$C48$^-$ LKS), ST-HSC (Flk2$^-$CD34$^+$ LKS or CD150$^+$C48$^+$ LKS), MPP (Flk2$^+$CD34$^+$ LKS or CD150$^-$C48$^+$ LKS), MPP1 (CD150$^+$CD48$^-$CD135$^-$CD34$^+$ LKS), MPP2 (CD150$^+$CD48$^+$CD135$^-$CD34$^+$ LKS), MPP3 (CD150$^-$CD48$^+$CD135$^-$CD34$^+$ LKS), MPP4 (CD150$^-$CD48$^+$CD135$^+$CD34$^+$ LKS), CLP (Lin$^-$c-Kit^{med}Sca1^{med}IL7R$^+$ Flk2$^+$ B220$^-$), pre-pro B (Lin$^-$c-Kit^{med}Sca1^{med}IL7R$^+$ Flk2$^+$ B220$^+$), CMP (Lin$^-$c-Kit$^+$Sca1$^-$CD34$^+$CD16/32$^-$), GMP (Lin$^-$c-Kit$^+$Sca1$^-$CD34$^+$CD16/32$^+$), and MEP (Lin$^-$c-Kit$^+$Sca1$^-$CD34$^-$CD16/32$^-$).

Stem Cell Cultures

Mouse LT-HSCs were cultured per manufacturer's instructions in PBS or in S-clone media (Sanko Junyaku) for 2 hr or 2–14 days, respectively. Human CD34$^+$ CB cells were cultured per manufacturer's instructions in PBS or in StemSpan SFEM (serum-free expansion medium) for 2 hr or 7 days, respectively.

Transplantation

Transplantation of conditional knockout donor cells (1:1 competitive), transplantation of B6.SJL donor BM into conditional knockout recipients, ex vivo reconstitution assays, serial and rescue cells, tiRNA-transfected donor cells, irradiated or 5-FU-treated donor cells, and treated human CD34$^+$ CB cells were performed as described in the Supplemental Experimental Procedures.

Statistical Analyses

All bar graphs represent mean ± SEM, and all heatmaps represent mean. All data are derived from two to four independent experiments. For comparisons of two experimental groups, an unpaired two-tailed Student's t test was used (Excel). Kaplan-Meier survival curves were analyzed by log rank tests (Prism 6). Heatmaps were generated with RStudio. Limiting dilution analysis (LDA) was assessed by ELDA (http://bioinf.wehi.edu.au/software/elda). For all analyses, p values are given in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.06.042.

AUTHOR CONTRIBUTIONS

K.A.G., L.S., D.T.S., and G.H. conceived the project, designed experiments, and analyzed data. K.A.G., L.S., S.L., N.S., M.G.H., and H.Y. performed experiments. K.A.G., D.T.S., and G.H. wrote the manuscript.

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Tufts Medical Center researchers find new functions of blood cell protein in transplant

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BOSTON (August 11) - Tufts Medical Center and Tufts University scientists have found exciting, new functions of the protein angiogenin (ANG) that play a significant role in the regulation of blood cell formation, important in bone marrow transplantation and recovery from radiation-induced bone marrow failure. Since current bone marrow transplantations have significant limitations, these discoveries may lead to important therapeutic interventions to help improve the effectiveness of these treatments. The findings were published in an article, "Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells," in the August 11, 2016 issue of the journal *Cell*.

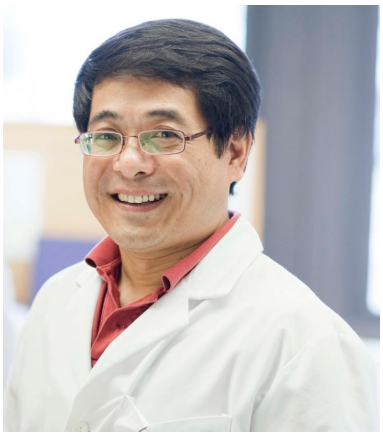

IMAGE: GUO-FU HU, PHD, IS AN INVESTIGATOR IN THE MOLECULAR ONCOLOGY RESEARCH INSTITUTE AT TUFTS MEDICAL CENTER **view more** ›

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In the paper, the researchers show for the first time that ANG simultaneously reduces proliferation of stem cells and promotes proliferation of myeloid progenitor cells that give rise to mature myeloid cells. They further report that these two-pronged processes are accomplished by a novel molecular regulating mechanism, a first-ever such finding.

These findings have significant implications for both human stem cell transplantation and for radiation exposure. Cancer patients undergoing stem cell transplantation face two hurdles: the short-term challenge of having enough white blood cells to fight possible infections immediately following the transplant and the long-term challenge of sustaining stem cell function to maintain immunity. People exposed to large doses of radiation face challenges due to bone marrow failure induced by such exposures.

"We knew that ANG was involved in promoting cell growth so it was not unexpected to find that ANG stimulates proliferation of myeloid progenitor cells," said Guo-fu Hu, PhD, Investigator in the Molecular Oncology Research Institute at Tufts Medical Center, and the paper's senior author. "But it was surprising to find that ANG also suppresses growth of stem cells and that it accomplishes these divergent promotion or suppression functions through RNA processing events specific to individual cell types. Our discoveries suggest considerable therapeutic potential."

Dr. Hu also serves as faculty in the Biochemistry; Cell, Molecular & Developmental Biology; and Cellular & Molecular Physiology programs at the Sackler School of Graduate Biomedical Sciences at Tufts.

In a series of experiments, the team from Tufts MC and the Sackler School at Tufts, which collaborated with scientists at Massachusetts General Hospital, isolated and described the divergent regulatory functions of ANG. They demonstrated how ANG stimulates proliferation of myeloid progenitor cells. They showed how ANG maintains stem cells by inducing a state of quiescence, or cellular dormancy, the first known evidence of ANG's suppressive activity. Quiescence preserves stem cells over time so that they will be available in the future to help maintain immunity.

In another novel finding, the team demonstrated that ANG achieves these dual functions by inducing RNA processing that is different in various cell types. In hematopoietic stem/progenitor cells, ANG induces processing of a specific type of RNA (tiRNA) that is quiescence-related whereas in myeloid progenitor cells, ANG induces processing of a specific type of RNA (rRNA) that is proliferation related. tiRNA is a type of small RNA that suppresses global protein synthesis, while rRNA or ribosomal RNA is a type of RNA molecule that enhances protein synthesis.

"Proper blood cell production is dependent on functioning hematopoietic stem and progenitor cells that are destroyed during conditioning procedures for transplantation or following bone marrow injury," said the study's first author Kevin A. Goncalves, who performed this research as part of his PhD studies in cellular and molecular physiology at the Sackler School. "Our study demonstrates that ANG regulates critical functions of both clinically-relevant cell types."

In further studies, the researchers tested the capacity of ANG to prevent and mitigate radiation-induced bone marrow failure, and in pre-clinical models, they found that survival following radiation exposure was increased after treatment with recombinant ANG protein.

###

A complementary paper, "Proximity-Based Differential Single-Cell Analysis of the Niche to Identify Stem/Progenitor Cell Regulators," published online on August 11, 2016 in the journal *Cell Stem Cell*, reports the discovery and confirmation of ANG as a niche regulator.

Additional authors are Shuping Li, MD, PhD, Miaofen G. Hu, MD, PhD from Tufts Medical Center; Hailing Yang, PhD, a recent graduate of the Sackler School; and Lev Silberstein, PhD and Nicolas Severe, PhD from Massachusetts General Hospital and Harvard University. David Scadden, MD, also from Massachusetts General Hospital and Harvard University is co-corresponding author.

This study was supported by the National Institutes of Health, specifically the National Cancer Institute (award R01CA105241), the National Institute of Neurological Disorders and Stroke (award R01NS065237), the National Heart, Lung, and Blood Institute (awards R01HL097794 and F31HL128127), and the National Institute of Diabetes and Digestive and Kidney Diseases (award R01DK050234); the United States Department of Defense (W81XWH-15-1-02070); the National Natural Science Foundation of China (81272674); the Leukemia & Lymphoma Research UK/Leukemia & Lymphoma Society fellowships; a Sackler Dean's Fellow award; and a Sackler Families Collaborative Cancer Biology award.

About Tufts Medical Center and Floating Hospital for Children

Tufts Medical Center is an exceptional, not-for-profit, 415-bed academic medical center that is home to both a full-service hospital for adults and Floating Hospital for Children. Conveniently located in downtown Boston, the Medical Center is the principal teaching hospital for Tufts

University School of Medicine. Floating Hospital for Children is the full-service children's hospital of Tufts Medical Center and the principal pediatric teaching hospital of Tufts University School of Medicine. Tufts Medical Center is affiliated with the New England Quality Care Alliance, a network of more than 1,800 physicians throughout Eastern Massachusetts. For more information, please visit http://www.tuftsmedicalcenter.org.

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Tufts University School of Medicine and the Sackler School of Graduate Biomedical Sciences are international leaders in medical and population health education and advanced research. Tufts University School of Medicine emphasizes rigorous fundamentals in a dynamic learning environment to educate physicians, scientists, and public health professionals to become leaders in their fields. The School of Medicine and the Sackler School are renowned for excellence in education in general medicine, the biomedical sciences, and public health, as well as for innovative research at the cellular, molecular, and population health level. The School of Medicine is affiliated with six major teaching hospitals and more than 30 health care facilities. Tufts University School of Medicine and the Sackler School undertake research that is consistently rated among the highest in the nation for its effect on the advancement of medical and prevention science.

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Title

TREATMENT WITH ANGIOGENIN TO ENHANCE HEMATOPOIETIC RECONSTITUTION

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TREATMENT WITH ANGIOGENIN TO ENHANCE HEMATOPOIETIC RECONSTITUTION

TECHNICAL FIELD

[0001] The technology described herein relates to use of Angiogenin in methods and compositions for enhancing hematopoietic reconstitution post-transplant and treatment of radiation injury.

FUNDING SUPPORT

[0002] This invention was made with government support under Grant No. R01DK050234, R01HL097794, R01CA105241, R01NS065237 and F31HL128127 awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

BACKGROUND

[0003] Hematopoietic stem cells possess the ability of both 'multi-potency" and "self-renewal". Multi-potency is the ability to differentiate into all functional blood cells and self-renewal is the ability to give rise to HSCs itself without differentiation. Since mature blood cells are predominantly short lived, HSCs continuously provide more differentiated progenitors while maintaining the HSCs pool size throughout life by precisely balancing self-renewal and differentiation.

[0004] Hematopoietic stem cell transplantation (HSCT) or bone marrow transplantation is a procedure to restore impaired bone marrow and its function and therefore the immune system of patients who have suffered a decrease in hematopoietic cells or mature blood cells due to a disease, radiation or chemotherapy. Low transplantation efficiency can result in poor survival outcome for patients undergoing HSCT. For e.g.,the number of hematopoietic stem and progenitor cells (HSPCs) in umbilical cord blood (CB) is often low and post-transplantation patient survival can be improved by doubling the number of CB units (Smith and Wagner, 2009). One potential strategy therefore for improved recovery can be to expand the numbers of HSPCs prior to administration (Boitano et al., 2010; Delaney et al., 2010; Fares et al., 2014; Frisch et al., 2009; Himburg et al., 2010; Hoggatt et al., 2009; North et al., 2007). This approach however results in loss of stem cell properties of "multi-potency" and "self-renewal" which are critical for

successful post-transplant reconstitution. Active cycling results in faster exhaustion due to differentiation into progressively more mature marrow cells and loss of proliferative, renewal, and reconstitution potential of the HSPCs to be transplanted (Nakamura-IsiZulu, A., Takizawa, H., and Soda, T. (2014). The analysis, roles and regulation of quiescence in hematopoietic stem cells. Development 141, 4656-4666.) (Passage, E., Wagers, A'S., Giuriato, S., Anderson, W.C., and Weissman, I.L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J. Exp. Med. 202, 1599-1611.).

[0005] In order to improve post-transplant hematopoietic reconstitution, efforts have been made to modulate the growth control properties of hematopoietic stem cells. Cell cycle and epigenetic regulators as well as pathways involved in growth control, including cyclin dependent kinases and inhibitors, Rb, PI3K, and p53, have been demonstrated as cell-intrinsic regulators of HSPC proliferation (Ito and Suda, 2014; Nakamura- Ishizu et al., 2014). A variety of secreted and cell-surface factors which are produced by bone marrow (BM), including angiopoetin-1, thrombopoietin, SCF, and CXCL12 (Ito and Suda, 2014; Mendelson and Frenette, 2014; Morrison and Scadden, 2014), has been shown to extrinsically regulate HSPC. Cytokines SCF and TPO can both support survival and proliferation of purified mouse HSCs assayed in serum-free culture at the single cell level (Seita J, et al. Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. Proc Natl Acad Sci U S A. 2007; 104(7):2349–2354). Functional effects of many cytokines including IL-3, IL-6, IL-11, Flt-3 ligand in combinations with either SCF and/or TPO have been reported. Although exposing HSCs to these cytokines resulted in survival and proliferation of cells, in most studies, these cells immediately lose long-term reconstitution potential as assessed in transplantation assays. The Flt-3 receptor is not expressed on HSCs (Adolfsson J, et al. Upregulation of Flt3 expression within the bone marrow Lin(−)Sca1(+)c-kit(+)stem cell compartment is accompanied by loss of self-renewal capacity. Immunity. 2001; 15(4):659–669. Similarly, the IL-11 receptor knockout mice showed normal hematopoiesis [84], questioning an essential functional role for this receptor-ligand system on HSC function. It has now become clear that many cytokines have redundant functions at the level of either receptor binding or intracellular signal transduction.

[0006] In vivo culture studies have revealed inhibitory effect of TGF-β on HSC proliferation without inducing apoptosis. Moreover, neutralization of TGF-β has been shown to facilitate rapid proliferation of HSPC in vivo by releasing them from quiescence (Hatzfeld J, et al. Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor beta 1 or Rb oligonucleotides. J Exp Med. 1991; 174(4):925–929.(US 6,841,542 B2). US 2010/0034778 A1 teaches the use of a modulator of the retinoic acid receptor RXR to enable stem cell expansion in vivo. Pleiotrophin is a growth factor shown to enhance HSC self-renewal and/or expansion in vivo (US 2011/0293574A1). CXCR4 antagonists have been shown to increase the rate of hematopoietic stem or progenitor cellular multiplication, self-renewal, expansion and proliferation (US 20020156034A1). Modulators of PI 3-kinase activity can be used to expand populations of renewable stem cells (US 2005/0054103 A1). Tie2/angiopoeitin-1 signaling regulates HSC quiescence in the bone marrow niche (Arai F, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004; 118(2):149–161.

[0007] The success of HSCT depends upon rapid reconstitution of mature blood cells to avoid infections and bleeding complications and long-term reconstitution of mature blood cells from durable restored source stem cells. (Doulatov et al., 2012; Smith and Wagner, 2009). Cell preparations intended for transplant are desired to comprise HSPCs who have their "multi-potency" and "self-renewal" capacities preserved and have retained an ability to achieve short-term recovery as well as improved long-term, multilineage hematopoietic reconstitution upon in vivo administration. Committed progenitors are responsible for the initial hematopoietic recovery, whereas the long-term repopulating HSCs (LT-HSCs) are responsible for establishing life-long multilineage hematopoiesis.

[0008] In contrast to high turnover of lineage-restricted progenitors, most of the HSCs reside in the "quiescent" G0 phase of the cell-cycle (Rossi DJ, et al. Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. Cell Cycle. 2007; 6(19):2371–2376.(Nakamura-Ishizu, A., Takizawa, H., and Suda, T. (2014). The analysis, roles and regulation of quiescence in hematopoietic stem cells. Development 141, 4656-4666.). Quiescence contributes to HSC longevity and function, perhaps by minimizing

stresses due to cellular respiration and genome replication (Eliasson, P., and J.-I. Jönsson. 2010. The hematopoietic stem cell niche: low in oxygen but a nice place to be. J. Cell. Physiol. 222:17–22. http://dx.doi.org/10.1002/jcp.21908) (Disruption of HSC quiescence leads to defects in HSC self-renewal and often results in HSC exhaustion (Orford, K.W., and D.T. Scadden. 2008. Deconstructing stem cell self-renewal:genetic insights into cell-cycle regulation. Nat. Rev. Genet. 9:115–128. http://dx.doi.org/10.1038/nrg2269). Therefore it follows that a proper balance of pools of HSPCs with quiescence and proliferative properties can result in successful transplantation outcomes. However, a non-cell autonomous regulator of hematopoiesis with cell-context specific effects for e.g., a modulator, which simultaneously preserves HSC stemness by quiescence while enabling progenitor expansion, has not been identified till date. Such a modulator can enhance post-transplant reconstitution of the cells to be administered by promoting quiescence and self-renewal of primitive HSPC including LT-HSCs, and proliferative expansion of myeloid-restricted progenitors. As such there is an unmet need of methods of producing the hematopoietic stem cell composition which is characterized by preserved stemness of the HSC such that the compositions enable short-term recovery and enhanced long-term multilineage post-transplantation reconstitution and therefore successful outcome.

[0009] Enhanced hematopoietic reconstitution is also required after IR-induced hematopoietic failure, which is a primary cause of death after exposure to a moderate or high dose of total body irradiation (TBI). Within a few hours or days after exposure to a significant dose of TBI, a series of characteristic clinical complications termed the acute radiation syndrome (ARS) appear. The hematopoietic syndrome occurs at TBI doses in the range of 2–7.5 Gy in humans (3–10 Gy in rodents) and is caused by severe depletion of blood elements due to BM suppression; the gastrointestinal syndrome occurs after doses >5.5 Gy of TBI; and the neurovascular syndrome occurs following large doses of TBI (>20 Gy), indicating that the hematopoietic system is the most radiosensitive tissue of the body. In addition, exposure to a moderate- or high-dose TBI also induces residual (or long-term) BM injury manifested by a decrease in HSC reserves and fitness and impairment in HSC self-renewal. Currently, there are no FDA-approved drugs to treat severely irradiated individuals (Singh et al., 2015). A number of hematopoietic growth factors have been shown in various animal models to mitigate hematopoietic syndrome of acute radiation syndrome, however only pleiotrophin has been reported to improve survival when

administered 24 hours post-irradiation (Himburg et al., 2014). Moreover, current standard- of-care approaches, including granulocyte colony-stimulating factor (G-CSF) and its derivatives, target a limited progenitor cell pool and requires repeated doses to combat radiation-induced neutropenia (Singh et al., 2015). Therefore, there is an unmet need for a prophylactic and therapeutic to improve hematopoietic reconstitution and survival of subject post-exposure to radiation.

SUMMARY

[0010] The invention is based in part on the discovery that in vivo or ex vivo, exposure of HSPCs or a HSPCs population to ANG, results in enhanced hematopoietic reconstitution including repopulation of cells of all blood lineage and their functions as well as enhanced self-replication of the HSCs to repopulate and maintain the stem cell pool, after in vivo administration of the treated cells. Accordingly, one aspect, of the invention is directed to a population of the HSPC, that has been exposed or treated with ANG or an Angiogenin agonist ex vivo, which can be transplanted into a patient in need of improved hematopoietic regeneration. While not wishing to be bound by theory, the exposure to ANG results in restricted proliferation, maintenance of quiescence and self-renewal capacities of the primitive HSPC, while preserving their viability and differentiation state. Accordingly, in some embodiments, one aspect of the invention is directed towards a population of HSC generated after ex vivo exposure to ANG.

[0011] Another aspect of the present invention relates to use of ANG protein or an agonist thereof to treat subjects that have been exposed to or likely to be exposed to ionization radiation. Accordingly, the invention relates to a pharmaceutical composition comprising ANG or a functional fragment thereof, or an agonist thereof for preventing radiation induced hematopoietic injury, e.g., as a result of radio-or chemotherapy as a treatment for a disease or a result of accidental exposure to radiation, wherein the pharmaceutical composition is administered in an therapeutically effective amount.

[0012] Described herein are uses, methods and compositions comprising of Angiogenin as a regulator of hematopoietic regeneration. In one aspect, the technology described herein relates to ex vivo or in vitro cultured hematopoietic stem and progenitor cell compositions comprising

HSPCs treated with Angiogenin or an agonist thereof and to methods for producing the same, wherein the compositions are characterized by reduced proliferation and maintenance of primitive HSPC in quiescent state and enhancing their self-renewal while increased proliferation and therefore expansion of myeloid restricted progenitors without differentiation. The invention also relates to methods to enhance the short term and long term hematopoietic reconstitution upon in vivo administration of the said compositions. In another aspect, the invention relates to methods and use of Angiogenin or an agonist thereof for treatment of irradiation injury in vivo.

[0013] Thus in one aspect, described herein is a method of increasing hematopoietic reconstitution in a human subject, the method comprising: (i) contacting a population of hematopoietic stem cells (HSCs), ex vivo, with an effective amount of an Angiogenin (ANG) protein or an Angiogenin agonist; (ii)administering cells from step (i) to a subject, wherein the subject is in need of hematopoietic reconstitution.

[0014] In some embodiments, the population of HSCs is obtained from bone marrow, peripheral blood, cord blood, amniotic fluid, placental blood, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs). In some embodiments, the population of HSCs is human. In some embodiments, the population of HSCs comprises at least one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs). In some embodiments, the population of HSCs is autologous or allogeneic to the subject.

[0015] In one aspect, the methods described herein further comprises culturing the population of HSCs in presence of Angiogenin prior to step (ii). In some embodiments, the population of HSCs are cultured in presence of Angiogenin for at least 2 hrs. In another embodiment, the population of HSCs are cultured in presence of Angiogenin for about 2 days or more. In another embodiment, the population of HSCs are cultured in presence of Angiogenin for at least 7 days. In some embodiments, the population of HSCs are cryopreserved prior to, or after, the contacting with ANG. In some embodiments, the subject is susceptible to, or has decreased HSC levels as compared to a healthy subject.

[0016] In some embodiments, the subject has, or will undergo bone marrow or stem cell transplantation, or has, or will undergo chemotherapy or radiation therapy. In some embodiments, the subject has a disease or disorder selected from the group: leukemia, lymphoma, myeloma, solid tumor, a blood disorder (e.g., myelodysplasia), immune disorders or anemia.

[0017] In some embodiments of the technology described herein, ANG protein is human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1 or a functional fragment thereof with a biological activity of at least 80% of human ANG protein to increase hematopoietic reconstitution in a human subject. In some embodiments, the functional fragment is a human recombinant ANG polypeptide. In some embodiments, the human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1 comprises a mutation K33A. In some embodiments, the functional fragment comprises an amino acid sequence of at least 80% of human ANG of SEQ ID NO: 1. In some embodiments, the functional fragment of human ANG protein comprises at least 80% sequence identity to amino acids 1-147 of SEQ ID NO 1. In other embodiments, the functional fragment of human ANG protein comprises at least 90% sequence identity to amino acids 1-147 of SEQ ID NO 1. In other embodiments, the functional fragment of human ANG protein comprises at least 95% sequence identity to amino acids 1-147 of SEQ ID NO 1. In other embodiments, the functional fragment of human ANG comprises at least 98% sequence identity to amino acids 1-147 of SEQ ID NO 1.

[0018] In some embodiments of the foregoing aspects the hematopoietic reconstitution is multi-lineage hematopoietic reconstitution. In some embodiments, the hematopoietic reconstitution is long-term multi-lineage hematopoietic reconstitution. In some embodiments, the hematopoietic reconstitution comprises reconstitution of short-term hematopoietic stem cells (ST-HSC) and/or long-term (LT-HSC) hematopoietic stem cells.

[0019] In another aspect, described herein are methods for expanding a population of hematopoietic cells in a biological sample, the method comprising contacting the hematopoietic cells with an Angiogenin (ANG) protein or an ANG agonist, wherein the population comprises

primitive hematopoietic stem cells and myeloid restricted progenitors, and wherein the contacting is for a sufficient amount of time to allow for primitive hematopoietic stem cells quiescence and myeloid restricted progenitor proliferation.

[0020] In some embodiments, the biological sample is selected from the group of: cord blood, bone marrow, peripheral blood, amniotic fluid, or placental blood.

[0021] In another aspect, described herein is method for expanding a population of hematopoietic cells in a biological sample further comprises collecting the population of hematopoietic cells.

[0022] In another aspect, described herein is a population of primitive hematopoietic stem cells produced by the methods disclosed herein.

[0023] In another aspect, described herein is a population of myeloid restricted progenitors produced by the methods disclosed herein.

[0024] In another aspect, described herein is a cryopreserved population of hematopoietic cells comprising primitive hematopoietic stem cells and/or myeloid restricted progenitors in the presence of an angiogenin protein.

[0025] In another aspect, disclosed herein is a blood bank comprising the said population of hematopoietic cells.

[0026] In another aspect, disclosed herein is a method of administering a population of hematopoietic cells to a subject, comprising administering an effective amount of the population of hematopoietic cells to the subject, wherein the population of hematopoietic cells have been contacted ex vivo or in vitro with an Angiogenin (ANG) protein or ANG agonist, wherein the population of hematopoietic stem cells comprises primitive hematopoietic stem cells and myeloid restricted progenitors, and wherein the Angiogenin protein increases primitive hematopoietic stem cells quiescence and increases myeloid restricted progenitor proliferation.

[0027] In another aspect, disclosed herein is a method of increasing reconstitution potential of transplanted hematopoietic stem cells in a subject, the method comprising the step of administering Angiogenin (ANG) protein or an ANG agonist to the subject, prior to, during or after transplantation of HSCs, wherein the subject is a candidate for bone marrow or stem cell transplant.

[0028] In another aspect, disclosed herein are uses of Angiogenin (ANG) protein to increase hematopoietic reconstitution in a human subject in need thereof. In some embodiments, the population of HSCs are obtained from bone marrow, peripheral blood, cord blood, amniotic fluid, placental blood, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs). In some embodiments, the population of HSCs are human. In some embodiments, the population of HSCs comprises at least one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs). In some embodiments of the foregoing aspects, the population of HSCs are autologous or allogeneic to the subject.

[0029] In some embodiments, the population of HSCs is cultures in presence of Angiogenin prior to administering to the subject. In some embodiments, of the use of Angiogenin, the population of HSCs are cultured in presence of Angiogenin for at least 2 hrs. In some embodiments, the population of HSCs are cultured in presence of Angiogenin for about 2 days or more. In some embodiments, the population of HSCs are cultured in presence of Angiogenin for at least 7 days. In some embodiments, the population of HSCs are cryopreserved prior to, or after, the contacting with ANG. In some embodiments, the population of HSCs are cryopreserved in the presence of ANG.

[0030] In some embodiments, the subject is susceptible to, or has decreased HSC levels as compared to a healthy subject. In some embodiments, the subject has, or will undergo bone marrow or stem cell transplantation, or has, or will undergo chemotherapy or radiation therapy. In some embodiments, the subject has a disease or disorder selected from the group: leukemia,

lymphoma, myeloma, solid tumor, a blood disorder (e.g., myelodysplasia), immune disorders or anemia. In some embodiments, the anemia is sickle cell anemia, thalassemia or aplastic anemia.

[0031] In some embodiments, of the use of Angiogenin in the methods disclosed herein, ANG protein is human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1, a functional fragment thereof with a biological activity of at least 80% of human ANG protein to increase hematopoietic reconstitution in a human subject. In some embodiments, the functional fragment is a human recombinant ANG polypeptide. In some embodiments, the functional fragment comprises at least amino acids 1-147 of SEQ ID NO 1. In some embodiments, the human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1 comprises a mutation K33A. In some embodiments, the functional fragment comprises an amino acid sequence of at least 80% of human ANG of SEQ ID NO: 1. In some embodiments, the functional fragment of human ANG protein comprises at least 80% sequence identity to amino acids 1-147 of SEQ ID NO 1. In some embodiments, the functional fragment of human ANG protein comprises at least 90% sequence identity to amino acids 1-147 of SEQ ID NO 1. In some embodiments, the functional fragment of human ANG protein comprises at least 95% sequence identity to amino acids 1-147 of SEQ ID NO 1. In some embodiments, the functional fragment of human ANG comprises at least 98% sequence identity to amino acids 1-147 of SEQ ID NO 1.

[0032] In some embodiments, the hematopoietic reconstitution is multi-lineage hematopoietic reconstitution. In some embodiments, the hematopoietic reconstitution is long-term multi-lineage hematopoietic reconstitution. In some embodiments, the hematopoietic reconstitution comprises reconstitution of short-term hematopoietic stem cells (ST-HSC) and/or long-term (LT-HSC) hematopoietic stem cells.

[0033] In one aspect, described herein is a method of treating a subject that has been exposed to ionizing radiation, the method comprising administering an effective amount of an Angiogenin (ANG) protein or Angiogenin agonist to the subject. In some embodiments of the foregoing aspects, the subject has been exposed to, will be exposed to or is at a risk of exposure to ionizing radiation. In some embodiments, the subject is a mammal. In some embodiments, the subject will, or has undergone, radiation therapy for the treatment of a disease or disorder. In some

embodiments, the subject will, or has undergone radiation therapy as part of an ablative regimen for hematopoietic stem cell or bone marrow transplant or chemotherapy. In some embodiments, the subject will, or has under gone total body radiation. In some embodiments, the subject will, or has been exposed to a radiation accident, chemotherapy or transplantation.

[0034] In some embodiments, the hematopoietic cells are selected from the group consisting of Long-term hematopoietic stem cells (LT-HSCs), Short-term hematopoietic stem cells (ST-HSCs), Multipotent progenitor cells (MPPs), Common myeloid progenitor (CMPs), CLPs, Granulocyte-macrophage progenitor (GMPs) and Megakaryocyte-erythroid progenitor (MEPs).

[0035] In some embodiments, the ANG is administered to the subject prior to, during or after exposure, or a combination thereof, to an ionizing radiation. In some embodiments, the ANG protein is administered for between 12 hours and 3 days prior to exposure to ionizing radiation. In some embodiments, the exposure to ionizing radiation occurs within about 24 hours after the last administration of ANG protein. In some embodiments, the ANG protein is administered immediately after the exposure to ionizing radiation. In some embodiments, the ANG protein is administered about 24 hours after exposure to ionizing radiation.

[0036] In some embodiments, the ANG protein is administered for at least 3 days or more.

[0037] In some embodiments, the administration of ANG results in improved hematopoietic regeneration after exposure to radiation as compared to in absence of administration of Angiogenin. In some embodiments, the administration of Angiogenin maintains hematopoietic stem and/or progenitor cells in a state of quiescence.

[0038] In some embodiments, the ANG protein is human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1, a functional fragment thereof with a biological activity of at least 80% of human ANG protein to increase hematopoietic reconstitution in a human subject. In some embodiments, the functional fragment is a human recombinant ANG polypeptide. In some embodiments, the functional fragment comprises at least amino acids 1-147 of SEQ ID NO 1.In some embodiments, the human ANG protein of at least 85% amino acid

sequence identity to SEQ ID NO: 1 comprises a mutation K33A. In some embodiments, the functional fragment comprises an amino acid sequence of at least 80% of human ANG of SEQ ID NO: 1. In some embodiments, the functional fragment of human ANG protein comprises at least 80% sequence identity to amino acids 1-147 of SEQ ID NO 1. In some embodiments, the functional fragment of human ANG protein comprises at least 90% sequence identity to amino acids 1-147 of SEQ ID NO 1. In some embodiments, the functional fragment of human ANG protein comprises at least 95% sequence identity to amino acids 1-147 of SEQ ID NO 1. In some embodiments, the functional fragment of human ANG comprises at least 98% sequence identity to amino acids 1-147 of SEQ ID NO 1.

[0039] In another aspect, disclosed herein is a method, of increasing the dose of an ionizing radiation treatment, comprising administering to the subject an effective amount of an Angiogenin (ANG) protein or Angiogenin agonist before, after or during the ionizing radiation, wherein the dose of the ionizing radiation treatment is higher as compared to the dose in absence of Angiogenin (ANG) protein or Angiogenin agonist administration.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Fig. 1. Proximity based single cell analysis of the bone marrow niche. (A) Experimental schema. DiI-labeled adult bone marrow LKS CD34-Flk2- LT-HSCs were intravenously injected into irradiated col2.3GFP pups (P2). Forty-eight hours later, fresh sections of the femori were obtained, individual proximal and distal OLCs were identified and harvested for single cell RNA-Seq analysis. Selected differentially expressed genes were validated in vivo. (B) Micropipette aspiration of proximal OLC. Shown are overlaid single color (GFP and DiI) images before and after retrieval of proximal OLC (i) The proximal GFP+ OLC (green was identified based on proximity to the DiI-labeled HSPC (red). (ii) Following in-situ enzymatic dissociation, the HSPC was dislodged from its original location, other hematopoietic cells became loose and OLCs partially detached from the endosteal surface. (iii) Proximal OLC was aspirated into a micropipette.

[0041] Fig. 2. (A) Bayesian approach to estimate the posterior distribution of expression levels in individual proximal and distal OLCs (colored lines). The joint posteriors (black lines) describe the overall estimation of likely expression levels in each group and are used to estimate the

posterior of the expression-fold difference (middle plot). The shaded area under the fold-difference posterior shows 95% confidence region. Expression of Vcam-1 gene is shown as an example. (B) Gene set enrichment analysis (GSEA) of differentially expressed genes between proximal and distal OLCs. GSEA plots referring to expression of gene sets "Surface proteins" and "Immune response" in proximal OLCs ($p<0.0005$) are shown.

[0042] Fig. 3. Proximal and distal OLCs are transcriptionally distinct. (A) Classification of individual OLCs based on the top 200 differentially expressed genes. Each row represents a gene, with the most likely gene expression levels indicated by color (blue – high, white – low absent. (B) An unbiased genome-wide classification of proximal and distal OLCs. The receiver-operator curve is shown for the Support Vector Machine classification where all successive pairs of cells (one proximal and one distal were classified based on the training data provided by other cells ($P<0.005$. (C, D) Expression analysis of known niche-derived HSPC regulators and OLC maturation genes. The violin plots show the posterior distribution of the expression fold-difference (y-axis, log2 scale for each gene, with the shaded area marking the 95% confidence region). The horizontal solid red lines show the most likely fold-change value.

[0043] Fig. 4. Conditional deletion of Ang from niche cell subsets leads to the loss of quiescence in LT HSCs and CLPs. (A) Comparison of Ang expression in proximal and distal OLCs. (B) LT-HSC number per femur and (C) LT-HSC cell cycle status following conditional deletion of Ang from distinct niche cell subsets, as per the color-coded legend (n=4-10). Non-shaded graphs: control animals, shaded graphs: Ang-deleted animals. (D) CLP number per femur and (E) CLP cell cycle status following conditional deletion of Ang from distinct niche cell subsets (n=4-10). (F) Long-term reconstitution following competitive (1:1) transplantation of bone marrow from control animals (solid lines) and animals with conditional deletion of Ang (broken lines) into WT congenic recipients (n=8). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

[0044] FIG. 5. (A) FACS gating strategy used for quantification of primitive hematopoietic subsets. (B) The number (per femur) of STBHSC (i), MPP (ii) and common myeloid progenitors (CMP) following conditional deletion Ang from niche cell subsets, as indicated by the color scheme on the right (n=8). (C) FACS gating strategy used for cell cycle studies in primitive hematopoietic cells using Ki67/DAPI staining. (D) Cell cycle status of STBHSC (i), MPP (ii) and CMP (iii) following conditional deletion Ang from niche cell subsets, as indicated by the color scheme on the right (n=8).

[0045] Fig 6. In vivo analysis of Interleukin 18 function in HSPC regulation. (A) Comparison of IL18 expression in proximal and distal OLC. (B) BrdU incorporation by HSPC in IL18KO mice (n=5). (C) IL18 receptor expression in HSPC. Representative histograms are shown (n=3). A comparable cell population from IL18R KO mouse was used as a negative control (shaded histogram. (D) Flow cytometric assessment of multi-lineage response to 5-FU in IL18KO mice. The statistical significance was assessed by ANOVA. Boxplots illustrating log ratios of cell numbers between 5-FU-treated and vehicle-treated animals in WT and IL18 groups are shown (n=7). (E) Enumeration of apoptotic LKS cells and lin-negative cells in WT animals pre-treated with rIL18 prior to 5-FU exposure (n=5). (F) Myeloid and lymphoid reconstitution in IL18KO mice following transplantation of (WT) LKS cells (n=7). (G) Multi-lineage donor chimerism following transplantation of LKS cells from IL18R1KO or WT animals into WT hosts (n=8) per group. *P<0.05, **P<0.01.

[0046] Fig 7. (A) Peripheral blood analysis of IL18KO mice (n=12). (B,C) Quantification of primitive and mature cells in IL18KO mice (n=6). (D) Experimental schema and cumulative donor chimerism following noncompetitive transplantation of WT BM marrow cells into WT or IL18KO hosts (n=5-7). (E-G) Estimation of in vivo growth kinetics and localization following transplantation of fluorescently labeled LKS cells into WT or IL18KO host by intra-vital microscopy (n=6). (H) Survival of WT and IL18KO animals following limiting dose bone marrow transplantation. *P<0.05, **P<0.01, ns – not significant.

[0047] Fig 8. (A,B) Quantification and representative FACS plots from cell cycle studies in newborn IL18KO mice (n=6) (C) Flow cytometric assessment of primitive hematopoietic subsets in P1 pups following in-utero exposure to busulphan (n=6).*P<0.05, **P<0.01.

[0048] Fig 9. Expression of human IL18 receptor in primitive hematopoietic cells. Representative histograms of cord blood and bone marrow analysis are shown (shaded histogram – isotype control, n=3) .

[0049] Fig 10. Embigin regulates HSPC localization and homing. (A) Comparison of Embigin expression in proximal and distal OLC. (B,C) Enumeration of myeloid (kit+linSca1- progenitor cell frequency and CFC number in peripheral blood following treatment with anti-Embigin or isotype control antibody (n=5). (D, E) Quantification of HSPC homing to calvarial bone marrow 24 hours after transplantation using intravital microscopy. Animals which were either injected with anti-Embigin or isotype control antibody prior to transplantation of LKS cells (D), or

transplanted with anti-Embigin or isotype control-treated LKS cells (E) (cumulative of two independent experiments, 2 animals per condition in each experiment . Each dot on the calvarial map represents location of an individual cell and each color – an individual mouse (n=4). Representative images and quantification of cell number are shown below. (F) Proliferation of transplanted LKS cells in animals pre-treated with anti-Embigin (n=4) between 24 and 48 hours post-transplantation. *P<0.05, **P<0.01, ***P<0.001

[0050] Fig. 11. Embigin regulates HSPC quiescence. (A,B) The number of primitive hematopoietic cells and colony-forming cells 24 hours after treatment with anti-Embigin or isotype control antibody (n=5). (C, D) BrdU incorporation and cell cycle analysis of primitive hematopoietic cells following treatment with anti-Embigin or isotype control antibody (n=5 mice). (E) Competitive (1:1) transplant of the bone marrow from animals treated with anti-Embigin or isotype control antibody (n=10).

[0051] Fig. 12. Ang deficiency results in loss of HSPC quiescence and defective transplantation (A-B) Quantification of primitive hematopoietic cells (A, n=12) and cell cycle status (B, n=8) in Ang-/- mice. (C) Quantification of stem and progenitor in Ang-/- mice on day 7 post-exposure to 150 mg/kg 5- FU (n=8). (D) Survival of Ang-/- mice following weekly 5-FU (150 mg/kg) exposure (n=10). Arrows indicate day of injection. (E) Experimental schema of serial transplant using WT or Ang-/- hosts. (F-H) Multi-lineage donor cell chimerism (F), HSPC number (G), and HSPC cell cycle status (H) after competitive primary transplantation of LT-HSCs into lethally-irradiated WT or Ang-/- recipients (n=8). (I) Chimerism after secondary transplantation of sorted LT-HSCs from primary recipients into WT or Ang-/- secondary recipients (n=8). See also Figures 13 and Tables 1-2.

[0052] Fig. 13. Ang deficiency results in loss of HSPC quiescence and defective transplantation potential in young and aged mice, Related to Figure 12. (A) Representative gating schema of stem and progenitor cells. (B) BrdU incorporation in Ang-/- HSPC (n=5). (C) Frequency of apoptotic HSPCs, lymphoid-restricted progenitors, and myeloid-restricted progenitors in WT or Ang-/- mice (n=10). (D-E) Quantification of primitive hematopoietic cells (D, n=12) and cell cycle status (E, n=12) in Ang-/- mice using SLAM/CD48 staining. (F-G) Quantification of HSPC, lymphoid- and myeloid-restricted progenitors (F, n=5) and cell cycle status (G, n=5) in 22-month old WT or Ang-/- mice (F, n=5). (H) Colony formation of BM isolated from 22-month old WT or Ang-/- mice (n=5). (I) Serial re-plating of BM from 22-month old WT or Ang-/- mice

(n=5). Colonies were harvested on day 7 and re-plated in equal numbers. Colonies were then scored again on day 14. (J) Experimental schema for transplantation of BM from aged WT and Ang-/- mice. (K) Competitive transplant (1:1) of whole BM from 22-month old WT or Ang-/- donors (n=5). (L) Experimental schema for non-competitive whole BM primary and secondary transplants into 8-week old WT or Ang-/- mice. (M) Multi-lineage donor cell chimerism following non-competitive primary transplant of WT BM into WT or Ang-/- recipients (n=7-8). (N) Homing analysis following transplantation of CFSE-labeled WT CD45.1 lineage-negative cells into WT or Ang-/- recipients 16-hours post-transplant (n=5). (O) Survival of animals following secondary transplantation of BM from primary recipients into respective WT or Ang-/- secondary recipients (n=10).

[0053] Fig. 14. Dichotomous effect of ANG in LKS and myeloid-restricted progenitor cell cycling (A-B) Cell cycle status of LKS cells and myeloid-restricted progenitors (A, n=8) and MPP1-4 cells (B, n=6) from WT and Ang-/- mice. (C) qRT-PCR analysis of self-renewal transcripts from sorted LKS cells or myeloid-restricted progenitors treated with mouse ANG protein (0-600 ng/ml, n=6). See also Fig. 15.

[0054] Fig. 15. Effect of ANG on quiescence is cell-context specific, Related to Figure 14. (A) BrdU incorporation in WT or Ang-/- LKS cells and myeloid-restricted progenitors (n=5). (B-D) Lymphoid-restricted progenitor cell number (B, n=6), cell cycle status (C, n=6), and BrdU incorporation (D, n=5) in WT and Ang-/- mice. (E-G) Myeloid-restricted progenitor cell number (E, n=9), cell cycle status (F, n=6 mice), and BrdU incorporation (G, n=5) in WT and Ang-/- mice. (H-K) qRT-PCR analysis of self-renewal transcripts from sorted WT or Ang-/- LKS cells and myeloid-restricted progenitors (H), uncultured or cultured WT LT-HSCs in the presence of mouse ANG protein (0-600 ng/ml) for 2 h in PBS (I), uncultured or cultured WT LT-HSCs in the presence of mouse ANG protein (0-600 ng/ml) for 2 h, 48 h or 7 days in S-clone media (J), and WT or Ang-/- LT-HSCs cultured in the presence or absence of 300 ng/ml ANG (K) (n=6).

[0055] Fig. 16. ANG-mediated regulation of protein synthesis is cell context-specific. (A) In vivo OP-Puro incorporation in WT or Ang-/- LKS cells and myeloid-restricted progenitors. Cells were sorted 1 h after OP-Puro administration. Bar graphs are relative values to WT LKS (n=5). (B) In vivo OP-Puro incorporation following 2 h ANG treatment of LKS cells and myeloid-restricted progenitors. Bar graphs are relative values to untreated LKS (n=6). (C) qRT-PCR

analysis of rRNA species following 2 h ANG treatment of LKS cells and myeloid- restricted progenitors, using various primer sets (n=3). See also Figures 17-19.

[0056] Fig 17. ANG-mediated regulation of protein synthesis is correlated with cell context-specific RNA processing, Related to Figures 16 and 18. (A) OP-Puro incorporation in WT or Ang-/- stem, progenitor, and mature cell subsets 1 h after in vivo administration. Bar graphs are relative values to WT LKS (n=5). (B-C) BM cellularity (B) and LT-HSC frequency (C) 1 h after in vivo OP-Puro administration (n=5). (D) qRT-PCR analysis of rRNA species in WT or Ang-/- LT-HSCs, myeloid-restricted progenitors, or whole BM (n=3). (E) Small RNA production in WT Lin+ cells treated with or without 300 ng/ml ANG protein for 2 h, using 15 μg RNA for electrophoresis (n=3). (F) Small RNA production in WT or Ang-/- LKS cells (n=3). (G) Small RNA production in WT LKS cells and myeloid-restricted progenitors treated with or without sodium arsenite (500 μM) and/or ANG protein (300 ng/ml) for 2 h (n=3). (H) Colony formation of whole BM transfected with inactive (d)5'-P or active 5'-P tiRNA (n=3).

[0057] Fig. 18. ANG-mediated regulation of protein synthesis is correlated with cell context-specific tiRNA production (A and B) Small RNA production (A, n=3) and Northern blot analysis of tiRNA-Gly-GCC (B, n=3) following 2 h treatment of LKS cells and myeloid-restricted progenitors with ANG. Bar graphs are relative values to untreated LKS. (C-D) OP-Puro incorporation (C, n=5), and qRT-PCR analysis of self-renewal, pro-survival, and pro-apoptotic transcripts (D, n=5) in LKS cells and myeloid-restricted progenitors transfected with inactive (d)5'-P tiRNA or active 5'-P tiRNA. (E) Post-transplant reconstitution of LKS cells transfected with inactive (d)5'-P tiRNA or active 5'-P tiRNA (n=7). See also Fig.17-19.

[0058] Fig. 19. ANG is associated with RNH1 in the nucleus of HSPC and in the cytoplasm of myeloid-restricted progenitors, Related to Figures 16 and 18. (A) ANG (green) or PABP (red) localization in LKS cells and myeloid-restricted progenitors by immunofluorescence (n=5). (B) RNH1 (green) or PABP (red) localization in LKS cells and myeloid-restricted progenitors by immunofluorescence (n=5).(C) ANG (green) or RNH1 (red) localization in LKS cells and myeloid-restricted progenitors by immunofluorescence (n=5). (D) ANG/RNH1 FRET (n=10 cells from 3 mice). Scale bar: 1 μm. Increased sensitivity of Ang-/- mice to γ-irradiation, Related to Figure 20

[0059] Fig. 20. (A) Kaplan-Meier survival curves of WT or Ang-/- mice subjected to 7.5 Gy (left), 7.75 Gy (middle), or 8.0 Gy (right) radiation (n=12). (B) Blood leukocyte recovery on day

7 in WT or Ang-/- mice treated with 8.0 Gy (n=10). (C-K) BM cellularity (C), HSPC number (D), HSPC cycling (E), lymphoid-restricted progenitor number (F), lymphoid-restricted progenitor cycling (G), myeloid-restricted progenitor number (H), myeloid-restricted progenitor cell cycling (I), apoptotic activity (J), and colony formation (K) of WT or Ang-/- mice treated with 4.0 Gy TBI (n=6). Animals were sacrificed and analyzed on day 7 post-irradiation.

[0060] Fig 21. ANG enhances radioprotection and radioresistance. (A) Survival of WT or Ang-/- mice treated with ANG daily for three successive days 24 h pre- TBI (n=10). (B) Survival of WT or Ang-/- mice treated with ANG daily for three successive days 24 h post- TBI (n=10). (C-G) H&E and BM cellularity of femurs (C), LKS and myeloid-restricted progenitor cell number (D), cell cycling (E), apoptotic activity (F), and post-transplant reconstitution (G) of WT mice treated with ANG daily for three successive days 24 h post-TBI (n=6). Scale bar = 100 μm. (H) Survival of WT mice treated with ANG daily for three successive days 24 h prior or post-12 Gy. (I) H&E and BM cellularity of femurs of WT mice treated with ANG daily for three successive days 24 h post-12.0 Gy TBI (n=6). Scale bar = 100 μm. (J) LD50 of mice treated with ANG daily for three successive days beginning 24 h post-TBI (n=8). (K-L) qRT-PCR analysis of self-renewal, pro-survival, pro-apoptotic, and rRNA transcripts (K, n=6), and tiRNA production (L, n=3) in LKS or myeloid-restricted progenitors sorted from irradiated mice (4.0 Gy) and treated with 300 ng/ml ANG. See also Figures 19-21 and Tables 7-9.

[0061] Fig. 22. ANG enhances radioprotection and radioresistance, Related to Fig 21. (A-J) BM cellularity (A), HSPC number (B), HSPC cycling (C), lymphoid-restricted progenitor number (D), lymphoid-restricted progenitor cycling (E), myeloid-restricted progenitor number (F), myeloid-restricted progenitor cell cycling (G), apoptotic cell percentage (H), colony formation (I), and post-transplant reconstitution (J) of WT mice pre-treated with ANG daily for three successive days 24 h before 4.0 Gy TBI (n=6). Animals were sacrificed and analyzed on day 7 post-irradiation. (K) Kaplan-Meier survival curve of WT mice treated with ANG immediately following 8.0 Gy TBI (n=10). (L-S) HSPC number (L), HSPC cycling (M), lymphoid-restricted progenitor number (N), lymphoid-restricted progenitor cycling (O), myeloid-restricted progenitor number (P), myeloid- restricted progenitor cell cycling (Q), apoptotic cell percentage (R), and colony formation (S) of WT mice treated with ANG daily for three successive days beginning 24 h after 4.0 Gy TBI (n=6). Animals were sacrificed and analyzed on day 7 post-irradiation.

[0062] Fig. 23. ANG enhances post-transplant reconstitution. (A) Cell density on day 7 from sorted WT or Ang-/- LT-HSCs (1875 cells/ml) cultured in the presence of various doses of ANG (n=6). (B) tiRNA levels following 7 day culture with 0 or 300 ng/ml ANG. After culture, cells were harvested and again treated with 0 or 300 ng/ml ANG (indicated by + or -) for 2 h prior to analysis by electrophoresis (n=3). (C) Post-transplant reconstitution of LT-HSCs after 2 h ex vivo treatment with ANG (n=8-9). (D) Secondary transplant without further ex vivo ANG treatment (n=7-8). (E) Post-transplant reconstitution of WT or Ang-/- LT-HSCs which were cultured in the presence or absence of 300 ng/ml ANG for 2 h and competitively transplanted in WT hosts (n=7). See also Fig. 22.

[0063] Fig. 24. ANG enhances post-transplant reconstitution, Related to Figures 23 and 25. (A) Post-transplant reconstitution of human CD34+ CB cells following 2 h ex vivo treatment with 300 ng/ml ANG (n=7). (B-C) Cells were grown in culture for 7 days (2,500 cells/ml). At day 7, cells were harvested, washed with PBS, and replated in S-clone media without addition of ANG. Cell density (B) and self-renewal transcripts (C) were examined (n=6). (D) BM homing 16 h post-transplant with CFSE-labeled Lin− cells that were cultured in the presence or absence of 300 ng/ml ANG for 2 h (n=5). (E) qRT-PCR analysis of self-renewal transcripts in human CD34+ CB cells following 7-day culture with human WT ANG protein and variants (n=6). (F) Colony formation of human CD34+ CB cells plated in the presence or absence of 300 ng/ml human ANG (n=6). (G-H) Human CD19 (G) and human CD33 (H) frequencies in BM of NSG mice transplanted with human CD34+ CB cells treated with or without human ANG protein (300 ng/ml) for 2 hours (from Figure 7C-D). BM was harvested 16 weeks post-transplant.

[0064] Fig. 25. ANG enhances post-transplant reconstitution of human CD34+ CB cells.

[0065] (A) Cell density on day 7 from human CD34+ CB cells (2,500 cells/ml) cultured in the presence of various doses of ANG or ANG variants: K40Q (enzymatic variant), R70A (receptor-binding variant), or R33A (nuclear localization variant) at 300 ng/ml (n=6). (B) qRT-PCR analysis of self-renewal transcripts in human CD34+ CB cells following 2 h culture with human ANG protein (n=6). (C) Human CD45 cells in BM of NSG mice transplanted with human CD34+ CB cells treated with or without human ANG (300 ng/ml) for 2 h. BM was harvested 16 weeks post-transplant (n=9-10). (D) LT-HSC frequencies (black line) and 95% confidence intervals (shaded boxes) for each transplant condition from Figure 7C (p=8.28x10-5). See also Fig. 24.

DETAILED DESCRIPTION

[0066] Hematopoietic stem cells (HSCs) give rise to all other blood cells within the mammalian blood system, through the process of hematopoiesis. The HSCs can carry out this function as they possess the unique ability of both "multi-potency" and "self-renewal". Multi-potency is the ability to differentiate into all functional blood cells. Self-renewal is the ability to give rise to new HSC cells without differentiation. Since mature blood cells are predominantly short lived, HSCs continuously provide more differentiated progenitors while maintaining the HSCs pool size properly throughout life by precisely balancing self-renewal and differentiation. These properties together define the "stemness" of HSCs and are harnessed in the medical process of hematopoietic stem cells transplant which involves administration of HSCs in patients whose bone marrow or immune system is damaged or defective, in order to reestablish hematopoietic function. As described herein the present invention generally relates to methods and use of protein Angiogenin (ANG) to improve the post-transplantation hematopoietic reconstitution of HSPC compositions and methods of preparing the same. In another aspect, the current invention generally relates to use of Angiogenin as a prophylactic and/or therapeutic agent to enhance hematopoietic regeneration associated with radiation injury due to past, or predicted future exposure to radiation and enable survival of exposed subject.

Definitions

[0067] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0068] As used herein, the term "ex vivo" refers to a process in which cells are removed from a living organism and are treated outside the organism (e.g., in a test tube). The ex vivo conditions can involve providing the cells with nutrients (e.g. Cytokines). Methods of ex vivo culturing

stem cells of different tissue origins are well known in the art of cell culturing to this effect, see for example the text book :Culture of Animal cells –A manual of basic Technique" by Freshney, Wiley-Liss, N.Y. (1994), Third edition, the teachings of which are hereby incorporated by reference. Concomitant with treating the cells with conditions which allow for ex vivo the stem cells to proliferate, the cells are short-term treated or long-term treated with Angiogenin.

[0069] As used herein, the term "stem cell" refers to an undifferentiated cell which has the capacity to develop to any cell lineage present in the organism from which they are derived, given the right growth conditions, by the process of differentiation and can undergo self-renewal to produce daughter stem cell having the parental undifferentiated state and properties. In theory to self-renew, the stem cell can undergo an asymmetric cell division with one daughter cell maintaining the parental stem state and the other daughter expressing some distinct other specific function and phenotype (e.g., a progenitor cell). Alternatively, the stem cell can divide symmetrically into two daughter stem cells. Thus self-renewal maintains the number of stem cells in a population while other cells in the population give rise to differentiated progeny only. The stem cell therefore is capable of proliferation and giving rise to progenitor cells having the capacity to generate a large number of mother cells which in turn can give rise to differentiated or differentiable daughter cells. The daughter cells can further undergo proliferation to produce progeny that then can differentiate into one or more mature cell types. The capability of differentiation into a specialized cell type is defined as "potency". The more the cell types a cell can differentiate into, the more the potency. Stem cell can therefore be totipotent, pluripotent, and multipotent.

[0070] The term "Totipotent cells" refers to can grow and differentiate into any cell in the body, and thus can grow into an entire organism. They have the ability to give rise to all the cell types of the body plus all of the cell types that make up the extraembryonic tissues such as the placenta. These cells are not capable of self-renewal. In mammals, only the zygote and early embryonic cells are totipotent.

[0071] The term "Pluripotent cells" refers to are stem cells, with the potential to make nearly any differentiated cell in the body for e.g. Cells derived from any of the three germ layers namely

endoderm, mesoderm and ectoderm. They cannot however give rise to an entire organism like the totipotent cells.

[0072] "Multipotent cells can develop into more than one cell type, but are more limited than pluripotent cells; adult stem cells and cord blood stem cells are considered multipotent. Multipotent stem cells" are cells that self-renew as well as differentiate to regenerate adult tissues. They are able to give rise to a subset of cell lineages, but all within a particular tissue, organ or physiological system. For example, hematopoietic stem cells(HSC) can produce progeny that include HSC (by self-renewal), blood cell restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood. The term "stem cells", as used herein, refers to multipotent stem cells of mammalian origin capable of self-renewal and to generate differentiated progeny. "Oligopotent cells" can differentiate into only a few cell types e.g., lymphoid or myeloid progenitor cells.

[0073] The term "progenitor" or "precursor" cells are used interchangeably herein and refers to cells that have cellular phenotype that is more primitive (i.e. in earlier step along the developmental pathway) relative to the cell type it can give rise upon differentiation. They can also have high proliferative potential and can give rise to multiple distinct differentiated cell types or to a single differentiated cell type depending on the developmental pathway and on the environment in which the cells develop and differentiate.

[0074] The term "hematopoietic stem cells" or "HSCs" as used in the present invention refers to pluripotent stem cells or multipotent stem cells or lymphoid or myeloid (derived from bone marrow) that can differentiate into a progenitor cell of a lymphoid, erythroid or myeloid cell lineage or proliferate as a stem cell population without initiation of further differentiation. The "hematopoietic stem cells" as used in the invention can obtained from bone marrow, peripheral blood, umbilical cord blood, amniotic fluid, or placental blood or embryonic stem cells. HSCs are capable of self-renewal and differentiating into or starting a pathway to becoming a mature blood cell e.g. Erythrocytes (red blood cells), platelets, granulocytes (such as neutrophils, basophils and eosinophils), macrophages, B-lymphocytes, T-lymphocytes, and Natural killer

cells through the process of hematopoiesis. HSCs can also refer to long-term hematopoietic stem cells (LT-HSCs) or short-term hematopoietic stem cells (ST-HSCs).

[0075] The term "long-term hematopoietic stem cells" or LT-HSCs as used herein, refers to hematopoietic stem cell with long-term (typically more than three months) hematopoietic reconstitution potential. The LT-HSCs can have unlimited self-renewal lasting throughout adulthood, contribute to long-term multilineage reconstitution after transplant and can maintain reconstitution potential after serial transplantation into another subject. The LT-HSCs can be less actively dividing and/or quiescent relative to other HSCs. The LT-HSCs can be distinguished based on their surface markers known in the art, for example LT-HSCs can be CD34−, CD38−, SCA-1+, Thy1.1+/lo, C-kit+, lin−, CD135−, Slamf1/CD150+ (Lin−) and exhibit absence of Flk-2 (Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14541-6. Epub 2001 Nov 27).

[0076] The term "short-term hematopoietic stem cells" or ST-HSCs as used herein, refers to hematopoietic stem cell with hematopoietic reconstitution potential not exceeding three months and/or that is not multi-lineage. The ST-HSCs can be more actively dividing, more proliferating and less quiescent and have limited self-renewal capability relative to the LT-HSCs. ST-HSCs can be distinguished based on their surface markers known in the art, for example ST-HSCs can be CD34+, CD38+, SCA-1+, Thy1.1+/lo, C-kit+, lin−, CD135−, Slamf1/CD150+, Mac-1 (CD11b)lo and exhibit presence of Flk-2+ (Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14541-6. Epub 2001 Nov 27). Loss of Thy-1.1 expression with full expression of Flk-2 characterizes the next differentiation step to the multipotent progenitor (MPP).

[0077] The terms "hematopoietic progenitor cells" or "HPCs" as used herein refer to primitive hematopoietic cells that have differentiated to a developmental stage that, when the cells are further exposed to an appropriate cytokine or a group of cytokines, they will differentiate further along the hematopoietic cell lineage by the process of hematopoiesis. In contrast to HSCs, hematopoietic progenitor cells are only capable of limited self-renewal. "Hematopoietic progenitor cells" as used herein also include "precursor cells" that are derived from differentiation of hematopoietic progenitor cells and are the immediate precursors of mature differentiated hematopoietic cells. The term "hematopoietic progenitor cells", as used herein

include, but are not limited to, multipotent progenitors (MPPs), Common lymphoid progenitors (CMPs), Common myeloid progenitors (CMPs), Common Myelolymphoid Progenitors (CMLPs), common myeloid-erythroid progenitor (CMEPs), granulocyte-macrophage progenitor (GMPs), megakaryocyte-erythroid progenitors (MEPs), granulocyte-macrophage colony-forming cell (GM-CFC), megakaryocyte colony-forming cell (Mk-CFC), burst-forming unit erythroid (BFU-E), B cell colony-forming cell (B-CFC) and T cell colony-forming cell (T-CFC). "Precursor cells" include, but are not limited to, colony-forming unit-erythroid (CFU-E), granulocyte colony forming cell (G-CFC), colony-forming cell-basophil (CFC-Bas), colony forming cell-eosinophil (CFC-Eo) and macrophage colony forming cell (M-CFC) cells.

[0078] The term “Hematopoiesis" as used herein refers to the highly orchestrated process of blood cell development and homeostasis. Prenatally, hematopoiesis occurs in the yolk sack, then liver, and eventually the bone marrow. In normal adults it occurs in bone marrow and lymphatic tissues. All blood cells develop from pluripotent stem cells. Pluripotent cells differentiate into hematopoietic stem cells that are committed to three, two or one hematopoietic differentiation pathway.

[0079] The terms “hematopoietic stem and progenitor cells” or “HSPCs” as used herein refer to a mixture of hematopoietic stem cells and hematopoietic progenitor cells.

[0080] As used herein, the term “population of hematopoietic stem cells” refers to cell population comprising one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs). The cells may be contained in or obtained from bone marrow, peripheral blood, cord blood, amniotic fluid, or placental blood. The cells can be isolated using the defined cell surface markers. The markers and methods of isolation are known to those skilled in the art.

[0081] As used herein, the term differentiation refers to relatively generalized or specialized changes during development. Cell differentiation of various lineages is a well-documented

process and requires no further description herein. As used herein the term differentiation of hematopoietic stem cells and/or hematopoietic progenitors means both the change of hematopoietic stem cells into hematopoietic progenitors and the change of hematopoietic progenitors into unipotent hematopoietic progenitors and/or cells having characteristic functions, namely mature cells including erythrocytes, leukocytes and megakaryocytes. Differentiation of hematopoietic stem cells into a variety of blood cell types involves sequential activation or silencing of several sets of genes. Hematopoietic stem cells choose either a lymphoid or myeloid lineage pathway at an early stage of differentiation.

[0082] As used herein, the terms "hematopoietic reconstitution" or "hematopoietic repopulation" relates to the recovery of and/or repopulation of pool of HSCs by self-renewal and repopulation of all hematopoietic cell lineages for example; erythroid, myeloid and lymphoid lineages by differentiation of HSPCs and hematopoiesis within the bone marrow. Hematopoietic reconstitution in general therefore results in restoration of the normal functions of the bone marrow and immune system. Hematopoietic reconstitution comprises HSCs gaining access to the bone marrow (BM) in a process termed homing, take up residence in the BM, undergo self-renewing cell divisions to produce a larger pool of HSCs, and their differentiation into more committed progenitors, resulting in multilineage hematopoiesis. The reconstitution of a given cell type refers to its absolute count in the peripheral blood reaching a number of cells accepted by those of skill in the art as within the normal range for the subject. The reconstitution as referred herein can occur in a subject following a myeloablative regimen for example chemotherapy or radiation therapy and/or following in vivo administration of a population of hematopoietic stem cells for example bone marrow transplantation. Reconstitution efficiency depends upon several factors, including the underlying disease and disease status, patient's age, preparative regimen (myeloablative vs nonmyeloablative), the intensity of prior therapy such as chemotherapy or radiation therapy, and the stem cell source, transplant type (autologous vs allogeneic), major histocompatibility complex (HLA) disparity resulting in graft-versus-host disease (GVHD); and infection. Non-limiting examples of methods to measure successful hematopoietic reconstitution include measurement of complete blood count, differential blood counts, platelet counts, bone marrow biopsy tests, chest-x-rays which is would be known to those skilled in the art.

[0083] As used herein, the term "long-term hematopoietic reconstitution" refers to reconstitution for more than three months preferably for a lifetime of the subject. The HSCs contributing to long-term hematopoietic reconstitution can be for example LT-HSCs.

[0084] As used herein, the term "multi-lineage hematopoietic reconstitution" refers to the ability of HSCs to repopulate cells of all hematopoietic lineages for example; erythroid, myeloid and lymphoid lineages.

[0085] As used herein, "short-term hematopoietic reconstitution" refers to reconstitution for a period of less than three months. The HSCs contributing to short-term hematopoietic reconstitution can be for example ST-HSCs.

[0086] The phrase "expanding a population of hematopoietic cells" is used herein to describe a process of cell proliferation substantially devoid of cell differentiation. Cells that undergo expansion hence maintain their cell renewal properties and are oftentimes referred to herein as renewable cells, e.g., renewable stem cells.

[0087] As used herein, the term "culturing the population of HSCs" refers to maintaining the HSCs under in vivo culture conditions that facilitate expansion by proliferation, maintain their potency of the stem cells and preserve the viability of said stem cells. The viability can be determined by an assay for cell viability routinely used by those of skill in the art, e.g., a presidium iodide assay, by an in vivo culture assay in medium containing exogenously provided cytokines. With regard, maintaining the potency of "said stem cells", the term means preservation of HSCs into the same cell state as the cells used to initiate the culture, substantially devoid of cell differentiation e.g., an immunophenotype characteristic of human LT-HSC, for example, CD34−, CD38−, SCA-1+, Thy1.1+/lo, C-kit+, lin−, CD135−, Slamf1/CD150+ (Lin−), Flk-2-. The culture conditions can maintain potency of the cells by preserving them into a quiescence cell state for example LT-HSCs in the present invention or allowing cell proliferation devoid of cell differentiation for example proliferation of myeloid progenitors in the present invention.

[0088] As used herein the term “comprising” or “comprises” is used in reference to compositions, methods, and respective component(s) thereof, that are useful to an embodiment, yet open to the inclusion of unspecified elements, whether useful or not.

[0089] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[0090] As used herein the term “consisting of” refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0091] The terms “disease”, “disorder”, or “condition” are used interchangeably herein, refer to any alternation in state of the body or of some of the organs, interrupting or disturbing the performance of the functions and/or causing symptoms such as discomfort, dysfunction, distress, or even death to the person afflicted or those in contact with a person. A disease or disorder can also be related to a distemper, ailing, ailment, malady, disorder, sickness, illness, complaint, or affectation.

[0092] The term “in need thereof” when used in the context of a therapeutic or prophylactic treatment, means having a disease, being diagnosed with a disease, or being in need of preventing a disease, e.g.,,, for one at risk of developing the disease. Thus, a subject in need thereof can be a subject in need of treating or preventing a disease.

[0093] The term effective amount as used herein refers to an amount sufficient to affect a beneficial or desired clinical result upon treatment. Specifically, the term “effective amount” means an amount of compound e.g., Angiogenin, of this invention sufficient to measurably i. maintain HSCs in undifferentiated state and/or quiescent state ex vivo prior to transplantation, ii. allow self-renewal and expansion of HSCs prior to transplantation, iii) enhances short-term hematopoietic reconstitution and/or long-term hematopoietic reconstitution by at least 3 fold, at

least 2.5 fold, at least 2 fold, at least 1.5 fold upon transplantation of the treated HSCs relative to transplantation of the cells in absence of treatment. The enhanced hematopoietic reconstitution can result in a measurable effect in terms of repopulation of hematopoietic cells and functions thereof in a treated subject against for e.g., cancer of blood and bone marrow and/or hemaglobinopathy and/or thalassemia. The effective amounts may vary, as recognized by those skilled in the art, depending on the number of HSCs to be treated, the duration of treatment, source of HSCs, the specific underlying disease to be treated by transplantation, intensity of prior therapy such as chemotherapy or radiotherapy. Effects that can be measured are absolute counts for individual blood cell types (white blood cells, red blood cells and platelets) in the peripheral blood reaching a number of cells accepted by those of skill in the art as within the normal range for the subject. Methods of conducting a complete blood count are known to those skilled in the art.

[0094] An effective amount would therefore result in a clinical outcome of normalizing the numbers of HSCs and other blood cell types and their functions and cause treatment, reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of the disease resulting in improper functioning of the bone marrow and the immune system or their symptoms.

[0095] As used herein, a "subject", “patient”, “individual” and like terms are used interchangeably and refers to a vertebrate, preferably a mammal, e.g., a primate, e.g., a human. Mammals include, without limitation, humans, primates, rodents, wild or domesticated animals, including feral animals, farm animals, sport animals, and pets. Primates include, for example, chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include, for example, mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include, for example, cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, and canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. The terms, “individual,” “patient” and “subject” are used interchangeably herein. A subject can be male or female.

[0096] Mammals other than humans can be advantageously used as subjects that represent animal models of conditions or disorders associated with stem cell transplantation or disorders

associated with impaired bone marrow or immune system function. Such models are known in the art and are described in (Mouse Models of Bone Marrow Transplantation Biol Blood Marrow Transplant. 2008 Jan; 14(1 Suppl 1): 129–135.,) Biol Blood Marrow Transplant. 1999;5(1):1-7 Hematopoietic Stem Cell Function in a Murine Model of Sickle Cell Disease http://dx.doi.org/10.1155/2012/387385.

[0097] A subject can be one who has been previously diagnosed with or identified as suffering from or under medical supervision for a disorder causing damaged bone marrow or immune system function such as leukemias, lymphomas, myeloma, aplastic anemia, sickle cell anemia, thalassemia, immune deficiency disorders, and some solid tumor cancers. A subject can be one who is diagnosed and currently being treated for, or seeking treatment, monitoring, adjustment or modification of an existing therapeutic treatment, or is at a risk of developing such a disorder. A subject can be one who has undergone chemotherapy or radiation therapy. A subject can also be a person or individual has been exposed to, is being exposed to and/or to likely to be exposed to radiation or a radiation injury. (e.g. disaster response team members.)

[0098] In one aspect, the term "administering," refers to the placement of angiogenin treated HSCs as disclosed herein into a subject by a method or route that results in at least partial delivery of the cells at a desired site. Typically the HSCs are administered via intravenous route through a catheter much like blood transfusion. If the HSCs are cryopreserved, they are thawed prior to administration. In another aspect, “administering” relates to delivering angiogenin to a subject who has being, is being or is likely to be exposed to radiation.

[0099] As used herein, the terms “protein", “peptide” and “polypeptide" are used interchangeably to designate a series of amino acid residues connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", “peptide” and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g.,,,, phosphorylated, glycated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and “polypeptide” are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but

usage of these terms in the art overlaps. The terms "protein", “peptide” and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof.

[00100] The term “agonist” as defined herein is a compound capable of mimicking the biological activity of ANG upon binding to a receptor (e.g., Plexin-B2 or PlXNB2). The “biological activity” can be defined herein as enhancing the hematopoietic reconstitution potential of the HSCPs and/or maintaining primitive HSCs quiescence and/or enabling progenitor proliferation, upon contact with the HSPC population or source containing the HSPC population. The activity of the agonist can be for example at least 80%, at least 85%, at least 90%, at least 95%, at least 97% or at least 99% of the biological activity of human ANG of SEQ ID NO:1.

[00101] As used herein the term "ionizing radiation" refers to radiation of sufficient energy that, when absorbed by cells and tissues, typically induces formation of reactive oxygen species and DNA damage. Ionizing radiation can include X-rays, gamma rays, and particle bombardment (e.g., neutron beam, electron beam, protons, mesons, and others), and is used for purposes including, but not limited to, medical testing and treatment, scientific purposes, industrial testing manufacturing and sterilization, and weapons and weapons development. Radiation is generally measured in units of absorbed dose, such as therador gray (Gy), orin units of dose equivalence, such as rem or sievert (Sv).

[00102] By "at risk of exposure to ionization radiation" is meant a subject scheduled for (such as by scheduled radiotherapy sessions) exposure to ionizing radiation (IR) in the future, or a subject at risk of being exposed to IR inadvertently in the future. Inadvertent exposure includes accidental or unplanned environmental or occupational exposure (e.g., terrorist attack with a radiological weapon or exposure to a radiological weapon on the battlefield or exposure of a member of a disaster response team).

[00103] As used herein the phrase “an effective amount of an Angiogenin protein or Angiogenin agonist” refers to amount capable of reducing or eliminating the toxicity associated with radiation in healthy hematopoietic stem/progenitor cells in the subject. In some

embodiments, the effective amount is the amount required to temporarily (e.g., for a few hours or days) inhibit the proliferation of hematopoietic stem cells (i.e., to induce a quiescent state in hematopoietic stem cells) in the subject.

[00104] The terms "increased" ,"increase", "increasing" or "enhance" are all used herein to generally mean an increase by a statically significant amount; for the avoidance of doubt, the terms "increased", "increase", or "enhance", mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 10%, at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

[00105] The terms, "decrease", "reduce", "reduction", "lower" or "lowering," or "inhibit" are all used herein generally to mean a decrease by a statistically significant amount. For example, "decrease", "reduce", "reduction", or "inhibit" means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (e.g., absent level or non-detectable level as compared to a reference level), or any decrease between 10-100% as compared to a reference level. In the context of a marker or symptom, by these terms is meant a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 20%, at least 30%, at least 40% or more, and is preferably down to a level accepted as within the range of normal for an individual without a given disease.

[00106] The term "statistically significant" or "significantly" refers to statistical significance and generally means a difference of two standard deviations (2SD) or more.

[00107] Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Sambrook et al., Molecular Cloning: A Laboratory

Manual (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1995); Current Protocols in Protein Science (CPPS) (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.), Current Protocols in Cell Biology (CPCB) (Juan S. Bonifacino et. al. ed., John Wiley and Sons, Inc.), and Culture of Animal Cells: A Manual of Basic Technique by R. Ian Freshney, Publisher: Wiley-Liss; 5th edition (2005), Animal Cell Culture Methods (Methods in Cell Biology, Vol. 57, Jennie P. Mather and David Barnes editors, Academic Press, 1st edition, 1998) which are all incorporated by reference herein in their entireties.

[00108] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages means ±1% of the value being referred to. For example, about 100 means from 99 to 101.

[00109] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g.,," is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g.,," is synonymous with the term "for example."

[00110] As used in this specification and appended claims, the singular forms "a," "an", and "the" include plural references unless the context clearly dictates otherwise. Thus for example, reference to "the method" included one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[00111] In this application and the claims, the use of the singular includes the plural unless specifically stated otherwise. In addition, use of "or" means "and/or" unless stated otherwise. Moreover, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both

elements and components comprising one unit and elements and components that comprise more than one unit unless specifically stated otherwise.

[00112] The invention is based in part on the discovery that in vivo or ex vivo, exposure of HSPCs or a HSPCs population to ANG, results in enhanced hematopoietic reconstitution including repopulation of cells of all blood lineage and their functions as well as enhanced self-replication of the HSCs to repopulate and maintain the stem cell pool, after in vivo administration of the treated cells. Accordingly, one aspect, of the invention is directed to a population of the HSPC, that has been exposed or treated with ANG ex vivo, which can be transplanted into a patient in need of improved hematopoietic regeneration. While not wishing to be bound by theory, the exposure to ANG results in restricted proliferation, maintenance of quiescence and self-renewal capacities of the primitive HSPC, while preserving their viability and differentiation state. Accordingly, in some embodiments, one aspect of the invention is directed towards a population of HSC generated after ex vivo exposure to ANG.

[00113] Another aspect of the present invention relates to use of ANG protein or an agonist thereof to treat subjects that have been exposed to or likely to be exposed to ionization radiation. Accordingly, the invention relates to a pharmaceutical composition comprising ANG or a functional fragment thereof, or an agonist thereof for preventing radiation induced hematopoietic injury, e.g., as a result of radio-or chemotherapy as a treatment for a disease or a result of accidental exposure to radiation, wherein the pharmaceutical composition is administered in an therapeutically effective amount.

Hematopoietic stem and progenitor cells (HSPCs)

[00114] Hematopoietic stem cell is a multipotent immature cell that can differentiate into a progenitor cell and therefore can develop into all types of blood cells, including white blood cells, red blood cells, and platelets and can self-renew. Classic studies in mice describe two populations of HSCs; LT-HSCs and ST-HSCs. A long-term stem cell typically includes the long-term contribution to multi-lineage reconstitution after transplantation, which is for more than at least three months. The LT-HSCs can be less actively dividing and/or quiescent relative to other

HSCs. A short-term stem cell is typically anything that confers hematopoietic restoration for shorter than three months and/or is not multi-lineage. The ST-HSCs can be more actively dividing, more proliferating and less quiescent and have limited self-renewal capability relative to the LT-HSCs.

[00115] Hematopoietic progenitor cells are a class of hematopoietic stem cells that have limited self-renewal capacity but remain multipotent and thus can differentiate into all mature cell types found in the blood. These are called multipotent progenitor (MPP) or also can be called LMPP (lymphoid-primed multipotent progenitor) or CMLP cells (common myelolymphoid progenitor cells). LT-HSCs, ST-HSCs and MPP can also be called primitive hematopoietic stem cells. Hematopoietic progenitor cells, as used herein can include, but are not limited to, multipotent progenitors (MPPs), Common lymphoid progenitors (CMPs), Common myeloid progenitors (CMPs), Common Myelolymphoid Progenitors (CMLPs), common myeloid-erythroid progenitor (CMEPs), granulocyte-macrophage progenitor (GMPs), megakaryocyte-erythroid progenitors (MEPs), granulocyte-macrophage colony-forming cell (GM-CFC), megakaryocyte colony-forming cell (Mk-CFC), burst-forming unit erythroid (BFU-E), B cell colony-forming cell (B-CFC) and T cell colony-forming cell (T-CFC). "Precursor cells" include, but are not limited to, colony-forming unit-erythroid (CFU-E), granulocyte colony forming cell (G-CFC), colony-forming cell-basophil (CFC-Bas), colony forming cell-eosinophil (CFC-Eo) and macrophage colony forming cell (M-CFC) cells. Due to lack of long-term self-renewal capacity, hematopoietic progenitor cells cannot sustain long-term reconstitution, and are important for recovery in the period immediately following a hematopoietic stem cell transplant in an individual. Hematopoietic progenitor cells are useful for transplantation and therefore for use in methods and compositions of the present invention can be obtained from a variety of sources including, for example, bone marrow, peripheral blood, and umbilical cord blood.

[00116] HSPCs mostly live in the bone marrow (the spongy center of certain bones), where they divide to make new blood cells. Once blood cells mature, they leave the bone marrow and enter the bloodstream. A small number of stem cells also get into the bloodstream. These are called peripheral blood stem cells. In some embodiments hematopoietic stem cells encompassed for use in the methods and compositions disclosed herein include one or more of the cell types

described above. In some embodiments, the stem cells of the present invention can thus be a heterogeneous population of one or more of these cell types. In some embodiments, the stem cells encompassed for use in the methods and compositions disclosed herein comprises of a population of hematopoietic cells enriched in one or more cells types described above. In some embodiments the stem cells used in the methods and compositions described herein preferably comprise LT-HSCs or myeloid restricted progenitors or a combination thereof.

[00117] The stem cells of the present invention can thus be a heterogeneous population of one or more these cell types or can be a population which is enriched for a one or more of these cell types. The different types of hematopoietic stem and progenitor cells can be distinguished and isolated and enriched from any of their sources for example, bone marrow, peripheral blood, cord blood, prior to transplantation and for use in the present invention by using surface markers specific for the known stem/progenitor cell type which are known in the art. Numerous methods for human hematopoietic stem cell enrichment/isolation are known in the art and generally include obtaining bone marrow, newborn cord blood, fetal liver or adult human peripheral blood which contains hematopoietic stem cells. Once obtained, the hematopoietic stem cell component may be enriched by performing various separation techniques such as density gradient separation, immunoaffinity purification using positive and/or negative selection by panning, FACS, or magnetic bead separation. FACS-based cell sorting allows the recognition, quantification and purification of a small population of HSC and/or lineage committed progenitor cells and/or fully matured hematopoietic cells in a heterogeneous population of cells. Previous studies have also demonstrated that primitive hematopoietic cells, characterized as high proliferative potential colony-forming cells (HPP-CFC, in vivo) may be isolated by selecting a fraction of density gradient-enriched, lineage-depleted marrow cells, further selecting a cell population based on a single step fluorescence-activated cell sorter (FACS) fractionation for cells that bind low levels of the DNA binding dye, Hoechst 33342 (Hoechstlo) and low levels of the mitochondrial binding dye, Rhodamine 123 (Rholo; Wolf et al., 1993). The methods for stem cell isolation and enrichment can comprise selection of the required population based on identity of known markers on their surface for example by using commercially available magnetic beads coupled surface marker specific monoclonal antibodies for e.g.,anti-CD34 beads (Dynal, Lake success, NY) and/or using techniques such as flow cytometry. The heterogeneous population of

cells or enriched stem cells can be expanded in vivo prior to transplantation using the methods and compositions disclosed herein. In other aspects, they can be frozen in liquid nitrogen and stored for long periods of time, such that they can be thawed and used later.

[00118] The phenotypic markers which characterize the HSC are reported in the literature. Murine HSC are defined as KSL cells, which are c-Kit+, Sca-1+, and negative for lineage markers of mature blood cell types. The addition of the Flk-2/Flt3 receptor tyrosine kinase to the KSL markers enhances separation of ST-HSC (Flk-2+) from LT-HSC (Flk-2−). There is no human homolog for murine Sca-1. Instead, human HSC are identified on the basis of CD34 expression. Interestingly, more primitive HSC in mice have low or absent expression of CD34. The DNA-binding dye Hoechst 33342 can be used to identify low staining "side populations" (SP) of HSPC. Hoechst staining is often combined with KSL markers to further enrich HSC numbers, so called SPKLS cells. The purity of HSC in sorted SP, KSL or CD34+ HSPC can be increased by using the signaling lymphocyte activation molecule (SLAM) family proteins CD150, CD244, and CD48. The presence of CD150 distinguishes HSC from HPC; multipotent progenitors are CD150−CD244+CD48− and more committed progenitors are CD150−CD244+CD48+ [13], though there is even variability among CD150+ HSC in their ability to provide balanced repopulation of irradiated bone marrow in mice.

[00119] In humans, for example, CD34 is an adhesion molecule that is expressed on HSC and progenitor cells. It plays a central role in HSC and progenitor cell recognition. CD90 is another important cell surface marker expressed on early stage hematopoietic cells. On the other hand, the absence of CD38 is normally associated with an earlier stage of hematopoiesis. CD10 and CD7 are important markers for early lymphoid lineage development. CD123, an interleukin-3 receptor, and CD135 (which is also called Flt3) have been shown to be important for myeloid lineage development. CD110, a thrombopoietin receptor, is important for platelet development. The CD34+ fraction of human bone marrow contains lineage-committed progenitors as well as long-term multi-lineage HSC, many laboratories have sought additional markers to further enrich the CD34+ population for long-term HSC. CD90/Thy1, Tie, CD117/c-kit, and CD133/AC133 have been found as positive markers to enrich long-term-HSC whereas several negative markers including CD38 have been reported. A recent report has demonstrated human HSC from cord

blood with a marker set of Lin- CD34+ CD38- CD45RA CD90/Thy1+ Rhodamin123Low CD49f+ with long-term multilineage engraftment capablities in NOD/SCID/IL2 receptor common-γ chain null mice (Notta et al., 2011). Non-limiting examples of characteristic marker combinations for humans include; CD34+CD38–CD90+CD45RA–CD49f+ (HSC), CD34+CD38–CD90–CD45RA–CD49f– (MPP). CD34+CD10+CD7+ (CLP), CD34+CD38+CD123medCD135+CD45RA– (CMP), CD34+CD38+CD123medCD135+CD45RA+ (GMP), CD34+CD38+CD123–CD135–CD45RA–CD110+ (MEP). An accurate detection, enumeration and isolation of subpopulations bearing these surface marker compositions can be achieved using flow cytometry. The enumeration of these cells within the blood post-transplantation can be indicator of successful hematopoietic reconstitution. The markers used for different hematopoietic stem and progenitor cell types in the methods and compositions in the present invention are disclosed in the examples.

[00120] Following such enrichment steps, the cell population is typically characterized both phenotypically and functionally. In vivo assays generally measure HPC rather than primitive HSC, while long-term in vivo assays are a measure of LT-HSC. Colony-forming cell (CFC) assays determine the capacity of cells to form lineage-restricted colonies in a semi-solid, usually methylcellulose-based, media, but do not identify HSC, rather only HPC. The colony forming cell (CFC) assay, also referred to as the methylcellulose assay, is an in vivo assay used in the study of hematopoietic stem cells. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to modulator for example Angiogenin. The colonies formed can be enumerated and characterized according to their unique morphology. While proliferation, and expansion can be measured by increase in cell number, loss of quiescence can be assayed by increase in actively dividing cells. A loss of quiescence can result in; (i) increase in cell numbers of the same type of HSC by self-renewal as assayed by proliferation assays or FACS analysis, (ii) active cell division and proliferation as assayed for example by incorporation of BrDU into newly synthesizing DNA and/or (iii) differentiation of HSC into lineage committed cells, which can be assayed by increase in the numbers of lineage committed cells by FACs analysis. The gold standard for differentiating LT-HSC from ST-HSC and progenitors is their ability to engraft in vivo into

irradiated hosts and maintain multilineage hematopoiesis indefinitely and through serial transplantation into new hosts for example the NOD/SCID mouse model.

Sources of HSCs

[00121] Blood products – HSCs can be obtained from blood products. A blood product includes a product obtained from the body or an organ of the body containing cells of hematopoietic origin. Examples of such sources include but are not limited to unfractionated bone marrow, peripheral blood mononuclear cells, umbilical cord blood, umbilical cord tissue, peripheral blood (e.g., G-CSF mobilize peripheral blood), liver, thymus, lymph and spleen. In some embodiments, the aforementioned blood products can be directly used in the methods and compositions of the present invention. In some embodiments, the aforementioned crude or unfractionated blood products can be enriched for cells having hematopoietic stem cell characteristics in a number of ways, for example, the mature differentiated cells can be selected against based on the surface markers that they express, as described above. Exemplary method includes fractionation of the blood product by selecting CD34+ cells. CD34+ cells include a sub-population of cells capable of self-renewal and multi-potentiality. Such selection can be done for example by using commercially available magnetic anti-CD34 beads. Unfractionated blood products can be obtained directly from a donor or retrieved from a cryopreservative storage. In some embodiments, the population of HSCs comprise of CD34+ cells.

[00122] Bone marrow - Bone marrow can be obtained or harvested by anesthetizing the stem cell donor, puncturing bone with a needle and harvesting bone marrow cells with a syringe. Most sites used for bone marrow harvesting are located in the hip bones and the sternum. The bone marrow aspirate can contain, LT-HSC, stromal cells, stromal stem cells, hematopoietic progenitor cells, mature and maturing white and red blood cells and their progenitors. Once obtained the bone marrow aspirate can be treated as a whole using the methods described herein, or HSPCs can be isolated prior to use in the methods by using surface specific markers for the HSCs and progenitor cells known to those skilled in the art, also described in previous sections. Alternatively the harvested bone marrow or cells isolated from bone marrow can be cryopreserved for later use in the current invention.

[00123] Peripheral blood - HSPCs can be contained in or obtained from peripheral, circulating blood. Prior to harvesting, stem cells can be mobilized from marrow into the blood stream by injecting the donor with compounds including cytokines. Such mobilization can be accomplished by using for example, one or more of granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), thrombopoietin (Tpo), and a chemotherapeutic agent (i.e.,cyclophosphamide). Typically, the donor is injected a few days prior to the harvest. To collect the cells, an intravenous tube is inserted into the donor's vein and donor's blood is passed through a filtering system that pulls out CD34+ white blood cells and returns the red blood cells to the donor. The methods of collection are well known to those skilled in the art. Once collected, the cells can be used as a whole or can be further fractionated into specific cell types and/or cryopreserved for later use in the methods and compositions described herein.

[00124] Umbilical cord and/or placental blood - HSPCs can be obtained from umbilical cord and/or placental blood, i.e. the blood that remains in the placenta and in the attached umbilical cord after childbirth Nakahata & Ogawa, 70 J. Clin. Invest. 1324-28 (1982); Prindull et al., 67 Acta. Paediatr. Scand. 413-16 (1978); Tchernia et al., 97(3) J. Lab. Clin. Med. 322-31 (1981)). Several methods of cord blood collections are known in the art. The blood remaining in the delivered placenta is safely and easily collected and stored. The predominant collection procedure currently practiced involves a relatively simple venipuncture, followed by gravity drainage into a standard sterile anti-coagulant-filled blood bag, using a closed system, similar to the one utilized on whole blood collection. After aliquots have been removed for routine testing, the units can be cryopreserved and stored in liquid nitrogen See, e.g.,U.S. Pat. No. 7,160,714; No. 5,114,672; No. 5,004,681; U.S. patent application Ser. No. 10/076,180, Pub. No. 20030032179. Stem and progenitor cells in cord blood appear to have a greater proliferative capacity in culture than those in adult bone marrow. Salahuddin et al., 58 Blood 931-38 (1981); Cappellini et al., 57 Brit. J. Haematol. 61-70 (1984). Umbilical cord blood stem cells have been used to reconstitute hematopoiesis in children with malignant and nonmalignant diseases after treatment with myeloablative doses of chemo-radiotherapy. Sirchia & Rebulla, 84 Haematologica 738-47 (1999). See also Laughlin 27 Bone Marrow Transplant. 1-6 (2001); U.S. Pat. No. 6,852,534. The placenta and umbilical cord tissues are also a source of hematopoietic

stem and progenitor cells (Robin, C. et al. Cell Stem Cell. 2009 Oct 2; 5(4): 385–395.). CN104711226A; U.S. Pat. No. 7,045,148; U.S. Pat No. 8673547B2.

[00125] Alternatively, fetal blood can be taken from the fetal circulation at the placental root with the use of a needle guided by ultrasound (Daffos et al., 153 Am. J. Obstet. Gynecol. 655 60 (1985); Daffos et al., 146 Am. J. Obstet. Gynecol. 985-87 (1983), by placentocentesis (Valenti, 115Am. J. Obstet. Gynecol. 851-53 (1973); Cao et al., 19 J. Med. Genet. 81 87 (1982)), by fetoscopy (Rodeck, in Prenatal Diagnosis, (Rodeck & Nicolaides, eds., Royal College of Obstetricians & Gynaecologists, London, 1984)). Indeed, the chorionic villus and amniotic fluid, in addition to cord blood and placenta, are sources of pluripotent fetal stem cells (see WO 2003 042405) that may be treated by the ANG of the present invention.

[00126] Various kits and collection devices are known for the collection, processing, and storage of cord blood. See, e.g.,U.S. Patents No. 7,147,626; No. 7,131,958. Collections should be made under sterile conditions, and the blood may be treated with an anticoagulant. Such an anticoagulants include citrate-phosphate-dextrose, acid citrate-dextrose, Alsever's solution (Alsever & Ainslie, 41 N. Y. St. J. Med. 126-35 (1941), DeGowin's Solution (DeGowin et al., 114 J.A.M.A. 850-55 (1940)), Edglugate-Mg (Smith et al., 38 J. Thorac. Cardiovasc. Surg. 573-85 (1959)), Rous-Turner Solution (Rous & Turner 23 J. Exp. Med. 219-37 (1916)), other glucose mixtures, heparin, or ethyl biscoumacetate. See Hurn Storage of Blood 26-160 (Acad. Press, NY, 1968).

[00127] Various procedures are known in the art and can be used to enrich collected cord blood for HSCs. These include but are not limited to equilibrium density centrifugation, velocity sedimentation at unit gravity, immune rosetting and immune adherence, counterflow centrifugal elutriation, T lymphocyte depletion, and fluorescence-activated cell sorting, alone or in combination. See, e.g.,U.S. Patent No. 5,004,681. Typically, collected blood is prepared for cryogenic storage by addition of cryoprotective agents such as DMSO (Lovelock & Bishop, 183 Nature 1394-95 (1959); Ashwood-Smith 190 Nature 1204-05 (1961)), glycerol, polyvinylpyrrolidine (Rinfret 85 Ann. N.Y. Acad. Sci. 576 94 (1960)), polyethylene glycol (Sloviter & Ravdin 196 Nature 899-900 (1962)), albumin, dextran, sucrose, ethylene glycol, i-

erythritol, D-ribitol, D-mannitol (Rowe, 3(1) Cryobiology 12-18 (1966)), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender et al., 15 J. Appl. Physiol. 520 24 (1960)), amino acids (Phan & Bender, 20 Exp. Cell Res. 651-54 (1960)), methanol, acetamide, glycerol monoacetate (Lovelock, 56 Biochem. J. 265-70 (1954)), and inorganic salts (Phan & Bender, 104 Proc. Soc. Exp. Biol. Med. (1960)). Addition of plasma (e.g.,to a concentration of 20-25%) may augment the protective effect of DMSO.

[00128] Collected blood should be cooled at a controlled rate for cryogenic storage. Different cryoprotective agents and different cell types have different optimal cooling rates. See e.g.,Rapatz, 5(1) Cryobiology 18-25 (1968), Rowe & Rinfret, 20 Blood 636-37 (1962); Rowe, 3(1) Cryobiology 12-18 (1966); Lewis et al., 7(1) Transfusion 17-32 (1967); Mazur 168 Science 939 49 (1970). Considerations and procedures for the manipulation, cryopreservation, and long-term storage of HSC sources are known in the art. See e.g.,U.S. Patents No. 4,199,022; No. 3,753,357; No. 4,559,298; No. 5,004,681. There are also various devices with associated protocols for the storage of blood. U.S. Patents No. 6,226,997; No. 7,179,643. Accordingly, in some embodiments the HSPC populations used in the methods and composition disclosed herein are obtained or enriched from or are contained in biological source such as bone marrow, peripheral blood, cord blood, amniotic fluid, or placental blood or tissues such as the placenta.

[00129] Considerations in the thawing and reconstitution of HSC sources are also known in the art. U.S. Patents No. 7,179,643; No. 5,004,681. The HSC source blood may also be treated to prevent clumping (see Spitzer, 45 Cancer 3075-85 (1980); Stiff et al., 20 Cryobiology 17-24 (1983), and to remove toxic cryoprotective agents (US Patent No. 5,004,681). Further, there are various approaches to determining an engrafting cell dose of HSC transplant units. See U.S. Patent No. 6,852,534; Kuchler Biochem. Methods in Cell Culture & Virology 18-19 (Dowden, Hutchinson & Ross, Strodsburg, PA, 1964); 10 Methods in Medical Research 39-47 (Eisen, et al., eds., Year Book Med. Pub., Inc., Chicago, IL, 1964). Thus, not being limited to any particular collection, treatment, or storage protocols, an embodiment of the present invention provides for the addition of ANG to the source of HSPCs. This may be done at collection time, or at the time of preparation for storage, or upon thawing and before infusion. For example, stem cells isolated from a subject, e.g.,with or without prior treatment of the subject with ANG, may

be incubated in the presence of ANG to maintain HSC quiescence, prevent differentiation, progenitor proliferation and/or expand the number of HSCs. Treated and/or expanded HSCs may be subsequently reintroduced into the subject from which they were obtained (autologous transplantation) or may be introduced into another subject (allogeneic transplantation).

[00130] A subject from whom a source of HSC can be derived can include anyone who is a candidate for autologous stem cell or bone marrow transplantation during the course of treatment for malignant disease or as a component of gene therapy. Other possible candidates are subjects who donate stem cells or bone marrow to patients for allogeneic transplantation for malignant disease or gene therapy. Subjects may have undergone irradiation therapy, for example, as a treatment for malignancy of cell type other than hematopoietic. Subjects may be suffering from anemia, e.g.,sickle cell anemia, thalessemia, aplastic anemia, or other deficiency of HSC derivatives.

Angiogenin (ANG)

[00131] Angiogenin, a 14.1-kD protein, is a potent inducer of neovascularization in vivo. ANG, also known as ribonuclease 5 (RNase5), is a member of the secreted vertebrate specific ribonuclease superfamily, with a 33% sequence homology to the pancreatic ribonuclease A. Angiogenin has angiogenic (Fett et al., 1985), neurogenic (Subramanian and Feng, 2007), neuroprotective (Subramanian et al., 2008), and immune-regulatory functions (Hooper et al., 2003). RNase activity of ANG is important for its angiogenic activity. Endogenous ANG is required for cell proliferation induced by other angiogenic proteins such as vascular endothelial growth factor (VEGF; 192240). Like VEGF, ANG is induced by hypoxia to elicit angiogenesis and is expressed in motor neurons (Lambrechts et al., 2003). The role of Angiogenin as a regulator of hematopoiesis is not known.

[00132] "Angiogenin protein" refers to a full length ANG polypeptide or to a fragment or derivative thereof that retains the ability, at a minimum, to maintain quiescence of primitive HSC preferably that of LT-HSC and promote proliferation of progenitor cells preferably that of myeloid progenitor cells. The ANG of the compositions and methods described herein can be

full length human ANG and/or functional fragments thereof, a species homologue and/or functional fragments thereof, an ortholog of human ANG and/or functional fragments thereof. The ANG polypeptide can be a mammalian ANG protein. The ANG polypeptide can also be a functional isoform of the full length ANG or functional fragment thereof.

[00133] In some embodiments, the ANG polypeptide includes or is derived from human ANG having the following amino acid sequence (SEQ ID NO:1)., or a functional fragment thereof. A "functional fragment" refers to fragment of the full length ANG of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140 consecutive amino acids of SEQ ID NO:1, that has at least about 70%, 80%, 90%, 100% or more than 100% of the function of wild type ANG of SEQ ID NO:1 at reconstituting HSC in vivo or in vitro. The functional activity can be tested by one of ordinary skill in the art by the assays described in the examples.
1 MVMGLGVLLL VFVLGLGLTP PTLAQDNSRY THFLTQHYDA KPQGRDDRYC ESIMRRRGLT
61 SPCKDINTFI HGNKRSIKAI CENKNGNPHR ENLRISKSSF QVTTCKLHGG SPWPPCQYRA
121 TAGFRNVVVA CENGLPVHLD QSIFRRP
(See GenBank Accession No. AAA51678.1, which is incorporated herein by reference in its entirety).

[00134] The polypeptide and coding nucleic acid sequences of ANG and of other members of the family of human origin and those of a number of animals are publically available, e.g., from the NCBI website. Examples include, but are not limited to, Mouse (GenBank Accession No. AAA91366.1), Rat (GenBank Accession No. AAR28758.1), Bovine (GenBank Accession No. AAG47631.1).

[00135] In some embodiments, the ANG polypeptide is a mammalian homolog of human FGF1 or a functional fragment thereof. In some embodiments, the ANG polypeptide has an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97% or at least 99% identical to the amino acid sequence of SEQ ID NO:1 and maintains quiescence of HSPCs and

promotes proliferation of progenitors preferably myeloid restricted progenitors. In some embodiments, the ANG polypeptide has an amino acid sequence that has at least 85%, at least 90%, at least 95%, at least 97% or at least 99% amino acid sequence homology to amino acid sequence of SEQ ID NO: 1 and maintains quiescence of HSPCs and promotes proliferation of progenitors preferably myeloid restricted progenitors. In some embodiments, the ANG is a functional fragment of SEQ ID NO:1 of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140 consecutive amino acids of SEQ ID NO:1, that has at least about 70%, 80%, 90%, 100% or more than 100% of the function of wild type ANG of SEQ ID NO:1 at reconstituting HSC in vivo or in vitro. The functional activity can be tested by one of ordinary skill in the art by the assays described in the examples. Percent (%) amino acid sequence identity for a given polypeptide sequence relative to a reference sequence is defined as the percentage of identical amino acid residues identified after aligning the two sequences and introducing gaps if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent (%) amino acid sequence homology for a given polypeptide sequence relative to a reference sequence is defined as the percentage of identical or strongly similar amino acid residues identified after aligning the two sequences and introducing gaps if necessary, to achieve the maximum percent homology. Non identities of amino acid sequences include conservative substitutions, deletions or additions that do not affect the blood sugar reducing or normalizing activity of FGF1. Strongly similar amino acids can include, for example, conservative substitutions known in the art. Percent identity and/or homology can be calculated using alignment methods known in the art, for instance alignment of the sequences can be conducted using publicly available software software such as BLAST, Align, ClustalW2. Those skilled in the art can determine the appropriate parameters for alignment, but the default parameters for BLAST are specifically contemplated.

[00136] The ANG polypeptide can be recombinant, purified, isolated, naturally occurring or synthetically produced. The term "recombinant" when used in reference to a nucleic acid, protein, cell or a vector indicates that the nucleic acid, protein, vector or cell containing them have been modified by introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or a protein, or that the cell is derived from a cell so modified. The term

"heterologous" (meaning 'derived from a different organism') refers to the fact that often the transferred protein was initially derived from a different cell type or a different species from the recipient. Typically the protein itself is not transferred, but instead the genetic material coding for the protein (often the complementary DNA or cDNA) is added to the recipient cell. Methods of generating and isolating recombinant polypeptides are known to those skilled in the art and can be performed using routine techniques in the field of recombinant genetics and protein expression. For standard recombinant methods, see Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY (1989); Deutscher, Methods in Enzymology 182:83-9(1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, NY (1982).

Biological activity of Angiogenin

[00137] The minimum, central biological activity and/or biological effect of the ANG protein as described herein is maintaining primitive HSCs quiescence and/or enabling progenitor proliferation, upon contact with the HSPC population or source containing the HSPC population. In some embodiments, the ANG protein restricts proliferation of the primitive hematopoietic stem cells and/or lymphoid-biased progenitors e.g.,MPP4s. In some embodiments, the ANG protein increases proliferation of myeloid restricted progenitors for example CMP, GMP, MEP, myeloid biased progenitors e.g., MPP3. In some embodiments, the ANG protein maintains LT-HSCs in a quiescent state. In some embodiments, ANG protein restricts proliferation and/or differentiation of the LT-HSCs. In some embodiments the ANG protein enables in vivo expansion of a population of hematopoietic stem and/or progenitor cells. In some embodiments the ANG protein enhances the regenerative capacity of the transplanted HSPC population. In some embodiments the ANG protein results in enhanced hematopoietic reconstitution upon in vivo administration of the exposed HSC and/or progenitor cell population. In some embodiments, ANG is a regulator of HSPC stemness. In some embodiments, ANG maintains the self-renewal capacity of the HSPCs. In some embodiments, ANG results in multi-lineage hematopoietic reconstitution of the treated HSPC population. In some embodiments, the ANG results in short-term reconstitution of the treated HSPCs upon their administration in vivo. In some embodiments, the ANG results in long-term reconstitution of the treated HPSCs upon their

administration in vivo. Methods for determining hematopoietic reconstitution are known in the art and disclosed above. In some embodiments, the ANG protein is a regulator of HSPCs. In some embodiments, the ANG is a regulator of hematopoiesis. In some embodiments, the ANG polypeptide retains at least 85%, at least 90%, at least 95%, at least 97% or at least 99% of the biological activity of human ANG of SEQ ID NO:1.

[00138] In some embodiments, the ANG polypeptide or fragment thereof used in the methods and compositions disclosed herein retains the ribonucleolytic activity. The ribonucleolytic activity of the ANG used for example can be at least 80%, 85%, 90%, 95%, 99%, 100% of that of the native full length polypeptide of SEQ ID NO:1. In some embodiments, the ANG polypeptide or fragment thereof used in the present invention retains receptor binding activity. The receptor binding activity of the ANG used for example can be at least 80%, 85%, 90%, 95%, 99%, 100% of that of the native full length polypeptide of SEQ ID NO:1.

[00139] Without wishing to be bound by theory, ANG has been reported in other cell types to regulate global protein synthesis. A higher rate of protein synthesis was observed Ang-/- LKS cells, while Ang-/- myeloid-restricted progenitors demonstrated reduced protein synthesis (Figure 16A). Accordingly, in some embodiments, ex vivo contact with ANG results in decrease in protein synthesis in HSPC and increase in protein synthesis of myeloid restricted progenitor cells. While not wishing to be bound by theory, ANG has been shown to reprogram protein synthesis as a stress response to promote survival under adverse conditions. This function of ANG is mediated by tiRNA, a noncoding small RNA that specifically permits translation of anti-apoptosis genes while global protein translation is suppressed so that stressed cells have adequate time and energy to repair damage, collectively promoting cell survival (Emara et al., 2010; Fu et al., 2009; Ivanov et al., 2011; Yamasaki et al., 2009). Addition of ANG led to markedly elevated tiRNA levels in LKS cells (Figure 18A). Accordingly, in some embodiments, the methods disclosed herein comprise increasing the tiRNA levels in the HSCs for example LT-HSC and/or decreasing tiRNA levels in myeloid restricted progenitor cells. In some embodiments, the increasing of tiRNA levels in LT-HSCs and decreasing of tiRNA levels in myeloid restricted progenitors comprises of contact of the said HSPCs population with an effective amount of ANG.

Exposing HSPCs and/or source containing HSPCs to ANG ex vivo

[00140] The invention is based in part on the discovery that in vivo or ex vivo, exposure of HSPCs or a HSPCs population to ANG, results in enhanced hematopoietic reconstitution including repopulation of cells of all blood lineage and their functions as well as enhanced self-replication of the HSCs to repopulate and maintain the stem cell pool, after in vivo administration of the treated cells. Accordingly, one aspect, of the invention is directed to a population of the HSPC, that has been exposed or treated with ANG ex vivo, which can be transplanted into a patient in need of improved hematopoietic regeneration. While not wishing to be bound by theory, the exposure to ANG results in restricted proliferation, maintenance of quiescence and self-renewal capacities of the primitive HSPC, while preserving their viability and differentiation state. Accordingly, in some embodiments, one aspect of the invention is directed towards a population of HSC generated after ex vivo exposure to ANG. In some embodiments, the invention relates to method of generating the said population of quiescent HSC. Furthermore, ex vivo exposure to ANG results in promotion of proliferation and expansion of progenitor cells, preferably that of myeloid restricted progenitor. Accordingly, in some embodiments, one aspect of the invention is directed towards a population of progenitor cells with enhanced proliferative capacity after exposure to ANG ex vivo. In some embodiments, the invention relates to methods of generating said population of proliferative progenitor cells. A further embodiment of the present invention provides a method for expanding a population of cells comprising primitive hematopoietic stem cells and progenitors, preferably myeloid restricted progenitors ex vivo upon contacting with an effective amount of ANG for a sufficient time such that the contacting results in quiescence of primitive HSPC and proliferation of myeloid restricted progenitors.

[00141] The HSC populations obtained after ex vivo exposure with ANG can be administered to the subject in need of HSC transplantation and/or improved hematopoietic reconstitution. In some embodiments, the invention relates to method of administering to a subject a population of HSPCs that has been treated/exposed ex vivo to ANG. In some embodiments, the HSC population obtained upon treatment with ANG can be cryopreserved,

such that they can later be thawed and used, e. g., for administration to a patient. In general, the cells are stored in a typical freezing medium, e.g., 10% DMSO, 50% fetal calf serum (FCS), and 40% cell culture medium. The exposed cell population or a source containing exposed cell population e.g., blood product can be deposited into a blood bank. Accordingly, in some embodiments, the invention relates to the blood bank comprising the populations of HSPCs obtained upon ex vivo exposure to ANG. Another embodiment provides for a kit comprising a container suitable for HSC source sample storage in which the container is preloaded with an effective amount of ANG. An additional embodiment provides a kit comprising a container suitable for HSC source sample storage and a vial containing a suitable amount of ANG.

[00142] Methods of culturing HSPCs in vivo are well known in the art. The cells can be cultured for example in Phosphate buffered saline, or a commercially available media such as StemSpan SFEM (Stem Cell Technologies). The media can be further supplemented with other known modulators of HSPCs. Non-limiting examples of other modulators include one or more of interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-11 (IL-11), interleukin-12 (IL-12), stem cell factor (SCF), fms-like tyrosine kinase-3 (fit-3), transforming growth factor-β (TGF-B), an early acting hematopoietic factor, described, for example in WO 91/05795, and thrombopoietin (Tpo). The effective dosage of ANG used in in vivo culture can be described as a dosage necessary to maintain HSPCs in an undifferentiated state and/or quiescent state, and/or a dosage necessary to enhance proliferation and expansion of myeloid restricted progenitor cells and/or a dosage necessary to enhance the post-transplant reconstitution of the treated cell upon in vivo administration. The effective amounts may vary, as recognized by those skilled in the art, depending on the number of HSCs to be treated, the duration of treatment, source of HSCs, the specific underlying disease to be treated by transplantation, intensity of prior therapy such as chemotherapy or radiotherapy.

[00143] The effective duration of ex vivo contact with ANG can be determined by those of skill in the art. For example, the population of HSC can be maintained in contact with ANG for a period of about 2 hours, about 4 hours, about 6 hours, about 24 hours, about 2 days or longer, at least 7 days. In one embodiment, cells can be treated for at least 2 hours prior to changing to medium without ANG.

[00144] In some embodiments, the cells can be maintained in culture in absence of ANG before addition of ANG, and then transplanted in vivo. In some embodiments, the cells can be cultured in presence of ANG and then can be maintained in absence of ANG prior to transplantation in vivo. In some embodiments, the cells may be administered in vivo along with ANG. In some embodiments, in addition to ANG, the cells can be cultured in combination with one or more regulators disclosed in the present invention for example, Embigin. IL8. The effective concentration and duration of treatment can vary for each of the regulators and can be easily determined by one skilled in the art. The cells can be treated simultaneously with these factors or on different times.

[00145] The contacting with ANG in methods disclosed herein can be done at initial collection of the source and/or the cells, during processing, at storage, upon thawing, prior to in vivo administration, or during in vivo administration. Methods to determine cellular proliferation and/or loss of quiescence and/pr expansion are known in the art. Briefly the cell number of a desired cell population can be enumerated using a hemocytometer before and after the treatment with ANG. Cellular expansion and proliferation is indicated by an increase in cell number.

Stem cell transplants

[00146] Stem cell transplants are used to restore the stem cell reservoir when the bone marrow has been destroyed by disease, chemotherapy (chemo), or radiation. Depending on the source of the stem cells, this procedure may be called a bone marrow transplant, a peripheral blood stem cell transplant, or a cord blood transplant. They can all be called hematopoietic stem cell transplants (HSCT). Hematopoietic stem cells (HSC) and progenitors are commonly used to replace the hematopoietic system in patients with hematopoietic malignancies, or patients undergoing high dose chemotherapy. Hematopoietic reconstitution after transplantation encompasses the recovery of optimal numbers of hematopoietic stem cells and hematopoietic cells of both the myeloid and lymphoid lineages and their functions, thereby restoring a functional bone marrow. In one aspect, the methods and compositions described herein result in enhanced hematopoietic reconstitution in vivo. In some embodiments the reconstitution potential

obtained using the methods and compositions described herein is multi-lineage. Multi-lineage reconstitution or repopulation or differentiation can be defined as an ability to differentiate in multiple mature blood cell types. Exemplary method for assessment of HSCs multi-potentiality and/or multi-lineage reconstitution, includes detection of human CD45+ cells, represented by at least myeloid and lymphoid lineages in blood or/and in bone marrow. Commonly used set of lineage markers in combination with human pan-leukocyte CD45can include myeloid lineage: CD33 or CD13, B-cell lymphoid: CD19, T-cell lymphoid: CD4+CD8 or CD3, erythroid: GlyA (CD235a). In some embodiments the post-transplantation hematopoietic reconstitution can be short-term recovery or sustained long-term reconstitution. In human patients, sustained and/or long-term reconstitution can be assessed by persistence of human-derived lymphoid and myeloid cells in the blood or/and HSCs and their mature progeny in bone marrow at least 12-20 weeks after primary transplant. In some embodiments, the long-term hematopoietic reconstitution can be for example at least 12 weeks (or 3 months), at least 13 weeks, at least 14 weeks, at least 15 weeks, at least 16 weeks (or 4 months), at least 17 weeks, at least 18 weeks, at least 18 weeks, at least 20 weeks (or 5 months), at least 6 months, at least 1 year or more. In some embodiments, the methods and compositions disclosed herein can result in result in sustained hematopoietic reconstitution after a single transplant. In some embodiments, the hematopoietic reconstitution is short-term i.e. for a period not exceeding three months. The short-term reconstitution can be for example less than 3 months (12 weeks), less than 11 weeks, less than 10 weeks, less than 9 weeks, less than 8 weeks (or 2 months), less than 7 weeks, less than 6 weeks, less than 5 weeks, 4 weeks or less. In some embodiments, the methods and compositions disclosed herein can enhance the self-renewal capacity of the HSC population after transplantation in vivo. Self-renewal can be defined as an ability of human-derived cells to multilineage repopulation and/or engraftment in bone marrow in serial transplantation (at least after secondary).

Methods to assess hematopoietic reconstitution

[00147] Methods to determine successful transplant and therefore hematopoietic reconstitution are known in the art. The long term repopulating ability of candidate hematopoietic stem cells can be evaluated, e.g., in an in vivo sheep model or an in vivo NOD-SCID mouse model for human HSC. The NOD/SCID mouse is an immunodeficient recipient,

which allows the introduction of human, NHP or mouse cells and the determination of stem cell functionality through engraftment, proliferation and differentiation into at least two distinct lineages (typically myeloid and lymphoid). This in vivo reconstitution assay is typically known as the Competitive Repopulating Unit (CRU) or SCID Repopulating Cell (SRC) assay. In humans, for example, successful hematopoietic reconstitution can be determined, by measurement of absolute counts for individual blood cell types (white blood cells, red blood cells and platelets) in the peripheral blood, reaching a number of cells accepted by those of skill in the art as within the normal range for the subject. Methods of conducting a complete blood count, differential leukocyte count i.e. including counts of each type of white blood cell, for e.g.,neutrophils, eosinophils, basophils, monocytes, and lymphocytes, and platelet counts are known to those skilled in the art. Briefly, post-transplantation, the blood can be collected at regular intervals in a tube containing an anti-coagulant like the EDTA, the cells can be counted using an automated blood count analyzer or manually using a hemocytometer. Neutrophils are a type of white blood cell that are a marker of engraftment; the absolute neutrophil count (ANC) must be at least 500 for three days in a row to say that engraftment has occurred. This can occur as soon as 10 days after transplant, although 15 to 20 days is common for patients who are given bone marrow or peripheral blood cells. Umbilical cord blood recipients usually require between 21 and 35 days for neutrophil engraftment. Platelet counts are also used to determine when engraftment has occurred. The platelet count must be between 20,000 and 50,000 (without a recent platelet transfusion). This usually occurs at the same time or soon after neutrophil engraftment, but can take as long as eight weeks and even longer in some instances for people who are given umbilical cord blood.

[00148] Alternatively analysis of chimerism status can be monitored for example following allogeneic transplantation. Analysis of chimerism involves discrimination between donor- and recipient-derived hematopoiesis based on molecular methods for example using cytogenetics, isoenzyme analysis, blood group phenotyping, sex chromosome differentiation using fluorescence in situ hybridization, or using PCR-based methods relying on the amplification of highly polymorphic repetitive DNA sequences such as short tandem repeats (STR), variable number of tandem repeat (VNTR) sequences. The methods for whole blood chimerism analysis are known to those skilled in the art. Exemplary method involves, obtaining

blood samples at routine points post-transplant, or when there is a suspicion of disease relapse. DNA is extracted from EDTA blood sample for example using a magnetic purification method (Qiagen EZ1). Forensic kits, comprising, PCR reactions using three STR markers are commercially available (Promega PwerPlex16 Monoplex System). The differentially sized PCR products can be detected and analyzed on a capillary system genetic analyser (Applied Biosystems 3130xl). Lineage specific chimerism analysis can be done by separating the leukocyte lineages by cell separation using AutoMACS immune magnetic separation technology. Positive chimerism analysis performed on patients who underwent transplant to ameliorate a malignant disease can indicate signal of appearance of malignant cells or give a measure of efficiency of transplantation.

[00149] In some embodiments the enhanced hematopoietic reconstitution results cause treatment, reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of the disease resulting in improper functioning of the bone marrow and the immune system or their symptoms. The efficacy of a given therapeutic regimen involving methods and compositions described herein, may be monitored, for example by convention FACS assays for phenotypes of cells in the blood circulation of the subject under treatment. Such analysis is useful to monitor changes in the numbers of cells of various lineages, particularly

Patient selection and treatment

[00150] While the methods and compositions described herein can be used to enhance the post-transplant hematopoietic reconstitution, in some embodiments, they can be described to be of use in one or more of the following situations; (1) Replace diseased, nonfunctioning bone marrow with healthy functioning bone marrow (for example in conditions such as leukemia, aplastic anemia, and sickle cell anemia), (2) Regenerate a new immune system that will fight existing or residual disorder for example leukemia or other cancers not killed by the chemotherapy or radiation, (3) Replace the bone marrow and restore its normal function after high doses of chemotherapy and/or radiation are given to treat a malignancy (for diseases such as lymphoma and neuroblastoma). This process can be called rescue or hematopoietic reconstitution. (4) Replace bone marrow with genetically healthy functioning bone marrow to

prevent further damage from a genetic disease process (for example Hurler's syndrome and adrenoleukodystrophy).

[00151] A subject having decreased HSC levels or is susceptible to decrease in HSC levels and/or blood cell deficiency can benefit from the methods and compositions of the present invention. Decreased HSC levels and/or blood cell deficiency can be caused due to a number of conditions, for example due to hematological diseases also called as blood disorders and hematological malignancies. In one embodiment, the invention relates to methods and compositions useful in the treatment and prevention of blood disorders and/or to ameliorate symptoms and disorders related to decrease in HSC levels and/or blood cell deficiency, for example hematological disorders.

[00152] Hemoglobinopathies and thalassemia can both be characterized as "blood disorders". Blood disorders include disorders that can be treated, prevented, or otherwise ameliorated by the administration of compositions of the invention. A blood disorder is any disorder of the blood and blood-forming organs. The term blood disorder includes nutritional anemias (e.g.,iron deficiency anemia, sideropenic dysphasia, Plummer-Vinson syndrome, vitamin B12 deficiency anemia, vitamin B12 deficiency anemia due to intrinsic factor, pernicious anemia, folate deficiency anemia, and other nutritional anemias), myelodysplastic syndrome, bone marrow failure or anemia resulting from chemotherapy, radiation or other agents or therapies, hemolytic anemias (e.g., anemia due to enzyme disorders, anemia due to phosphate dehydrogenase (G6PD) deficiency, favism, anemia due to disorders of glutathione metabolism, anemia due to disorders of glycolytic enzymes, anemias due to disorders of nucleotide metabolism and anemias due to unspecified enzyme disorder), thalassemia, α-thalassemia, β-thalassemia, δβ-thalassemia, thalassemia trait, hereditary persistence of fetal hemoglobin (HPFP), and other thalassemias, sickle cell disorders (sickle cell anemia with crisis, sickle cell anemia without crisis, double heterozygous sickling disorders, sickle cell trait and other sickle cell disorders), hereditary hemolytic anemias (hereditary spherocytosis, hereditary elliptocytosis, other hemoglobinopathies and other specified hereditary hemolytic anemias, such as stomatocyclosis), acquired hemolytic anemia (e.g., drug-induced autoimmune hemolytic anemia, other autoimmune hemolytic anemias, such as warm autoimmune hemolytic anemia, drug-

induced non-autoimmune hemolytic anemia, hemolytic-uremic syndrome, and other non-autoimmune hemolytic anemias, such as microangiopathic hemolytic anemia); aplastic anemias (e.g., acquired pure red cell aplasia (erythoblastopenia), other aplastic anemias, such as constitutional aplastic anemia and fanconi anemia, acute posthemorrhagic anemic, and anemias in chronic diseases), coagulation defects (e.g., disseminated intravascular coagulation (difibrination syndrome)), hereditary factor VIII deficiency (hemophilia A), hereditary factor IX deficiency (Christmas disease), and other coagulation defects such as Von Willebrand's disease, hereditary factor Xi deficiency (hemophilia C), purpura (e.g., qualitative platelet defects and Glanzmann's disease), neutropenia, agranulocytosis, functional disorders of polymorphonuclear neutrophils, other disorders of white blood cells (e.g., eosinophilia, leukocytosis, lymophocytosis, lymphopenia, monocytosis, and plasmacyclosis), diseases of the spleen, methemoglobinemia, other diseases of blood and blood forming organs (e.g., familial erythrocytosis, secondary polycythemia, essential thrombocytosis and basophilia), thrombocytopenia, infectious anemia, hypoproliferative or hypoplastic anemias, hemoglobin C, D and E disease, hemoglobin lepore disease, and HbH and HbS diseases, anemias due to blood loss, radiation therapy or chemotherapy, or thrombocytopenias and neutropenias due to radiation therapy or chemotherapy, sideroblastic anemias, myelophthisic anemias, antibody-mediated anemias, and certain diseases involving lymphoreticular tissue and reticulohistiocytic system (e.g., Langerhans' cell hystiocytosis, eosinophilic granuloma, Hand-Schuller-Christian disease, hemophagocytic lymphohistiocytosis, and infection-associated hemophagocytic syndrome).

[00153] In some embodiments, the blood deficiencies are acquired or genetic deficiencies. Genetic blood disorders are well known by persons of ordinary skill in the art, and include, without limitation, Thalassemias, Sickle cell disease, hereditary spherocytosis, G6PD Deficiency hemolytic anemia, Kostman’s syndrome, Swachman-Diamond Syndrome, Cyclic neutropenia, Hereditary neutropenia, Dyskeratosis Congenita, Hereditary thrombocytopenia syndromes, Wiskott-Aldrich Syndrome, May-Hegglin anomaly, Thrombocytopenia with Absent Radii Syndrome, Fanconi’s anemia and other hereditary blood disorders.

[00154] In some embodiments, the compositions and methods as disclosed herein can be used for the treatment of neutropenia. Neutrophenia is a disorder of low white blood cell count in

a subject, and is characterized by one or more of the following: an absolute neutrophil count (ANC) of less than 1500/microL. People suffering or diagnosed with neutrophia may result in hospitalization for treatment of fever, neutropenic sepsis, and can cause potentially fatal infection. Neutropenia is very common in subjects undergone or currently undergoing chemotherapy, transplants, radiation therapy and the like.

[00155] Accordingly, the methods and composition disclosed herein can be used for the treatment of low platelet count, for example but not limited to, a low platelet count occurring in thrombocytopenia and/or platelet dysfunction. There is currently no or inadequate drug therapy, and the only current treatment is a platelet transfusion. In some embodiments, the methods and compositions disclosed herein can be used for the treatment of low platelet count which is occurs as a consequence of other disorders, for example but not limited to, AIDS (acquired immunodeficiency syndrome); ITP (immune thrombocytopenic purpura); DIC (disseminated intravascular coagulation); TTP (thrombotic thrombocytopenic purpura) and the like.

[00156] In some embodiments, the methods and compositions as disclosed herein can be used for the treatment of cytopenias. Significant cytopenias are associated with radiation therapies and also occur after or during chemotherapy and chemo-radiation.

[00157] In some embodiments, the methods and compositions disclosed herein can be used to treat a subject suffering from malignancy for example hematological malignancy. Examples of malignanccis that can benefit from the technology detailed herein include but are not limited to lymphoma (Hodgkin's disease, Burkitt's lymphoma, Anaplastic large cell lymphoma, Splenic marginal zone lymphoma, Hepatosplenic T-cell lymphoma, Angioimmunoblastic T-cell lymphoma), myeloma (Plasmacytoma, Waldenstrom macroglobulinemia, Multiple myeloma), Leukemia (Aggressive NK-leukemia, T-cell large granular lymphocyte leukemia, Acute lymphocytic leukemia, Chronic lymphocytic leukemia, Acute myelogenous leukemia, Chronic myelogenous leukemia, Chronic idiopathic myelofibrosis, Chronic myelogenous leukemia, T-cell prolymphocytic leukemia, B-cell prolymphocytic leukemia, Chronic neutrophilic leukemia, Hairy cell leukemia). In some embodiments, the methods and compositions disclosed herein can be used to treat subjects suffering from solid tumors. Non-limiting examples of solid tumors to benefit from the present

invention can include solid tumors of childhood (Peripheral Neuroblastoma, Ewing's Sarcoma and the Ewing Family of Tumors, Rhabdomyosarcoma, Wilms Tumor, Osteosarcoma, Retinoblastoma), Lung cancer, any histology Colon cancer, Rectal cancer, Pancreas cancer, Stomach cancer, Esophageal cancer, Gall bladder cancer, Cancer of the bile duct, Renal cell cancer, Cervical cancer, Uterine cancer, Cancer of the fallopian tubes, Epithelial ovarian cancer, Breast cancer, Prostate cancer, Nasopharyngeal cancer, Paranasal sinus cancer, Neuroendocrine tumors, Soft tissue sarcomas, Thyroid tumors, Tumors of the thymus, Tumors of unknown primary origin, Malignant melanoma, Glioma.

[00158] Radiation therapy and chemotherapy are usually considered treatment options for patients suffering from cancer, which may result in ablation of bone marrow. Additionally chemotherapy or radiation therapy may be given prior to a stem cell transplant as part of the myeloablative conditioning regimen, in order to eradicate the patient's disease and suppress immune reaction prior to HSC transplant. Accordingly, in some embodiments, the methods and compositions described herein can be used to treat a subject who has or will undergo bone marrow transplantation, or has, or will undergo chemotherapy or radiation therapy.

[00159] In some embodiments, the methods and compositions can be used for accelerating the recovery of, or preventing the development of a blood cell deficiency or a blood disorder in a subject, where the subject has been exposed to any one of the following: radiation therapy, chemotherapy, and radiation as a pretreatment to ablate the immune system prior to transplantation. In some embodiments, the methods and compositions can also be used to treat a subject who is or will be treated with non-myeloablative transplantation, usually with allogeneic transplantation.

[00160] In some embodiments, the methods and compositions disclosed herein are used to treat a subject suffering from immune disorder. Non-limiting examples of immunodeficiencies include Ataxia telangiectasia, DiGeorge syndrome, Severe combined immunodeficiency (SCID). Wiskott-Aldrich syndrome, Kostmann syndrome, Shwachman-Diamond syndrome, Griscelli syndrome, type II, NF-Kappa-B Essential Modulator.

[00161] In some embodiments the subject can be a candidate for autologous transplantation, i.e. the stem cell population is obtained from the patient himself. The stem cells or the source containing stem cells can be collected prior to chemotherapy and/or radiation therapy. In some embodiments, the subject can be a candidate for allogeneic transplantation, i.e. the stem cells to be transplanted are obtained from another healthy person (the donor). The donor can be related or complete stranger to the patient undergoing transplantation. Accordingly in some embodiments the population of HSCs used in the current invention can be is autologous or allogeneic to the subject.

[00162] In preferred applications of the invention for patient treatment, the subject is a human subject. Further, in preferred application, the methods are applicable to treatment of any condition wherein promoting the hematopoietic reconstitution i.e. self-replication and differentiation by administering the HSPC compositions of the invention, would be effective to result in an improved therapeutic outcome for the subject under treatment. The invention provides a method of increasing the hematopoietic reconstitution of a HSC population in a human subject, upon contacting the population with an effective amount of protein ANG prior to in vivo administration.

Methods and use of Angiogenin for treatment of radiation injury

[00163] Another aspect of the present invention relates to use of ANG protein or an agonist thereof to treat subjects that have been exposed to or likely to be exposed to ionization radiation. Accordingly, the invention relates to a pharmaceutical composition comprising ANG or a functional fragment thereof, or an agonist thereof for preventing radiation induced hematopoietic injury, e.g., as a result of radio-or chemotherapy as a treatment for a disease or a result of accidental exposure to radiation, wherein the pharmaceutical composition is administered in an therapeutically effective amount.

[00164] In some embodiments, a composition comprising ANG or an agonist thereof can be used in methods for treatment of thrombocytopenia (deficiency in platelets), or neutropenia (deficiency in neurtrophils), anemia and the like, for example, where these disorders are a result

of any, or a combination of: exposure to radiation (e.g., accidental radiation exposure), radiation therapy, chemotherapy, and radiation as a pretreatment to ablate the immune system prior to a transplantation.

[00165] In some embodiments, a composition comprising ANG or an agonist thereof can be used in methods for accelerating the recovery of, or preventing the development of a blood cell deficiency or a blood disorder in a subject, where the subject has been exposed to any one of the following: radiation (e.g., accidental radiation exposure), radiation therapy, chemotherapy, and radiation as a pretreatment to ablate the immune system prior to a transplantation. Accordingly, in some embodiments, a composition comprising of ANG or an agonist thereof can be used in treating "first responders" or rescue personnel to assist a disaster recovery operation at a radiation accident, e.g., military and rescue personnel who attend to a the location of radiation accident, or are likely to be exposed to radiation at a site of a radiation accident or leakage.

[00166] In some embodiments, a composition comprising of ANG or an agonist thereof can be used in methods for treating a blood cell deficiency as a complication or side effect of where the subject has been exposed to any one of the following: radiation (e.g., accidental radiation exposure), radiation therapy, chemotherapy, and radiation as a pretreatment to ablate the immune system prior to a transplantation. In some embodiments, the blood cell deficiency is a complication or side effect of AIDS (acquired immunodeficiency syndrome); ITP (immune thrombocytopenic purpura); DIC (disseminated intravascular coagulation); TTP (thrombotic thrombocytopenic purpura) and the like.

[00167] In some embodiments, a composition comprising ANG or an agonist thereof can be administered to a subject prior to, during or after exposure to radiaition or a combination thereof. In some embodiments, treatment of a subject with a composition comprising of ANG or an agonist thereof can be according to the methods as disclosed herein can be therapeutic treatment, e.g., a method of treatment of a blood disorder in a subject, for example, a subject with neutropenia or low platelet count. In some embodiments, therapeutic treatment involves administration of a composition of ANG or an agonist thereof according to the methods as disclosed herein to a patient suffering from one or more symptoms of or having been diagnosed

as being afflicted with a blood disease or disorder. Relief and even partial relief from one or more of a symptom or a blood disorder may correspond to an increased life span or, simply, an increased quality of life. Further, treatments that alleviate a pathological symptom can allow for other treatments to be administered.

[00168] Accordingly, in some embodiments, a composition comprising ANG or an agonist thereof can be administered to the subject after exposure to ionizing radiation. In some embodiments a composition comprising an effective amount of ANG or an agonist thereof can be administered immediately, about 2 hrs, about 4 hrs, about 6 hrs, about 10 hrs, about 12 hrs, about 16 hrs, about 20 hrs, at least about 24 hrs after exposure to ionizing radiation. The time interval and duration for administration can be determined by those skilled in the art and among other factors can depend on the age of the subject, gender of the subject, strength of the ionizing radiation exposed, severity of the disease symptoms etc. In some embodiments for example, a composition comprising ANG or an agonist thereof can be administered every 2 hrs, every 4 hrs, every 6 hrs, every 10 hrs, at least every 24 hrs for a period of at least 1 day, at least 2 days, at least 3 days after starting the treatment post exposure to radiation. In some embodiments, the treatment is started 24 preferable 24 hrs after irradiation.

[00169] In alternative embodiments, a composition comprising ANG or an agonist thereof can be administered according to the methods as disclosed herein and can be a prophylactic treatment, for example, to prevent low platelet count of a subject with cancer who has undergone or will undergo a cancer treatment, such as for example chemotherapy, radiotherapy and the like. In some embodiments, a prophylactic treatment comprises administration of a composition comprising of ANG or an agonist thereof according to the methods described herein to a subject who has been recommended to have, or has undergone a cancer treatment, where it is desirable to prevent the loss or decrease of white blood cells in the subject as a side-effect of the cancer treatment. Administration of a composition comprising of ANG or an agonist thereof can begin at the start or after, or during (e.g., concurrent with) administration of a cancer therapy (e.g., chemotherapy, radiation therapy) etc., and can continue, if necessary, after cancer treatment, and if necessary for life. In some embodiments, prophylactic treatment is also useful where a subject is likely to be exposed to radiation, for example, subjects who are in or located near an area of a

radiation disaster accident, or subjects who are working in a recovery effort in an area that has had a radiation disaster or working in or near a radiation exposure.

[00170] Accordingly in some embodiments, administration of the compositions can be prior to or during the exposure to ionizing radiation. The time and interval of administering a composition comprising an effective amount of ANG or an agonist thereof can be determined by those skilled in the art and can depend for example on factor such as age, gender of the subject to be treated, the strength of the ionizing radiation that is expected to effect the subject. For example the composition comprising ANG or an agonist thereof can be administered at for example before 3 days, before 2 days, before 24 hrs (1 day), before 12 hrs, before 10 hrs, before 8 hrs, before 6 hrs, before 4 hrs, before 2 hrs, or immediately before exposure to ionizing radiation. In some embodiments the treatment can be carried out for at least 3 consecutive days, at least 2 consecutive days, at least 1 day prior to exposure to ionizing radiation. Exemplary schedule for treatment can be administering a composition comprising an effective amount of ANG or an agonist thereof for 3 consecutive days, at an interval of 24 hrs, until 24 hrs before the exposure to radiation.

[00171] In some embodiments, the administration of compositions disclosed herein can enhance the hematopoietic reconstitution, colony formation, cell survival, bone marrow cellularity, restrict proliferation of HSPC and/or enhance proliferation of myeloid restricted progenitor cells after exposure to radiation. In some embodiments, in vivo administration of ANG can increase hematopoietic reconstitution of cell administered during HSCT. The hematopoietic reconstitution of the transplanted hematopoietic cell compositions is enhanced with or without myeloablative radiation regimen as part of the treatment. In some embodiments, the subject undergoing HSCT transplant can be treated with ANG prior to, during or after transplantation or a combination thereof.

[00172] In some embodiments, a composition comprising of ANG or an agonist thereof can be used in methods for treating a subject who will or has undergone total body radiation (TBI). TBI doses used as a preparative regimen for HSCT typically ranges from 10 to higher than 12 Gy, which destroys the bone marrow function of the subject. The total dose of radiation

may be spread over multiple sessions between intervals of time between each session. Accordingly, the therapeutic administration of ANG can be done as a single dose or multiple doses for example, administered each time prior to multiple cycles of chemotherapy or radiation therapy. The non-myeloablative regimen uses low doses of chemotherapy and radiation, for example typically about 2 Gy, which do not destroy the subject's bone marrow. In some embodiments of the present invention, the compositions comprising ANG or an agonist there and methods comprising in vivo administration of the said composition can be used to enhance hematopoietic reconstitution post myeloablative regimen, non myeloablative regimen or in absence to radiation treatment prior to HSCT. In other aspect, the subject to be treated with composition and methods disclosed herein can be, will be or has been subject to single or multiple dose of for example 2 Gy, 4 Gy, 6 Gy, 8 Gy, 10 Gy, 12 Gy, lethal dose of irradiation. The LD50 dose is defined as a measure of a lethal dose of radiation required to kill half the members or a tested population after specified test duration. A lower LD50 is indicative of increased toxicity. In some embodiments, the treatment with compositions and methods disclosed herein can increase the LD50 for a specific dose of radiation. Accordingly, in some embodiments, the methods disclosed herein can be used to administer higher doses of ionizing radiation treatment than that would be feasible without treatment with ANG.

[00173] It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the invention. Further, all patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the

contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

EXAMPLES

[00174] The following examples illustrate some embodiments and aspects of the invention. It will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be performed without altering the spirit or scope of the invention as defined in the claims which follow. The technology described herein is further illustrated by the following examples which is no way should be construed as being further limiting.

Materials and Methods for Examples 1 to 3

[00175] **Mice**- All animal experiments were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital and Tufts University/Tufts Medical Center. Wild-type C57Bl/6, B6SJL, MTMG, IL18KO, IL18R1KO, NesCreERT2, NG2CreERT2, Col1a1CreERT2 mice were obtained from the Jackson laboratory. Col2.3GFP (Kalajzic et al., 2002) were previously described. Ang/ conditional/KO mice were generated by the Hu laboratory (manuscript in review . OsxCreERT2 mice were a kind gift of Dr H Kronenberg, Massachusetts General Hospital. For studies using ERT2 mice, tamoxifen (150 mg/kg, Sigma Aldrich) was injected intra-peritoneally daily for three days three times daily in both genotypes (Cre-positive +/+ or fl/fl , and BM was harvested 24 hours following the final injection. Age-matched (7-12 week old littermates were used)

[00176] **Single OLC harvesting and single cell RNA Seq** Newborn col2.3GFP animals were injected with DiI- labeled LKS CD34-Flk2- adult bone marrow cells, as described below, and sacrificed 48 hours after transplantation. Femurs were dissected, embedded in 10% low melting temperature agarose (Lonza) and sectioned at 100μ using a vibratome (Leica) . Single OLC harvesting was performed using a physiology microscope BX51 (Olympus) equipped with filters to detect GFP and DiI fluorescence, DIC optics, micromanipulators (Eppendorff) , real-time imaging camera, peristaltic pump, in-line heater, perfusion chamber (Harvard Apparatus

and SAS Air Syringe (Research Instruments) . Sections were pre-screened for the presence of rare GFP-labeled OLCs located next to single DiI-positive transplanted HSCPs, which were found in 1-2 out of 15 sections per animal. Once a target proximal OLC was identified, the section was rotated so that the target was directly opposite the aspiration pipette (Humagen). The section was secured against the bottom of the perfusion chamber using a horizontal portion of the holding pipette (Humagen). With the aspiration pipette just above the target, the section was perfused with warm (37°C cell dissociation solution (Liberase TM, Roche) for 8-10 minutes while the target cell was visually monitored. Then, applying positive pressure from the micropipette using Air Syringe, hematopoietic cells surrounding the target OLC were dislodged to create a 20-30 μ clearing. Finally, the aspiration pipette was lowered onto the target OLC, the cell was gently detached from the endosteal surface and aspirated. The presence of GFP fluorescence in the aspirated cell inside the aspiration pipette was confirmed, the contents of the pipette was ejected into a PCR tube with the lysis buffer for the single cell RNA-Seq protocol, and frozen immediately at -80°C. Reverse transcription, cDNA amplification, library preparation and SOLiD RNA-Seq were performed as described (Tang et al., 2009).

[00177] **FACS analysis and cell sorting** Gating strategies, phenotypic studies, chimerism analyses, cell cycle assays, BrdU incorporation assays, Annexin V assays, and cell sorting were done as described in Supplemental Experimental Procedures.

[00178] **BrdU incorporation** BrdU was administered in drinking water at 0.35 mg/ml for 3 days. Cells were stained with cells surface markers as detailed above and BrdU antibody using BrdU FITC kit (BD) following fixation and permeabilization, as per manufacturer's instructions.

[00179] **Bone marrow stem cell transplantation** Conditioning regimens and transplant procedures are described in Supplementary methods.

[00180] **5 fluorouracil treatment** 8-week old age and gender matched WT or IL18KO mice were injected with 5-fluorouracil (APP at 150 mg/kg intra-peritoneally. Bone marrow was analyzed on day 8 by flow cytometry. For serial 5FU exposure, animals received weekly intra-peritoneal injections at the same dose.

[00181] **Bioinformatics and statistical analysis** The differential expression estimates were obtained from single-cell RNA-seq data using the approach described (Kharchenko et al., 2014). The stability of differential expression signature/ distinguishing OLC-proximal and distal cells was tested using support vector machine (SVM) classifier as follows: the SVM classifiers were constructed using all genes for which expression was detected in any of the examined cells; the ability to distinguish OLC-proximal and distal cells was/tested using leave-two-out validation: one OLC-proximal and one OLC-distal cell was excluded, and a v-classification SVM was constructed based on all remaining cells using e1071 R package. All possible pairs of OLC-proximal and distal cells were tested to evaluate the classification performance (Fig. 3B). Gene set enrichment analysis (GSEA) was performed using mouse GO annotations from Mouse Genome Database (2013.12.27 version, see http://www.informatics.jax.org/ for gene listings). A total of 1590 GO categories (BP or CC) containing between 10 and 2000 genes were tested, taking into account the magnitude of the expression differences. In the analysis of the single-cell differential expression, the mode of the log-fold expression difference posterior distributions was used as a difference magnitude (with power factor p=0.5). The empirical P-values were determined based on 106 randomizations, with Q-values derived using Benjamini & Hochberg correction. RNA-Seq data from bulk-sorted samples was aligned to the NCBI mm9 annotation using TopHat. The expression fold-differences were estimated using HTSeq and DESeq. The GSEA was performed using signed expression difference Z-score (power factor p=2, 106 randomizations). To verify classification of the bulk samples based on the 200-gene signature (Fig. 3A), RPKM estimates were used, correcting for mouse batch effect using ComBat (Johnson et al., 2007) .The classification was calculated using Ward method hierarchical clustering, with a Euclidean distance metric. The single cell and bulk analysis RNA-Seq data has been deposited in GEO under accession number GSE52359. The full differential expression analysis can be viewed via the following URL,
http://pklab.med.harvard.edu/sde/viewpost.html?dataset=olc.

[00182] **Intravital microscopy** WT C57Bl/6 mice or IL18KO mice were irradiated 950 cGy the night before and were intravenously injected with 50,000 LKS cells obtained from MTMG mice (for tdTomato labeling). Intravital imaging of calvarial bone marrow and data

analysis were performed at 24 hours post- transplant, as previously described (Lo Celso et al., 2009).

[00183] **Anti Embigin mobilization** 10 week old C57Bl/6 mice were injected intravenously via tail vein with 2 mg/kg/day of functional grade anti-Embigin antibody (clone G7.43.1; E-bioscience) or IgG2b control antibody for 3 days. Twenty four hours after the last injection peripheral blood was collected via cardiac puncture and phenotypic progenitors determined by flow cytometry and functional progenitors determined by colony assays in methylcellulose as we have previously described (Hoggatt et al.)

[00184] **Cell sorting and flow cytometry** Whole bone marrow mononuclear cells (BMMNC) were collected by crushing tibias, femurs and hips and stained with the following monoclonal antibodies: c Kit APC, CD34 FITC (e Bioscience), Sca1 BV421, Flk2 PE, IL18Rα/CD218a (E Bioscience) , CD48 APCCy7 (BD) , lineage cocktail biotin (B220, Mac1, Ter119, CD3, CD4, CD8 at 1:1:1:1:1:1 followed by streptavidin Pacific Orange (Invitrogen). LT HSCs, ST HSCs and MPP were gated as described. For the lineage analysis, red cell depleted BMMNC or peripheral blood samples were stained with CD3 APC (e Bioscience), Mac1FITC, Gr1 PeCy7 and B220 PE (BD). For CLP enumeration, BMMNC were stained with FITC conjugated antibodies against Mac1, Gr1, CD19, Ter119, CD3 Pacific Blue, Flk2 PE, B220 PE Cy7 and biotin conjugated IL7R/CD127, followed by streptavidin PerCP Cy5.5 (all from BD . For CLP cell cycle analysis, BMMNC were stained with lineage cocktail biotin (B220, Mac1, Ter119, CD3, CD4, CD8 at 1:1:1:1:1:1 followed by PE Texas Red conjugate (Invitrogen) , B220 PE Cy5, CD127PE, Flk2APC and DAPI. For post-transplant chimerism analysis, CD45.1 AF700 and CD45.2 Pacific Blue (BD) were added. 7 AAD (BD or DAPI (Invitrogen) were used as viability dyes. At least 2×106 events per sample were acquired for progenitor analysis and 104 events for lineage analysis using a BD LSRII flow cytometer. For cell cycle analysis, BMMNC were stained with monoclonal antibodies for HSPC markers, as described above. The cells were permeabilized using Cytofix/Cytoperm Fixation/Permeabilization Kit (BD) according to the manufacturer's instructions and stained with Ki 67 FITC (BD), Hoechst 33342 or DAPI (Invitrogen). For FACS analysis/sorting of osteolineage cells, bone fragments were obtained by gently crushing tibiae, femora, humeri and pelvic bones of 4 6 weeks old col2.3GFP mice. After

rinsing away the bone marrow cells, the fragments were incubated with 0.25% Collagenase (Stem Cell Technologies) at 37°C with gentle agitation for 1 hour. The samples were vortexed several times during the incubation, then filtered through 0.45 micron mesh and stained with CD45 APC Cy7, Ter 119 APC Cy7 (BD) , Embigin PE (E Bioscence) and CD106 APC (R D Systems) . The samples were analysed using LSRII (BD) or FACS sorted using Aria (BD). Compensation and data analysis were performed using Flowjo 7.6 software. For the RNA Seq analysis of GFP+Embbright VCAM 1+ cells, lethally irradiated col2.3GFP mice were injected with 10,000 LKS CD34 Flk2 LT HSCs, lin kit+Sca progenitors or PBS and sacrificed 48 hours later. GFP+Embbright VCAM 1+ cells and remaining GFP+cells were sorted directly into the lysis buffer for the single cell RNA Seq protocol, and frozen immediately at 80°C. Reverse transcription, cDNA amplification, library preparation, SOLiD RNA Seq were performed as described for the single cell RNA Seq samples, except for the reduction in the initial PCR amplification cycle number from 20 to 18. Three biological replicates for each sample group were sequenced. For FACS analysis of IL18 receptor expression in human primitive hematopoietic cells, CD34 enriched bone marrow or cord blood cells were stained with the following antibodies: CD34 APC Cy7, CD38 FITC, CD45RA APC, CD10 BV510, CD49f BV650, CD90 BV421 (all from BD and CD218a/IL18R1 PE (E Bioscience), as described (Notta et al., 2011).

[00185] **Bone marrow/stem cell transplantation** Adult recipients (CD45.2) were irradiated 950 cGy the evening before and transplanted with 500K total bone marrow cells (CD45.1) via retro orbital injection. For LKS cell transplantation, lethally irradiated animals were intravenously injected with 8,000 CD45.1 LKS cells and CD45.2 support cells for IL18KO experiments, 8000 CD45.2 LKS cells and CD45.2 support cells from for IL18 receptor KO experiments. For the transplants which involved Ang conditional knock out strains, 500K bone marrow cells from Ang deleted animals (45.2) were co transplanted with 500K bone marrow cells from CD45.1 animals into lethally irradiated CD45.1 recipients. For non-competitive transplants, 106 CD45.1 bone marrow cells were used. Recipients' peripheral blood chimerism was assessed at 4 weekly intervals after transplantation. For neonatal transplantation, col2.3GFP P2 pups were irradiated 450 cGy the evening before. Adult bone marrow LKS 34 Flk2 cells were

isolated as described and labeled with DiI according to manufacturer's instructions. 5000-7000 cells per animal were injected in a 50 μl volume via anterior facial vein.

Methods for Examples 4 to 8

EXPERIMENTAL PROCEDURES

[00186] **Animal Studies** Ang-/- mice were generated in-house. B6.SJL and NSG mice were purchased from The Jackson Laboratory. For aged animal experiments, 22-month old WT (NIH/NIA) and Ang-/- mice were used. For all other studies, age-matched 7-12 week old mice were used. Littermates and gender-matched animals were used whenever possible. All procedures were performed in accordance with protocols approved by Institutional Animal Care and Use Committee of Tufts University/Tufts Medical Center.

[00187] **Statistical Analyses** All bar graphs represent mean ± SEM and all heatmaps represent mean. All data are derived from 2-4 independent experiments. For comparisons of two experimental groups, an unpaired two-tailed Student's t-test was used (Excel). Kaplan-Meier survival curves were analyzed using log rank tests (Prism 6). Heatmaps were generated using RStudio. LDA was assessed by ELDA (http://bioinf.wehi.edu.au/software/elda/). For all analyses, *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ns=not significant.

[00188] **Bone Marrow Cellularity** Femurs were dissected and flushed with 5 ml phosphate buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS, Mediatech). Cells were resuspended by pipetting and vortexing. White blood cell counts were obtained by VetScan HM5 instrumentation (Abaxis Veterinary Diagnostics).

[00189] **Generation of ANG** Mouse and human recombinant ANG protein were generated by a pET E. coli expression system and purified to homogeneity by HPLC in-house (Shapiro et al., 1988). Angiogenic and ribonucleolytic activity of each batch of ANG preparation was confirmed (data not shown). ANG variants (R33A, K40Q, and R70A) were generated

through site-directed mutagenesis followed by expression in pET system and purification by HPLC.

[00190] **In vivo and In vivo ANG Treatment** Unless otherwise indicated (in dose response experiments), 300 ng/ml ANG was used for in vivo treatments. For all in vivo ANG treatments, 1.25 mg/kg was injected intraperitoneally at the indicated time points.

[00191] **5-Fluorouracil (5-FU) Treatment** For 5-FU rebound experiments, 5-FU (150 mg/kg) was injected intraperitoneally once and BM harvested for analysis on Day 7. For serial 5-FU treatments, 5-FU (150 mg/kg) was injected intraperitoneally every 7 days until 100% animal mortality was achieved.

[00192] **Histology** Femurs were dissected from animals and fixed overnight in 10% neutral buffered formalin. Bones were prepared, decalcified, and stained with Hematoxylin and Eosin (H&E) by the Tufts Animal Histology Core.

[00193] **Genotyping** Genotyping was performed by PCR with Hot Start Green PCR Master Mix (Thermo Scientific), using standard PCR conditions on an iCycler PCR machine (Biorad). The Ang primers for Ang-/- mice were as follows: Forward, 5'-AGCGAATGGAAGCCCTTACA-3'; reverse, 5'- CTCATCGAAGTGGACAGGCA-3'. The primers for the LoxP site (F12/B6) were as follows: Forward, 5'-AGGGTGGAACTTCAGGATTCAAG-3'; reverse, 5'-GAAGTTATCCGCGGGAAGTTC-3'.

[00194] **Complete Blood Counts** Peripheral blood was harvested from mice by retro-orbital bleeding using heparinized micro-hematocrit capillary tubes (Fisherbrand). Blood was collected directly into EDTA-coated Microtainer tubes (BD) and automated complete blood counts were assessed by VetScan HM5 instrumentation.

[00195] **Flow Cytometry and Cell Sorting** Whole bone marrow mononuclear cells (BMMNC) were obtained by crushing tibias and femurs in PBS/2% FBS and straining cellular suspension through 0.45 μm mesh. Red blood cells were depleted using ACK Lysis Buffer

(Lonza). Briefly, 2 ml buffer was added to cell pellet and incubated on ice for 5 minutes with periodic vortexing. Cells were washed once and resuspended in 200 μl PBS/2% FBS for staining using 1:200 dilutions of primary antibodies unless otherwise indicated. Gating was established by the following phenotypic cell surface markers, based on standard gating approaches:

Methods Table 1. Surface markers for gating of various cell populations.

Cell Type	Cell Surface Markers
LKS	Lin−c-Kit+Sca1+
Myeloid-restricted progenitor	Lin−c-Kit+Sca1−
LT-HSC	Flk2−CD34− LKS
ST-HSC	Flk2−CD34+ LKS
MPP	Flk2+CD34+ LKS
HSC	CD150+CD48−CD135−CD34− LKS
MPP1	CD150+CD48−CD135−CD34+ LKS
MPP2	CD150+CD48+CD135−CD34+ LKS
MPP3	CD150−CD48+CD135−CD34+ LKS
MPP4	CD150+CD48+CD135+CD34+ LKS
CLP	Lin− IL7R+ Flk2+ B220−
Pre-pro B	Lin− IL7R+ Flk2+ B220+
CMP	Lin−c-Kit+Sca1−CD34+CD16/32−
GMP	Lin−c-Kit+Sca1−CD34+CD16/32+
MEP	Lin−c-Kit+Sca1−CD34−CD16/32−

[00196] For stem and progenitor staining, red cell-depleted BMMNCs were stained with antibodies against cKit BV711 (BD), Sca1 PE-Cy5 (eBioscience), Flk2 PE (BD), CD34 e660 (eBioscience), IL7R APC-Cy7 (eBioscience), B220 BV785 (Biolegend), CD16/32 AF700 (eBioscience) and a biotinylated lineage cocktail (B220, CD3, CD4, CD8, Mac1, and Ter119 at 1:1:1:1:1:1). Cells were stained for 90 minutes on ice, followed by streptavidin PE-Cy7 (Biolegend) for 15 minutes on ice. Cells were analyzed using a FACSAria flow cytometer (BD).

[00197] For lineage analysis, red cell-depleted BMMNCs were stained for 30 minutes on ice with antibodies against CD11b PE-Cy7 (Biolegend), Gr1 PE (eBioscience, 1:400), CD45R/B220

[00198] FITC (BD), CD3ε APC-Cy7 (Biolegend), and Ter119 APC (eBioscience). Cells were analyzed using a LSRII flow cytometer (BD).

[00199] For chimerism studies, peripheral blood was obtained by retro-orbital bleeding and depleted of red blood cells. Samples were stained for 30 minutes on ice with antibodies against CD45.1 APC (eBioscience), CD45.2 Pacific Blue (Biolegend), CD11b PE-Cy7, Gr1 PE, CD45R/B220 FITC, and CD3ε APC-Cy7. Cells were analyzed using a LSRII flow cytometer.

[00200] For sorting LKS cells or myeloid-restricted progenitors, red cell-depleted BMMNCs were stained with antibodies against cKit APC (eBioscience), Sca1 PE (eBioscience), and a FITC lineage cocktail for 30 minutes on ice. Cells were sorted using FACSAria or MoFlow Astrios (Beckman Coulter) flow cytometers. For sorting LT-HSCs, red cell-depleted BMMNCs were stained with antibodies against cKit APC-eF780 (eBioscience), Sca1 PE-Cy5, Flk2 PE, CD34 e660, and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin PE-Cy7 (Biolegend) for 15 minutes on ice. Cells were sorted using a FACSAria flow cytometer.

[00201] For all analyses, 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) or 7-aminoactinomycin d (7-AAD, BD) were used as viability dyes, per manufacturer's instructions. At least 2 x 106 events per sample were acquired for bone marrow stem and progenitor analysis and 3 x 104 events for lineage analysis. Data were analyzed using FlowJo X (Tree Star).

[00202] **Cell Cycle Analysis** For cell cycle, 1 x 107 red cell-depleted BMMNCs were stained with cell surface markers as described above and fixed and permeabilized using Cytofix/Cytoperm Fixation/Permeabilization Kit (BD) per manufacturer's instructions. Cells were then stained with Ki67 FITC (BD, 1:10 in BD Perm/Wash buffer) and DAPI (2 μg/ml for 10 minutes prior to analysis), and analyzed using a FACSAria flow cytometer, acquiring 2 x 106 events per sample.

[00203] **BrdU Incorporation** BrdU was administered in drinking water (0.35 mg/ml) for 3 days. Volume of drinking water was assessed to confirm equal water intake among cages. Mice were sacrificed and red cell- depleted BMMNCs were stained with antibodies against cell surface markers (1:200) as follows:

[00204] For HSPCs, cells were stained with c-Kit APC-eF780, Sca1 PE-Cy5, Flk2 PE, CD34 e660 and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin Pacific Orange (Invitrogen) for 15 minutes on ice.

[00205] For lymphoid-restricted progenitors, cells were stained with c-Kit APC-eF780, Sca1 PE-Cy7 (Biolegend), IL7R PE (eBioscience), B220 PE-Cy5 (eBioscience), and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin Pacific Orange for 15 minutes on ice.

[00206] For myeloid-restricted progenitors, cells were stained with c-Kit APC-eF780, Sca1 PE-Cy5, CD16/32 BV605 (BD), CD34 e660 and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin Pacific Orange for 15 minutes on ice.

[00207] Following cell surface stain, cells were fixed and permeabilized, and stained with BrdU FITC (BD), per manufacturer's instructions. For all stains, cells were analyzed using a FACSAria flow cytometer, acquiring 2 x 106 events per sample. BrdU gating was established by cells isolated from mice not administered BrdU and BrdU fluorescence-minus-one controls.

[00208] **Annexin V Analysis** To assess apoptotic activity, red cell-depleted BMMNCs were stained for cell surface markers as above, and stained with Annexin V FITC (BD), per manufacturer's instructions. Briefly, cells were resuspended in 1x Binding buffer (BD) at 1 x 106 cells/ml and stained for 15 min at room temperature (RT) in the dark. Four hundred µl of 1x Binding buffer was added to each tube analyzed on a LSRII or FACSAria flow cytometer within 1 hour. Annexin V-positive gates were established by Annexin V fluorescence-minus-one controls.

[00209] **Mouse and Human Methylcellulose Colony Assays** For myeloid progenitor quantification, 2 x 104 whole BMMNCs were plated in MethoCult M3434 methylcellulose (Stem Cell Technologies), per manufacturer's instructions. Colonies were scored by visualization on Day 12.

[00210] For serial re-plating assays, 2 x 104 whole BMMNCs were plated in MethoCult M3434 methylcellulose and colonies were scored at Day 7. Colonies were the harvested, per manufacturer's instructions, 2 x 104 whole BMMNCs were again plated in methylcellulose. Colonies were subsequently scored on Day 14.

[00211] For pre-pro B progenitor quantification, 5 x 104 whole BMMNCs were plated in MethoCult M3630 methylcellulose (Stem Cell Technologies), per manufacturer's instructions. Colonies were scored by visualization on Day 7.

[00212] For human progenitor quantification, 2 x 104 human CD34+ cord blood cells (Stem Cell Technologies, mixed donors) were plated in MethoCult H4034 methylcellulose in the presence or absence of 300 ng/ml human ANG. Colonies were scored by visualization on Day 15.

[00213] All assays were cultured in untreated 35-mm culture dishes (Stem Cell Technologies) and maintained for the duration of the experiment at 37°C/5% CO2, per manufacturer's instructions. For all experiments, data were presented as frequency of total number of plated cells.

[00214] **Quantitative RT-PCR Analyses** Total RNA was extracted from sorted or treated hematopoietic cell populations using RNeasy Plus Micro Kit (Qiagen), and was reverse transcribed into cDNA with Quantitech Reverse Transcription Kit (Qiagen), per manufacturer's instructions. For qRT-PCR analysis of rRNA species, random primers (IDT) were used during reverse transcription. For all other analyses, Oligo(d)T primers (IDT) were used. qRT-PCR analysis was performed on a LightCycler 480 II (Roche) using SYBR Green PCR mix (Roche). Relative expression was determined by the 2-ΔΔCt method, using β-actin as an internal control. Primer sequences were adapted from the following sources: mouse p21, p27, and p57 (Chakkalakal et al., 2014); mouse GATA3, Bmi1, and vWF (Kent et al., 2009); mouse a1, Bcl2, Bcl-xl, Mcl1, Bak, Bax, Bid, Bim, Noxa, Puma, and β-Actin (Mohrin et al., 2010); human p21 (Zhu et al., 2011); human p27 (Bryant et al., 2006); human p57 (Giovannini et al., 2012); human

GATA3 (Wang et al., 2014); human vWF (Yoon et al., 2012); human Bmi1 (Abdouh et al., 2009); human cyclin D1 (Ding et al., 2009); and human β-Actin (Sheng et al., 2014). Tables 2 and 3, below, for primer information.

[00215] **Mouse LT-HSC Culture** For 2 hour treatments in PBS, LT-HSCs were sorted directly into PBS and cultured in the presence or absence of 300 ng/ml ANG. For other cell proliferation and qRT-PCR analyses, LT- HSCs were sorted into 96-well plates and cultured in S-clone SF-O3 (Sanko Junyaku), supplemented with 0.5% bovine serum albumin (Gibco Life Technologies), 50 ng/ml thrombopoietin (Peprotech), 50 ng/ml stem cell factor (Peprotech) and 50 μM 2-mercaptoethanol (Gibco Life Technologies), in the presence or absence of 300 ng/ml ANG. For 2- or 7 day treatments, 1X Penicillin/Streptomycin (Corning) was included in culture medium. Cells were cultured at 37°C/5% CO2.

[00216] For proliferation studies, cell number was determined by hemocytometer. For qRT-PCR studies, cells were harvested and analyzed as described under "Quantitative RT-PCR Analyses". For BM transplantation, cells were harvested, washed with PBS, and counted. Equal cell numbers were transplanted as described under "Mouse Bone Marrow Transplantation."

[00217] **Human CD34+ Cord Blood Cell Culture** Human CD34+ cord blood cells (Stem Cell Technologies) were thawed per manufacturer's instructions. For 2 hour treatments, cells were cultured in PBS in the presence or absence of 300 ng/ml hANG. For 7 day culture, cells were cultured in StemSpan SFEM (Stem Cell Technologies), supplemented with stem cell factor, Flt3 ligand, IL6, and thrombopoietin (100 ng/ml, R&D), in the presence or absence of 300 ng/ml hANG. Cells were cultured at 37°C/5% CO2. Commercial human ANG (R&D) was also tested at 300 ng/ml and shown to neither have as strong induction of candidate self-renewal transcripts nor as strong reduction in proliferation, consistent with our previous findings that the biological activity of commercial ANG is about 10% of our in house ANG preps (data not shown). Human ANG variants (K40Q, R70A, R33A) were used at the same concentration of 300 ng/ml. For proliferation studies, cell number was determined by hemocytometer. For qRT-PCR studies, cells were harvested and analyzed as described under "Quantitative RT-PCR Analyses".

[00218] **Mouse Bone Marrow Transplantation** For all mouse transplant studies, recipient mice were lethally-irradiated 16 hours prior to transplantation with 12 Gy total body irradiation (TBI, split dose 3 hours apart). All mice were irradiated in a pie cage (Braintree Scientific) with rotation (JL Shepherd irradiator). For each experiment, mice from different experimental groups were simultaneously irradiated to ensure equal irradiation among groups.

[00219] For serial transplantation of LT-HSCs into ANG-deficient hosts, 400 sorted LT-HSCs from CD45.1 donor mice were co-injected with 1 x 106 CD45.2 whole BM support cells into lethally- irradiated WT or Ang-/- (CD45.2) recipient mice. After 24 months, BM was harvested, 400 LT- HSCs were re-sorted and transplanted again into WT or Ang-/- (CD45.2) secondary recipients with 1 x 106 CD45.2 whole BM support cells.

[00220] For serial transplantation of WBM into ANG-deficient hosts, 1 x 106 whole BM cells were transplanted into lethally-irradiated WT or Ang-/- (CD45.2) recipient mice. After 24 months, BM was harvested and 1 x 106 whole BM cells (CD45.1) were transplanted again into WT or Ang-/- (CD45.2) secondary recipients.

[00221] For direct 1:1 competitive transplantation studies using 22 month old WT or Ang-/- mice, 5 x 105 whole BMMNCs (CD45.2) were intravenously co-injected with 5 x 105 B6.SJL (CD45.1) support cells into lethally-irradiated B6.SJL (CD45.1) recipient mice.

[00222] For ex vivo reconstitution assays, WT and Ang-/- LT-HSCs (CD45.2), either freshly sorted or cultured with or without 300 ng/ml ANG for 2 hours or 7 days, were washed in PBS, and 400 donor cells were intravenously co-injected with 1 x 106 B6.SJL (CD45.1) support cells into lethally-irradiated B6.SJL (CD45.1) recipient mice. For secondary transplantation in ex vivo reconstitution assays, C57BL/6 LT-HSCs (CD45.2) were sorted from primary recipients that were transplanted with fresh LT-HSCs or LT-HSCs treated with or without ANG for 2 hours. Four hundred LT-HSCs from primary recipients were then intravenously co-injected with 1 x 106 B6.SJL (CD45.1) support cells into lethally-irradiated B6.SJL (CD45.1) recipient mice.

[00223] For transplantation of tiRNA-transfected LKS cells, 3,000 sorted C57BL/6 LKS (CD45.2) were transfected as described under "tiRNA Transfection", and intravenously co-injected with 1 x 106 B6.SJL (CD45.1) support cells into lethally-irradiated B6.SJL (CD45.1) recipient mice.

[00224] For transplantation of irradiated BM (pre-treatment group), C57BL/6 (CD45.2) mice were pretreated daily for three successive days with ANG and irradiated (4 Gy TBI) 24 hours following the final ANG treatment. BM was harvested at Day 7, donor BMMNCs were pooled and intravenously co-injected with B6.SJL (CD45.1) support cells (1:1) into lethally-irradiated B6.SJL (CD45.1) recipient mice. For the delayed treatment group, C57BL/6 (CD45.2) mice were irradiated (4 Gy) and treated with ANG daily for three successive days, beginning 24 hours post- irradiation. BMMNCs were harvested and transplanted as in the pre-treatment group.

[00225] For all transplants, except for irradiation reconstitution assays, peripheral blood was taken by retro-orbital bleeding at 4-week time intervals, up through 16 or 24 weeks, as indicated. For irradiation assays, peripheral blood was taken by retro-orbital bleeding at 16 weeks post- transplant. Reconstitution units (RU) per femur, corresponding to the HSC content per 1 x 105 BM cells, was calculated as previously described (Purton and Scadden, 2007; Winkler et al., 2012).

[00226] **Human CD34+ Cord Blood Cell Transplantation** NSG mice were purchased from The Jackson Laboratory and maintained in sterile housing. Recipient NSG mice were sublethally irradiated (2.5 Gy TBI) 16 hours prior to transplantation. Human CD34+ cord blood cells from mixed donors were treated with or without 300 ng/ml human ANG for 2 hours in PBS at 37°C/5% CO2. Cells were washed once in PBS and intravenously injected in three doses: 100, 1,000, and 10,000 cells. Both male and female mice were used as recipients for all treatments and doses. No significant differences were observed among experimental groups between male and female mice, different from a previous report (McDermott et al., 2010). At 16 weeks post-transplant, red cell-depleted BMMNCs were surface stained with the following antibodies for 30 minutes on ice (1:200 dilution): human CD45 Pacific Blue (Biolegend), Mouse CD45 APC-e780 (eBioscience), Human CD19 PE-Cy7 (BD), Human CD33 PE (BD). Samples were analyzed

using a FACSAria flow cytometer. Engraftment was assessed by the frequency of human CD45 cells. All samples demonstrating greater than or equal to 0.1% hCD45 expression were considered to be positively-engrafted, in keeping with prior studies (Boitano et al., 2010).

[00227] **Homing Assay** Homing assays were performed as described previously (Hoggatt et al., 2009). For homing assays using WT or Ang-/- mice as recipients, 2 x 106 CD45.1 Lin− cells were labeled with CFSE (Molecular Probes) per manufacturer's instructions, and transplanted into lethally-irradiated WT or Ang-/- (CD45.2) recipient mice. Cells were harvested 16 hours post-transplant, stained with antibodies against cell-surface markers as described above, and analyzed on a FACSAria flow cytometer. Percent CFSE-positive LKS cells and myeloid-restricted progenitors was determined. For homing assays using ANG-treated cells, 2 x 106 CD45.2 Lin− cells were treated with 300 ng/ml ANG in PBS for 2 hours at 37 °C/5% CO2. Cells were labeled with CFSE, as above, and transplanted into lethally-irradiated B6.SJL (CD45.1) recipient mice. Cells were harvested 16 hours post-transplant, stained with antibodies against cell-surface markers as described above, and analyzed on a FACSAria flow cytometer. Percent CFSE-positive LKS cells and myeloid- restricted progenitors was determined.

[00228] **Protein Synthesis Analyses** Determination of protein synthesis rates in BM cells was done using OP-Puro as described in reference (Signer et al., 2014). For in vivo analyses, LKS cells or myeloid-restricted progenitors were sorted as described above, and plated in DMEM (Sigma) in the presence or absence of 300 ng/ml ANG. Cells were cultured for 2 hours at 37°C/5% CO2. Cells were washed once with Ca2+- and Mg2+-free PBS and cultured for 1 hour with OP-Puro (50 μM, Medchem Source). Cells were fixed in 0.5 ml of 1% paraformaldehyde (Affymetrix) in PBS for 15 minutes on ice, washed once with PBS, and then permeabilized with 200 μl PBS supplemented with 3% FBS and 0.1% saponin (Sigma) for 5 minutes at room temperature (RT). Click-iT Cell Reaction Buffer Kit (Life Technologies) was used for azide-alkyne cycloaddition of AF488-conjugated azide (5 μM, Life Technologies), per manufacturer's instructions. Cells were washed twice in PBS/3% FBS/0.1% saponin and analyzed using a FACSAria flow cytometer.

[00229] For in vivo analyses, OP-Puro was injected intraperitoneally (50 mg/kg in PBS). One hour post- injection, BM was collected from sacrificed mice and red cell-depleted BMMNCs were stained as follows. Unless otherwise indicated, primary antibodies were used at 1:200 dilution. For stem and progenitor staining, 5 x 106 cells were stained with cKit BV711, Sca1 APC-Cy7 (Biolegend, 1:80), Flk2 APC (Biolegend, 1:50), CD34 e450 (eBioscience, 1:50), and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin Pacific Orange for 15 minutes on ice.For lymphoid-restricted progenitor staining, 5 x 106 cells were stained with cKit BV711, Sca1APC-Cy7, Flk2 APC, IL7R PerCP-Cy5.5 (eBioscience, 1:80), B220 BV650 (Biolegend, 1:80) and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin Pacific Orange for 15 minutes on ice. For myeloid-restricted progenitor staining, 5 x 106 cells were stained with cKit BV711, Sca1 APC-Cy7, CD16/32 BV605 (BD. 1:80), CD34 e450 and a biotinylated lineage cocktail. Cells were stained for 90 minutes on ice, followed by streptavidin Pacific Orange for 15 minutes on ice.

[00230] For lineage staining, 5 x 105 cells were stained with Mac1 APC (eBioscience), Gr1 PE (1:400), CD3ε Pacific Blue (Biolegend, 1:100), and Ter119 APC-Cy7 (Biolegend, 1:100) for 30 minutes on ice. Following surface staining, cells were washed twice with Ca2+- and Mg2+-free PBS and resuspended in 1 ml PBS. One µl UV-fixable eFluor 455 viability dye was added (eBioscience), cells were incubated for 30 minutes at 4°C in the dark, and washed once with PBS, per manufacturer's instructions. Following staining, cells were fixed and permeabilized and cycloaddition of AF488-conjugated azide (Life Technologies) was performed as described above. Cells were analyzed using a FACSAria flow cytometer, acquiring 2 x 106 events per sample for BM stem and progenitor analysis and at least 3 x 104 events for lineage analysis. Treated samples were compared to mice or cells not administered OP-Puro and/or OP-Puro fluorescence-minus-one controls. Relative rate of protein synthesis was determined as described previously (Signer et al., 2014). Briefly, background fluorescence was subtracted from OP- Puromycin AF488 geometric means and normalized relative to whole BM or WT controls for in vivo and in vivo experiments, respectively.

[00231] **tiRNA Gel Electrophoresis** For all RNA work, equipment was sterilized according to standard laboratory protocol and diethylpyrocarbonate-treated water was used for

all procedures. Total RNA was isolated and pooled from sorted LKS cells, myeloid-restricted progenitors, or lineage-positive cells for each experimental parameter. Total RNA was diluted in 2X Novex TBE-Urea sample buffer (Invitrogen), heated to 65°C for 5 minutes and cooled briefly to RT prior to loading. A 15% TBE-Urea Gel (Invitrogen) was pre-run at 74 V for 60 minutes and samples were electrophoresed to the bottom of the gel at 100 V in 0.5X TBE running buffer. A low molecular weight marker (10-100 nt, Affymetrix) was simultaneously run to compare RNA band sizes.

[00232] Following electrophoresis, the gel was equilibrated in 0.5X TBE for 5 minutes and stained with SYBR Gold solution (Invitrogen) diluted in 20 ml of 0.5X TBE buffer for 60 minutes with agitation, per manufacturer's instructions. Gels were imaged on a Kodak Electrophoresis Documentation and Analysis System 120 using UV illumination. Images were quantified by Image J software (NIH) and multiple independent experiments were normalized and averaged. For oxidative stress experiments, cells were treated with 500 μM sodium arsenite (Sigma Aldrich) for 2 hours in the presence or absence of 300 ng/ml ANG. For irradiation experiments, WT C57BL/6 mice were irradiated with 4.0 Gy TBI. Twenty four hours post-TBI, LKS cells or myeloid-restricted progenitors were sorted and treated in vivo with 300 ng/ml ANG for 2 hours in PBS at 37°C/5% CO2. For culture experiments, sorted LKS cells were either immediately stimulated with ANG or cultured for 7 days in the presence or absence of ANG in S-clone media, as indicated above. On Day 7, cells cultured in the presence or absence of ANG were harvested, washed once in PBS, and again stimulated with or without 300 ng/ml ANG for 2 hours in PBS at 37°C/5% CO2.

[00233] **Northern Blotting** Total RNA was isolated from ANG-treated LKS cells or myeloid-restricted progenitors and subjected to electrophoresis, as described above. RNA was transferred to a Pall Biodyne nylon membrane (Promega) using wet transfer. Briefly, a transfer cassette was assembled with the following pre-wet components: sponge, 3 pieces Whatman chromatography paper, gel, membrane, 3 pieces Whatman chromatography paper, and sponge. The apparatus was then transferred in pre-chilled 0.5X TBE at 80 V for 60 minutes at 4°C. Following transfer, the apparatus was disassembled and the membrane rinsed in 1X TBE. Transfer efficiency was confirmed by post-transfer staining of the gel with SYBR Gold, as

described above. RNA was fixed to the blot by baking at 80°C for 2 hours. The membrane was rinsed in pre-warmed digoxigenin (DIG) Easy Hyb buffer (Roche) for 30 minutes at 50°C with rotation and then hybridized in DIG Easy Hyb buffer containing DIG-labeled DNA Probe (IDT) at 25 ng/ml. For 5'-Gly-GCC the HPLC-purified DIG-labeled probe with the sequence of 5'-GGCGAGAATTCTACCACTGAACCACCAA-3' was used. The probe was heat-denatured for 5 minutes prior to hybridization. Following overnight hybridization, membranes were rinsed once in 2X SSC/0.1% SDS for 10 minutes at 60°C, twice in 0.5X SSC/0.1% SDS for 20 minutes at 60°C and once for 5 minutes in Washing Buffer (Roche) at RT, all with agitation. Following stringency washes, the membranes were blocked for 30 minutes, rocking at RT in blocking solution (Roche), probed with alkaline phosphatase-labeled anti-DIG antibody (Roche) for 30 minutes at RT, washed twice for 20 minutes per wash with washing buffer (Roche), equilibrated for 5 minutes in detection buffer (Roche), and visualized with CSPD (Roche), per manufacturer's instruction.

tiRNA Transfection

[00234] Active 5'-P-tiRNA-Gly-GCC (5'-P-AUUGGUGGUUCAGUGGUAGAAUUCUCGCCUGCC-3') was commercially synthesized (IDT). Inactive, 5'-dephosphorylated (d)5'-P-tiRNA was generated by treating active 5'-P-tiRNA with acid phosphatase (Sigma). Sorted LKS cells or myeloid-restricted progenitors were transfected with 1 μM of 5'-P-tiRNA-Gly-GCC or (d)5'-P- tiRNA-Gly-GCC using Lipofectamine 2000 (Invitrogen), as previously described (Yamasaki et al., 2009; Ivanov et al., 2011).

[00235] **Immunofluorescence and Confocal Microscopy** LKS cells or myeloid-restricted progenitors were sorted directly onto poly-L-lysine coated slides (Thermo Scientific). Cells were allowed to settle onto the slide for 20 minutes, fixed in methanol at RT for 10 minutes, washed once with PBS, and blocked with 30 mg/ml BSA/PBS at 37°C for 1 hour. Cells were stained with primary antibody in a humidified chamber at 4°C overnight. For ANG/PABP localization, cells were stained with R163 rabbit polyclonal antibody (pAb) of ANG (10 μg/ml) and F-20 goat pAb of PABP (Santa Cruz #sc-18611, 1:50 dilution), followed with AF488-conjugated goat anti-rabbit (Thermo Scientific A11070, 1:600 dilution) and AF555- conjugated

donkey anti-goat (Thermo Scientific A21432, 1:600 dilution). For RNH1/PABP localization, cells were stained with R127 rabbit pAb of RNH1 (5 μg/ml) and F-20 goat pAb of PABP followed with AF488-conjugated goat anti-rabbit AF488 and AF555-conjugated donkey anti-goat. For ANG/RNH1 localization, cells were stained with an in-house made mouse ANG-specific C527 monoclonal antibody (10 μg/ml) and R127 rabbit pAb of RNH1 (5 μg/ml), followed with AF488-conjugated rabbit anti-mouse (Thermo Scientific A11059, 1:600 dilution) and AF555-conjugated goat anti-rabbit (Thermo Scientific A21428, 1:600 dilution). Appropriate isotype controls were used at the same concentration. Images were acquired using Nikon A1R confocal microscopy.

[00236] **Fluorescence Resonance Energy Transfer (FRET)** FRET was performed using the acceptor photo-bleaching method, as previously described (Pizzo et al., 2013). Briefly, AF488 was used as the donor and AF555 as the acceptors. Signals were photobleached to less than 10% of the initial fluorescent measurement. ROI measurements from LKS cells and myeloid-restricted progenitors were taken from 10 individual cells. FRET effiiency was calculated using the formula E=(IDA-ID)/ID, where ID and IDA are fluorescence intensities before and after photobleaching, respectively. FRET was performed using Leica SP2 confocal microscopy.

Methods Table 2. Mouse qRT-PCR Primer Sequences

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
p21	TGGAGTCAGGCGCAGATCCAC	CGCCATGAGCGCATCGCAATC
p27	AGGCAAACTCTGAGGACCGGCA	TGCTCCACAGTGCCAGCGTTC
p57	CGAGGAGCAGGACGAGAATC	GAAGAAGTCGTTCGCATTGGC
GATA3	GGTATCCTCCGACCCACCAC	CCAGCCAGGGCAGAGATCC
vWF	GGCGAGGATGGAGTTCGACA	TGACAGGGCTGATGGTCTGG
Bmi1	AAACCAGACCACTCCTGAACA	TCTTCTTCTCTTCATCTCATTTTTGA
Cyclin D1	GCGTACCCTGACACCAATCTCCTC	ACCTCCTCTTCGCACTTCTGCTCC
47S	TCCCGACTACTTCACTCCTG	CAAGAGAACACAACGAGCGAC
28S	CGCGACCTCAGATCAGACGT	GCTCTTCCCTGTTCACTCGC
A1	GCTTGTTTCTCCCGATTGCG	ACACATCCACAAGGACCACG
A1-CT	GCGCACTTTTCTCAAGTGGT	TGAAACACGTGAGGGCACAA
a1	CCCTGGCTGAGCACTACCTT	CTGCATGCTTGGCTTGGA
Bcl2	TGGGATGCCTTTGTGGAACT	ACAGCCAGGAGAAATCAAACAG

Bcl-xl	GGCTGGGACACTTTTGTGGAT	GCGCTCCTGGCCTTTCC
Mcl1	CCCTCCCCCATCCTAATCAG	AGTAACAATGGAAAGCATGCCAAT
Bak	AATGGCATCTGGACAAGGAC	GTTCCTGCTGGTGGAGGTAA
Bax	TGGAGCTGCAGAGGATGATTG	AGCTGCCACCCGGAAGA
Bid	GAAGACGAGCTGCAGACAGATG	AATCTGGCTCTATTCTTCCTTGGTT
Bim	TTGGAGCTCTGCGGTCCTT	CAGCGGAGGTGGTGTGAAT
Noxa	GGAGTGCACCGGACATAACT	TTGAGCACACTCGTCCTTCA
Puma	GCGGCGGAGACAAGAAGA	AGTCCCATGAAGAGATTGTACATGAC
β-Actin	GACGGCCAGGTCATCACTATTG	AGGAAGGCTGGAAAAGAGCC

Methods Table 3. Human qRT-PCR Primer Sequences

Gene	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**
p21	GTCACTGTCTTGTACCCTTGTG	CGGCGTTTGGAGTGGTAGAAA
p27	TGCAACCGACGATTCTTCTACTCAA	CAAGCAGTGATGTATCTGATAAACAAGG
p57	AGAGATCAGCGCCTGAGAAG	GGGCTCTTTGGGCTCTAAAC
GATA3	ACCACAACCACACTCTGGAGGA	TCGGTTTCTGGTCTGGATGCCT
vWF	CGGCTTGCACCATTCAGCTA	TGCAGAAGTGAGTATCACAGCCATC
Bmi1	AATCCCCACCTGATGTGTGT	GCTGGTCTCCAGGTAACGAA
Cyclin D1	AGCTCCTGTGCTGCGAAGTGGAAAC	AGTGTTCAATGAAATCGTGCGGGGT
β-Actin	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT

EXAMPLE 1

Experimental platform for proximity based study of HSPC niche.

[00237] To test our hypothesis, we adapted the experimental platform used in the above-mentioned in vivo imaging experiments (Lo Celso et al., 2009 by intravenously injecting adult bone marrow LT-HSCs (lineage-negative (lin- kit+ Sca1+ [LKS] CD34−Flk2− fluorescently labeled with a lipophilic membrane-bound dye, DiI, into irradiated col2.3GFP mice (Kalajzik et al, 2002) (Fig. 1, top panel . However, we performed our experiments in neonatal col2.3GFP recipients, which offered a technical advantage of being able to isolate OLCs without bone decalcification, which would have made the samples unsuitable for the transcriptome analysis. Forty-eight hours after LT-HSC injection, the animals were sacrificed; femoral bones were dissected and immediately sectioned on a vibratome. Upon examination of multiple sections, we identified rare instances where single DiI-positive transplanted HSPCs were seen immediately adjacent to individual OLCs at the endosteal surface. Contrary to other transplanted cells, these cells had not formed clusters forty-eight hours after transplantation; we therefore assumed that

they remained quiescent throughout this time and would therefore serve as precise spatial "pointers" towards putative quiescence-regulating OLCs.

[00238] In order to retrieve OLCs directly from a section of neonatal trabecular bone, we modified the standard patch clump microscopy platform by introducing additional steps for tissue immobilization and in/situ enzymatic digestion under direct visual control [see Methods]. The tip diameter and micropipette geometry were optimized to enable aspiration of intact OLCs without cell membrane damage (as verified by the presence of cytoplasmic GFP signal to prevent mRNA leakage. We harvested individual proximal and distal OLCs as shown (Fig. 1, bottom panel, and performed comparative transcriptome analysis by single cell RNA-Seq (Tang et al., 2009).

Proximal OLCs have a distinct transcriptional signature

[00239] Given the rarity of proximal OLCs in tissue sections, a maximum of two proximal OLCs (and distal OLCs controls were harvested per each transplanted animal. In total, sixteen proximal OLCs and sixteen distal OLCs were retrieved. Following cDNA amplification and quality control [see Methods], eight cells from each group were selected for single cell RNA-Seq analysis. In order to accommodate for biological and technical noise commonly observed in single cell RNA- Seq experiments, we developed a probabilistic method, which uses Bayesian approach to estimate the likelihood of expression magnitude based on the observed reads for a gene in question and the overall error characteristics within the transcriptome of a particular single cell sample – Single Cell Differential Expression (SCDE (Kharchenko et al., 2014). By comparing combined probabilistic estimates from single cell transcriptomes across the samples in each group, the method estimated the likelihood that the level of expression of a given gene differed between proximal and distal OLCs (Vcam-1 gene shown as a representative example, Fig. 2A . Using the top 200 differentially expressed genes, we found that profiles of proximal OLCs are clustered separately from the profiles of distal OLCs (Fig. 3A). To test whether proximal and distal OLCs could be distinguished in an unbiased manner based on a genome-wide transcriptional signature, we performed cross-validation tests using the "leave-two-out" strategy. Specifically, transcriptional signatures of one proximal and one distal OLC were "left out" from the 16-cell dataset, a machine-learning classifier was trained on the remaining cells, and the ability of the classifier to correctly assign the transcriptomes of the "left-out" cells to

either proximal or distal group was evaluated (Rizzo, 2007) . The process was repeated for all proximal-distal cell pairs (64 possible combinations in total). Despite a small sample size, the majority of "left-out" samples were correctly classified (Fig.3B), area under the curve [AUC]=0.854, $p<10\text{-}5$ indicating that the proximal and distal OLCs displayed stable genome-wide transcriptional differences. In particular, gene set enrichment analysis showed that proximal OLCs displayed a significant up-regulation of genes encoding cell surface proteins (p-value $6.8\times10\text{-}4$, Q-value 0.048; top genes: Vcam1, Adam9, Amot and those involved in immune response (p-value $3.1\times10\text{-}6$, Q-value 0.0090; top genes: Map3k14, Cxcl12, Il18, supporting their role in intercellular communications (Fig. 2B). At the level of individual genes, we found that with the exception of c-kit, proximal OLCs had significantly higher expression levels of niche-associated molecules (most notably Cxcl12 and Vcam-1 as compared to distal OLCs. Further, in accordance with prior studies of a regulatory OLC phenotype, proximal OLCs were lineage-committed (Runx2+, Sp7/osterix+, col1a1+ but less mature (Spp1/osteopontinlow, Bglap/osteocalcinlow, Dmp1low than distal OLCs (Fig. 3C,D).

[00240] Taken together, these data demonstrate that a proximity-based approach enabled identification of the OLC fraction which is transcriptionally distinct from the remaining OLCs and whose signature is consistent with HSPC regulatory function. Our ability to detect consistent transcriptional features of proximal OLCs despite a limited sample number and inter-sample variability indicates that cellular proximity acts as a powerful and reliable discriminator between molecularly distinct subset within an apparently homogeneous, lineage-restricted cell population.

[00241] Based on these findings, we set out to test whether the proximal OLC signature could be used as a resource for identification of novel non cell-autonomous HSPC regulators in vivo. Among membrane-bound and secreted factors that were preferentially expressed in proximal OLCs, we chose three molecules from distinct functional groups for further validation. These included secreted RNase angiogenin (ANG), pro-inflammatory cytokine interleukin 18 (IL18, and cell adhesion molecule Embigin. ANG derived from committed osteoprogenitors, mesenchymal progenitors and peri arteriolar sheath cells, but not mature osteoblasts, regulates LT HSC quiescence. ANG is a secreted ribonuclease with established roles in promoting tumor

angiogenesis and cellular proliferation (Kishimoto et al., 2005). It also acts as a neuronal pro-survival factor in the context of amyotrophic lateral sclerosis (ALS (Greenway et al., 2006).

[00242] We found that Ang was expressed at a higher level in proximal OLCs (Figure 4A) and undertook a functional evaluation of its role in the bone marrow niche using AngKO mice (as described in the accompanying manuscript by Goncalves et al or mice in which Ang/was conditionally deleted from distinct niche cell subsets. We crossed Ang "floxed" mice with animals in which tamoxifen-inducible Cre-recombinase was driven by the promoters targeting specific mesenchymal cells – committed osteoprogenitors (Osx) (Mizoguchi et al., 2014), mesenchymal progenitors (nestin (Mendez-Ferrer et al., 2010), periarteriolar sheath cells (NG2) (Zhu et al.,2011) and mature osteoblasts (Col1a1 (Kim et al., 2004). Ang transcripts were detectable in Osx+ cells by Q-PCR (data not shown); Ang expression in other niche cell subsets mentioned above has been previously documented (Kunisaki et al., 2013) (Paic et al., 2009).

[00243] All conditional knock-outs demonstrated no significant changes in peripheral blood or bone marrow changes, apart from mild lymphocytosis (Table 1). However, immunophenotypic analysis of primitive hematopoietic cells (Figure 5A) revealed that deletion of Ang from Osx+, Nes+ and NG2+ cells resulted in an increase of the number of LT-HSC and more active cycling of LT-HSC, short-term HSC (ST-HSC) and multi-potent progenitors (MPP) (Figure 4B,C and Figure 5Bi,ii, C, Di,ii). In contrast, Ang deletion with col1a1Cre had no effect on these cell populations, but was associated with an increase in number and more active cycling of common lymphoid progenitors (CLP), as was also seen upon Ang/deletion from Nes+ and NG2+ cells (Figure 4D,E). The number and cell cycle status of the myeloid progenitors in any of the above strains were unaffected by the Ang deletion (Figure 5Biii, Diii).

Table 4: Baseline bone marrow and peripheral blood profiles of conditional Ang-deleted mouse strains.

			Osx-creER^{T2}		*Nestin-creER^{T2}*		*NG2-creER^{T2}*		*Col1a1-creER^{T2}*	
Organ	Parameter	Unit	*Ang$^{+/+}$*	*Ang$^{fl/fl}$*	*Ang$^{+/+}$*	*Ang$^{fl/fl}$*	*Ang$^{+/+}$*	*Ang$^{fl/fl}$*	*Ang$^{+/+}$*	*Ang$^{fl/fl}$*
Blood	WBC	10^3/μl	11.2 ± 1.13	13.3 ± 0.70	11.4 ± 0.88	13.6 ± 0.65*	9.99 ± 1.27	13.8 ± 1.10*	11.0 ± 0.96	13.8 ± 0.70*
	LYM	10^3/μl	9.61 ± 1.05	11.4 ± 0.73	8.90 ± 0.8	10.9 ± 0.38*	9.11 ± 1.16	12.7 ± 1.07*	9.22 ± 1.32	12.1 ± 0.62*
	MON	10^3/μl	0.20 ± 0.02	0.20 ± 0.07	0.18 ± 0.02	0.15 ± 0.03	0.34 ± 0.09	0.42 ± 0.07	0.23 ± 0.01	0.30 ± 0.07
	NEU	10^3/μl	1.22 ± 0.08	1.67 ± 0.31	2.32 ± 0.29	2.56 ± 0.36	0.54 ± 0.14	0.63 ± 0.08	1.58 ± 0.50	1.34 ± 0.15
	RBC	10^6/μl	9.92 ± 0.97	9.76 ± 0.85	9.67 ± 0.54	10.6 ± 1.20	9.51 ± 1.34	9.21 ± 1.37	8.82 ± 0.74	9.37 ± 0.22
	HGB	g/dl	13.6 ± 1.15	13.1 ± 1.17	11.6 ± 1.00	12.1 ± 0.76	12.5 ± 0.44	11.6 ± 0.55	13.2 ± 0.55	13.0 ± 0.22
	HCT	%	44.0 ± 1.92	45.1 ± 2.43	38.7 ± 1.04	39.7 ± 4.28	42.3 ± 0.73	41.6 ± 2.28	41.8 ± 0.15	42.3 ± 0.78
	MCV	fL	44.2 ± 0.40	43.6 ± 0.71	42.6 ± 1.02	42.7 ± 1.69	43.5 ± 1.23	44.3 ± 0.56	43.5 ± 0.87	42.6 ± 0.96
	MCH	pg	14.3 ± 0.17	14.1 ± 0.52	15.3 ± 0.38	14.9 ± 0.41	13.4 ± 0.51	12.3 ± 0.74	13.8 ± 0.37	13.5 ± 0.50
	MCHC	g/dl	33.2 ± 0.72	32.1 ± 0.56	33.4 ± 0.60	32.7 ± 0.90	32.1 ± 0.51	29.9 ± 2.68	33.0 ± 0.90	32.6 ± 0.73
	RDWc	%	20.9 ± 1.85	20.4 ± 0.37	19.0 ± 0.34	19.5 ± 0.44	19.5 ± 0.80	21.3 ± 0.99	19.8 ± 0.46	21.0 ± 0.79
	PLT	10^3/μl	663 ± 60	606 ± 76	721 ± 65.4	647 ± 76.6	579 ± 109	617 ± 81.1	694 ± 154	774 ± 72.5
	Mac1+Gr1+	10^3/μl	1.34 ± 0.20	1.15 ± 0.12	1.33 ± 0.19	1.63 ± 0.10	1.05 ± 0.21	1.61 ± 0.33	1.14 ± 0.03	1.47 ± 0.28
	B220+	10^3/μl	6.69 ± 0.75	8.83 ± 0.56*	6.36 ± 0.44	8.43 ± 0.50**	4.34 ± 0.86	6.65 ± 0.58*	6.16 ± 0.83	8.63 ± 0.37**
	CD3e+	10^3/μl	2.19 ± 0.32	2.42 ± 0.27	2.20 ± 0.26	2.90 ± 0.16*	1.12 ± 0.25	2.04 ± 0.46	1.55 ± 0.19	2.33 ± 0.34
Bone Marrow	Cellularity	10^6 / femur	25.8 ± 1.40	26.8 ± 1.12	25.5 ± 1.09	26.0 ± 1.30	25.2 ± 1.21	26.9 ± 1.20	25.6 ± 0.92	25.6 ± 1.52
	Mac1+Gr1+	10^6 / femur	13.6 ± 0.86	14.0 ± 0.82	12.1 ± 0.79	11.2 ± 0.99	11.4 ± 0.56	11.7 ± 1.08	12.3 ± 0.65	12.0 ± 1.21
	Ter119+	10^6 / femur	2.92 ± 0.28	2.70 ± 0.32	3.03 ± 0.24	3.54 ± 0.52	3.28 ± 0.95	3.20 ± 1.21	1.39 ± 0.39	1.55 ± 0.31
	B220+	10^6 / femur	5.36 ± 0.47	5.84 ± 0.76	5.67 ± 0.77	6.65 ± 0.62	4.55 ± 0.56	6.77 ± 0.71*	4.87 ± 0.23	6.66 ± 0.52*
	CD3e+	10^6 / femur	0.55 ± 0.07	0.66 ± 0.10	0.49 ± 0.07	0.52 ± 0.06	0.87 ± 0.30	0.88 ± 0.36	0.63 ± 0.05	0.75 ± 0.06

Data represent mean ± SEM. Statistical significance was assessed by two-tailed Student's t-test.

*$p<0.05$, **$p<0.01$

Osx-creERT2 n = 8-9

Nes-creERT2 n = 9-10

NG2-creERT2 n = 6

Cola1a-creERT2 n = 4-8

[00244] To assess the effect of the above-noted changes on long-term hematopoietic reconstitution, we competitively transplanted the bone marrow from Angfl/flOsxCre, Angfl/flNesCre, Angfl/flNG2Cre, and Angfl/flCol1a1Cre mice and corresponding controls into congenic WT recipients (Figure 4F). We observed significantly reduced long-term multi-lineage reconstitution in the recipients of the bone marrow from Angfl/flOsxCre, Angfl/flNesCre, Angfl/flNG2Cre mice while the animals which were transplanted with Angfl/flCol1a1Cre bone marrow displayed only a lymphoid reconstitution defect.

[00245] Taken together, our observations reveal the role of ANG as a niche-derived quiescence regulator of LT-HSC, ST-HSC, MPP and CLP and highlight differences in the target cell populations depending on a cellular source: ANG produced by mesenchymal progenitors, committed osteoprogenitors and peri-arteriolar sheath cells regulates quiescence and repopulating ability of LT-HSC, while ANG derived from mature osteoblasts regulates lymphoid progenitors. IL-18 regulates quiescence of short-term, hematopoietic, progenitors; IL 18 is a pro-inflammatory cytokine, which acts as a regulator of T-cell function through induction of interferon-gamma production (Okamura et al., 1995). It also serves as a regulator of stress response by the immune system. IL18 is expressed in multiple cell types within and outside the bone marrow (Novick et al., 2013; Sugama and Conti, 2008). Proximity-based analysis revealed IL18 expression in proximal OLCs, while none of the distal OLCs had detectable IL18 transcripts (Fig. 6A).

[00246] We used IL18 knock-out (IL18KO mice) to investigate a functional role of IL18 in hematopoiesis. These animals displayed no apparent abnormalities in the bone marrow and peripheral blood, apart from modest neutrophilia (Fig. 7A-C). However, BrdU incorporation studies showed an increased uptake in short-term hematopoietic progenitors - ST-HSC and MPP - but not in LT-HSC (Fig. 6B). These changes mirrored the pattern of the IL18 receptor (IL18R1)

expression, which was undetectable in LT-HSCs but present in short-term progenitors (Fig. 6C). These observations indicated that IL18 regulates quiescence of short-term progenitors.

[00247] Functionally, these cells are critical for replenishing blood cells following bone marrow injury. Quantification of progenitor cell subsets on 7 days post-exposure to 5-FU (Broxmeyer et al., 2012 showed a significantly increased frequency of LKS cells, lin-kit+Sca1- myeloid progenitors and CLPs in IL18KO mice, as compared to 5-FU-treated WT controls (Fig. 6D). In newborn IL18KO animals, loss of HSPC quiescence at baseline and exaggerated response to genotoxic injury (busulphan exposure in utero (Bruscia et al., 2006) were also observed (Fig. 8A-C). Taken together, these data demonstrate that IL18 normally constrains progenitor proliferation. Consistent with this, exogenous administration of recombinant IL18 protected LKS cells from 5- FU-induced apoptosis, but also resulted in decreased frequency of lineage-negative cells in rIL18-treated animals (Fig. 6E), indicating the IL18 can suppress progenitor response to injury and restrain hematopoietic recovery.

[00248] To test if the quiescence-inducing effect of IL18 on short-term progenitors is exerted in a non-cell-autonomous fashion, WT (CD45.1) bone marrow cells were transplanted into lethally irradiated IL18KO or WT recipients (CD45.2). We found that IL18-deficient microenvironment in the recipient animals conferred a significantly faster short-term hematopoietic recovery without affecting long-term reconstitution (Fig. 7D). In keeping with this, transplantation of progenitor- enriched WT bone marrow fraction (LKS cells into IL18KO hosts was accompanied by approximately 2-fold increase in both myeloid (week 2) and lymphoid (week 4) cells in peripheral blood of the recipient animals, which was no longer detectable at week 16 (Fig. 6F). The finding of enhanced early post-transplant reconstitution in the absence of IL18 signaling was recapitulated in a reciprocal experiment, when sorted LKS cells from IL18 receptor knock-out animals were transplanted into WT hosts (Fig. 6G), indicating that the effect of IL18 on short-term progenitors is likely to be direct. Interestingly, faster proliferation of transplanted LKS cells in IL18KO recipients was already evident at 24 hours, as shown by intra-vital imaging studies, and was associated with homing further away from the endosteal surface indicating that IL18 also regulates progenitor localization in the niche (Fig. 7E-G).

[00249] To test if the effect of IL18 on post-transplant progenitor expansion can be explored therapeutically, we transplanted lethally irradiated IL18KO and WT recipients with a limiting dose of WT bone marrow and found improved survival in the IL18KO group (Fig. 7H). This raises a possibility that IL18 neutralization might be a means of reducing post-transplant cytopenias – a major cause of morbidity and mortality in patients. Given that in humans, the highest level of IL18R expression is observed in the most primitive HSPC (Fig. 9), IL18 blockade may have an additional effect on post-transplant long-term HSC expansion.

Embigin regulates localization and quiescence of long-term HSC and short-term progenitors

[00250] Embigin is a cell adhesion molecule of immunoglobulin superfamily (Huang et al., 1990, 1993). Embigin is thought to enhance integrin-dependent cell substrate adhesion and was also shown to promote neuromuscular synapse formation (Lain et al., 2009). Embigin is widely expressed within the hematopoietic system, including primitive hematopoietic cells (Pridans et al., 2008), but its function remains obscure.

EXAMPLE 2

[00251] Our proximity-based analysis showed that proximal OLCs had a significantly higher level of Embigin expression compared to distal OLCs (Fig. 10A), and we undertook in vivo functional studies to evaluate its role as a hematopoietic regulator. In the absence of an established genetic model, we used a neutralizing antibody against Embigin for these experiments (Pridans et al.,2008).

[00252] Given that Embigin is a cell adhesion molecule, the inventors assessed the effect of Embigin on HSPC localization. We found that injection of anti-Embigin resulted in mobilization of myeloid progenitors and colony-forming cells (CFC into the blood (Fig. 10B,C). On the other hand, intra-vital microscopy studies revealed that pre-transplant Embigin blockade – either by in vivo incubation of LKS cells [known to express Embigin] (Forsberg et al., 2010 with anti-Embigin or by injecting anti-Embigin into lethally irradiated hosts - resulted in a significantly lower number of transplanted LKS cells reaching calvarial bone marrow as compared to an isotype control (Fig. 10D,E), thus identifying Embigin as a homing molecule.

We also observed that WT LKS cells transplanted into anti-Embigin pre-treated recipients displayed a higher proliferation rate (Fig. 10F), indicating that Embigin may also regulate HSPC quiescence. To examine this further, we performed cell cycle and BrdU incorporation studies following injection of WT animals with anti- Embigin or isotype-control antibody and found an approximately 2-fold increase in the frequency of LT-HSCs, ST-HSCs, MPP and colony-forming cells in anti-Embigin treated animals (Fig. 11A,B). This was associated with increased BrdU incorporation by primitive hematopoietic cells (Fig.11C), a reduction in the proportion of cells in G0 phase of the cell cycle (Fig.11D) with a corresponding increase in S/G2/M phase. Consistent with the above findings, we found that bone marrow from anti-Embigin treated animals reconstituted poorly when competitively transplanted into irradiated recipients as compared to isotype-control treated marrow, likely due to the impaired HSPC homing and increased cell cycling (Fig. 11E). Taken together, these results identify Embigin as a regulator of HSPC homing and quiescence and create the rationale for future mechanistic studies to examine the role of Embigin in HSPC regeneration.

EXAMPLE 3

[00253] Our approach illustrates several important methodological and biological principles. First, it applies single cell approach to the study of the bone marrow niche and by doing so, identifies a subset of osteolineage cells (proximal OLCs which are highly enriched for membrane-bound and secreted molecules, including known HSPC regulatory molecules and those characterized by us as niche factors in the current manuscript. Thus, we show that by using single cell transcriptome comparison between individual cells which belong to the same lineage but differ only by their proximity to HSPC, a previously unrecognized heterogeneity within a cell lineage can be revealed, and a molecularly relevant and highly specialized cell subset can be defined. More fundamentally, we demonstrate that positional relationship to a heterologous cell type serves as a powerful predictor of cellular heterogeneity in vivo.

[00254] Secondly, our approach to niche factor identification was unbiased. Of the factors that were identified herein as niche regulators, none has been previously implicated in extrinsic regulation of hematopoiesis. By comparing the effect of these factors on HSPC in/vivo, we find that despite marked functional distinctions between them (cytokine, cell adhesion molecule,

secreted RNase) they converge on the same role in the niche as regulators of HSPC quiescence. Notably, Embigin and ANG regulate quiescence of all primitive hematopoietic cells while IL18 acts predominantly on short-term progenitors, yet all of them are derived from the same proximal OLC signature. This demonstrates that bone marrow niches may not be restricted to a specific cell type, but rather control a distinct cellular state, such as quiescence. Moreover, this control is achieved through multiple, previously unappreciated molecular pathways, some of which have been uncovered by our unbiased proximity-based approach. From a purely technical angle, we demonstrate that combining micropipette-assisted single cell extraction from a defined location in a tissue section with single cell RNA-Seq is feasible and enables generation of single cell cDNA libraries whose complexity closely matches that of freshly dissociated or sorted single cells (Patel et al., 2014 (Shalek et al., 2014). Further, it has several advantages over laser capture microscopy (LCM, an established method for transcriptional analysis of spatially-defined cells (Espina et al., 2006). Firstly, it enables preservation of fluorescent labeling, which/would have been lost during ethanol fixation and subsequent drying of the section in preparation for LCM procedure. Secondly, the tissue architecture and micro- anatomical relationship between the cells are more accurately represented since our method uses thicker tissue sections as compared to LCM. Finally, the ability to harvest the whole intact cell, as opposed to the cell which would have been transected during tissue preparation for LCM, reduces cross-contamination from the neighboring cells and RNA loss, which have been noted as major technical drawbacks of the LCM procedure (Shapiro et al., 2013).

[00255] The inventors focused on bone marrow transplantation herein because of its clinical relevance and the importance of finding new ways to enhance post-transplant bone marrow recovery, for example IL18-blockade. We found that all three factors which we characterized act as regulators of HSPC quiescence in the transplant context. Surprisingly, the inventors also discovered that they have a measurable effect on HSPC quiescence under homeostatic conditions, indicating that despite marked differences in unconditioned and post-irradiation bone marrow niche, our platform is suitable for identification of niche factors which are active not only under conditions of stress but also in steady-state hematopoiesis.

[00256] As disclosed herein, the inventors have identified HSPC regulators based on the analysis of OLCs. IL18,/Embigin/and/Ang transcripts are detectable in several other niche cell types found in close apposition to HSPC, such as perivascular cells (Kunisaki et al., 2013), which likely act as non-redundant sources of these factors, as has been previously demonstrated for CXCL12(Ding and Morrison, 2013; Greenbaum et al., 2013), stem cell factor (Ding et al., 2012) and now shown for ANG in the current manuscript. Whether proximal OLCs also serve as a source of unique, OLC-specific niche factors remains an open question, which will be addressed by functional validation of multiple other candidate molecules which are present in the proximal OLC signature.

[00257] In summary, the inventors demonstrate that single cell proximity-based analysis serves as unbiased strategy for identification of niche-derived regulators, offers new insights into the molecular regulation of HSPC quiescence and opens unexplored avenues for translational approaches to enhance HSPC regeneration. Recent advances in in/situ transcriptome analysis methodology offered by TEVA (Lovatt et al., 2014), Fisseq (Lee et al., 2014)or MERFISH (Chen et al., 2015), will facilitate application of the proximity-based analysis which was designed and validated by the current study, to define the molecules and cell subsets intimately involved in inter-cellular communications in healthy and diseased tissue.

EXAMPLE 4

ANG is a non-cell autonomous regulator of LT-HSC quiescence and self-renewal

[00258] To functionally and mechanistically characterize the role of ANG in hematopoiesis, we first profiled HSPC in the BM of Ang knockout (Ang-/-) mice and found a 2-fold increase in the number of LT-HSCs (Flk2−CD34− Lin−c-Kit+Sca1+ [LKS]), but not short-term (ST)-HSCs (Flk2−CD34+ LKS) or multi-potent progenitors (MPP; Flk2+CD34+ LKS) in Ang-/- BM (Figure 12A; detailed gating scheme in Figure 13A). Consistently, a reduction in G0 phase and a corresponding increase in S/G2/M phases of the cell cycle (Figure 12B), as well as enhanced BrdU incorporation (Figure 13B) was observed in Ang-/- LT-HSCs. Ang-/- ST-HSCs and MPPs also displayed increased cycling (Figure 12B, 13B) but curiously no difference in cell number (Figure 12A), which could be attributed, at least in part, to elevated apoptosis across hematopoietic lineages in Ang-/- mice (Figure 13C). This observation is consistent with the anti-

apoptotic function of ANG in other cell types (Kieran et al., 2008; Li et al., 2010). These patterns were also observable by other commonly used cell surface markers (Figure 13D-E), confirming that LT-HSCs in Ang-/- BM cycle more actively than in WT BM. Despite the dramatic increase in LT-HSC number in Ang-/- BM (Figure 12A, 13D), only mild lymphocytosis was apparent at baseline in 8-12 week old mice (Table 5). However, under conditions of stress, progenitor response to the genotoxic agent, 5-fluorouracil (5-FU), was markedly exaggerated in Ang-/- mice (Figure 12C). Further, exposure of these animals to serial proliferative stress, such as weekly injections of 5-FU, resulted in excess animal morality (Figure 12D). Consistent with the phenotype of stress-induced exhaustion (Orford and Scadden, 2008), aged 22 month old Ang-/- mice developed leukopenia (Table 6) and showed a marked reduction in the number of primitive hematopoietic cells in the BM (Figure 13F), accompanied by more active HSPC cycling (Figure 13G). Aged Ang-/- mice also displayed reduced functional capabilities by in vivo methylcellulose assays (Figure 13H-I) and in vivo competitive transplantation (Figure 13J-K). To further characterize the functional significance of ANG-deficiency-induced loss of HSPC quiescence, transplant experiments were performed by injecting either total BM (Figure 13L) or purified LT-HSCs (Figure 12E) into lethally-irradiated WT or Ang-/- hosts. In both experiments, impaired long-term multi-lineage reconstitution was observed in Ang-/- hosts (Figure 12F, 13M) with particularly pronounced impairment at later time points. Notably, WT HSPC in the ANG-deficient microenvironment displayed dramatically reduced HSPC number, accompanied by more active cycling (Figure 12G-H). To rule out a homing defect as a cause of impaired reconstitution in Ang-/- hosts, CD45.1 lineage-negative cells were injected into irradiated WT or Ang-/- recipients, and no difference in the percentage of LKS cells or Lin− c- Kit+ Sca1− myeloid-restricted progenitors in the BM of these animals was observed 16 hours after transplantation (Figure 13N). In order to evaluate the effect of niche-derived ANG on HSC self-renewal, we carried out serial transplantation experiments. When performed non-competitively, injection of an equal number of whole BM cells from primary Ang-/- recipients strikingly resulted in death of all secondary Ang-/- recipients (Figure 13O), while competitive transplantation demonstrated no detectable hematopoietic contribution by LT-HSCs that had been passaged through ANG- deficient primary recipients (Figure 12I). The marked inability to reconstitute in both transplant settings indicates severe loss of HSC self-renewal capacity in ANG-deficient hosts. Taken together, these data demonstrate that ANG acts as a non-cell

autonomous regulator of quiescence and self-renewal of primitive hematopoietic cells, particularly LT-HSC.

Table 5: Cell counts for 8-12 week old Ang-/- mice

Organ	Parameter	Unit	WT	*Ang*$^{-/-}$
Blood	WBC	x 10^3/µl	8.60 ± 1.07	12.0 ± 1.18 *
	LYM	x 10^3/µl	5.57 ± 0.89	8.90 ± 1.21 *
	MON	x 10^3/µl	0.93 ± 0.30	0.80 ± 0.24
	NEU	x 10^3/µl	2.10 ± 0.38	2.29 ± 0.39
	PLT	x 10^3/µl	568 ± 80.2	665 ± 103
	Mac1$^+$Gr1$^+$	x 10^3/µl	0.77 ± 0.08	0.53 ± 0.06 *
	B220$^+$	x 10^3/µl	4.21 ± 0.54	6.36 ± 1.88 *
	CD3e$^+$	x 10^3/µl	2.88 ± 0.34	2.74 ± 0.35
Bone Marrow	Cellularity	x 10^6 / femur	21.0 ± 0.65	20.2 ± 1.59
	Mac1$^+$Gr1$^+$	x 10^6 / femur	12.3 ± 0.65	9.78 ± 0.96 *
	Ter119$^+$	x 10^6 / femur	2.15 ± 0.35	2.03 ± 0.26
	B220$^+$	x 10^6 / femur	4.69 ± 0.31	6.23 ± 0.45 *
	CD3e$^+$	x 10^6 / femur	0.57 ± 0.04	0.58 ± 0.06

Data represent mean ± SEM.
*p<0.05
n = 9

Table 6: Cell counts for 22 month old Ang-/-mice

Organ	Parameter	Unit	WT	*Ang*$^{-/-}$
Blood	WBC	x 10^3/µl	9.03 ± 1.72	4.67 ± 0.56 *
	LYM	x 10^3/µl	6.89 ± 1.28	3.27 ± 0.64 *
	MON	x 10^3/µl	0.22 ± 0.08	0.14 ± 0.02
	NEU	x 10^3/µl	1.92 ± 0.55	1.26 ± 0.12
	PLT	x 10^3/µl	960 ± 71.9	1038 ± 89.1
	Mac1$^+$Gr1$^+$	x 10^3/µl	0.38 ± 0.07	0.17 ± 0.02 *
	B220$^+$	x 10^3/µl	6.18 ± 1.34	2.63 ± 0.37 *
	CD3e$^+$	x 10^3/µl	0.69 ± 0.13	0.60 ± 0.11
Bone Marrow	Cellularity	x 10^6 / femur	31.0 ± 1.17	27.3 ± 1.01 *
	Mac1$^+$Gr1$^+$	x 10^6 / femur	16.3 ± 0.77	12.6 ± 0.40 **
	Ter119$^+$	x 10^6 / femur	4.05 ± 0.44	2.73 ± 0.28 *
	B220$^+$	x 10^6 / femur	5.01 ± 0.35	3.03 ± 0.34 **
	CD3e$^+$	x 10^6 / femur	1.05 ± 0.14	0.49 ± 0.08 **

Data represent mean ± SEM.
*p<0.05, **p<0.01
n = 5

EXAMPLE 5

ANG enhances myeloid-restricted progenitor cell proliferation while keeping HSPC quiescent

[00259] The finding that ANG restricts cell cycling of HSPC is the first evidence for a suppressive activity of ANG on cell proliferation, as all previous studies showed that ANG promotes cell proliferation (Li and Hu, 2010). We therefore examined cell-type specific effects of ANG in various cells of the hematopoietic lineage. We observed that while Ang-/- LKS cells cycle more actively, Ang-/- myeloid-restricted progenitors showed restricted, rather than enhanced, cycling (Figure 14A). Consistently, we observed an increase of in vivo BrdU incorporation in LKS cells but a marked decrease in myeloid-restricted progenitors in Ang-/-

mice, relative to WT controls (Figure 15A). The cell-context specificity of ANG was further illustrated by analyzing lymphoid- restricted and myeloid-restricted progenitors including common lymphoid progenitors (CLP; Lin−IL7R+Flk2+B220−), pre-pro B cells (Lin−IL7R+Flk2+B220+), common myeloid progenitors(CMP; Lin−c-Kit+Sca1−CD34+CD16/32−), granulocyte-macrophage progenitors (GMP; Lin−c-Kit+Sca1−CD34+CD16/32+), and megakaryocyte-erythroid progenitors (MEP; Lin−c-Kit+Sca1−CD34−CD16/32−). The inventors discovered that Ang-/- CLPs and pre-pro B cells (Figure 15B) resemble HSPC by displaying more active cycling (Figure 15C) and incorporating more BrdU(Figure 15D), demonstrating that ANG restricts lymphoid progenitor proliferation. In contrast, myeloid- restricted progenitors, including CMP, GMP, and MEP, all displayed less active cycling (Figure 15F) and reduced BrdU incorporation (Figure 15G), accompanied by a reduction of CMP and GMP number (Figure 15E) in Ang-/- mice. Importantly, restricted proliferation of myeloid-biased MPP3s (CD150−CD48+CD135−CD34+LKS) was detected and more active cycling of lymphoid-biased MPP4s (CD150+CD48+CD135+CD34+LKS; Figure 14B) (Cabezas-Wallscheid et al., 2014) in Ang-/- mice was observed. Together, these data indicate that the function of ANG is cell context- specific: while ANG restricts cell proliferation in primitive HSCs and lymphoid-restricted progenitors, it promotes proliferation of myeloid-restricted progenitors. This transition occurs within the earliest phenotypically-defined lineage-biased progenitor cell types between MMP3 and MPP4.

[00260] Cell context-specific regulation of ANG was confirmed by the fact that Ang deletion resulted in decreased expression of cycle checkpoint or self-renewal genes including p21, p27, p57, GATA3, vWF, Bmi1 (Cheng et al., 2000; Frelin et al., 2013; Kent et al., 2009; Matsumoto et al., 2011; Park et al., 2003) in LKS cells but not in myeloid-restricted progenitors (Figure 15H). In contrast, the cell cycle-related gene, cyclin D1, was decreased in myeloid-restricted progenitors but not in LKS cells upon Ang deletion (Figure 15H). Testing whether they might be clinically relevant to these findings, the inventors assessed the effect of recombinant ANG protein on cultured stem and progenitor cells. Remarkably, culture with ANG for 2 hours in PBS led to a dose-dependent increase in the expression of pro-self-renewal genes in LKS cells (Figure 14C). No such change was noted in myeloid-restricted progenitors. In contrast, cyclin D1 was enhanced by ANG in myeloid-restricted progenitors but not in LKS cells (Figure 14C). A

similar pattern was observed in LT-HSCs cultured with ANG for 2 hours in PBS (Figure 15I) or under longer culture conditions in S-clone media (Figure 15J). Notably, addition of exogenous ANG rescued the reduced pro-self-renewal transcripts observed in Ang-/- LKS cells (Figure 15K). Together, these data demonstrate that ANG differentially regulates gene expression in HSC and progenitors, including genes relevant for proliferation and self-renewal.

ANG dichotomously regulates protein synthesis in LKS and myeloid-restricted progenitor cells

[00261] ANG has been shown in other cell types to regulate global protein synthesis, a housekeeping function recently shown to be tightly regulated in primitive HSCs (Signer et al., 2014). To determine whether ANG regulates protein synthesis in HSPC, we assessed in vivo protein synthesis in Ang-/- mice by a fluorogenic assay using O-propargyl-puromycin (OP-Puro) (Signeret al., 2014). Consistent with increased cell cycling, Ang-/- LKS cells showed a higher rate of protein synthesis while Ang-/- myeloid-restricted progenitors demonstrated reduced protein synthesis (Figure 16A). This cell context specificity was also evident when BM was analyzed with more specific markers for HSPC, lineage-restricted progenitors, and mature hematopoietic cells (Figure 17A). In vivo administration of OP-Puro did not alter BM cellularity or LT-HSC frequency (Figures 17B-C). Significantly, in vivo culture of LKS cells with ANG led to reduced protein synthesis, while ANG addition to myeloid-restricted progenitors enhanced protein synthesis (Figure 16B). Together, these data demonstrate that the effect of ANG on protein synthesis is cell-context specific.

EXAMPLE 6

The restrictive function of ANG in HSPC is mediated by tiRNA

[00262] To reveal the biochemical mechanism for this dichotomous effect of ANG on protein synthesis, we first assessed rRNA transcription, which is stimulated by ANG in other cell types (Ibaragi et al., 2009; Kishimoto et al., 2005; Tsuji et al., 2005). Addition of ANG led to enhanced rRNA transcription in myeloid-restricted progenitors and whole BM cells, but not in LKS cells (Figure 16C). Further, Ang deletion resulted in a reduction in rRNA transcription in myeloid-restricted progenitors and whole BM but not in LKS cells (Figure 17D). These findings are consistent with the elevated protein synthesis rate and pro-proliferative status of myeloid-restricted progenitors following ANG treatment.

[00263] ANG has been shown to reprogram protein synthesis as a stress response to promote survival under adverse conditions. This function of ANG is mediated by tiRNA, a noncoding small RNA that specifically permits translation of anti-apoptosis genes while global protein translation is suppressed so that stressed cells have adequate time and energy to repair damage, collectively promoting cell survival (Emara et al., 2010; Fu et al., 2009; Ivanov et al., 2011; Yamasaki et al., 2009). To assess whether ANG-mediated regulation of protein synthesis is tiRNA-dependent, we assessed bulk small RNA production by electrophoresis. LKS cells exhibited dramatically higher small RNA production over myeloid-restricted progenitors at baseline (Figure 18A). tiRNA was undetectable in differentiated cell types under these conditions and was visible only when 15 μg total RNA was loaded (Figure 17E). Importantly, addition of ANG led to markedly elevated tiRNA levels in LKS cells (Figure 18A). Equal loading was affirmed by tRNA levels (indicated by arrows, Figure 18A). Addition of ANG to lineage-positive cells did not result in an increase in tiRNA levels, in contrast to significantly elevated tiRNA levels following ANG treatment of HSPC (Figure 17E, compared to Figure 18A). Consistently, Ang-/- LKS cells exhibited reduced levels of tiRNA relative to WT LKS cells (Figure 17F).

[00264] Further, an increase in tiRNA production in myeloid-restricted progenitors, but not in LKS cells, was observed following oxidative stress induced by sodium arsenite (Figure 17G). Interestingly, ANG enhanced tiRNA in LKS cells under oxidative stress, but rather suppressed oxidative stress-induced tiRNA in myeloid-restricted progenitors. These results demonstrate that ANG differentially regulates tiRNA in LKS and myeloid-restricted progenitors under both homeostatic and stress conditions.

[00265] To ensure that the bulk small RNA reflect tiRNA, we analyzed the levels of a representative tiRNA, tiRNA-Gly-CCC, by Northern blotting in ANG-treated LKS cells and myeloid-restricted progenitors. tiRNA-Gly-GCC was previously shown to be expressed in hematopoietic tissues, including BM and spleen, but was neither examined in primitive hematopoietic cells nor functionally-validated (Dhahbi et al., 2013). Figure 18B shows that tiRNA-Gly-GCC was significantly elevated in LKS cells, relative to myeloid-restricted

progenitors, and was further enhanced by exogenous ANG. Together, these data identify tiRNA as a distinct RNA species that is abundantly expressed in HSPC and that is regulated by ANG.To determine whether tiRNA is responsible for restricted protein synthesis in HSPC, we transfected synthetic tiRNA-Gly-GCC in LKS and myeloid-restricted progenitors, and assessed protein synthesis in vivo using OP-Puro. As tiRNA requires its 5'-phosphate to suppress protein synthesis (Ivanov et al., 2011), we used an inactive, dephosphorylated synthetic tiRNA-Gly-GCC, termed (d)5'-P-tiRNA, as a negative control. Expectedly, transfection of active 5'-P tiRNA, but not of inactive (d)5'-P-tiRNA, led to a significant reduction in the rate of protein synthesis in both LKS cells and myeloid-restricted progenitors (Figure 18C). Thus, tiRNA transfection phenocopies exogenous ANG on restriction of protein synthesis in LKS cells, as has been shown in Figure 3B. We also found that myeloid and lymphoid progenitor colony formation was restricted upon transfection of whole BM with active 5'-P tiRNA (Figure 17H). Moreover, transfection of active tiRNA led to upregulation of self-renewal and pro-survival genes, and downregulation of pro-apoptotic genes, in both LKS cells and myeloid-restricted progenitors (Figure 18D).

[00266] The exact subcellular compartment where tiRNA is produced by ANG is currently unknown, but it has been shown that tiRNA production is correlated to SG localization of ANG in stressed cells (Pizzo et al., 2013). The finding that ANG produces tiRNA and restricts protein synthesis only in LKS cells prompted us to examine differential localization of ANG in SGs between LKS and myeloid-restricted progenitors. We found that ANG was colocalized with PABP, a SG marker, in LKS cells, but not in myeloid-restricted progenitors (Figure 19A). Further, we found that RNase/ANG inhibitor 1 (RNH1), an endogenous ANG inhibitor that has been shown to regulate subcellular localization of ANG and tiRNA production (Pizzo et al.,2013), is localized in SGs in myeloid-restricted progenitors, but not in LKS cells (Figure 19B). This opposing localization pattern of RNH1 and ANG was further examined by double immunofluorescence (Figure 19C) and fluorescence resonance energy transfer (FRET, Figure 19D), which showed that ANG and RNH1 colocalize and interact in the nucleus, but not cytoplasm of LKS cells, and in the cytoplasm but not nucleus of myeloid-restricted progenitors.

[00267] Thus, RNH1, which is known to stoichiometrically inhibit ANG with a femto-molar Kd (Lee et al., 1989), likely inhibits nuclear ANG but not cytoplasmic ANG in LKS cells, permitting tiRNA production, whereas it inhibits cytoplasmic ANG but not nuclear ANG in myeloid-restricted progenitors to allow rRNA transcription. It is conceivable that RNH1 is an integral player in the dichotomous regulation of ANG in HSPC versus myeloid-restricted progenitor cells. To assess whether tiRNA-mediated regulation of protein synthesis affects HSPC function, we transfected LKS cells with synthetic tiRNA and competitively transplanted those cells into WT hosts. Significantly, the inventors discovered enhanced long-term multi-lineage post-transplant reconstitution of cells transfected with synthetic tiRNA, relative to untreated LKS cells or cells transfected with inactive tiRNA (Figure 18E). As ANG stimulates tiRNA production in LKS cells, these data strongly demonstrate that ANG may enhance the regenerative potential of HSPC by tiRNA-mediated alterations of protein synthesis.

ANG is a pro-regenerative factor after radio-damage

[00268] To begin to assess the pro-regenerative role of ANG, we first examined the function of ANG in the context of radiation-induced cell damage. Ang-/- mice displayed reduced survival following exposure to various doses of γ-radiation (Figure 20A), accompanied by decreased blood leukocyte recovery, reduced total BM cellularity, reduced HSPC and lymphoid-restricted progenitor number, and more active cycling (Figures 20B-G, Table 7). These data are consistent with the quiescence-inducing effect of ANG on HSPC, as discussed previously. In contrast, myeloid-restricted progenitors in Ang-/- mice showed reduced cell number, but restricted proliferation following total body irradiation (TBI) (Figure 20H-I) indicating that, normally, ANG would promote myeloid reconstitution. Ang-/- mice also demonstrated increased apoptosis in all cell types, as well as reduced lymphoid and myeloid colony formation in response to γ- radiation (Figure 20J-K). Together, these data demonstrate that ANG deficiency leads to reduced animal survival, accompanied by diminished cell number, perturbed cell cycling, and elevated apoptotic activity in hematopoietic cells. To determine whether treatment with ANG enhances survival, WT or Ang-/- mice were pretreated with ANG daily for three successive days and irradiated mice with 8.0 Gy 24 hours following the final ANG treatment. Significantly, the 30-day survival rate increased from 20% to 90% after ANG treatment, indicating that ANG is radioprotective (Figure 21A). Importantly, 80% of Ang-/- mice also

survived following ANG pretreatment whereas 100% of untreated Ang-/- mice died. Pre-treatment with ANG protected against TBI (4 Gy)-induced loss of cell number and increase in cycling of HSPC and lymphoid-restricted progenitors (Figure 22A-E, Table 8). In contrast, ANG pre-treatment not only prevented the loss of myeloid-restricted progenitors but also promoted their proliferation (Figure 22F-G), again demonstrating a dichotomous effect of ANG in regulating HSPC and myeloid-restricted progenitors under stress conditions. Moreover, ANG protected against TBI-induced apoptosis in all cell types, and led to enhanced colony formation and post-transplant reconstitution (Figure 22H-J). Together, these data demonstrate the protective function of ANG against radiation-induced BM damage, likely through induction of HSPC quiescence and promotion of myeloid-restricted progenitor proliferation.

[00269]

[00270] To assess a potential therapeutic use of ANG as a radio-mitigating agent, we irradiated mice with 8.0 Gy and began ANG treatment 24 hours later. Significantly, the majority of ANG- treated mice survived, including ANG-treated Ang-/- mice, suggesting that ANG has radio- mitigating capabilities (Figure 21B). A similar enhancement of survival was observed when ANG treatment was begun immediately following irradiation (Figure 22K). Importantly, treatment with ANG 24 hours post-irradiation prevented TBI-induced reduction of overall BM cellularity, as well as LKS cells and myeloid-restricted progenitors (Figure 21C-D, Table 8). Consistent with its dichotomous role in cell cycle kinetics, ANG restricted proliferation of LKS cells, and simultaneously enhanced proliferation of myeloid-restricted progenitors (Figure 21E). Further, ANG prevented TBI-induced apoptosis in both LKS cells and myeloid-restricted progenitors (Figure 21F). These effects on cell number, cycling, and apoptosis were also apparent using more specific cell-surface markers for stem and progenitor cell populations (Figure 22L-R). Significantly, defects in colony formation and post-transplant reconstitution can be rescued by in vivo ANG treatment (Figure 21G, 22S). We also assessed the protective and mitigative effect of ANG in lethally-irradiated animals and found that ANG treatment either before or after lethal irradiation improved survival, and enhanced BM cellularity, as well as peripheral blood content (Figure 21H-I, Table 9). Moreover, ANG significantly increased the LD50 when treatment was begun 24 hours post-TBI (Figure 21J). Further, treatment with ANG upregulated pro-self-renewal genes in LKS cells and led to enhanced pro-survival transcript levels and reduced pro-apoptotic transcripts in both LKS cells and myeloid-restricted progenitors

(Figure 21K). Importantly, ANG treatment enhanced rRNA transcription only in myeloid-restricted progenitors (Figure 21K) and tiRNA production only in LKS cells (Figure 21L) following TBI, consistent with its dichotomous role in promoting and restricting cell proliferation in these two cell types. Together, these results establish a model by which ANG simultaneously stimulates proliferation of rapidly-responding myeloid-restricted progenitors and preserves HPSC stemness, in association with enhanced hematopoietic regeneration and improved survival.

Table 7: Cell counts for irradiated Ang-/- mice

Cohort	Organ	Parameter	Unit		
				WT	*Ang*$^{-/-}$
WT vs *Ang*$^{-/-}$	Bone Marrow	Mac1^{+}Gr1^{+}	10^6 / femur	2.79 ± 0.54	0.98 ± 0.19 *
		Ter119^{+}	10^6 / femur	1.22 ± 0.17	0.65 ± 0.10 *
		B220^{+}	10^6 / femur	2.15 ± 0.29	1.24 ± 0.28 *
		CD3e^{+}	10^6 / femur	0.22 ± 0.03	0.11 ± 0.02 *

p-value relative to WT group
n=6

Table 8: Cell counts for irradiated mice

Cohort	Organ	Parameter	Unit				
				Untreated	+ANG	4 Gy	4 Gy + ANG
ANG Treatment Pre-irradiation	Bone Marrow	Mac1+Gr1+	10^6 / femur	11.3 ± 0.65	11.2 ± 1.17	6.39 ± 1.13 **	10.7 ± 1.54
		Ter119+	10^6 / femur	3.80 ± 0.17	3.49 ± 0.43	1.64 ± 0.38 ***	2.27 ± 0.55 *
		B220+	10^6 / femur	6.04 ± 0.33	5.10 ± 0.72	3.26 ± 0.45 **	4.57 ± 0.65
		CD3e+	10^6 / femur	0.58 ± 0.02	0.58 ± 0.17	0.36 ± 0.06 ***	0.62 ± 0.18
				Untreated	+ANG	4 Gy	4 Gy + ANG
ANG Treatment Post-irradiation	Bone Marrow	Mac1+Gr1+	10^6 / femur	11.9 ± 0.44	11.4 ± 1.38	3.19 ± 0.23 ***	7.98 ± 1.92
		Ter119+	10^6 / femur	2.99 ± 0.57	2.89 ± 0.41	0.78 ± 0.07 **	1.55 ± 0.41
		B220+	10^6 / femur	5.68 ± 0.33	4.54 ± 0.58	2.17 ± 0.20 **	4.50 ± 1.14
		CD3e+	10^6 / femur	0.54 ± 0.05	0.51 ± 0.15	0.28 ± 0.03 ***	0.41 ± 0.10

p-value relative to untreated group
n=6

Table 9: Cell counts for lethally-irradiated mice with ANG pre-treatment

Organ	Parameter	Unit	Day 0		Day 5		Day 10	
			Vehicle	+ANG	Vehicle	+ANG	Vehicle	+ANG
Blood	WBC	10^3/µl	6.93 ± 0.89	6.76 ± 0.69	0.99 ± 0.36	3.50 ± 0.82**	0.99 ± 0.59	4.23 ± 1.09**
	LYM	10^3/µl	4.99 ± 0.67	4.92 ± 0.46	0.54 ± 0.20	1.18 ± 0.28	0.57 ± 0.32	1.40 ± 0.36
	MON	10^3/µl	0.47 ± 0.11	0.42 ± 0.08	0.03 ± 0.01	0.27 ± 0.06**	0.09 ± 0.05	0.30 ± 0.08*
	NEU	10^3/µl	1.47 ± 0.29	1.44 ± 0.21	0.41 ± 0.15	2.05 ± 0.48**	0.32 ± 0.21	2.53 ± 0.65**
	PLT	10^3/µl	836 ± 55	845 ± 69	243 ± 53	780 ± 97***	176 ± 57	555 ± 122*
	Mac1+Gr1+	10^3/µl	0.75 ± 0.09	0.75 ± 0.08	0.06 ± 0.02	0.48 ± 0.11 **	0.004 ± 0.001	0.83 ± 0.22 **
	B220+	10^3/µl	3.61 ± 0.448	3.55 ± 0.34	0.04 ± 0.01	0.72 ± 0.25 *	0.050 ± 0.036	0.88 ± 0.26 **
	CD3e+	10^3/µl	1.94 ± 0.25	1.88 ± 0.18	0.02 ± 0.01	1.49 ± 0.48 **	0.005 ± 0.004	1.73 ± 0.58 **

n=10
Dose: 12.0 Gy
ANG Treatment: 1.25 mg/kg, three times daily pre-irradiation

EXAMPLE 8

Ex vivo treatment of LT-HSCs with recombinant ANG enhances post-transplant reconstitution

[00271] The in vivo (Figure 14C, 15H-K) and in vivo (Figure 20, 21, 22) activity of ANG in preserving HSPC stemness and in enhancing regeneration prompted us to assess its capacity in improving SCT and its potential for clinical development. Treatment of LT-HSCs with ANG in culture for 7 days led to a dose-dependent decrease of cell proliferation in WT and Ang-/- cells (Figure 23A), consistent with its ability to restrict HSC proliferation. Significantly, LKS cells cultured in the absence of ANG resulted in a reduction of tiRNA expression relative to uncultured cells (Figure 23B). In contrast, cells cultured in the presence of ANG not only maintained baseline tiRNA levels, but also their responsiveness to further ANG treatment.

[00272] To test whether restriction of proliferation would enhance transplantation efficiency, we competitively transplanted LT-HSCs that were either freshly isolated or had been cultured with or without 300 ng/ml ANG for 2 hours. Significantly, treatment with ANG led to a dramatic increase in multi-lineage post-transplant reconstitution over 24 weeks (Figure 23C). A similar enhancement in transplant efficiency was observed with LT-HSCs cultured with ANG for 7 days (Figure 24A). Enhanced regeneration was observed over 16 weeks upon secondary transplant without further ANG treatment (Figure 23D). Significantly, removal of ANG from the media after 7 days in culture did not induce proliferation (Figure 24B) and enhanced levels of pro-self- renewal transcripts were retained (Figure 24C). To confirm that improved reconstitution is not due to enhanced homing of ANG-treated cells, we transplanted ANG-treated, CFSE-labeled CD45.2 Lin- cells into irradiated CD45.1 recipients, and found no difference in homing capability, as indicated by a similar number of CFSE-positive LKS cells and myeloid-restricted progenitors in the BM 16 hours post-transplant (Figure 24D). Importantly, treatment of Ang-/-

[00273] LT-HSCs with exogenous ANG ameliorated post-transplant reconstitution defect of Ang-/- cells, and led to enhanced reconstitution over WT cells by week 16 (Figure 23E). Together, these data demonstrate that treatment of LT-HSCs with exogenous ANG significantly enhances their regenerative capabilities upon relatively short exposure, and this effect is long-lasting.

ANG improves regeneration of human cells

[00274] Given that ANG significantly improved transplantation efficiency of mouse LT-HSCs, we next examined whether human ANG has similar pro-regenerative capabilities in human cells. Consistent with the anti-proliferative effect of ANG on mouse LT-HSCs, treatment with human ANG led to a dose-dependent reduction of human CD34+ CB cell proliferation over 7 days (Figure 25A) and elevated level of pro-self-renewal transcripts (Figure 25E), whereas ANG variants that are defective in its ribonucleolytic activity (K40Q) or in receptor binding (R70A) were inactive (Figure 25A, 24E). Interestingly, R33A ANG, despite having a defective nuclear localization sequence, recapitulated the effect of WT ANG in restricting proliferation and enhancing self-renewal signature (Figure 25A, 24E). It is significant to note that a 2 hour exposure to human ANG is adequate for CD34+ human CB cells to up-regulate pro-self-renewal genes (Figure 25B), which greatly enhances the translational capability of ANG in improving SCT. The fact that R33A ANG variant is as active as WT ANG points to the dispensable role of nuclear ANG in HSPC, reinforcing the finding that cytoplasmic localization of ANG is important in preservation of HSPC stemness. Further, ANG treatment of CB cells led to slightly elevated numbers of primitive colonies (Figure 24F). Together, these data importantly indicate that in vivo properties of mouse ANG faithfully translate in a human setting, and suggest that the cellular mechanisms underlying mouse HSC regeneration may also translate into human cells.

[00275] To assess whether ANG improves transplantation efficiency of human cells, we transplanted CD34+ CB cells that had been cultured for 2 hours in the presence or absence of ANG into NSG mice at limit dilution and found that treatment with ANG led to elevated frequencies of human CD45+ cells across all doses examined in BM 16 weeks post-transplant (Figure 25C). Importantly, enhanced regeneration was multi-lineage, as confirmed by the presence of both CD19 B- lymphoid cells and CD33 myeloid cells in BM (Figure 24G-H). Remarkably, calculated LT-HSC frequency was 8.9-fold higher in ANG-treated human CD34+ CB cells relative to untreated cells (Figure 25D). Together, these data highlight the translational capacity of ANG in preservation and expansion of clinically-relevant human cells for transplantation.

EXAMPLE 9

[00276] The inventors have made several important discoveries. First, ANG has a cell context-specific role in regulating proliferation of HSPC versus myeloid-restricted progenitor cells: while promoting quiescence in the former, ANG stimulates proliferation in the latter. Second, recombinant ANG recapitulates the growth suppressive properties in vivo, and can remarkably improve post- transplant reconstitution of mouse LT-HSCs and human CD34+ CB cells in vivo. Previous studies have identified numerous factors that expand stem cell number in vivo by promoting cell proliferation (Boitano et al., 2010; Delaney et al., 2010; Fares et al., 2014; Frisch et al., 2009; Himburg et al., 2010; Hoggatt et al., 2009; North et al., 2007). However, it has been noted that cycling HSPC engraft less well upon transplantation and undergo faster exhaustion (Nakamura-Ishizu et al., 2014; Passegue et al., 2005), likely as a consequence of more active cycling, differentiation, and loss of stemness. Herein, the inventors demonstrate an improvement in regeneration by dichotomously restricting cell proliferation of more primitive HSPC while enabling increased proliferation of more mature myeloid-restricted progenitor cells. The success of SCT depends upon rapid reconstitution of mature blood cell pools to avoid infections and bleeding complications and long-term generation of mature cells from a durable cell source (Doulatov et al., 2012; Smith and Wagner, 2009). These two functions are provided by progenitor and stem cell populations, respectively.

[00277] Third, the ability of ANG to serve as a radio-mitigant is also of considerable interest, particularly given its in a model of IR injury to prevent IR injury and ability to rescue animals when administered 24 hours post-irradiation injury. Translation of this ability to humans to reduce mortality following radiation exposure is of considerable significance. Currently, there are no FDA-approved drugs to treat severely irradiated individuals (Singh et al., 2015). A number of hematopoietic growth factors have been shown in various animal models to mitigate hematopoietic syndrome of acute radiation syndrome, however only pleiotrophin has been demonstrated to improve survival when administered 24 hours post-irradiation (Himburg et al.,2014), an efficacy requirement mandated by The Radiation and Nuclear Countermeasures Program at the National Institute of Allergy and Infectious Diseases. Moreover, current standard-of-care approaches, including granulocyte colony-stimulating factor (G-CSF) and its derivatives, target a limited progenitor cell pool and requires repeated doses to combat radiation-induced

neutropenia (Singh et al., 2015). In this regard, the invention herein discovered that ANG can be used as a medical countermeasure for radiation exposure, as in a mouse model, only three ANG treatments are needed for improved animal survival, even if started 24 hours after a lethal radiation (12.0 Gy) dose.

[00278] A fourth important finding is that the technology herein identified a novel RNA-based mechanism by which hematopoiesis is regulated. Importantly, ANG promotes tiRNA production in LKS cells, in association with enhanced stemness in vivo and in vivo. Further, the invention here demonstrated that increased tiRNA production results in reduced levels of global protein synthesis in HSPC. In contrast, ANG stimulates rRNA transcription in myeloid-restricted progenitors, but not in HSPC, leading to increased protein synthesis and proliferation.

[00279] The discoveries herein are of particular importance given recent reports demonstrating tight regulation of protein synthesis in hematopoiesis, with HSCs demonstrating a reduced rate of protein synthesis relative to more lineage-restricted cell types (Signer et al., 2014). Further, a number of mutations or defects in ribosome function or protein synthesis have been shown to either promote or resist malignant hematopoiesis (Cai et al., 2015; Narla and Ebert, 2010).

[00280] Modulating tiRNA to alter protein synthesis and cell fate is unique among prior reports of regulatory mechanisms and is of particular interest because of its ability to be affected by a cell exogenous source. The notion that tiRNA can be cell state-specific in regulating hematopoiesis offers the possibility that similar distinct mechanisms may apply to other tissue types. This is of considerable biologic and, potentially, therapeutic interest.

CLAIMS

What is claimed is:

1. A method of increasing hematopoietic reconstitution in a human subject, the method comprising:
   (i) contacting a population of hematopoietic stem cells (HSCs), *ex vivo*, with an effective amount of an Angiogenin (ANG) protein or an ANG agonist,
   (ii) administering cells from step (i) to a subject, wherein the subject is in need of hematopoietic reconstitution.
2. The method of claim 1, wherein the population of HSCs are obtained from bone marrow, peripheral blood, cord blood, amniotic fluid, placental blood, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs).
3. The method of any one of claims 1-2, wherein the population of HSCs are human.
4. The method of any one of claims 1-3, wherein the population of HSCs comprises at least one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs).
5. The method of any one of claims 1-4, wherein the population of HSCs are autologous or allogeneic to the subject.
6. The method of any one of claims 1-5, further comprising culturing the population of HSCs in presence of Angiogenin prior to step (ii).
7. The method of claim 6, wherein the population of HSCs are cultured in presence of Angiogenin for at least 2 hrs.
8. The method of claim 6, wherein the population of HSCs are cultured in presence of Angiogenin for about 2 days or more.
9. The method of claim 6, wherein the population of HSCs are cultured in presence of Angiogenin for at least 7 days.
10. The method of claim 1, wherein the population of HSCs are cryopreserved prior to, or after, the contacting with ANG.
11. The method of claim 1, wherein the population of HSCs are cryopreserved in the presence of ANG.
12. The method of any one of claims 1-11, wherein the subject is susceptible to, or has decreased HSC levels as compared to a healthy subject.

13. The method of any one of claims 1-12, wherein the subject has, or will undergo a bone marrow or stem cell transplantation, or has, or will undergo chemotherapy or radiation therapy.
14. The method of any one of claims 1-13, wherein the subject has a disease or disorder selected from the group: leukemia, lymphoma, myeloma, solid tumor, a blood disorder (*e.g.,* myelodysplasia), immune disorders or anemia.
15. The method of any one of claims 14, wherein the anemia is sickle cell anemia, thalassemia or aplastic anemia.
16. The method of any one of claims 1-15, wherein ANG protein is human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1, a functional fragment thereof with a biological activity of at least 80% of human ANG protein to increase hematopoietic reconstitution in a human subject.
17. The method of claim 16 wherein the functional fragment is a human recombinant ANG polypeptide.
18. The method of any one of claims 16-17, wherein the functional fragment comprises at least amino acids 1-147 of SEQ ID NO 1.
19. The method of any one of claims 16-18, wherein the human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1 comprises a mutation K33A.
20. The method of any one of claims 16-19, wherein the functional fragment comprises an amino acid sequence of at least 80% of human ANG of SEQ ID NO: 1.
21. The method of claim 20, wherein the functional fragment of human ANG protein comprises at least 80% sequence identity to amino acids 1-147 of SEQ ID NO 1.
22. The method of claim 20, wherein the functional fragment of human ANG protein comprises at least 90% sequence identity to amino acids 1-147 of SEQ ID NO 1.
23. The method of claim 20, wherein the functional fragment of human ANG protein comprises at least 95% sequence identity to amino acids 1-147 of SEQ ID NO 1.
24. The method of claim 20, wherein the functional fragment of human ANG comprises at least 98% sequence identity to amino acids 1-147 of SEQ ID NO 1.
25. The method of any one of claims 1-24, wherein the hematopoietic reconstitution is multi-lineage hematopoietic reconstitution.
26. The method of any one of claims 1-25, wherein the hematopoietic reconstitution is long-term multi-lineage hematopoietic reconstitution.
27. The method of any one of claims 1-26, wherein the hematopoietic reconstitution comprises reconstitution of short-term hematopoietic stem cells (ST-HSC) and/or long-term (LT-HSC) hematopoietic stem cells.

28. A method for expanding a population of hematopoietic cells in a biological sample, the method comprising contacting the hematopoietic cells with an Angiogenin (ANG) protein or ANG agonist, wherein the population comprises primitive hematopoietic stem cells and myeloid restricted progenitors, and wherein the contacting is for a sufficient amount of time to allow for primitive hematopoietic stem cells quiescence and myeloid restricted progenitor proliferation.
29. The method of claim 28, wherein the biological sample is selected from the group of: cord blood, bone marrow, peripheral blood, amniotic fluid, or placental blood.
30. The method of claim 28, further comprising collecting the population of hematopoietic cells.
31. A population of primitive hematopoietic stem cells produced by the method of claim 28-30.
32. A population of myeloid restricted progenitors produced by the method of claim 28-30.
33. A cryopreserved population of hematopoietic cells comprising primitive hematopoietic stem cells and/or myeloid restricted progenitors in the presence of an angiogenin protein.
34. A blood bank comprising a population of hematopoietic cells according to claim 31 or 32.
35. A method of administering a population of hematopoietic cells to a subject, comprising administering an effective amount of the population of hematopoietic cells to the subject, wherein the population of hematopoietic cells have been contacted *ex vivo* or *in vivo* with an Angiogenin (ANG) protein or ANG agonist, wherein the population of hematopoietic stem cells comprises primitive hematopoietic stem cells and myeloid restricted progenitors, and wherein the Angiogenin protein increases primitive hematopoietic stem cells quiescence and increases myeloid restricted progenitor proliferation.
36. A method of increasing reconstitution potential of transplanted hematopoietic stem cells in a subject, the method comprising the step of administering Angiogenin (ANG) protein or an ANG agonist to the subject, prior to, during or after transplantation of HSCs, wherein the subject is a candidate for bone marrow or stem cell transplant.
37. Use of an Angiogenin (ANG) protein to increase hematopoietic reconstitution in a human subject in need thereof.
38. The use of claim 37, wherein the population of HSCs are obtained from bone marrow, peripheral blood, cord blood, amniotic fluid, placental blood, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs).
39. The use of any one of claims 37-38 wherein the population of HSCs are human.
40. The method of any one of claims 37-39, wherein the population of HSCs comprises at least one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common

lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs).

41. The use of any one of claims 39-40, wherein the population of HSCs are autologous or allogeneic to the subject.
42. The use of any one of claims 40-41, further comprising culturing the population of HSCs in presence of Angiogenin prior to step (ii).
43. The use of any one of claims 42, wherein the population of HSCs are cultured in presence of Angiogenin for at least 2 hrs.
44. The use of any one of claims 42, wherein the population of HSCs are cultured in presence of Angiogenin for about 2 days or more.
45. The use of any one of claims 42, wherein the population of HSCs are cultured in presence of Angiogenin for at least 7 days.
46. The use of any one of claims 37, wherein the population of HSCs are cryopreserved prior to, or after, the contacting with ANG.
47. The use of any one of claims 37, wherein the population of HSCs are cryopreserved in the presence of ANG.
48. The use of any one of claims 37-47, wherein the subject is susceptible to, or has decreased HSC levels as compared to a healthy subject.
49. The use of any one of claims 37-48, wherein the subject has, or will undergo a bone marrow or stem cell transplantation, or has, or will undergo chemotherapy or radiation therapy.
50. The use of any one of claims 37-49, wherein the subject has a disease or disorder selected from the group: leukemia, lymphoma, myeloma, solid tumor, a blood disorder (e.g., myelodysplasia), immune disorders or anemia.
51. The use of claim 50, wherein the anemia is sickle cell anemia, thalassemia or aplastic anemia.
52. The use of any one of claims 37-52, wherein ANG protein is human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1, a functional fragment thereof with a biological activity of at least 80% of human ANG protein to increase hematopoietic reconstitution in a human subject.
53. The use of claim 52 wherein the functional fragment is a human recombinant ANG polypeptide.
54. The use of any one of claims 52-53, wherein the functional fragment comprises at least amino acids 1-147 of SEQ ID NO 1.
55. The use of any one of claims 52-54, wherein the human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1 comprises a mutation K33A.

56. The method of any one of claims 52-55, wherein the functional fragment comprises an amino acid sequence of at least 80% of human ANG of SEQ ID NO: 1.
57. The use of claim 56, wherein the functional fragment of human ANG protein comprises at least 80% sequence identity to amino acids 1-147 of SEQ ID NO 1.
58. The use of claim 56, wherein the functional fragment of human ANG protein comprises at least 90% sequence identity to amino acids 1-147 of SEQ ID NO 1.
59. The use of claim 56, wherein the functional fragment of human ANG protein comprises at least 95% sequence identity to amino acids 1-147 of SEQ ID NO 1.
60. The use of claim 56, wherein the functional fragment of human ANG comprises at least 98% sequence identity to amino acids 1-147 of SEQ ID NO 1.
61. The use of any one of claims 37-60, wherein the hematopoietic reconstitution is multi-lineage hematopoietic reconstitution.
62. The use of any one of claims 37-61, wherein the hematopoietic reconstitution is long-term multi-lineage hematopoietic reconstitution.
63. The use of any one of claims 37-62, wherein the hematopoietic reconstitution comprises reconstitution of short-term hematopoietic stem cells (ST-HSC) and/or long-term (LT-HSC) hematopoietic stem cells.
64. A method of treating a subject that has been exposed to ionizing radiation, the method comprising administering an effective amount of an Angiogenin (ANG) protein or Angiogenin agonist to the subject.
65. The method of claim 64 wherein, the subject has been exposed to, will be exposed to or is at a risk of exposure to ionizing radiation.
66. The method of claim 64, wherein the subject is a mammal.
67. The method of claim 64, wherein the subject will, or has undergone, radiation therapy for the treatment of a disease or disorder.
68. The method of any of claims 64-67, wherein the subject will, or has undergone radiation therapy as part of an ablative regimen for hematopoietic stem cell or bone marrow transplant or chemotherapy.
69. The method of any one of claims 64-68, wherein the subject will, or has under gone total body radiation.
70. The method of any of claims 64-69, wherein the subject will, or has been exposed to a radiation accident, chemotherapy or transplantation.
71. The method claim 68, wherein the hematopoietic cells are selected from the group consisting of Long-term hematopoietic stem cells (LT-HSCs), Short-term hematopoietic stem cells (ST-

HSCs), Multipotent progenitor cells (MPPs), Common myeloid progenitor (CMPs), CLPs, Granulocyte-macrophage progenitor (GMPs) and Megakaryocyte-erythroid progenitor (MEPs).

72. The method of any one of claims 64-71, wherein the ANG is administered to the subject prior to, during or after exposure, or a combination thereof, to an ionizing radiation.
73. The method of claim 72, wherein the ANG protein is administered for between 12 hours and 3 days prior to exposure to ionizing radiation.
74. The method of claim 73, wherein the exposure to ionizing radiation occurs within about 24 hours after the last administration of ANG protein.
75. The method of claim 72, wherein the ANG protein is administered immediately after the exposure to ionizing radiation.
76. The method of claim 72, wherein the ANG protein is administered about 24 hours after exposure to ionizing radiation.
77. The method of claim 75-76, wherein the ANG protein is administered for at least 3 days or more.
78. The method of any one of claims 64-77, wherein the administration of ANG results in improved hematopoietic regeneration after exposure to radiation as compared to in absence of administration of Angiogenin.
79. The method of any one of claims 64-78, wherein the administration of Angiogenin maintains hematopoietic stem and/or progenitor cells in a state of quiescence.
80. The method of any one of claims 64-79, wherein ANG protein is human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1, a functional fragment thereof with a biological activity of at least 80% of human ANG protein to increase hematopoietic reconstitution in a human subject.
81. The method of claim 80 wherein the functional fragment is a human recombinant ANG polypeptide.
82. The method of any one of claims 80-81, wherein the functional fragment comprises at least amino acids 1-147 of SEQ ID NO 1.
83. The method of claim 80-82, wherein the human ANG protein of at least 85% amino acid sequence identity to SEQ ID NO: 1 comprises a mutation K33A.
84. The method of claim 80-83, wherein the functional fragment comprises an amino acid sequence of at least 80% of human ANG of SEQ ID NO: 1.
85. The method of claim 84, wherein the functional fragment of human ANG protein comprises at least 80% sequence identity to amino acids 1-147 of SEQ ID NO 1.

86. The method of claim 84, wherein the functional fragment of human ANG protein comprises at least 90% sequence identity to amino acids 1-147 of SEQ ID NO 1.
87. The method of claim 84, wherein the functional fragment of human ANG protein comprises at least 95% sequence identity to amino acids 1-147 of SEQ ID NO 1.
88. The method of claim 84, wherein the functional fragment of human ANG comprises at least 98% sequence identity to amino acids 1-147 of SEQ ID NO 1.
89. A method of increasing the dose of an ionizing radiation treatment, comprising administering to the subject an effective amount of an Angiogenin (ANG) protein or Angiogenin agonist before, after or during the ionizing radiation, wherein the dose of the ionizing radiation treatment is higher as compared to the dose in absence of Angiogenin (ANG) protein or Angiogenin agonist administration.

ABSTRACT

Aspects of the invention generally relates to (and in part) uses of Angiogenin for post-transplant hematopoietic reconstitution and protection against and treatment of radiation injury. One aspect relates to the angiogenin treated hematopoietic stem and progenitor cells (HSPCs) compositions and methods o fthier use in HSC transplantation. Treatment of HSPCs with angiogenin enhances self-renewal and preserves their potency by maintaining them in a quiescent state and reducing their proliferative capacity. Angiogenin also enables expansion of progenitor cells by increasing the proliferation of myeloid–restricted progenitor and Angiogenin treated HSPC demonstrate enhanced short-term and long-term multilineage post-transplant hematopoietic reconstitution. Another aspect relates to Angiogenin use in prophylactic and therapeutic treatment methods for radiation injury, as it prevents radiation induced reduction in bone marrow cellularity and therefore enables enhanced reconstitution of peripheral blood count post exposure to irradiation.

FIG. 1A

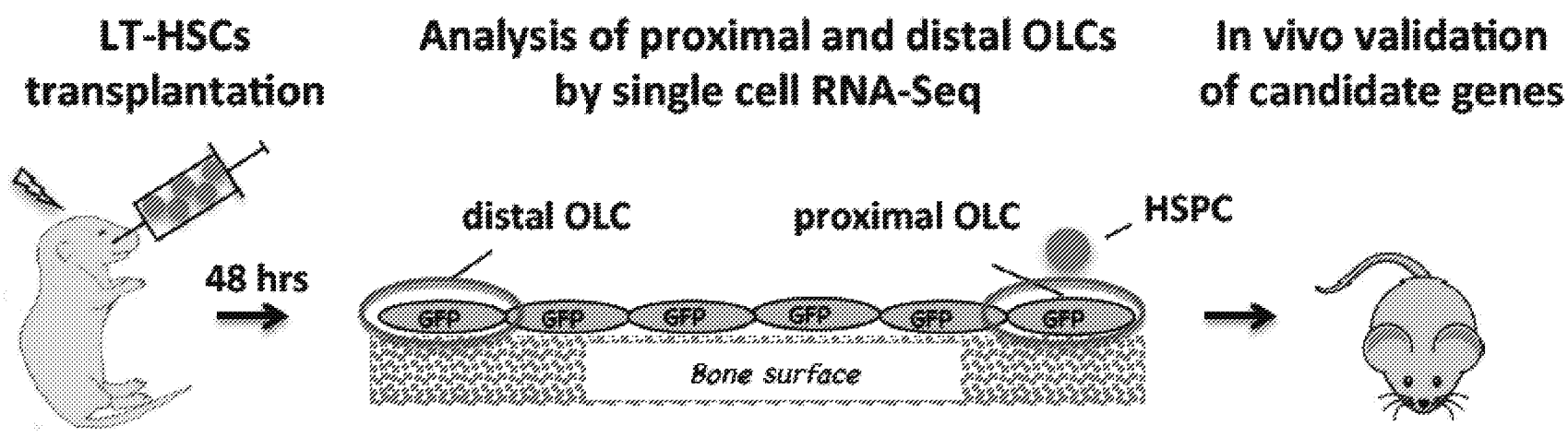

A
LT-HSCs transplantation
Analysis of proximal and distal OLCs by single cell RNA-Seq
In vivo validation of candidate genes
48 hrs
distal OLC
proximal OLC
HSPC
GFP
GFP
GFP
GFP
GFP
GFP
Bone surface

FIG. 1B

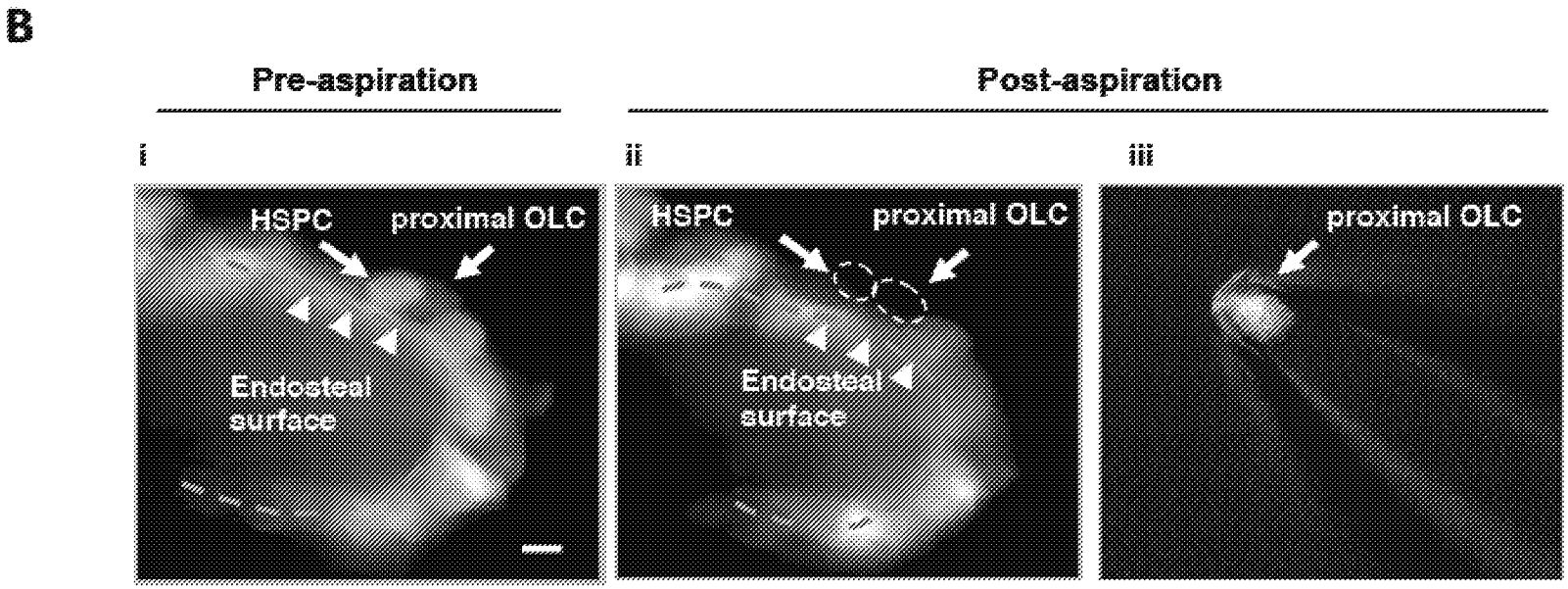

B
Pre-aspiration
Post-aspiration
i
ii
iii
HSPC
proximal OLC
Endosteal surface
HSPC
proximal OLC
Endosteal surface
proximal OLC

FIG. 2A

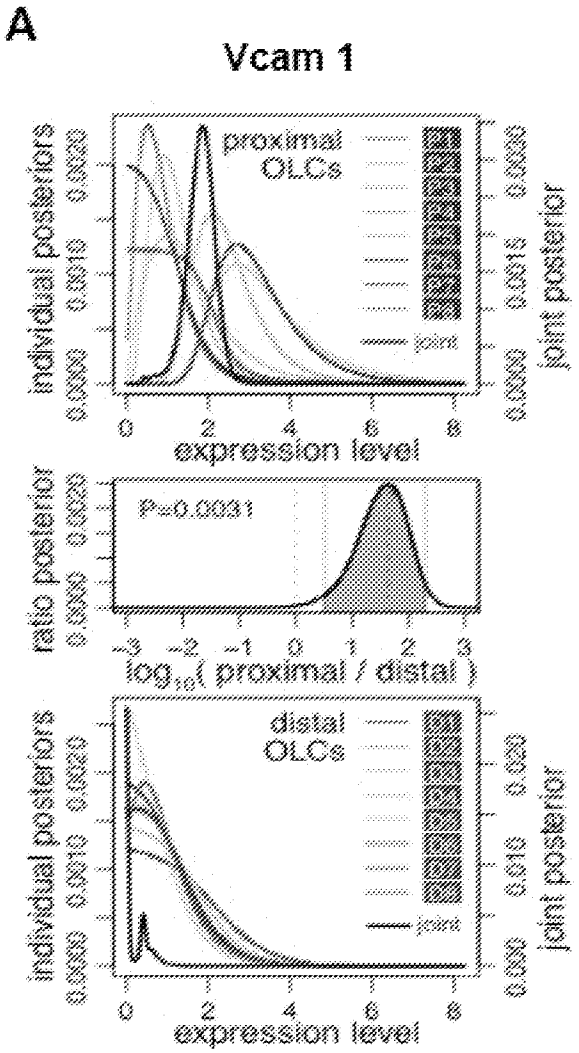
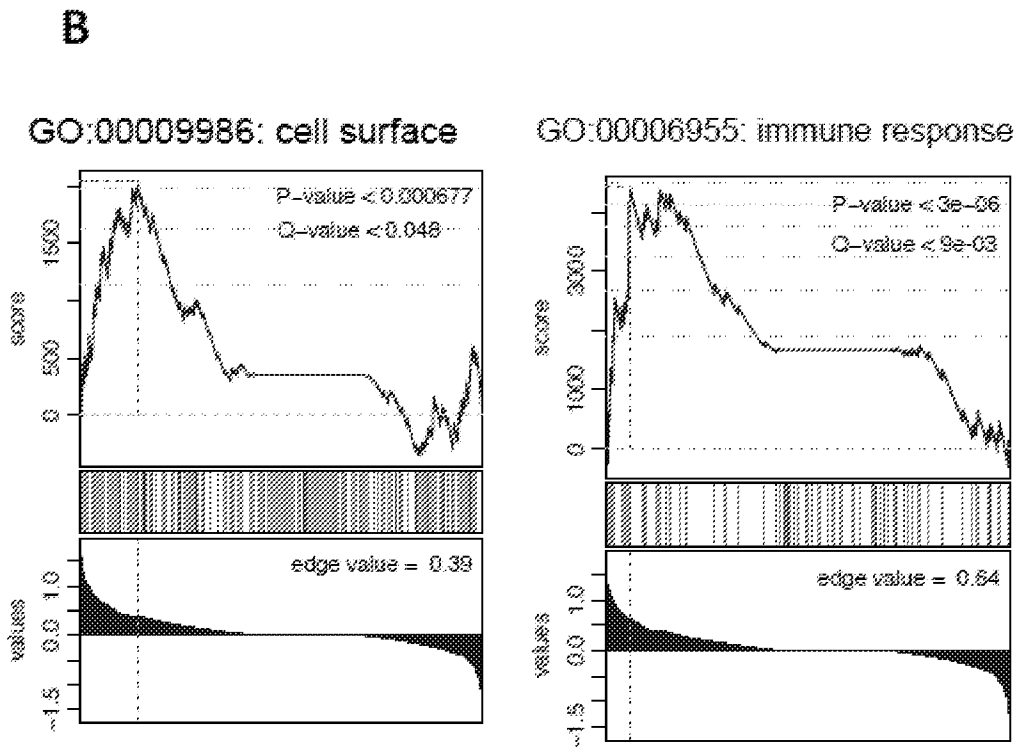
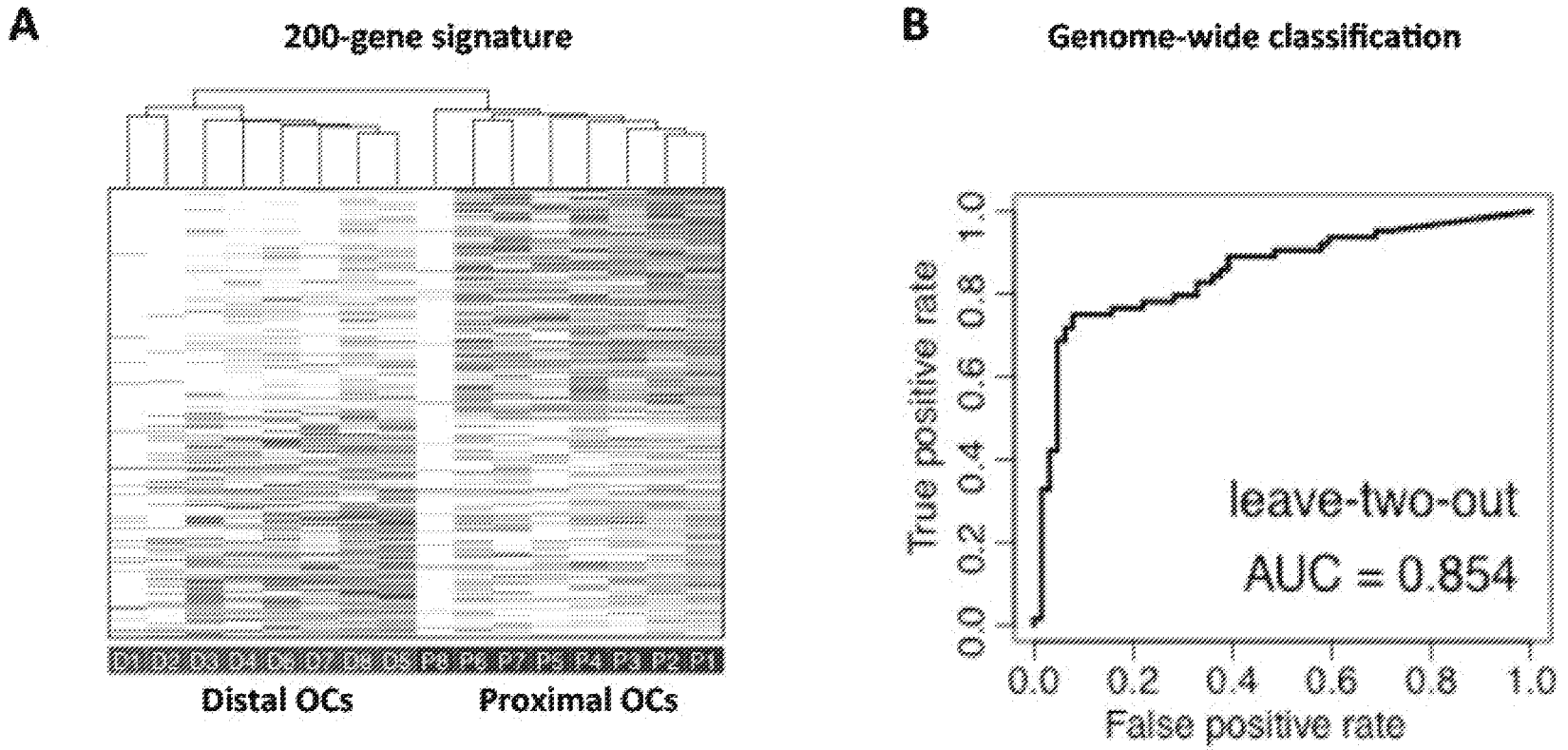

A
Vcam 1
proximal
OLCs
joint
individual posteriors
joint posterior
expression level
P=0.0031
ratio posterior
log10(proximal / distal)
distal
OLCs
joint
individual posteriors
joint posterior
expression level

FIG. 2B


B
GO:00009986: cell surface
P-value < 0.000677
Q-value < 0.048
score
1500
500
0
edge value = 0.39
values
1.0
0.0
-1.5
GO:00006955: immune response
P-value < 3e-06
Q-value < 9e-03
score
3000
1000
0
edge value = 0.64
values
1.0
0.0
-1.5

FIGs. 3A-3B


A
200-gene signature
Distal OCs
Proximal OCs
B
Genome-wide classification
True positive rate
0.0
0.2
0.4
0.6
0.8
1.0
leave-two-out
AUC = 0.854
False positive rate

FIGs. 3C-3D

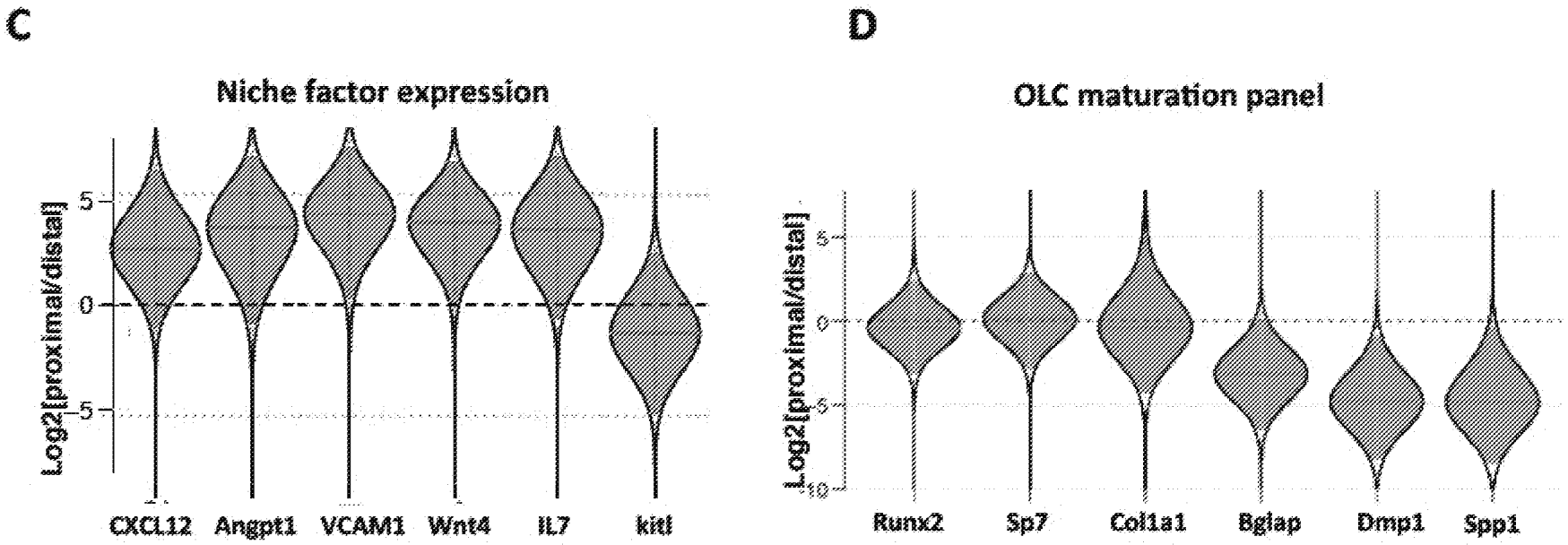

C
Niche factor expression
Log2[proximal/distal]
5
0
-5
CXCL12
Angpt1
VCAM1
Wnt4
IL7
kitl
D
OLC maturation panel
Log2[proximal/distal]
5
0
-5
-10
Runx2
Sp7
Col1a1
Bglap
Dmp1
Spp1

FIG. 4A

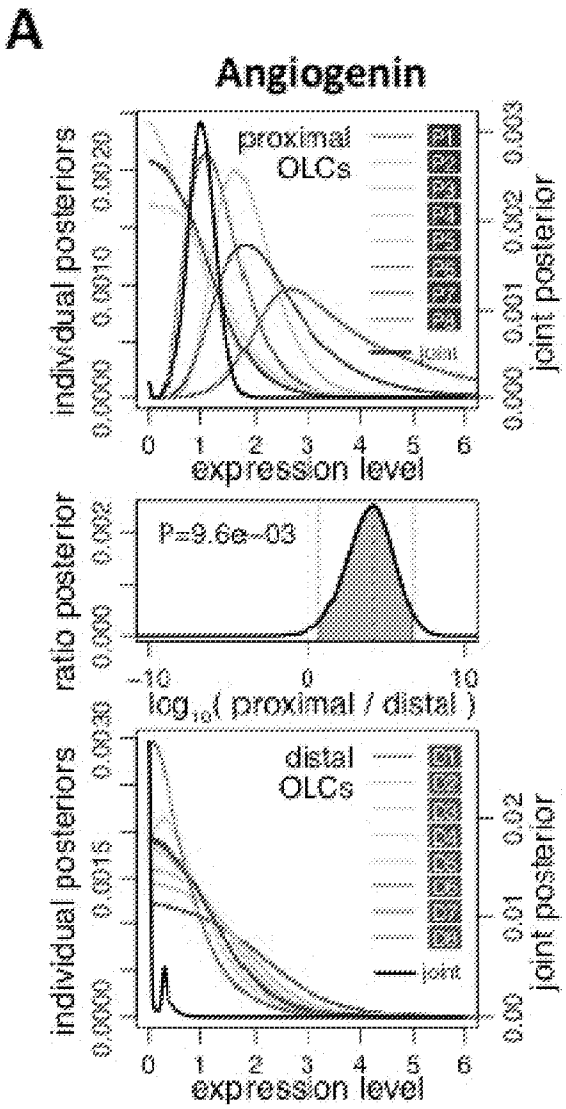

A
Angiogenin
individual posteriors
proximal
OLCs
joint
joint posterior
expression level
ratio posterior
P=9.6e-03
log10(proximal / distal)
individual posteriors
distal
OLCs
joint
joint posterior
expression level

FIG. 4B

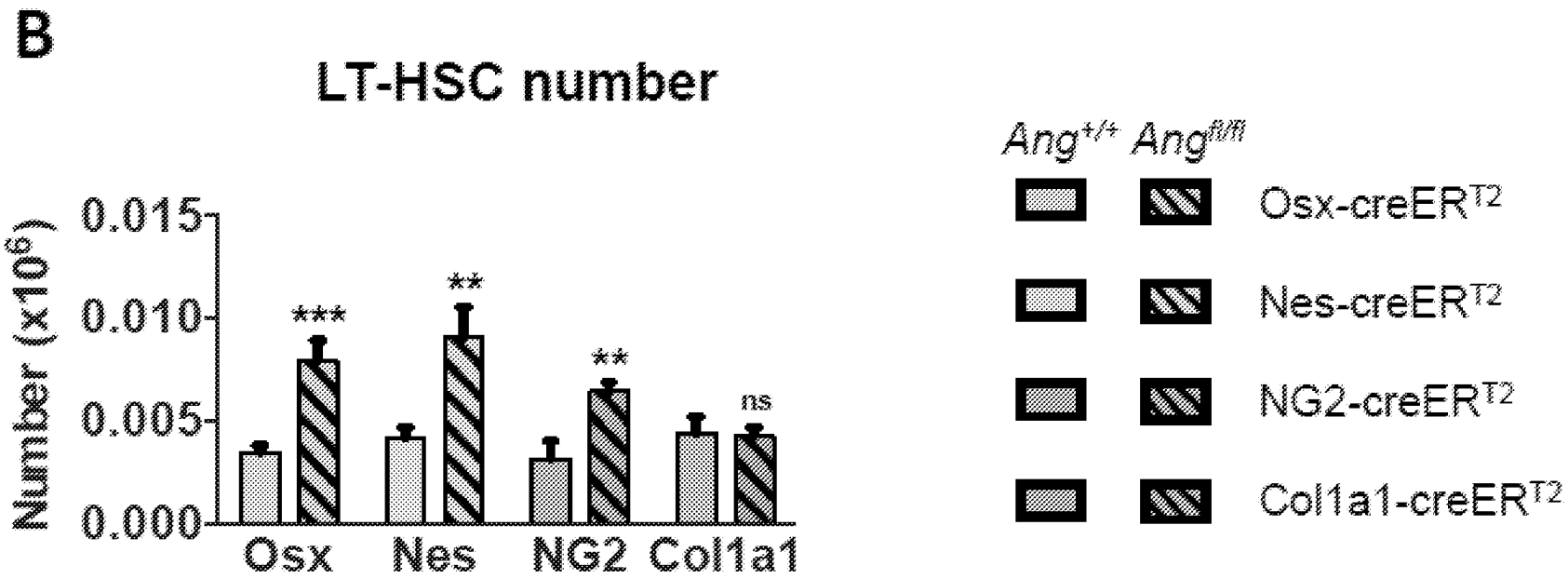
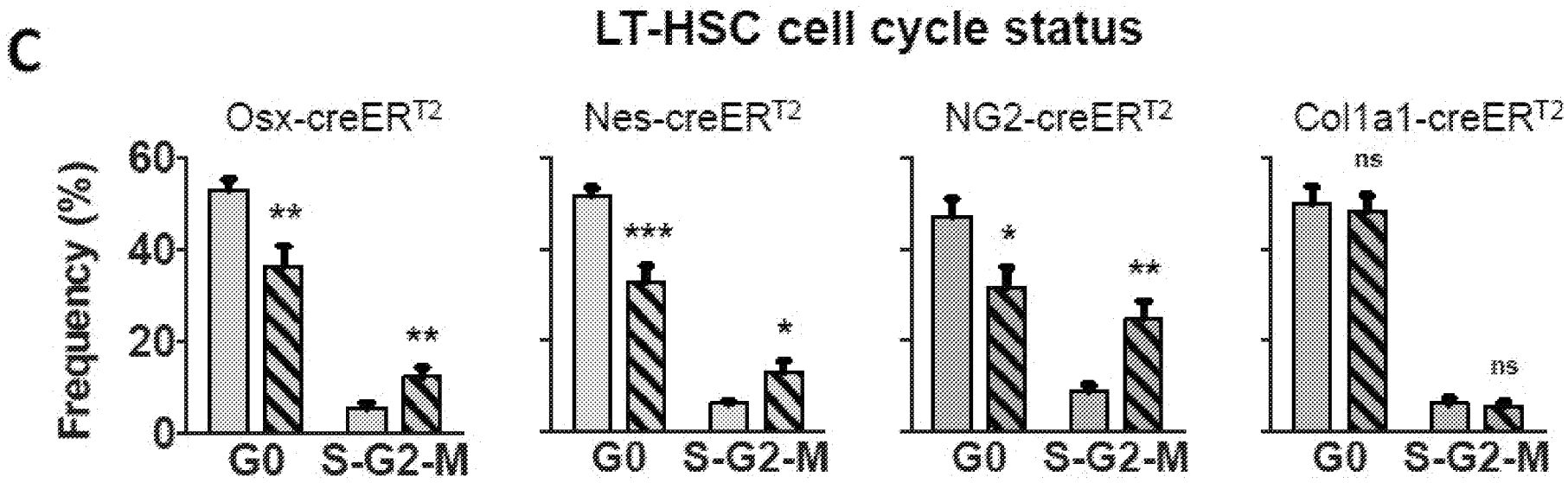

B
LT-HSC number
Number (x10^6)
0.015
0.010
0.005
0.000
***
**
**
ns
Osx
Nes
NG2
Col1a1
Ang+/+ Angfl/fl
Osx-creER^T2
Nes-creER^T2
NG2-creER^T2
Col1a1-creER^T2

FIG. 4C


C
LT-HSC cell cycle status
Osx-creER^{T2}
Nes-creER^{T2}
NG2-creER^{T2}
Col1a1-creER^{T2}
Frequency (%)
60
40
20
0
**
**
***
*
*
**
ns
ns
G0
S-G2-M
G0
S-G2-M
G0
S-G2-M
G0
S-G2-M

FIGs. 4D-4E

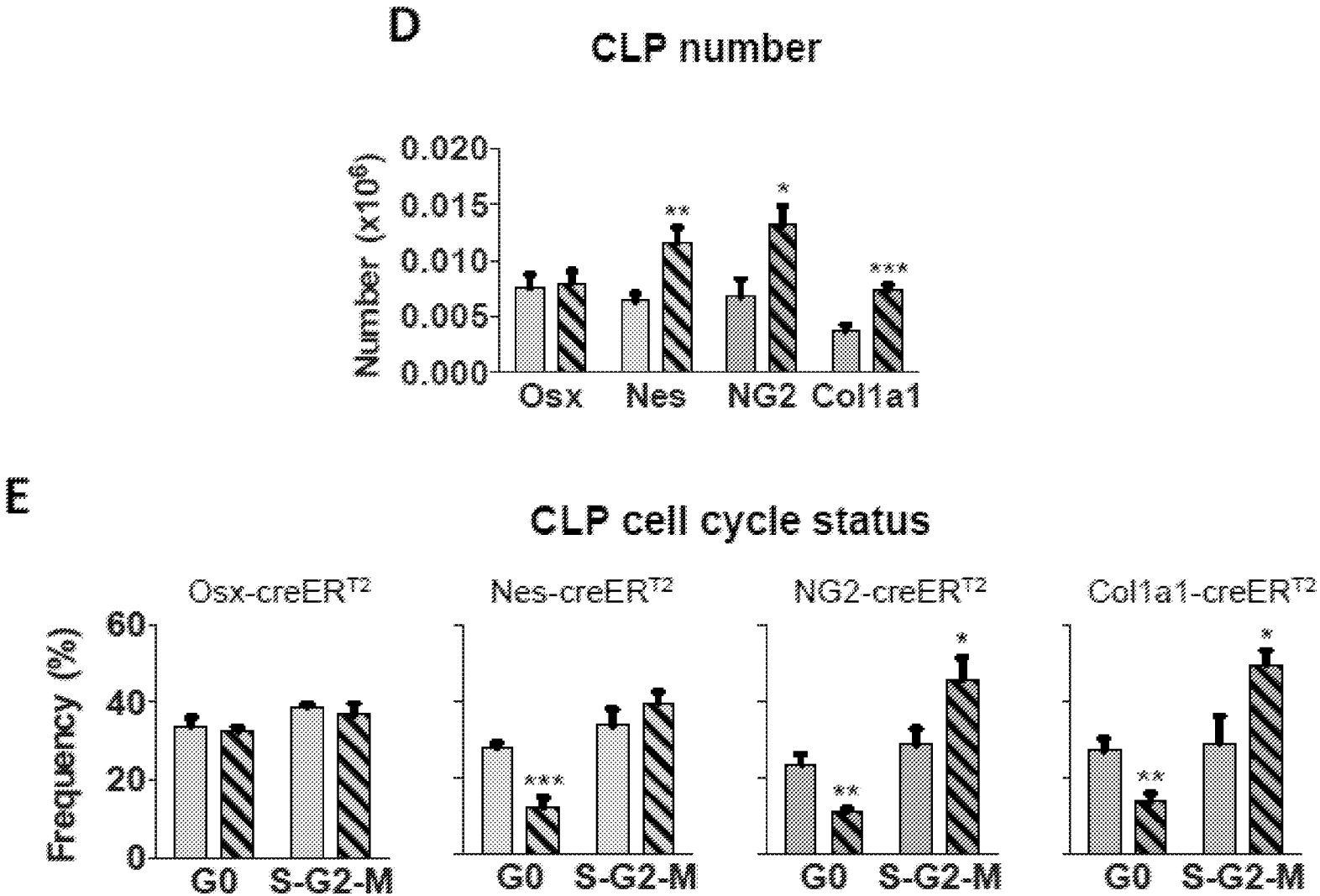

D
CLP number
Number (x10^6)
0.020
0.015
0.010
0.005
0.000
**
*
***
Osx
Nes
NG2
Col1a1
E
CLP cell cycle status
Osx-creER^T2
Nes-creER^T2
NG2-creER^T2
Col1a1-creER^T2
Frequency (%)
60
40
20
0
***
**
*
**
*
G0
S-G2-M

FIG. 4F

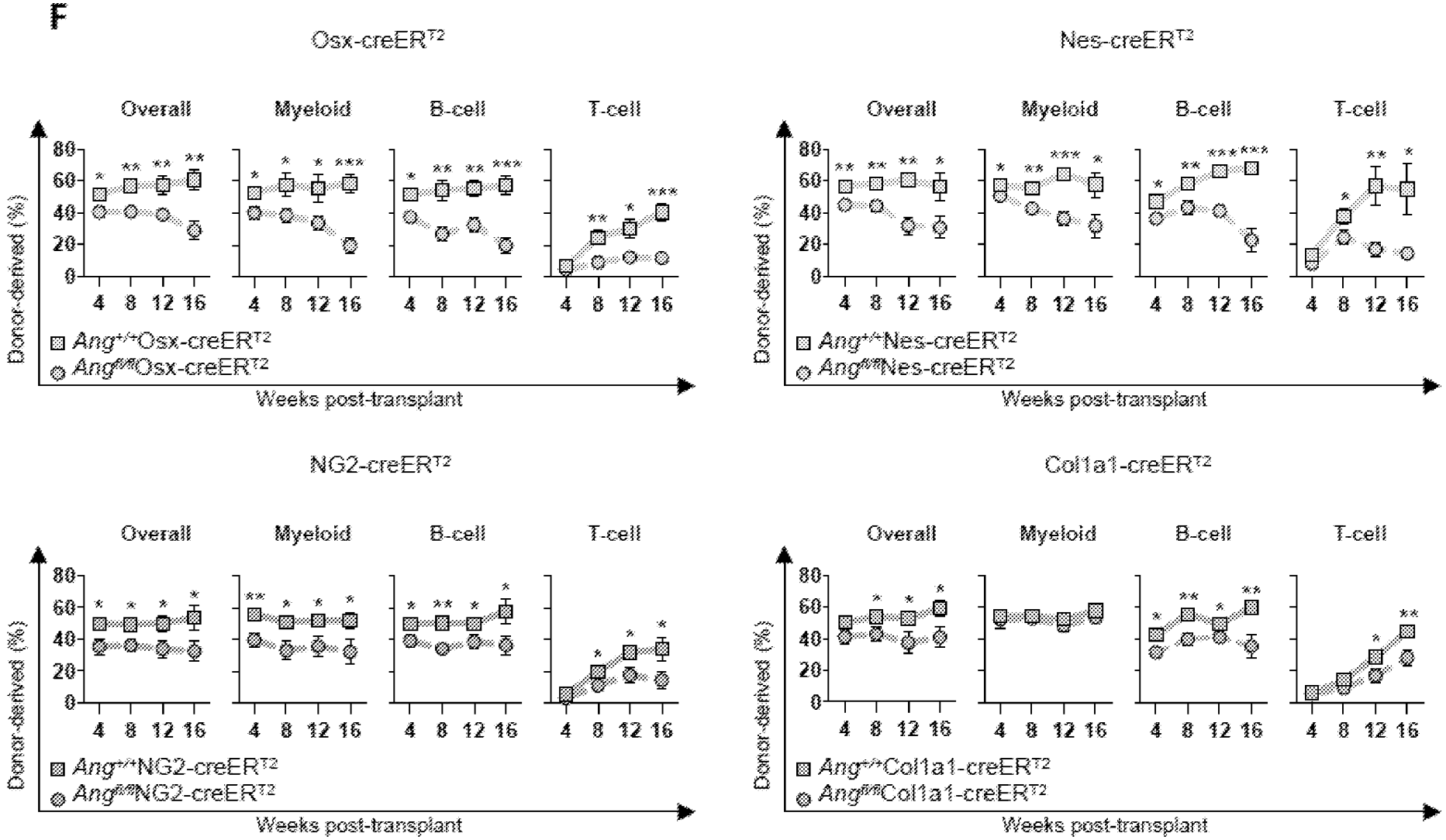

F
Osx-creER^{T2}
Nes-creER^{T2}
NG2-creER^{T2}
Col1a1-creER^{T2}
Overall
Myeloid
B-cell
T-cell
Donor-derived (%)
Weeks post-transplant
Ang$^{+/+}$Osx-creER^{T2}
Ang$^{fl/fl}$Osx-creER^{T2}
Ang$^{+/+}$Nes-creER^{T2}
Ang$^{fl/fl}$Nes-creER^{T2}
Ang$^{+/+}$NG2-creER^{T2}
Ang$^{fl/fl}$NG2-creER^{T2}
Ang$^{+/+}$Col1a1-creER^{T2}
Ang$^{fl/fl}$Col1a1-creER^{T2}

FIG. 5A

A

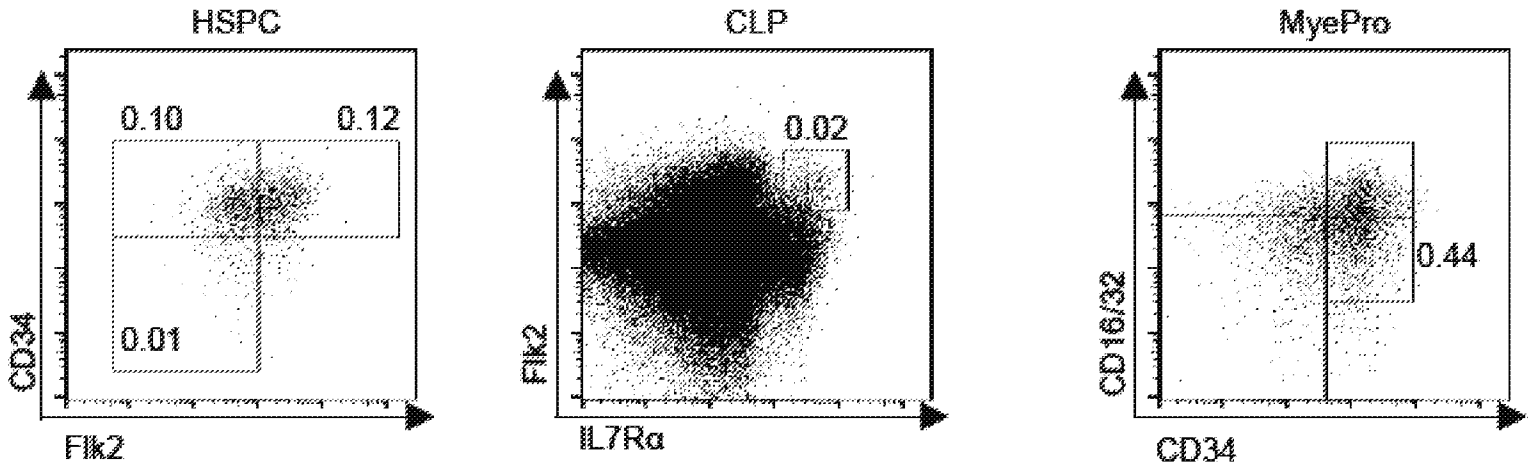

HSPC
0.10
0.12
0.01
CD34
Flk2
CLP
0.02
Flk2
IL7Ra
MyePro
0.44
CD16/32
CD34

FIG. 5B

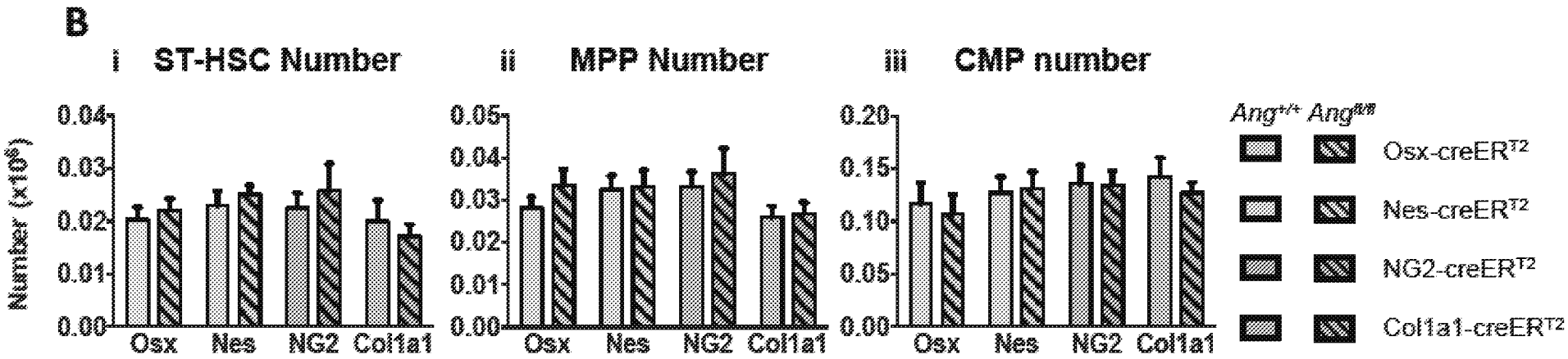

B
i ST-HSC Number
ii MPP Number
iii CMP number
Number (x10^6)
0.04
0.03
0.02
0.01
0.00
0.05
0.04
0.03
0.02
0.01
0.00
0.20
0.15
0.10
0.05
0.00
Osx
Nes
NG2
Col1a1
Ang+/+ Angfl/fl
Osx-creER^T2
Nes-creER^T2
NG2-creER^T2
Col1a1-creER^T2

FIG. 5C

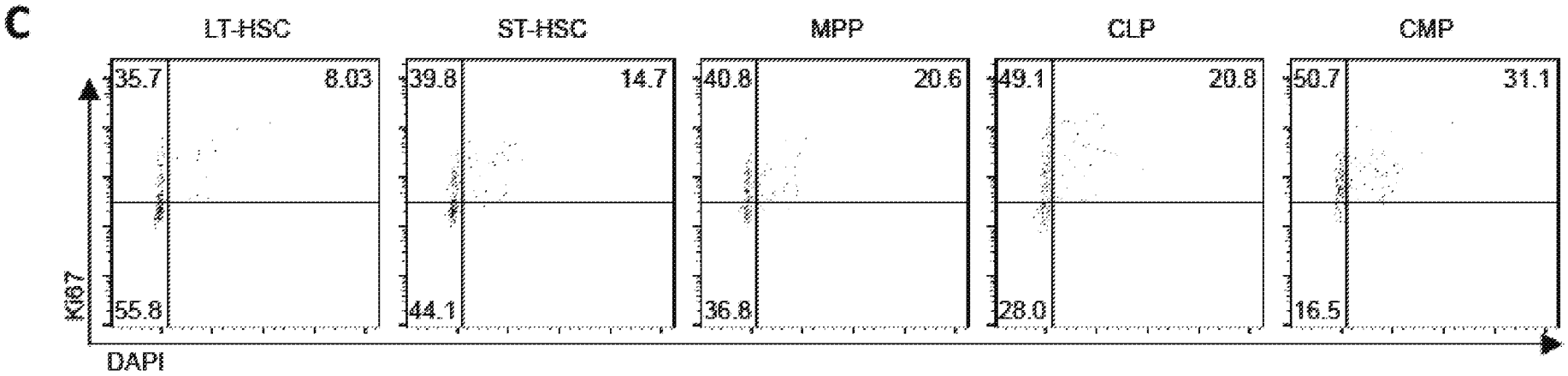

C
LT-HSC
ST-HSC
MPP
CLP
CMP
35.7
8.03
55.8
39.8
14.7
44.1
40.8
20.6
36.8
49.1
20.8
28.0
50.7
31.1
16.5
Ki67
DAPI

FIG. 5D

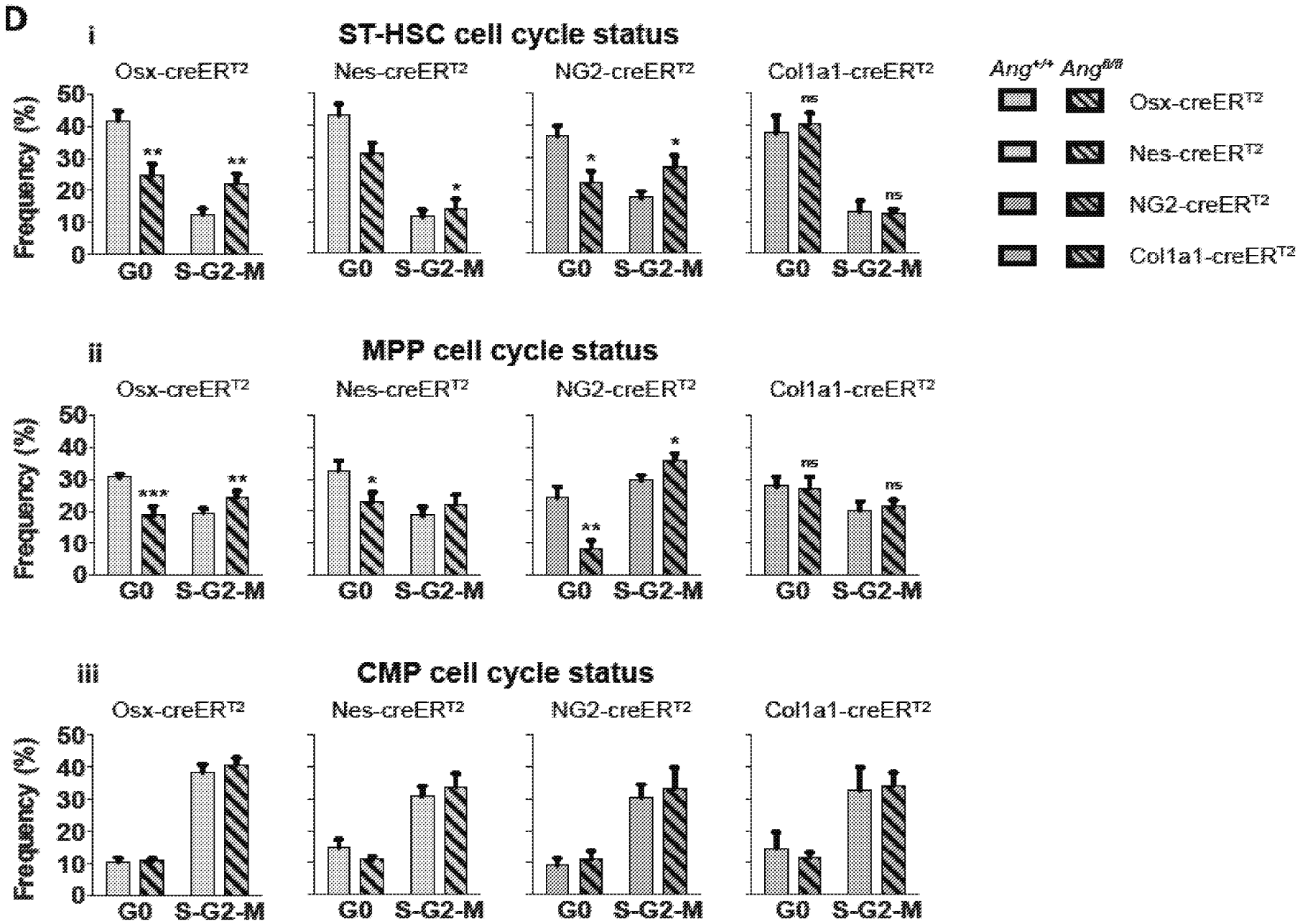
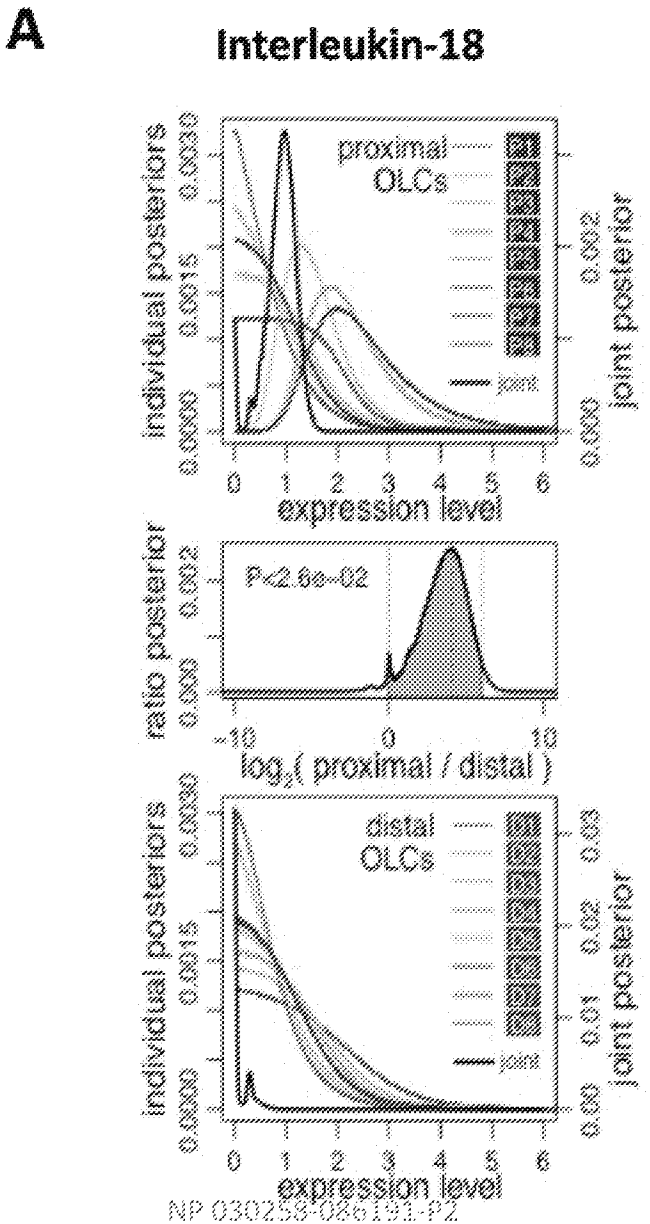

D
i
ST-HSC cell cycle status
Osx-creER^{T2}
Nes-creER^{T2}
NG2-creER^{T2}
Col1a1-creER^{T2}
Frequency (%)
50
40
30
20
10
0
**
**
*
*
*
ns
ns
G0
S-G2-M
$Ang^{+/+}$ $Ang^{fl/fl}$
Osx-creER^{T2}
Nes-creER^{T2}
NG2-creER^{T2}
Col1a1-creER^{T2}
ii
MPP cell cycle status
***
**
*
*
**
ns
ns
iii
CMP cell cycle status

FIG. 6A


A
Interleukin-18
proximal
OLCs
joint
individual posteriors
joint posterior
expression level
P<2.6e-02
ratio posterior
log2(proximal / distal)
distal
OLCs
joint
individual posteriors
joint posterior
expression level

FIGs. 6B-6C

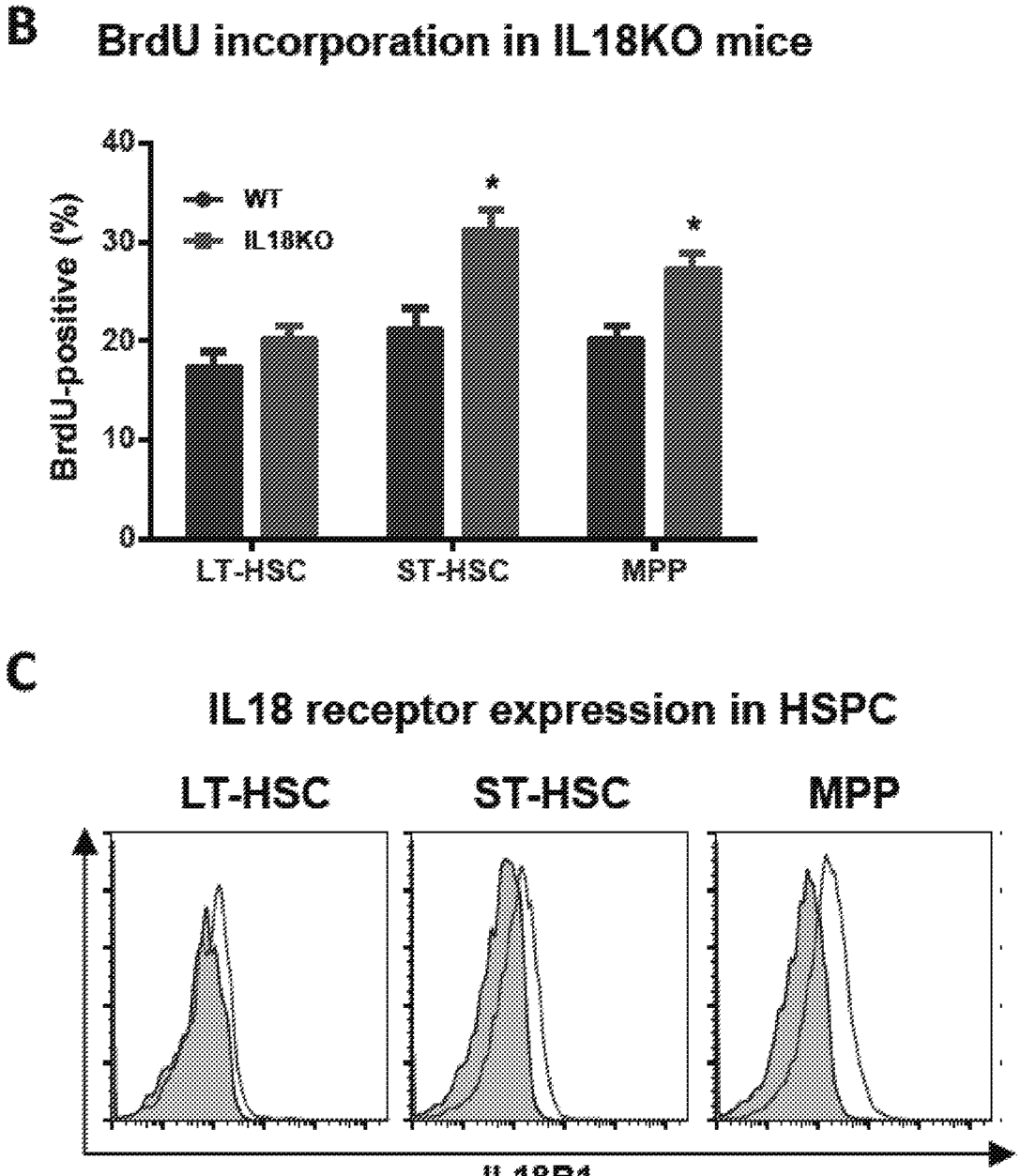

B
BrdU incorporation in IL18KO mice
BrdU-positive (%)
40
30
20
10
0
WT
IL18KO
*
*
LT-HSC
ST-HSC
MPP
C
IL18 receptor expression in HSPC
LT-HSC
ST-HSC
MPP
IL18R1

FIG. 6D

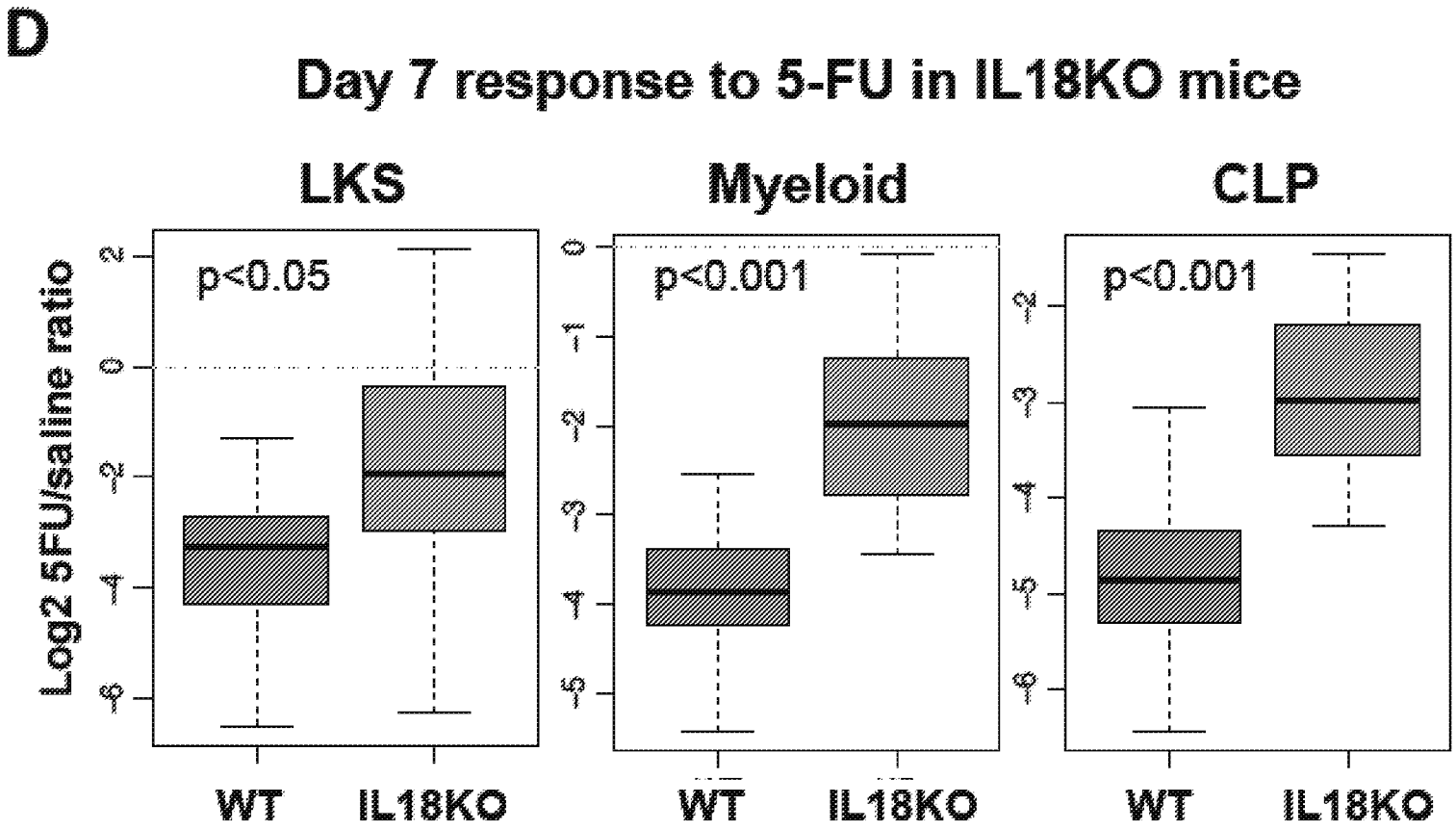

D
Day 7 response to 5-FU in IL18KO mice
LKS
Myeloid
CLP
Log2 5FU/saline ratio
p<0.05
p<0.001
p<0.001
WT
IL18KO
WT
IL18KO
WT
IL18KO

FIG. 6E

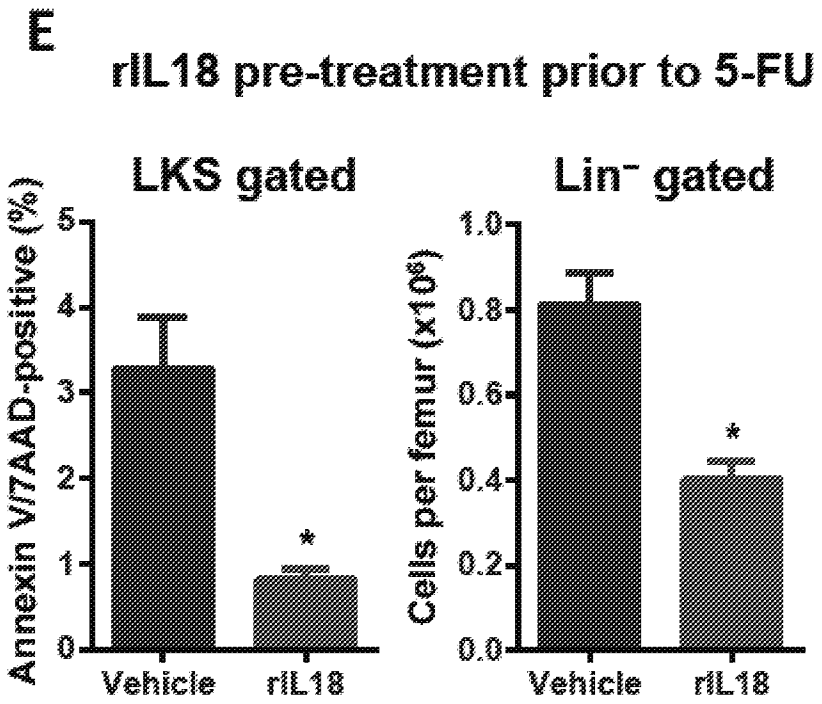

E
rIL18 pre-treatment prior to 5-FU
LKS gated
Lin⁻ gated
Annexin V/7AAD-positive (%)
Cells per femur (x10⁶)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
*
*
Vehicle
rIL18
Vehicle
rIL18

FIG. 6F

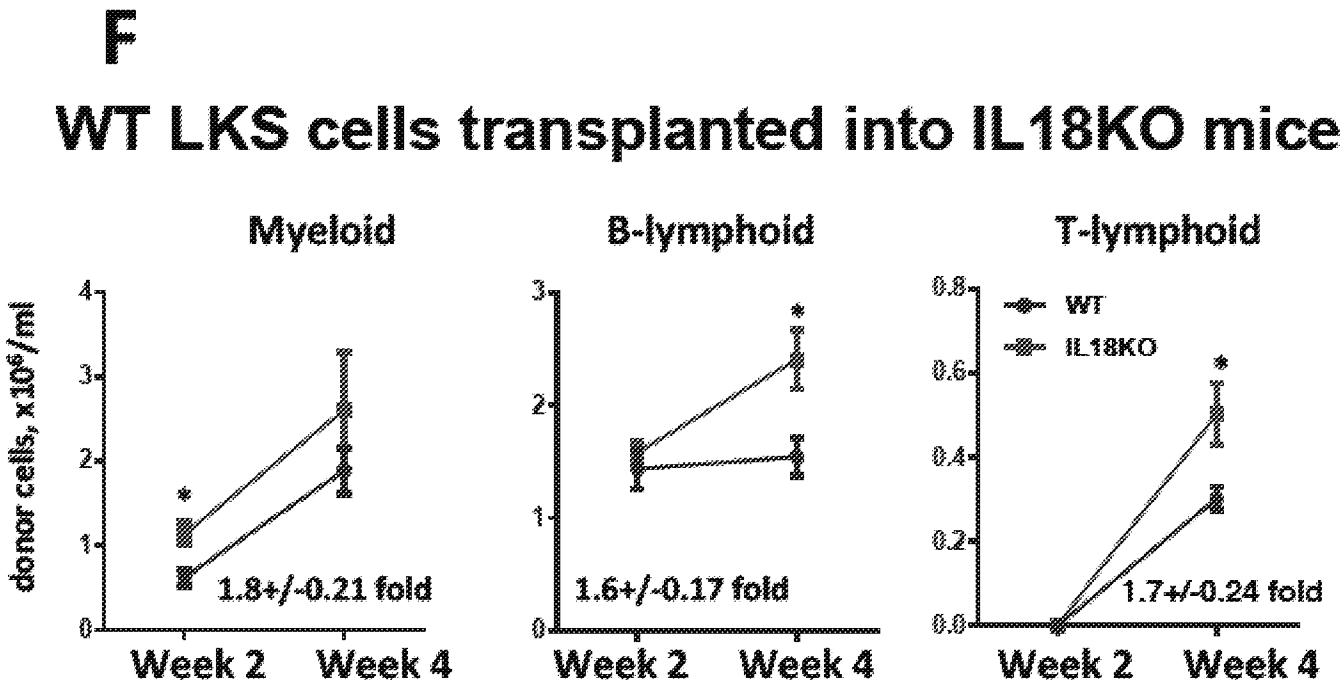

F
WT LKS cells transplanted into IL18KO mice
Myeloid
B-lymphoid
T-lymphoid
donor cells, x10^6/ml
WT
IL18KO
1.8+/-0.21 fold
1.6+/-0.17 fold
1.7+/-0.24 fold
Week 2
Week 4

FIG. 6G

G

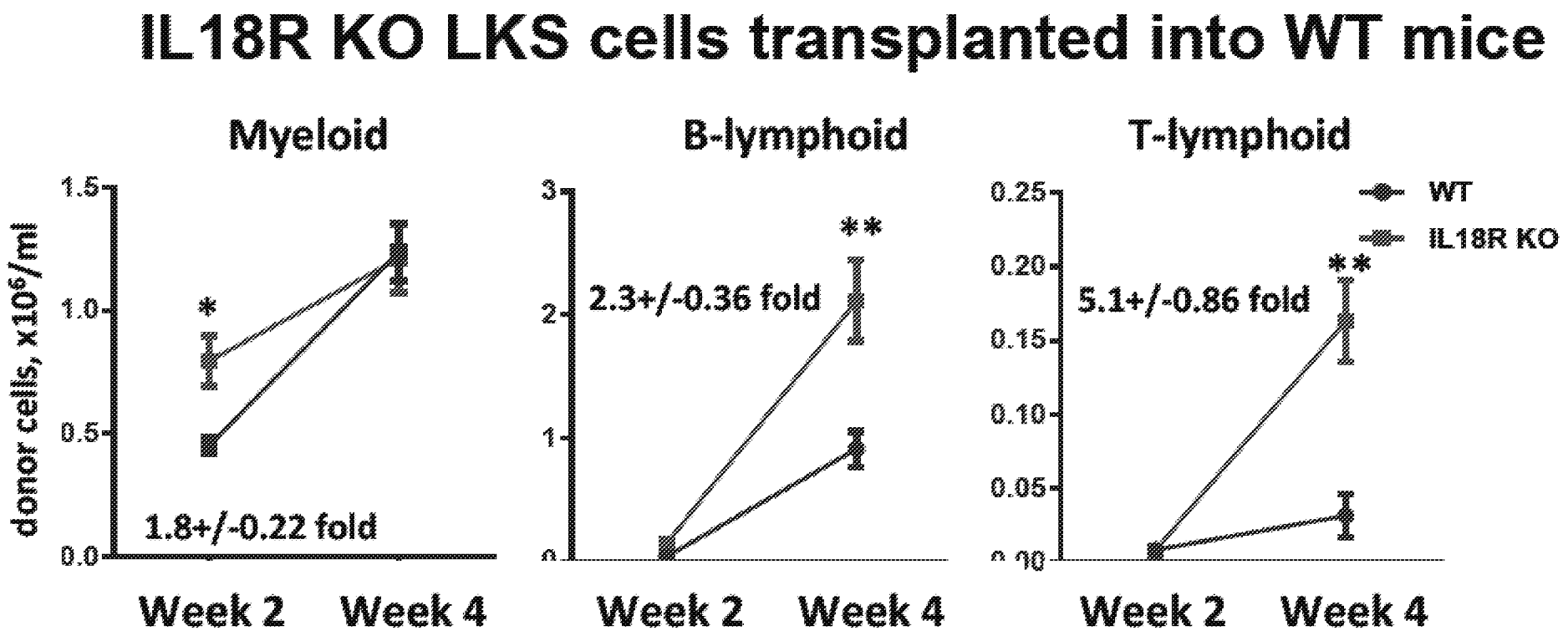

IL18R KO LKS cells transplanted into WT mice
Myeloid
B-lymphoid
T-lymphoid
donor cells, x10⁶/ml
1.5
1.0
0.5
0.0
*
1.8+/-0.22 fold
Week 2
Week 4
3
2
1
**
2.3+/-0.36 fold
0.25
0.20
0.15
0.10
0.05
**
5.1+/-0.86 fold
WT
IL18R KO

FIG. 7A

A

Parameter	WT	IL18KO	P-value
WBC ($x10^9/l$)	9.1 ± 1.08	10.6 ± 2.1	0.037
Neutrophils ($x10^9/l$)	0.49 ± 0.35	0.91 ± 0.36	0.005
Lymphocytes ($x10^9/l$)	8.5 ± 1.1	9.5 ± 2.2	0.11
Hemoglobin (g/dl)	13.17 ± 0.66	13 ± 0.81	0.54
Platelets ($x10^9/l$)	624 ± 44.9	596 ± 98	0.35

FIGs. 7B-7C

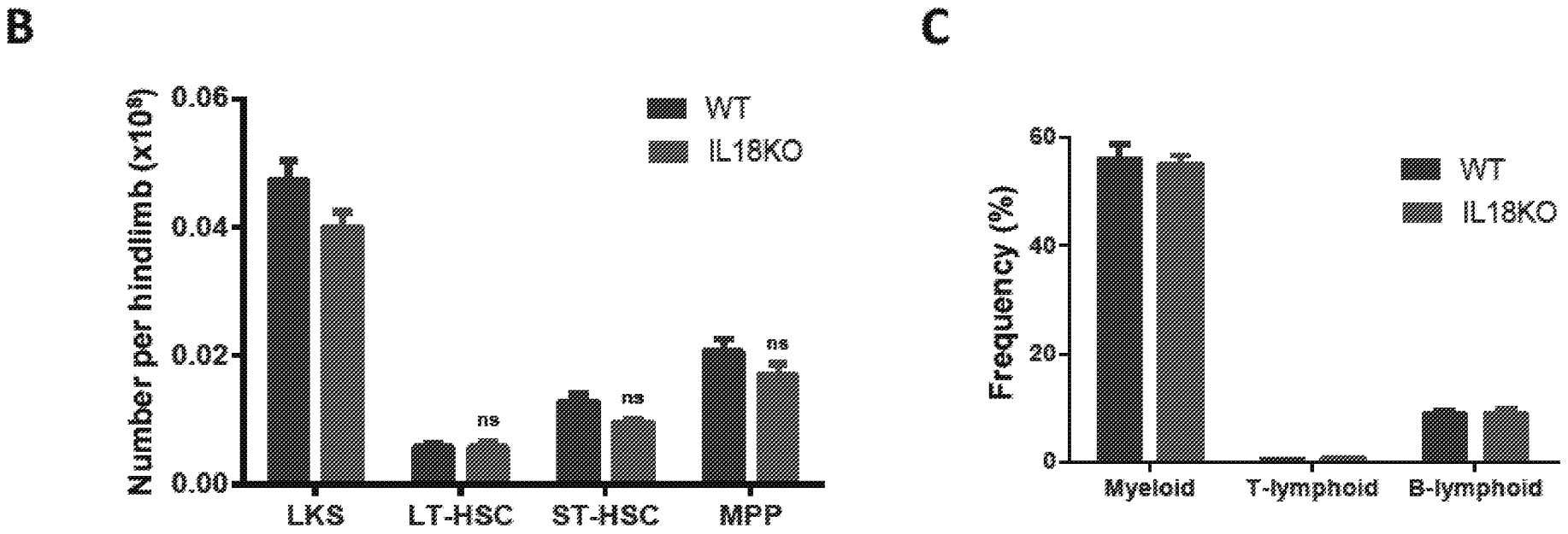
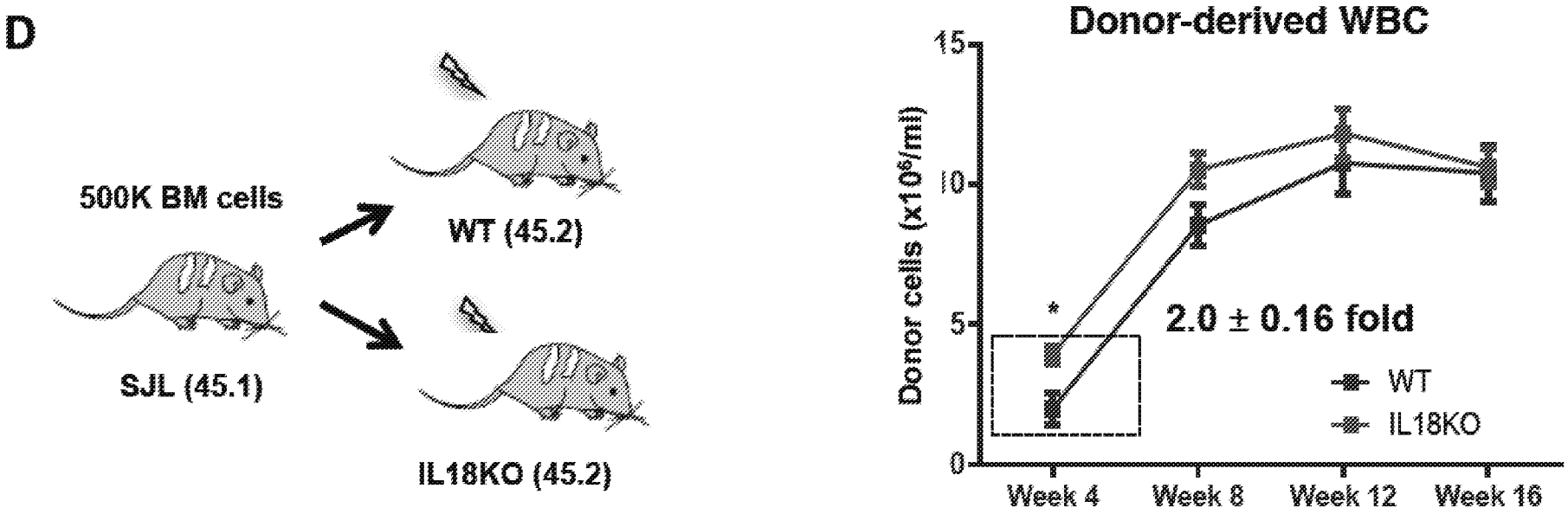

B
Number per hindlimb (x10⁸)
0.06
0.04
0.02
0.00
WT
IL18KO
ns
ns
ns
LKS
LT-HSC
ST-HSC
MPP
C
Frequency (%)
60
40
20
0
WT
IL18KO
Myeloid
T-lymphoid
B-lymphoid

FIG. 7D


D
500K BM cells
SJL (45.1)
WT (45.2)
IL18KO (45.2)
Donor-derived WBC
Donor cells (x10^6/ml)
15
10
5
0
*
2.0 ± 0.16 fold
WT
IL18KO
Week 4
Week 8
Week 12
Week 16

FIGs. 7E-7G

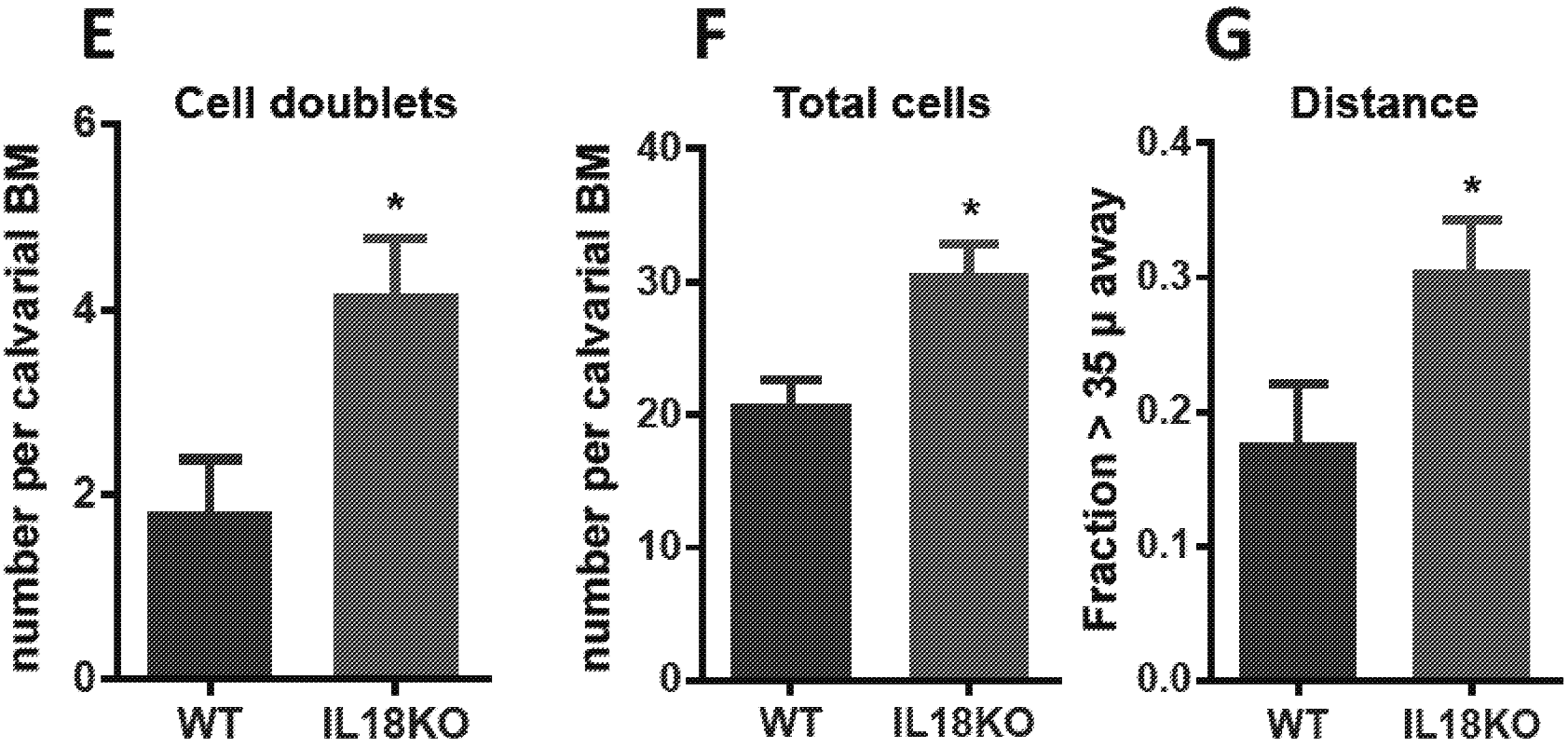

E
Cell doublets
number per calvarial BM
0
2
4
6
*
WT
IL18KO
F
Total cells
number per calvarial BM
0
10
20
30
40
*
WT
IL18KO
G
Distance
Fraction > 35 µ away
0.0
0.1
0.2
0.3
0.4
*
WT
IL18KO

FIG. 7H

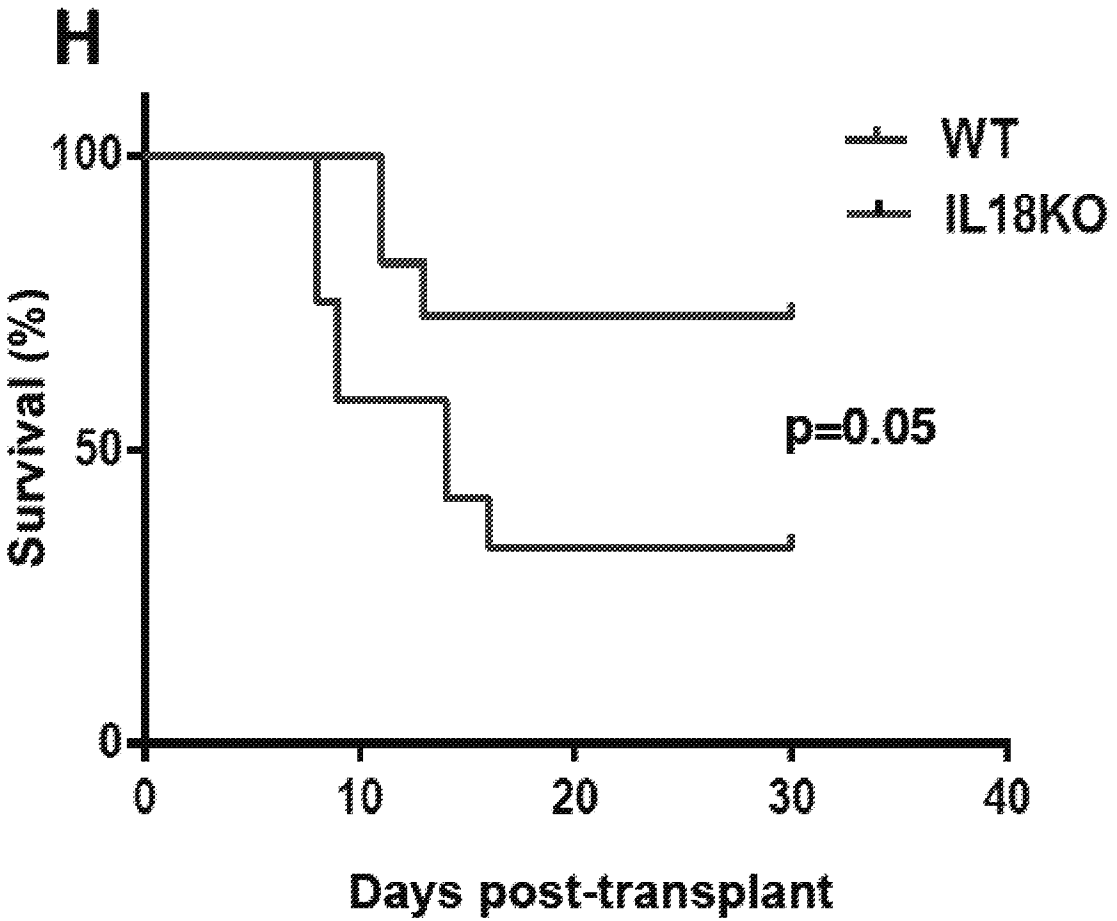

H
100
50
0
Survival (%)
0
10
20
30
40
Days post-transplant
WT
IL18KO
p=0.05

FIG. 8A

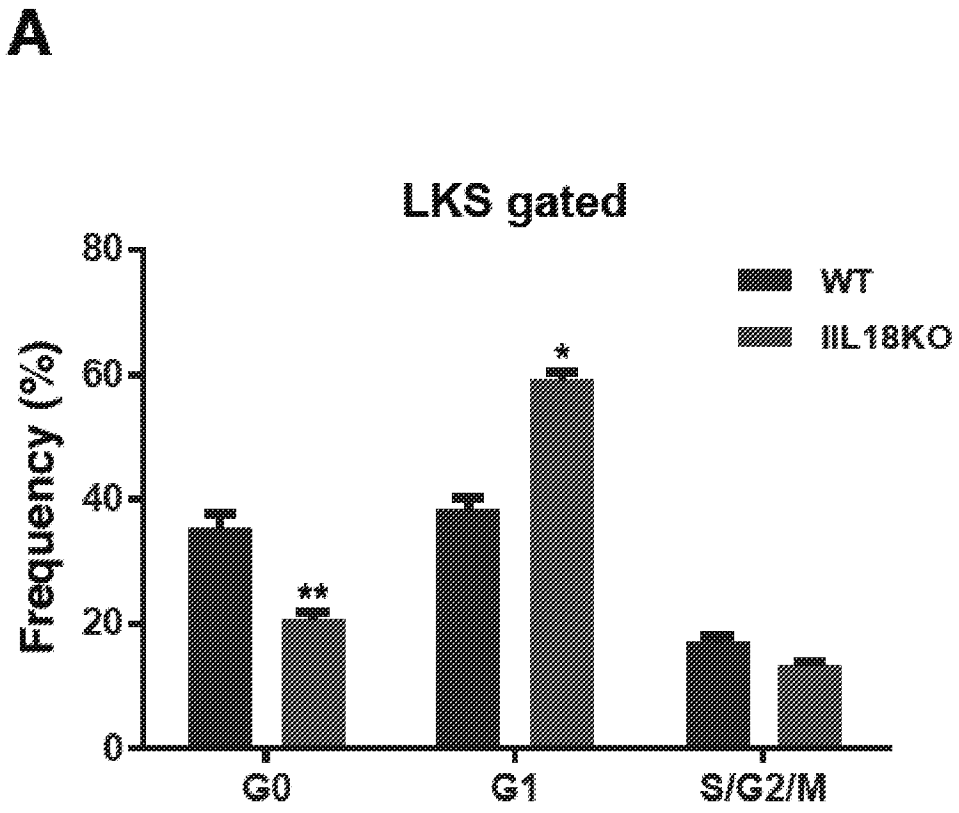


FIG. 8B

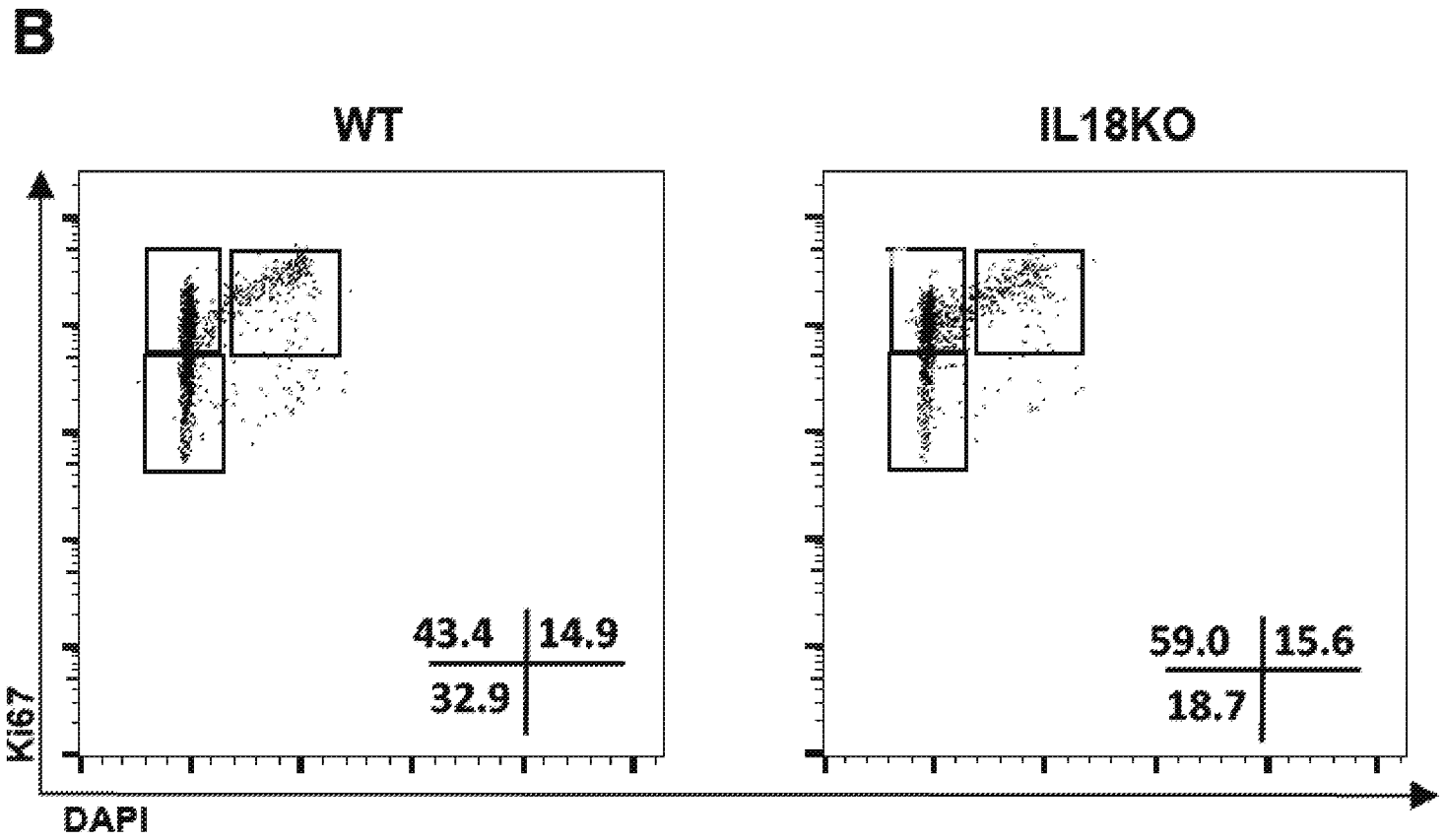


FIG. 8C

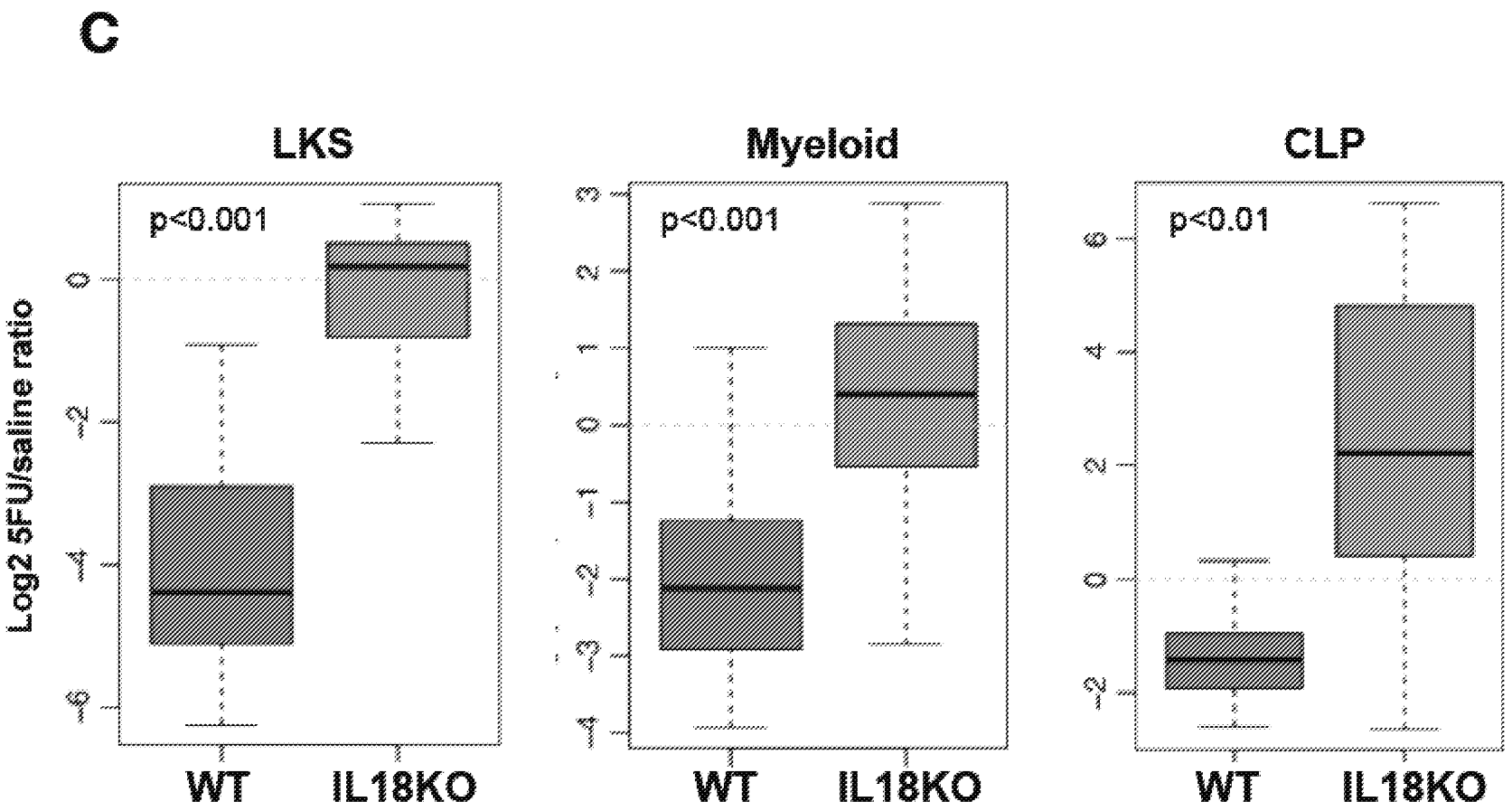

C
LKS
p<0.001
Log2 5FU/saline ratio
0
-2
-4
-6
WT
IL18KO
Myeloid
p<0.001
3
2
1
0
-1
-2
-3
-4
WT
IL18KO
CLP
p<0.01
6
4
2
0
-2
WT
IL18KO

FIG. 9A

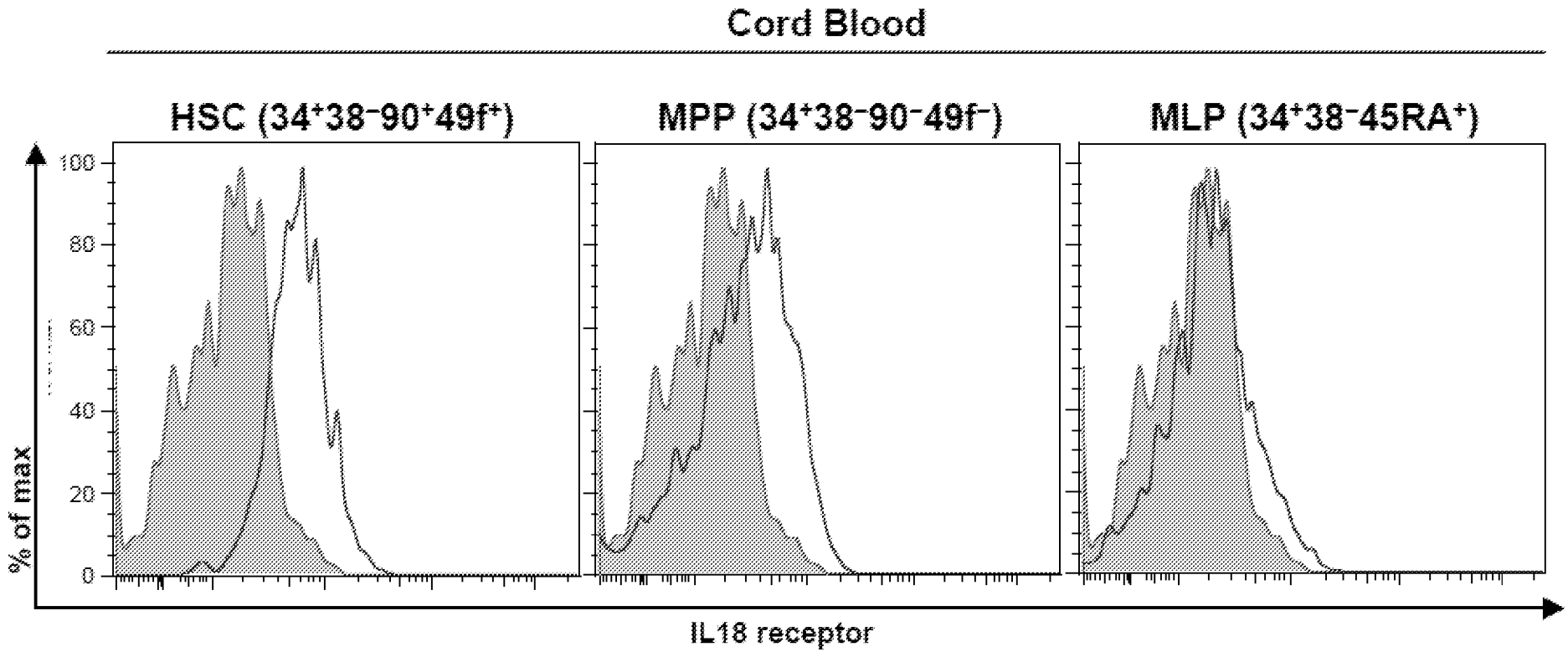

Cord Blood
HSC (34+38-90+49f+)
MPP (34+38-90-49f-)
MLP (34+38-45RA+)
% of max
100
80
60
40
20
0
IL18 receptor

FIG. 9A cont'd

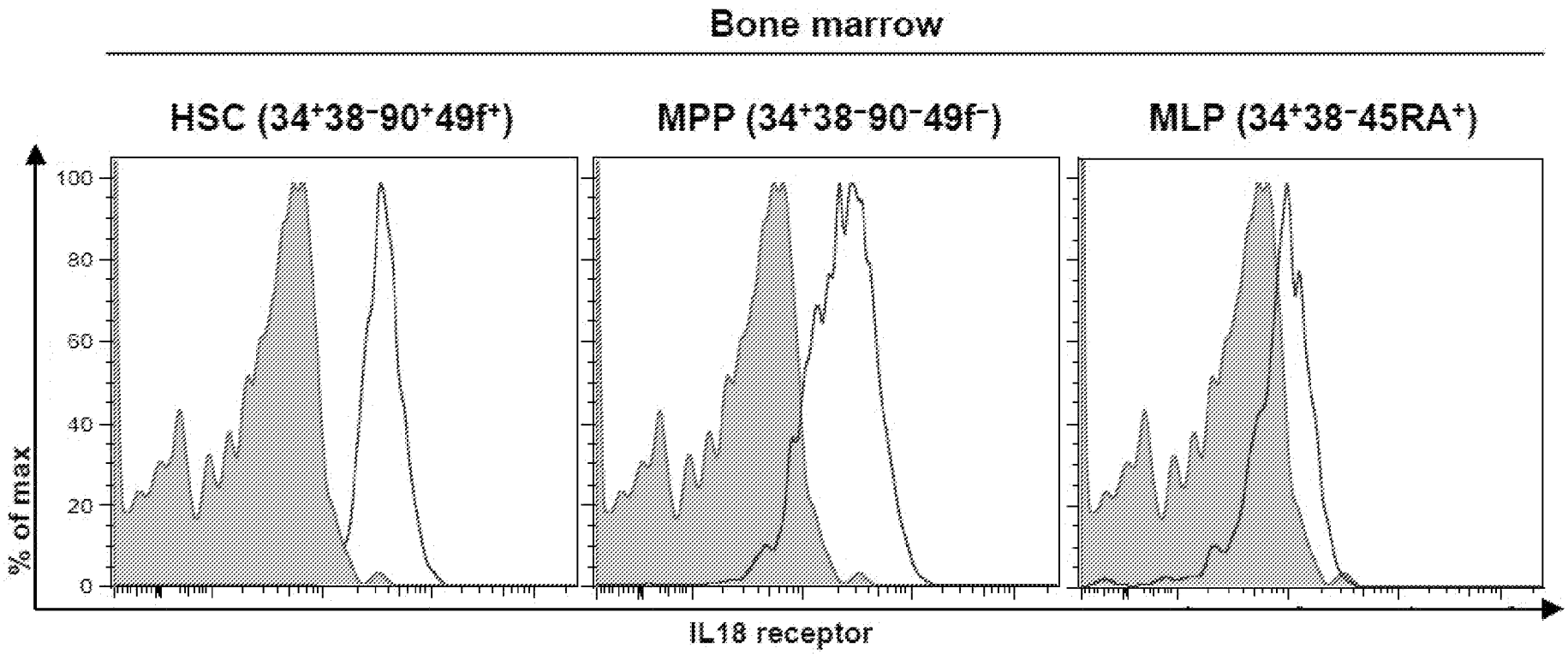
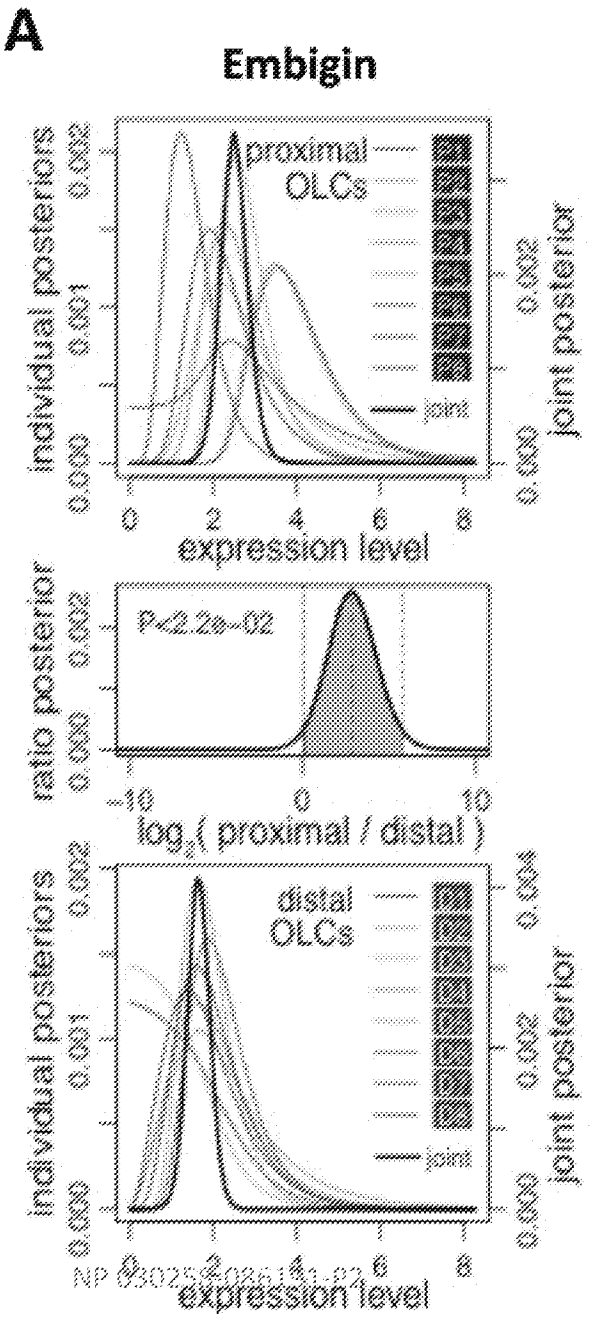
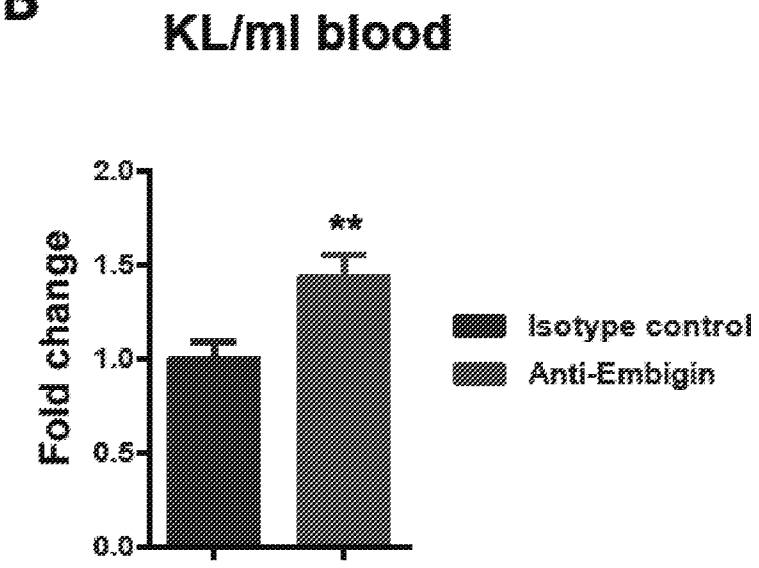

Bone marrow
HSC (34+38-90+49f+)
MPP (34+38-90-49f-)
MLP (34+38-45RA+)
% of max
100
80
60
40
20
0
IL18 receptor

FIG. 10A


A
Embigin
individual posteriors
0.000
0.001
0.002
proximal
OLCs
joint
joint posterior
0.000
0.002
0
2
4
6
8
expression level
ratio posterior
0.000
0.002
P<2.2e-02
−10
0
10
log2(proximal / distal)
individual posteriors
0.000
0.001
0.002
distal
OLCs
joint
joint posterior
0.000
0.002
0.004
0
2
4
6
8
expression level

FIGs. 10B-10C

B



C

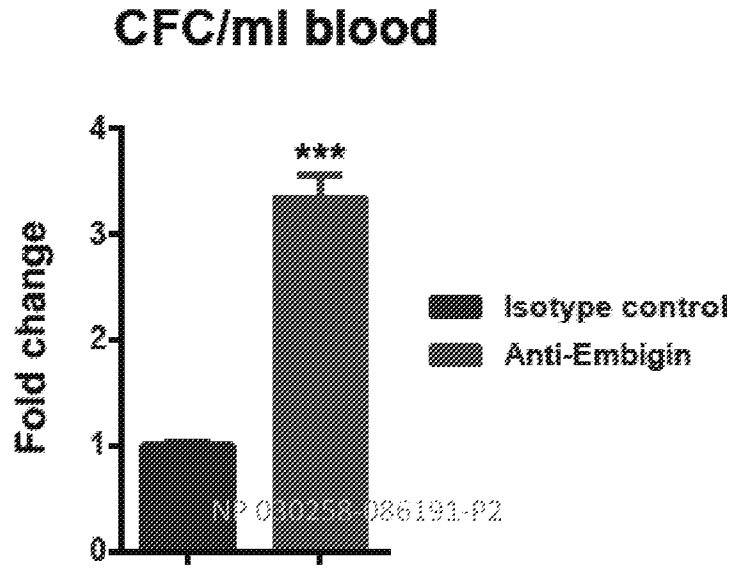


FIG. 10D

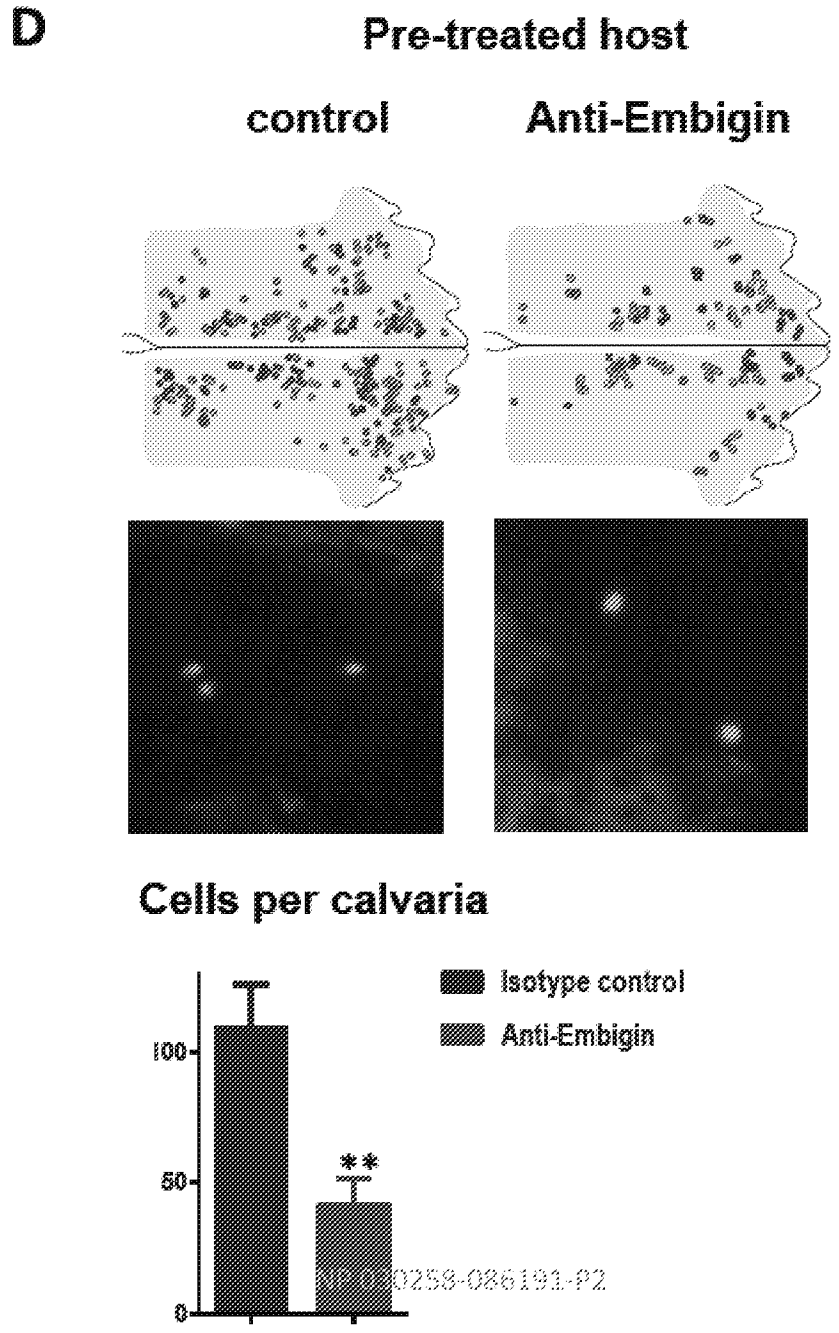

D
Pre-treated host
control
Anti-Embigin
Cells per calvaria
Isotype control
Anti-Embigin
100
50
0
**

FIG. 10E

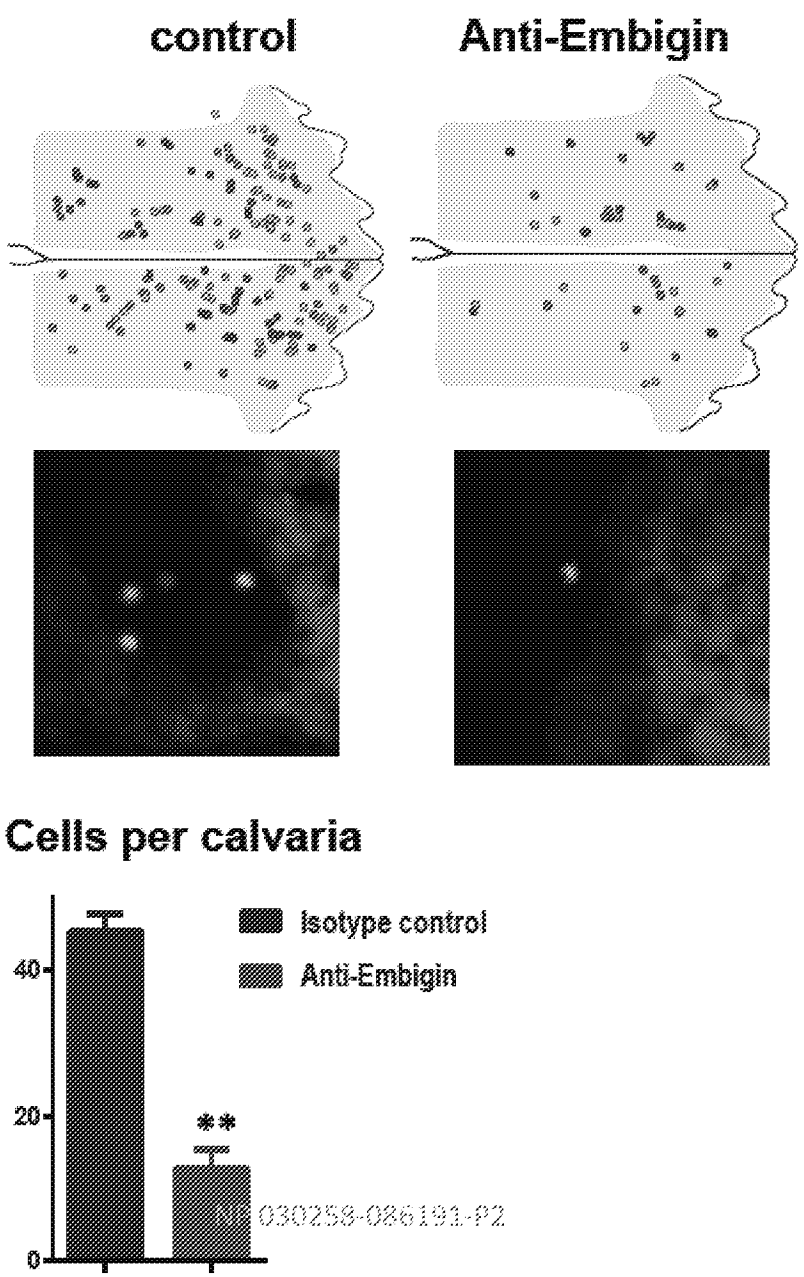

E
Pre-treated cells
control
Anti-Embigin
Cells per calvaria
Isotype control
Anti-Embigin
40
20
0
**

FIG. 10F

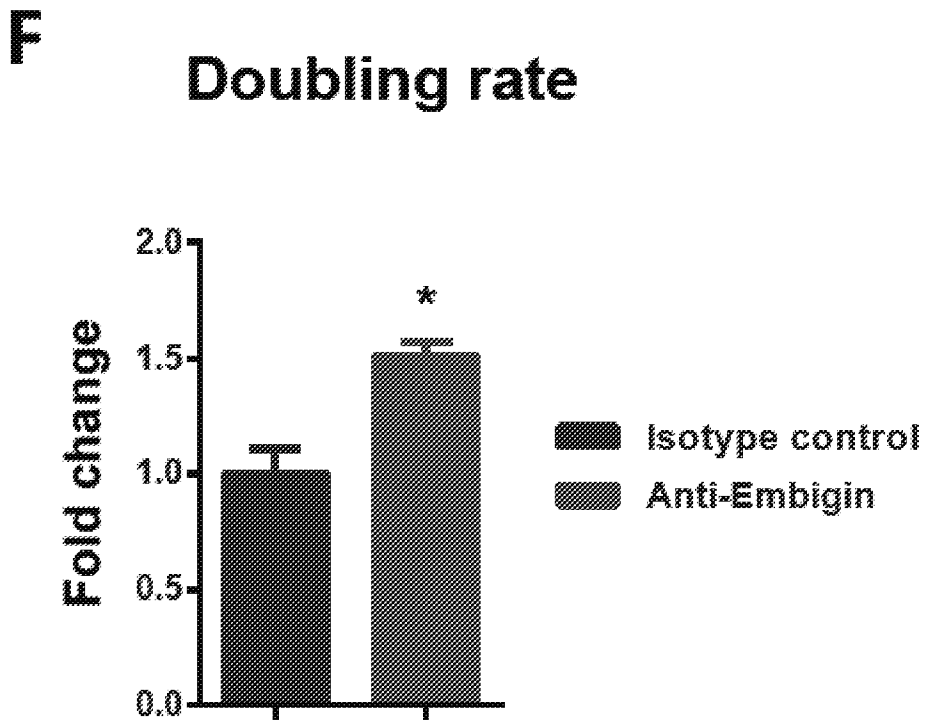

F
Doubling rate
Fold change
2.0
1.5
1.0
0.5
0.0
*
Isotype control
Anti-Embigin

FIG. 11A

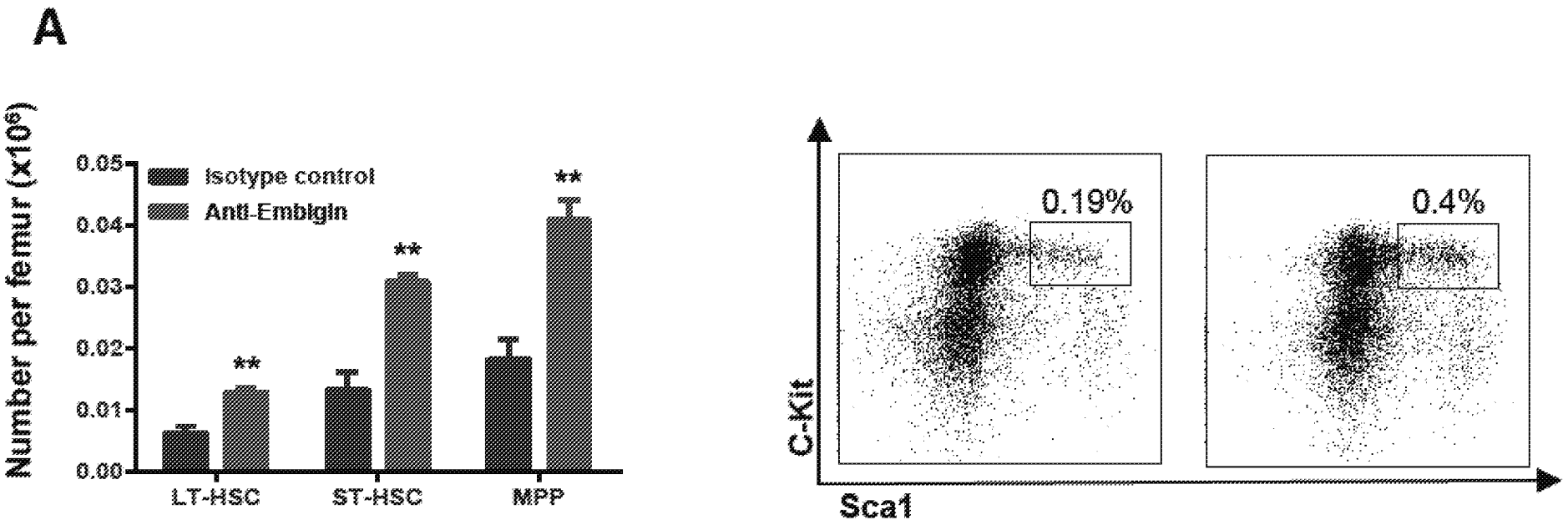

A
Number per femur (x10^6)
0.05
0.04
0.03
0.02
0.01
0.00
Isotype control
Anti-Embigin
**
**
**
LT-HSC
ST-HSC
MPP
0.19%
0.4%
C-Kit
Sca1

FIG. 11B

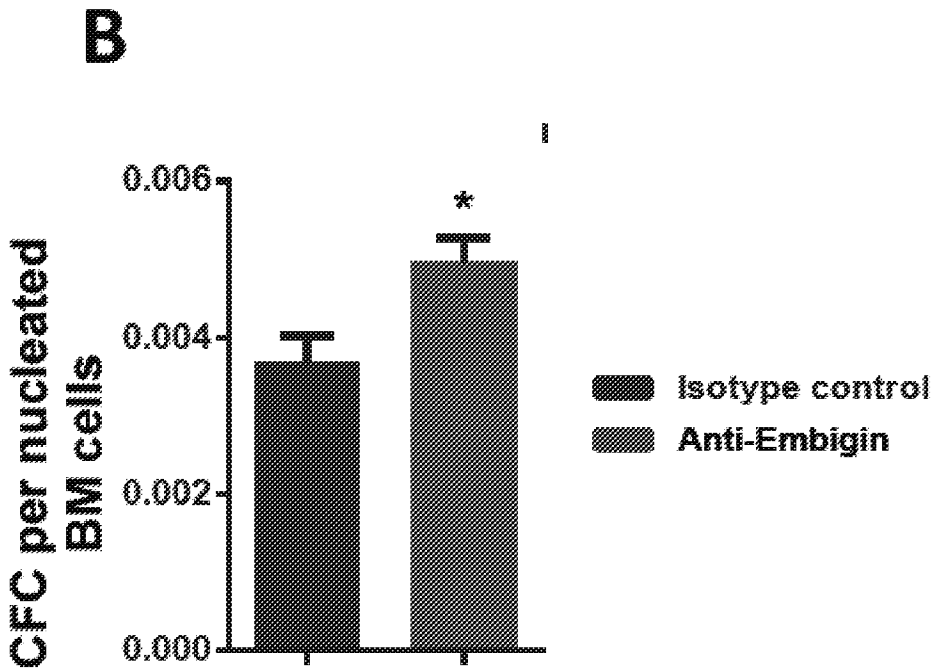

B
0.006
0.004
0.002
0.000
CFC per nucleated BM cells
*
Isotype control
Anti-Embigin

FIG. 11C

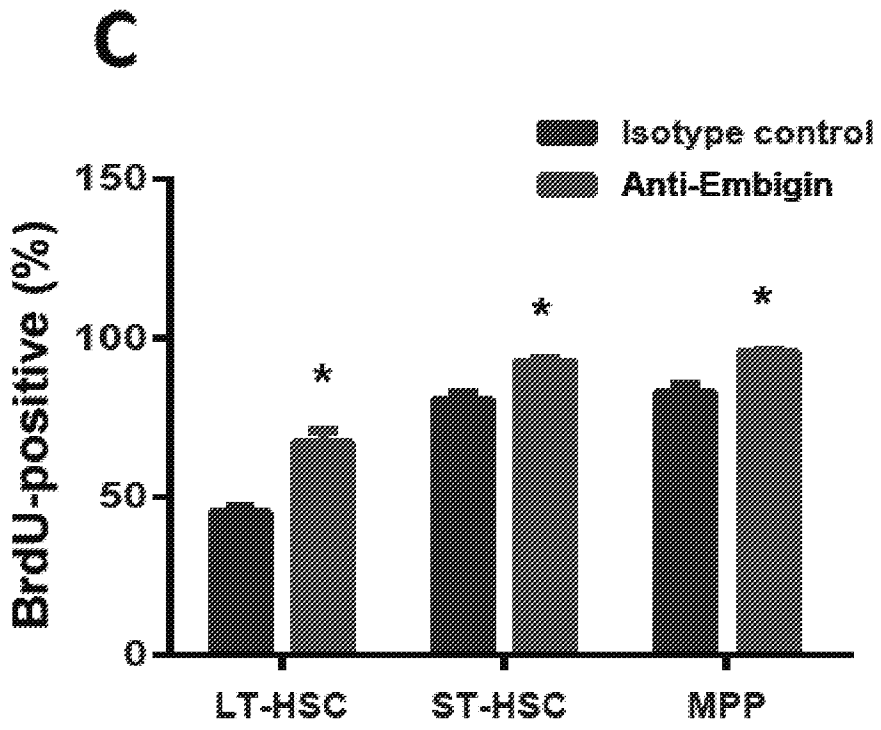

C
Isotype control
Anti-Embigin
BrdU-positive (%)
150
100
50
0
*
*
*
LT-HSC
ST-HSC
MPP

FIG. 11D

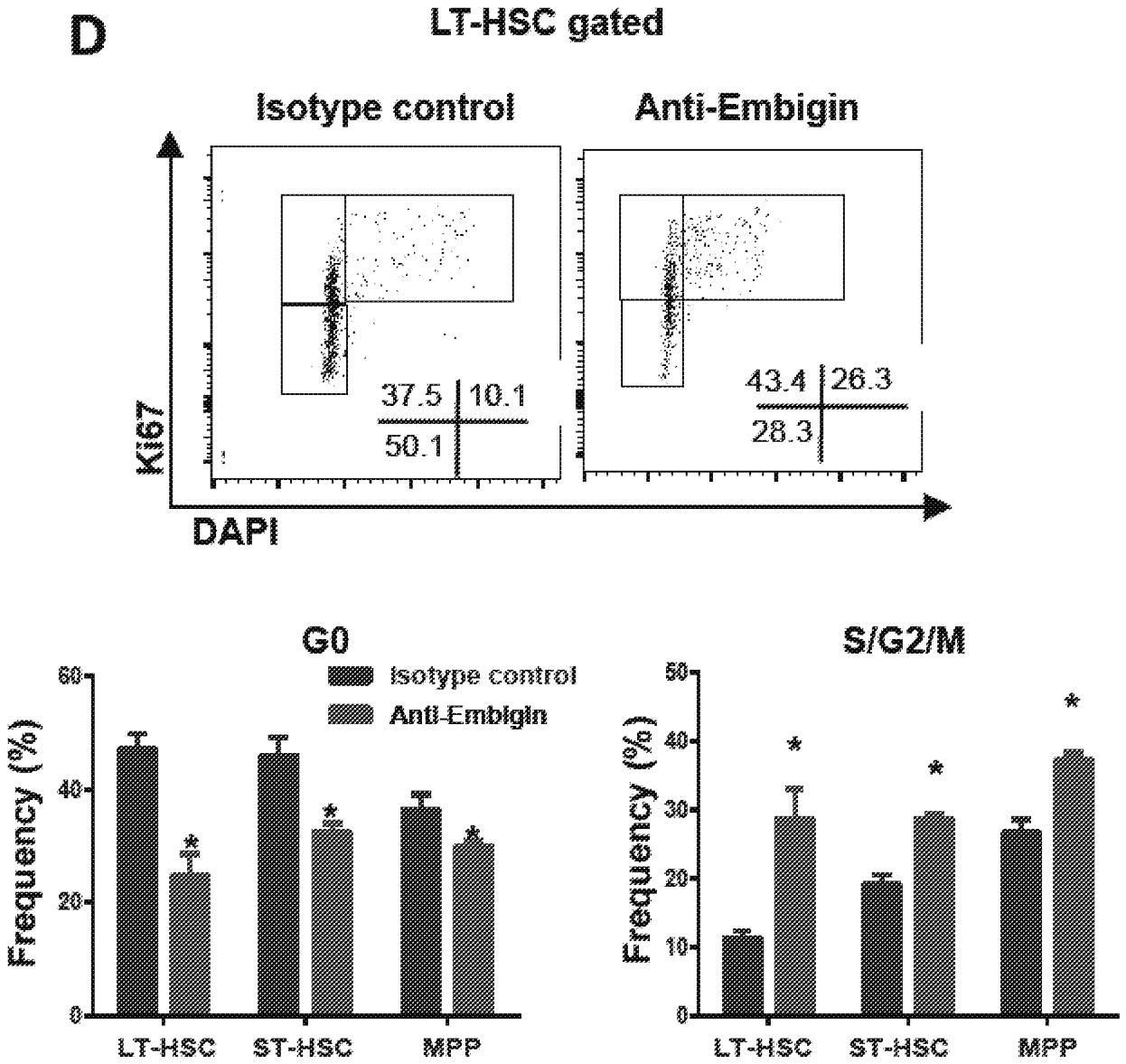

D
LT-HSC gated
Isotype control
Anti-Embigin
Ki67
DAPI
37.5
10.1
50.1
43.4
26.3
28.3
G0
S/G2/M
Isotype control
Anti-Embigin
Frequency (%)
LT-HSC
ST-HSC
MPP

FIG. 11E

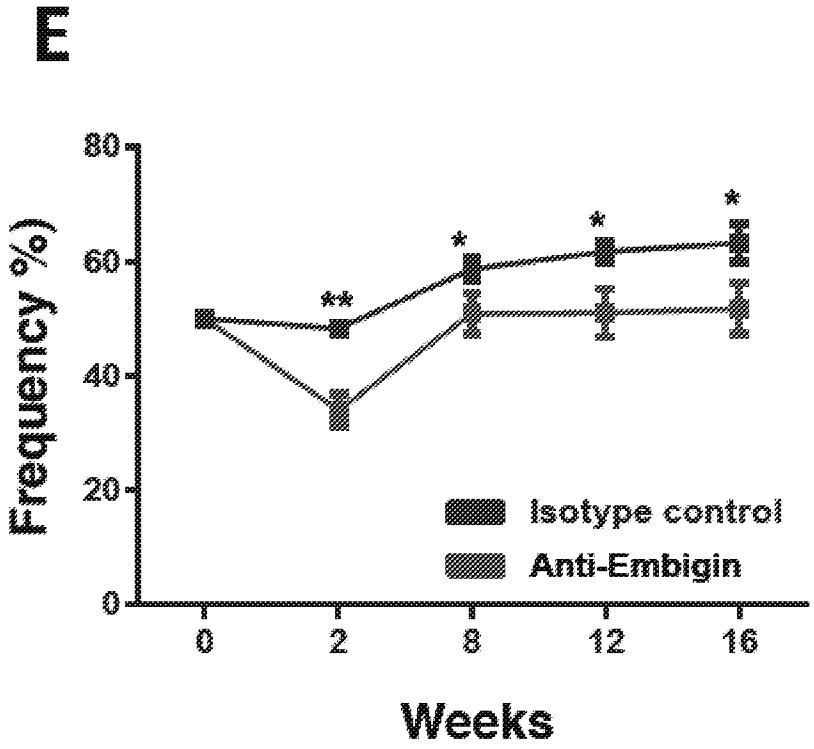

E
Frequency %)
80
60
40
20
0
**
*
*
*
Isotype control
Anti-Embigin
0
2
8
12
16
Weeks

FIG. 12A

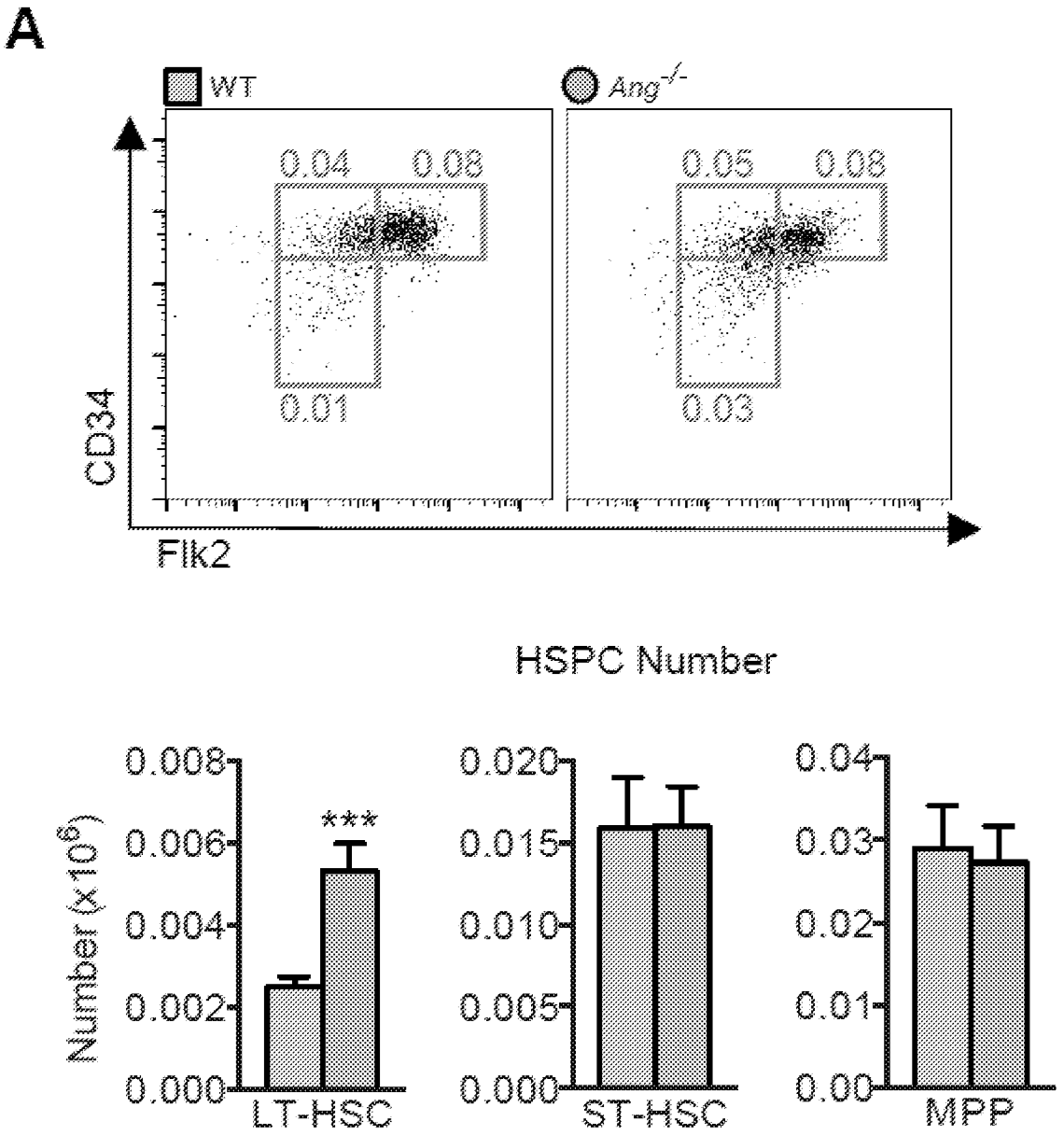

A
WT
Ang-/-
0.04
0.08
0.01
0.05
0.08
0.03
CD34
Flk2
HSPC Number
Number (x10^6)
0.008
0.006
0.004
0.002
0.000
***
LT-HSC
0.020
0.015
0.010
0.005
0.000
ST-HSC
0.04
0.03
0.02
0.01
0.00
MPP

FIG. 12B

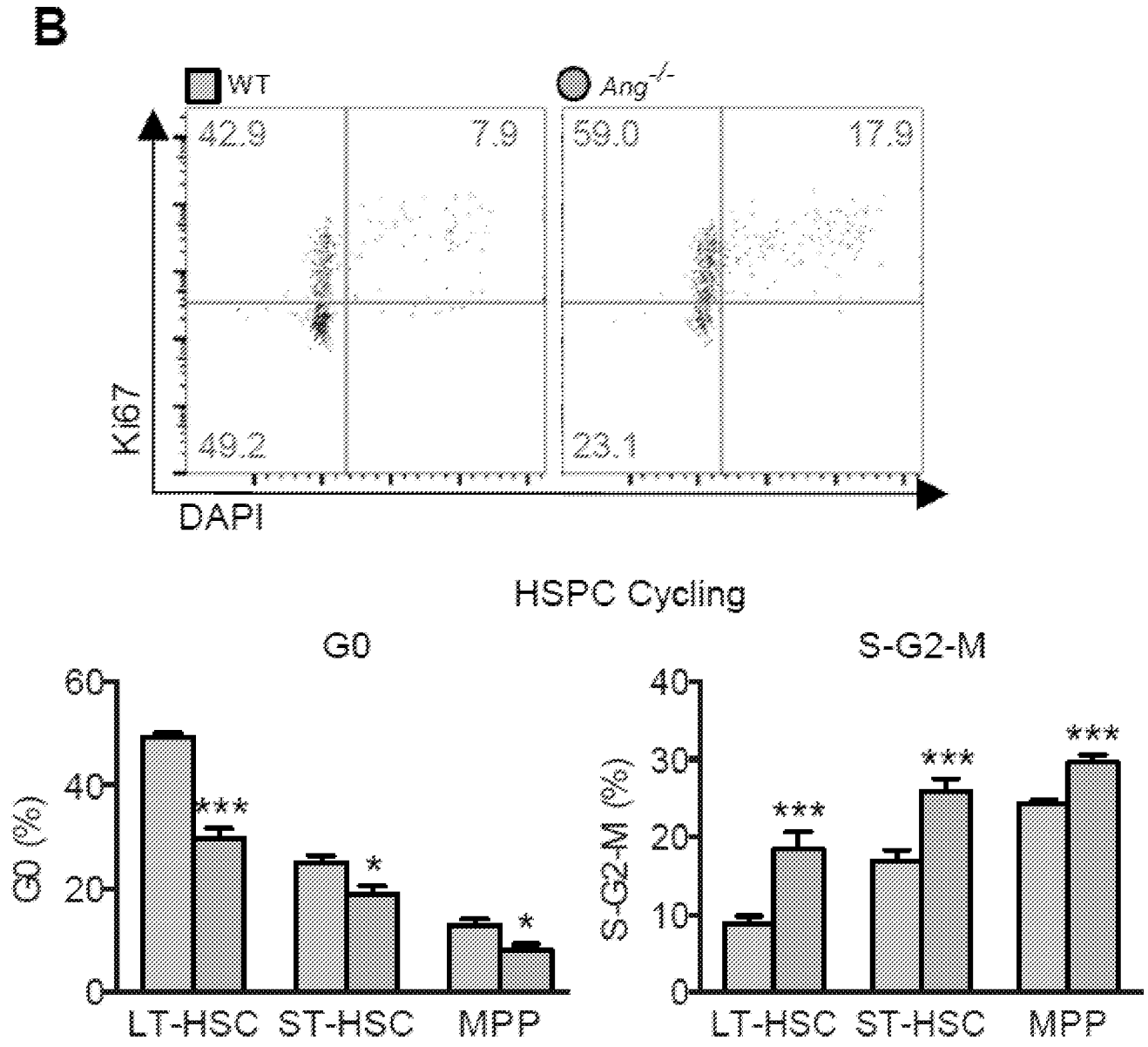

B
WT
Ang-/-
42.9
7.9
59.0
17.9
49.2
23.1
Ki67
DAPI
HSPC Cycling
G0
S-G2-M
G0 (%)
S-G2-M (%)
60
40
20
0
30
10
***
*
LT-HSC
ST-HSC
MPP

FIG. 12C

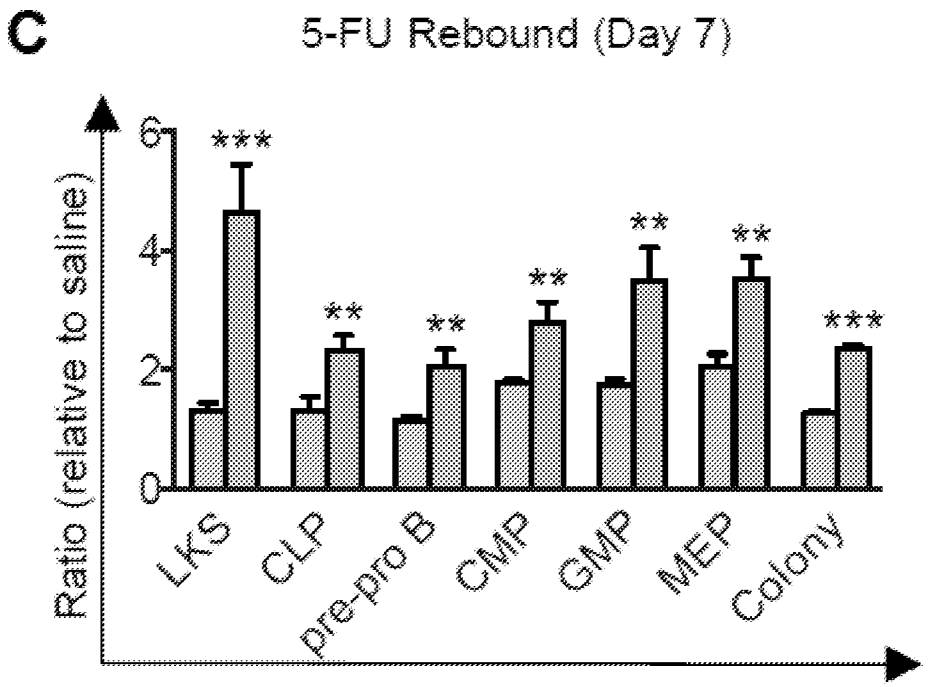
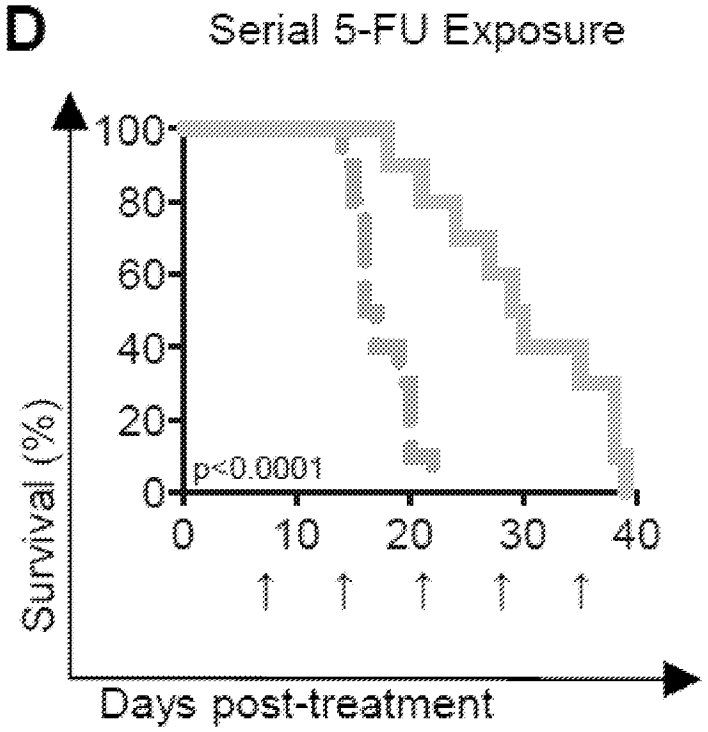

C
5-FU Rebound (Day 7)
Ratio (relative to saline)
0
2
4
6
***
**
**
**
**
**
***
LKS
CLP
pre-pro B
CMP
GMP
MEP
Colony

FIG. 12D-12E


D
Serial 5-FU Exposure
100
80
60
40
20
0
Survival (%)
p<0.0001
0
10
20
30
40
Days post-treatment

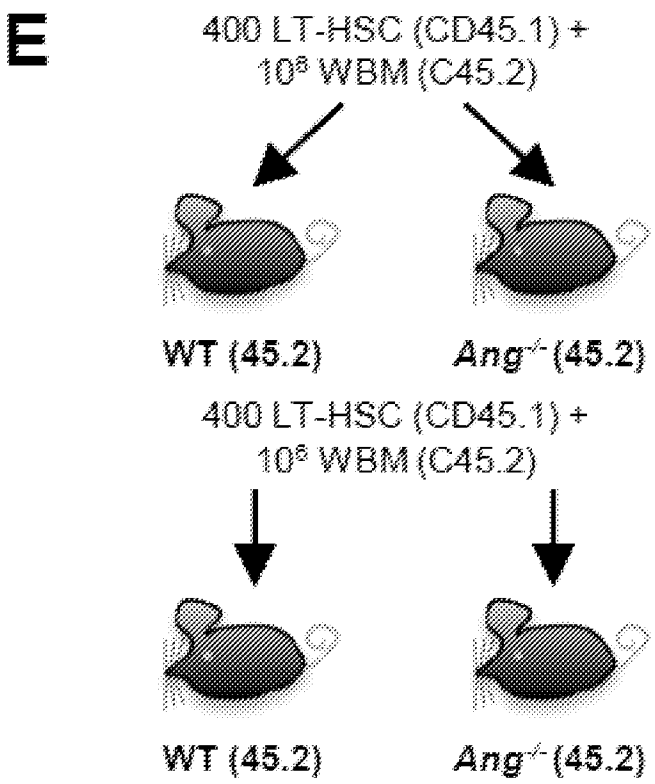

E
400 LT-HSC (CD45.1) +
10⁶ WBM (C45.2)
WT (45.2)
Ang-/- (45.2)
400 LT-HSC (CD45.1) +
10⁶ WBM (C45.2)
WT (45.2)
Ang-/- (45.2)

FIG. 12F

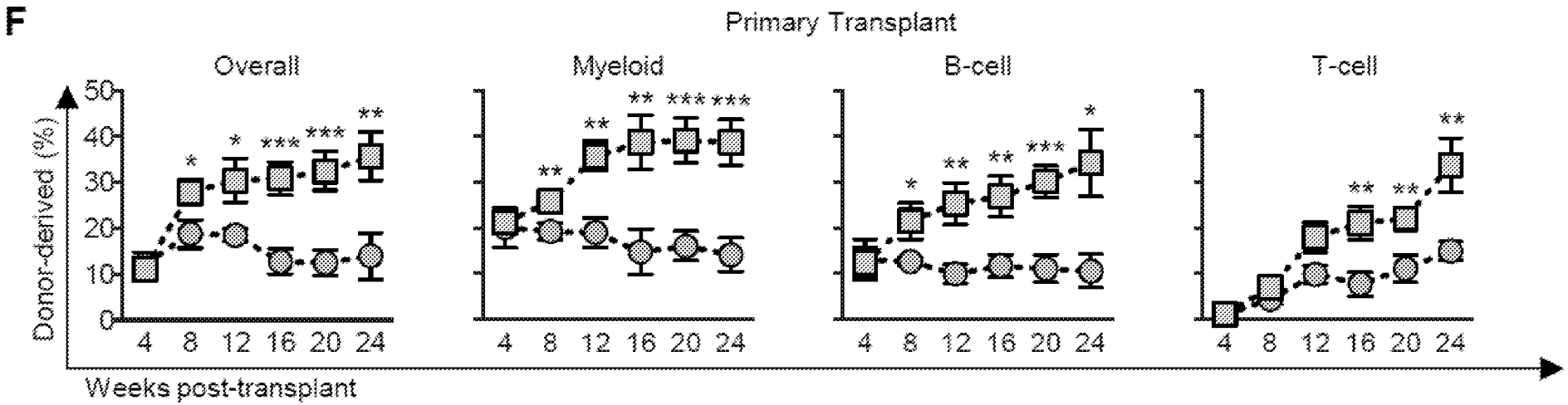

F
Primary Transplant
Overall
Myeloid
B-cell
T-cell
Donor-derived (%)
0
10
20
30
40
50
4 8 12 16 20 24
Weeks post-transplant

FIG. 12G

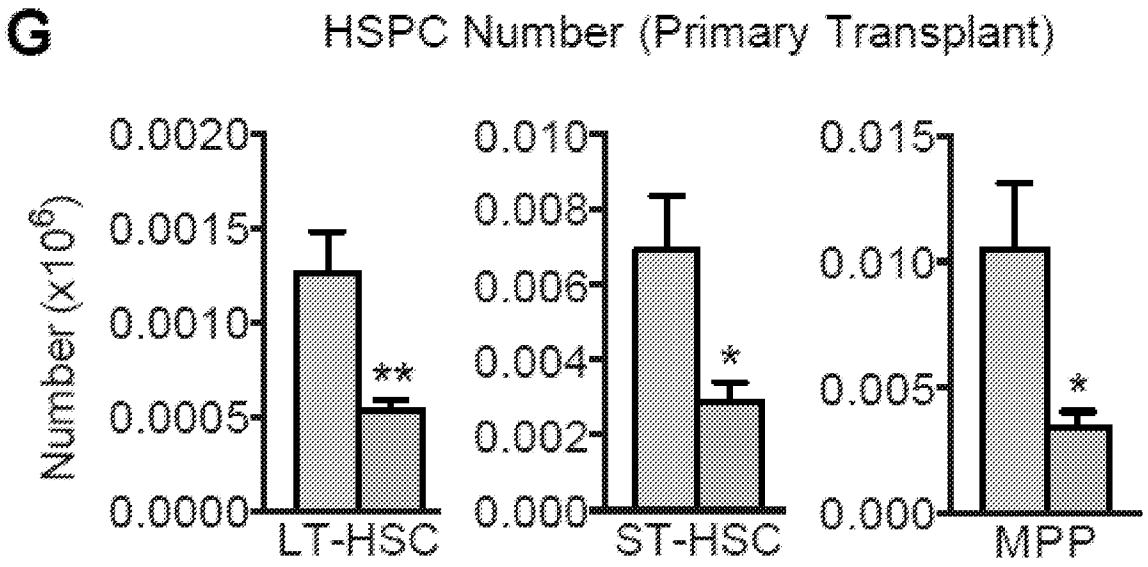

G
HSPC Number (Primary Transplant)
Number (x10^6)
0.0020
0.0015
0.0010
0.0005
0.0000
**
LT-HSC
0.010
0.008
0.006
0.004
0.002
0.000
*
ST-HSC
0.015
0.010
0.005
0.000
*
MPP

FIG. 12H

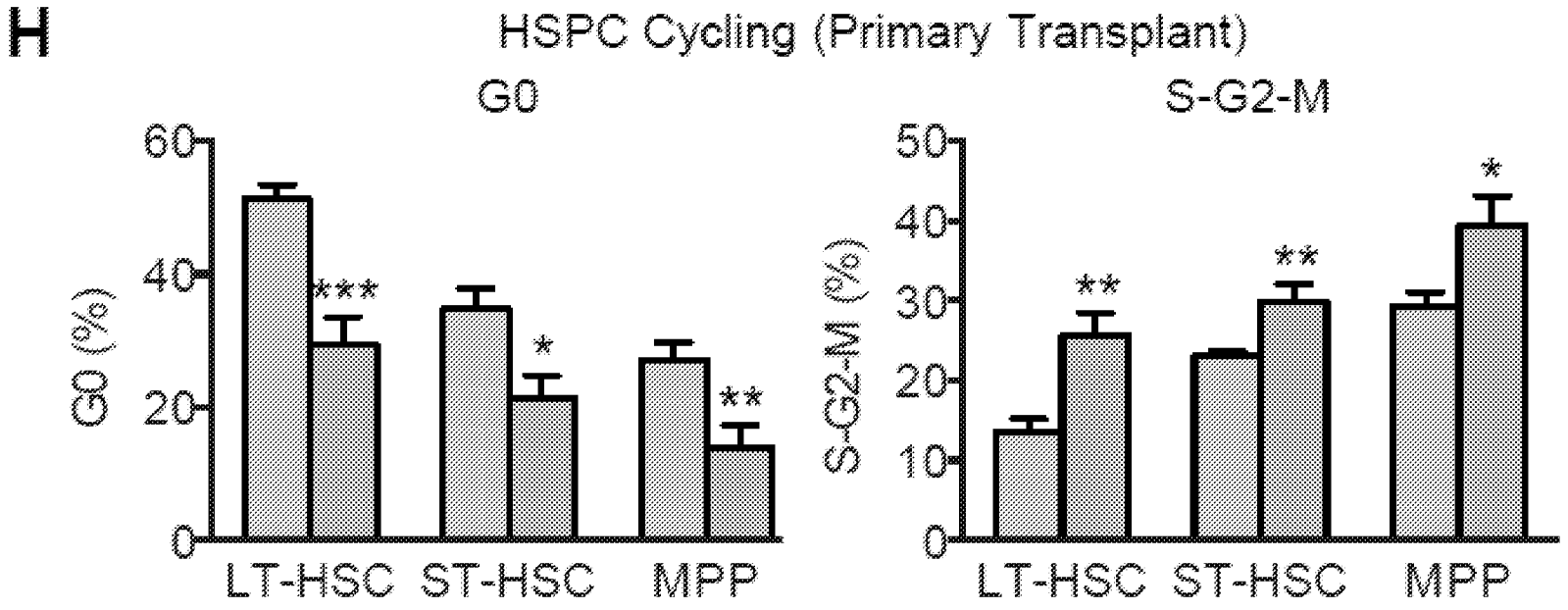

H
HSPC Cycling (Primary Transplant)
G0
G0 (%)
60
40
20
0
***
*
**
LT-HSC
ST-HSC
MPP
S-G2-M
S-G2-M (%)
50
40
30
20
10
0
**
**
*
LT-HSC
ST-HSC
MPP

FIG. 12I

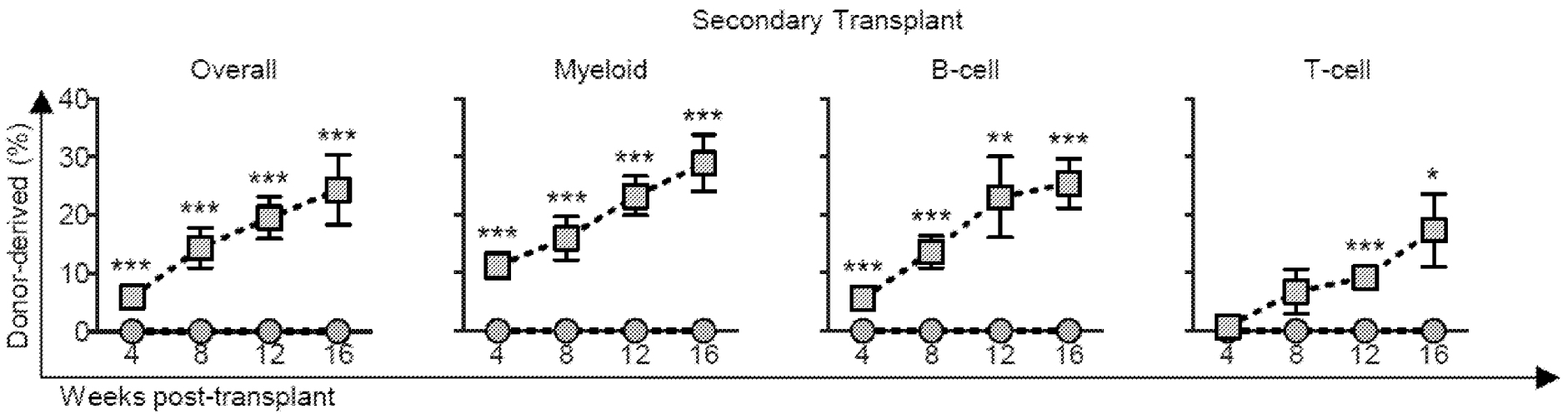
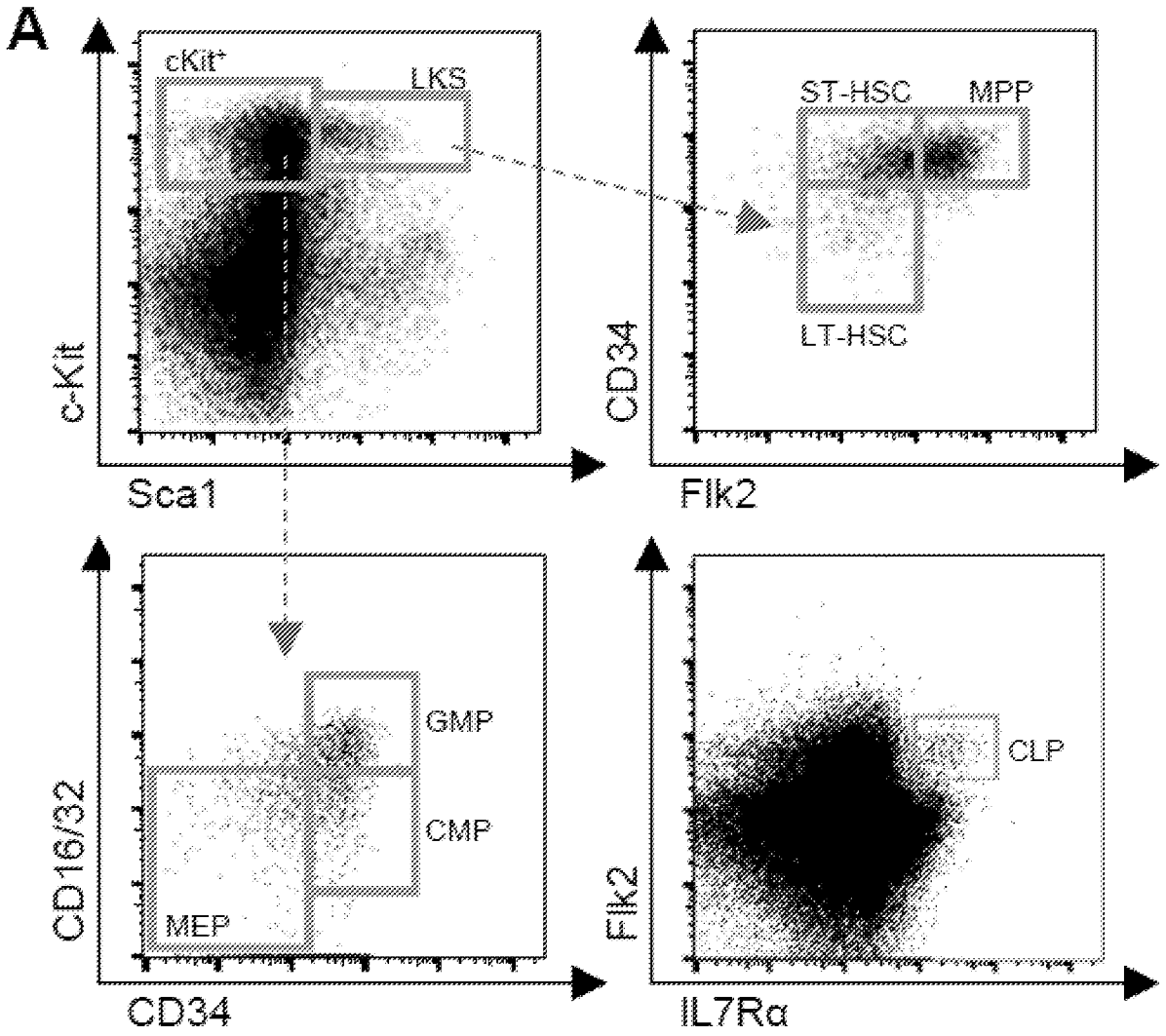
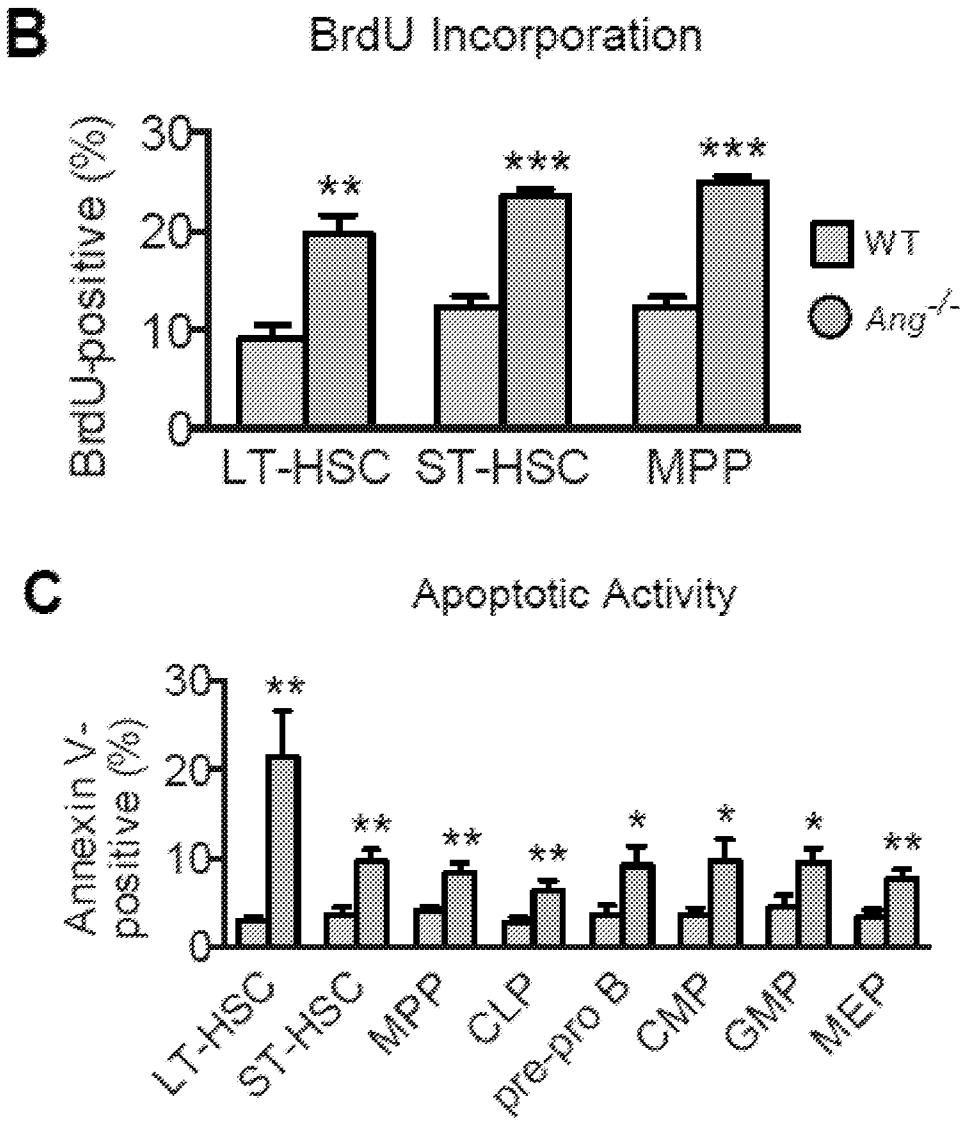

Secondary Transplant
Overall
Myeloid
B-cell
T-cell
Donor-derived (%)
40
30
20
10
0
4
8
12
16
***
**
*
Weeks post-transplant

FIG. 13A


A
cKit+
LKS
c-Kit
Sca1
ST-HSC
MPP
LT-HSC
CD34
Flk2
GMP
CMP
MEP
CD16/32
CD34
CLP
Flk2
IL7Rα

FIGs. 13B-13C


B
BrdU Incorporation
BrdU-positive (%)
30
20
10
0
**
***
***
WT
Ang-/-
LT-HSC
ST-HSC
MPP
C
Apoptotic Activity
Annexin V-positive (%)
30
20
10
0
**
**
**
**
*
*
*
**
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP

FIGs. 13D-13E

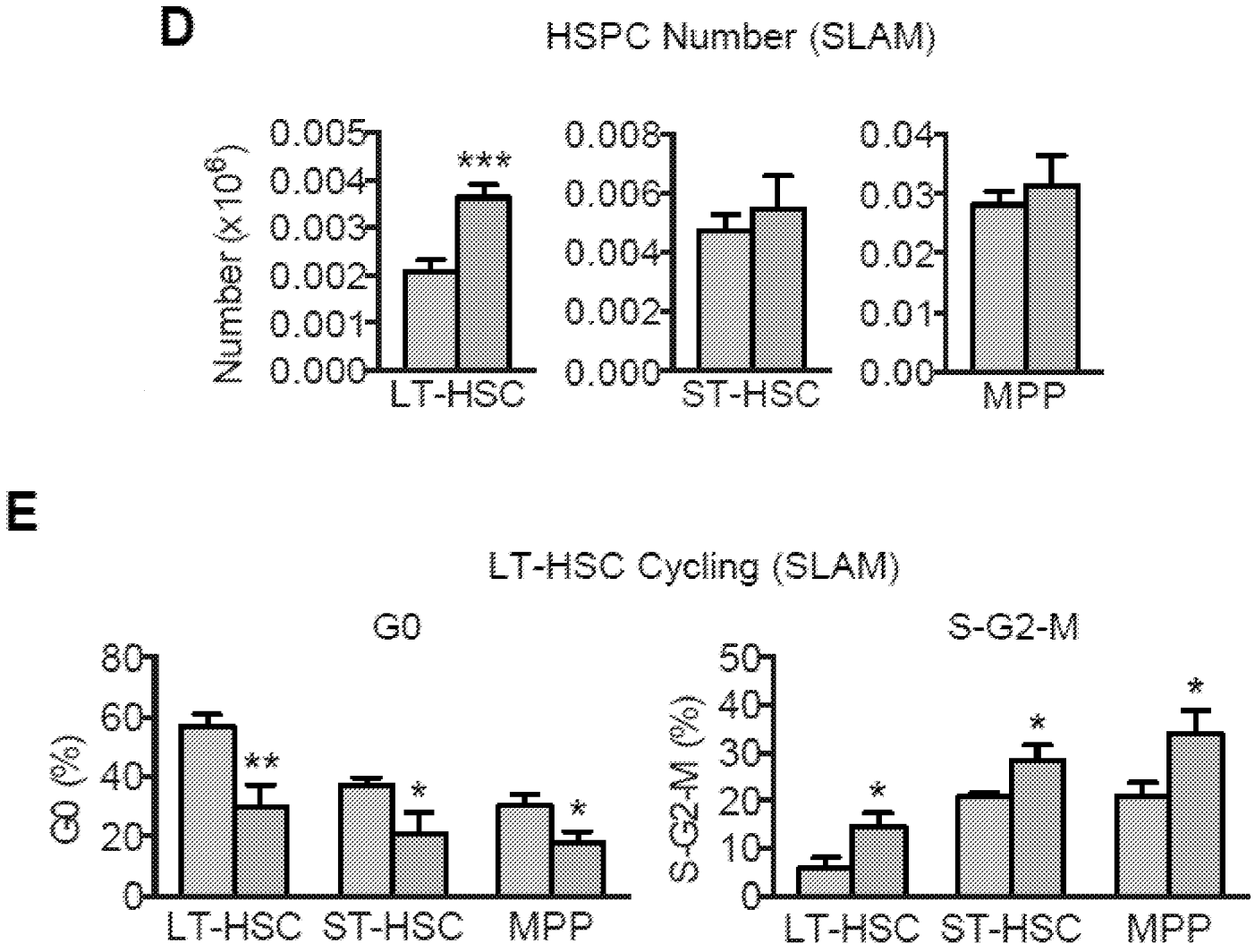

D
HSPC Number (SLAM)
Number (x10^6)
0.005
0.004
0.003
0.002
0.001
0.000
***
LT-HSC
0.008
0.006
0.004
0.002
0.000
ST-HSC
0.04
0.03
0.02
0.01
0.00
MPP
E
LT-HSC Cycling (SLAM)
G0
G0 (%)
80
60
40
20
0
**
*
*
LT-HSC
ST-HSC
MPP
S-G2-M
S-G2-M (%)
50
40
30
20
10
0
*
*
*
LT-HSC
ST-HSC
MPP

FIG. 13F

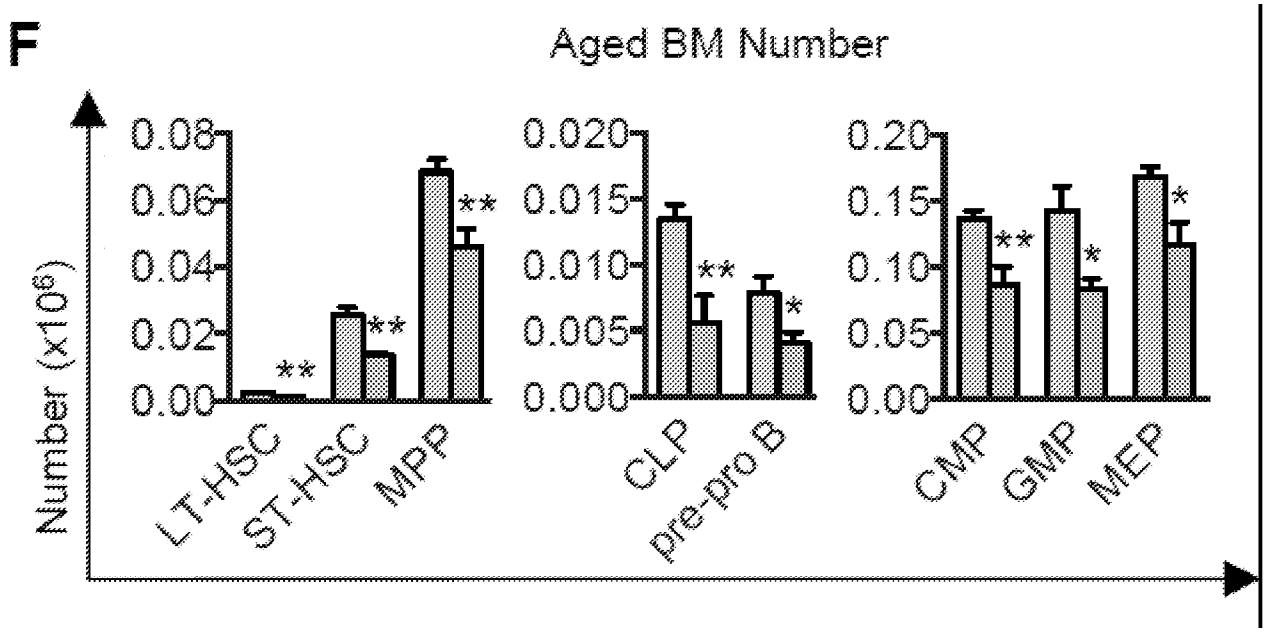

F
Aged BM Number
Number (x10^6)
0.08
0.06
0.04
0.02
0.00
0.020
0.015
0.010
0.005
0.000
0.20
0.15
0.10
0.05
0.00
**
**
**
**
*
**
*
*
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP

FIG. 13G

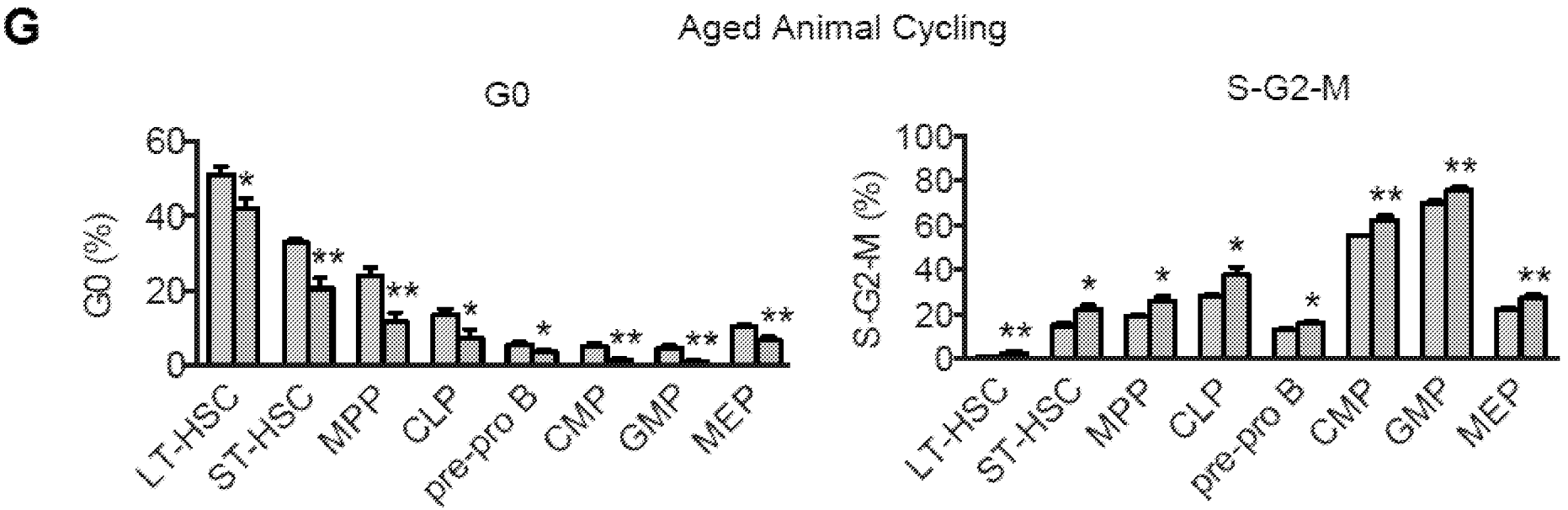
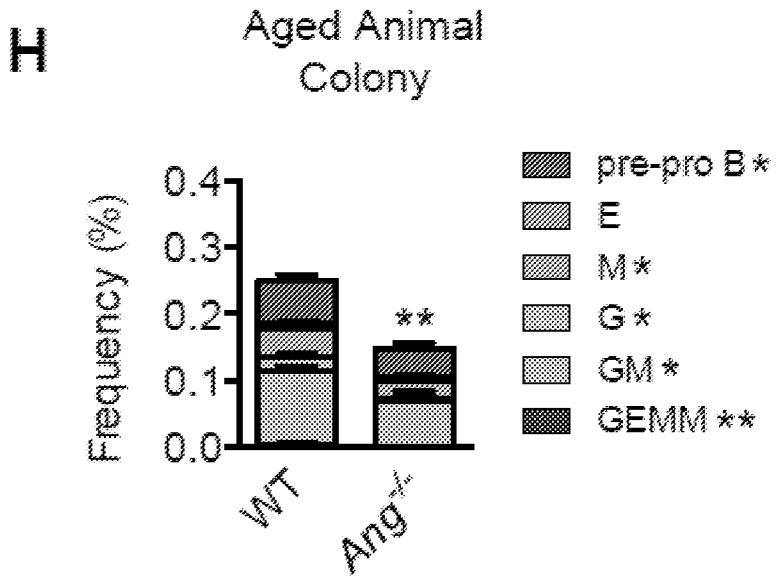

G
Aged Animal Cycling
G0
G0 (%)
60
40
20
0
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP
S-G2-M
S-G2-M (%)
100
80
60
40
20
0

FIG. 13H


H
Aged Animal Colony
Frequency (%)
0.4
0.3
0.2
0.1
0.0
**
WT
Ang-/-
pre-pro B *
E
M *
G *
GM *
GEMM **

FIGs. 13I-13J

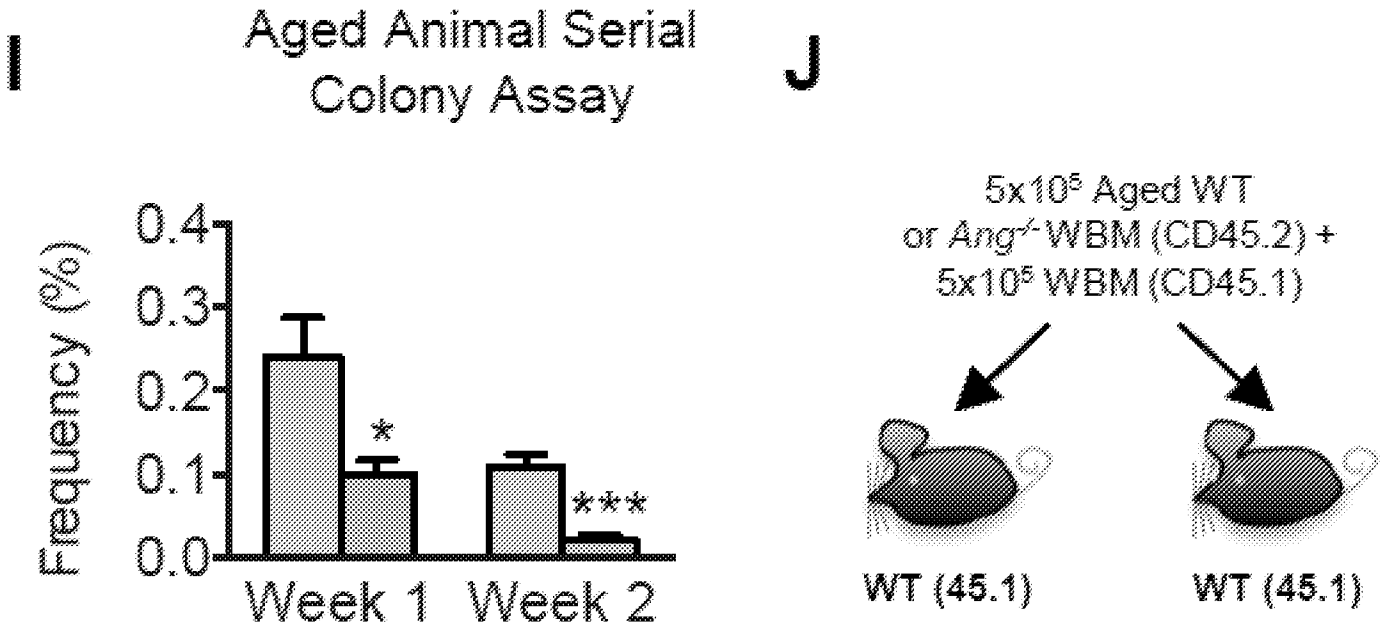

I
Aged Animal Serial Colony Assay
Frequency (%)
0.4
0.3
0.2
0.1
0.0
*
***
Week 1
Week 2
J
5x10^5 Aged WT or Ang^-/- WBM (CD45.2) + 5x10^5 WBM (CD45.1)
WT (45.1)
WT (45.1)

FIG. 13k

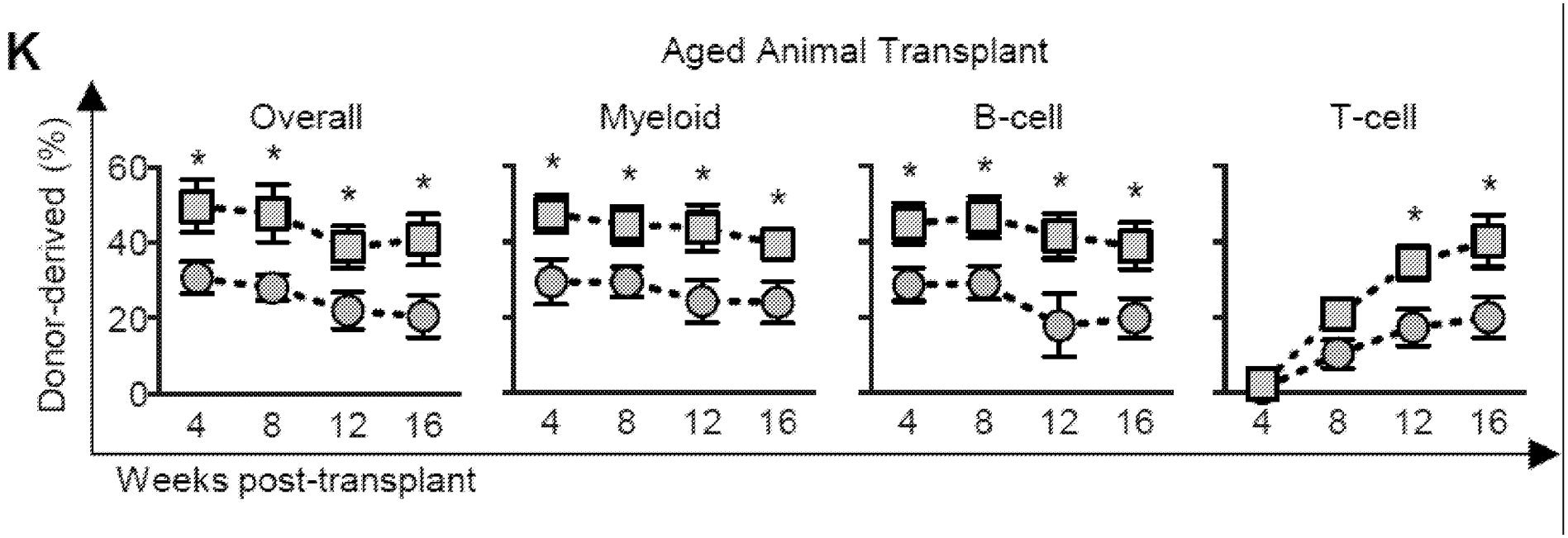

K
Aged Animal Transplant
Overall
Myeloid
B-cell
T-cell
Donor-derived (%)
60
40
20
0
4
8
12
16
Weeks post-transplant

FIG. 13L

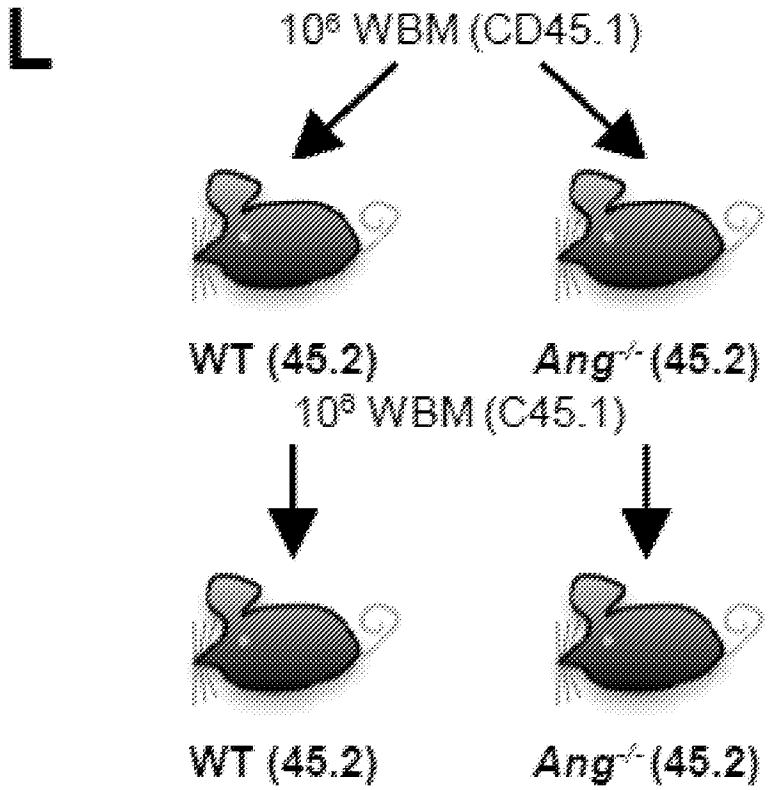
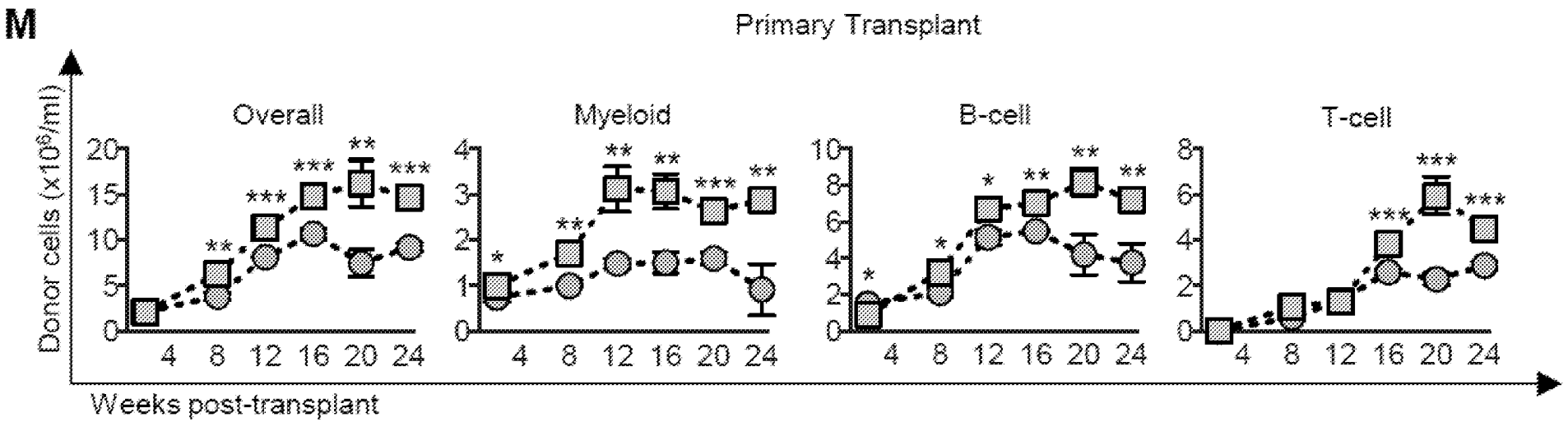


FIG. 13M


M
Primary Transplant
Overall
Myeloid
B-cell
T-cell
Donor cells (x10^6/ml)
Weeks post-transplant
4 8 12 16 20 24

FIGs. 13N-13O

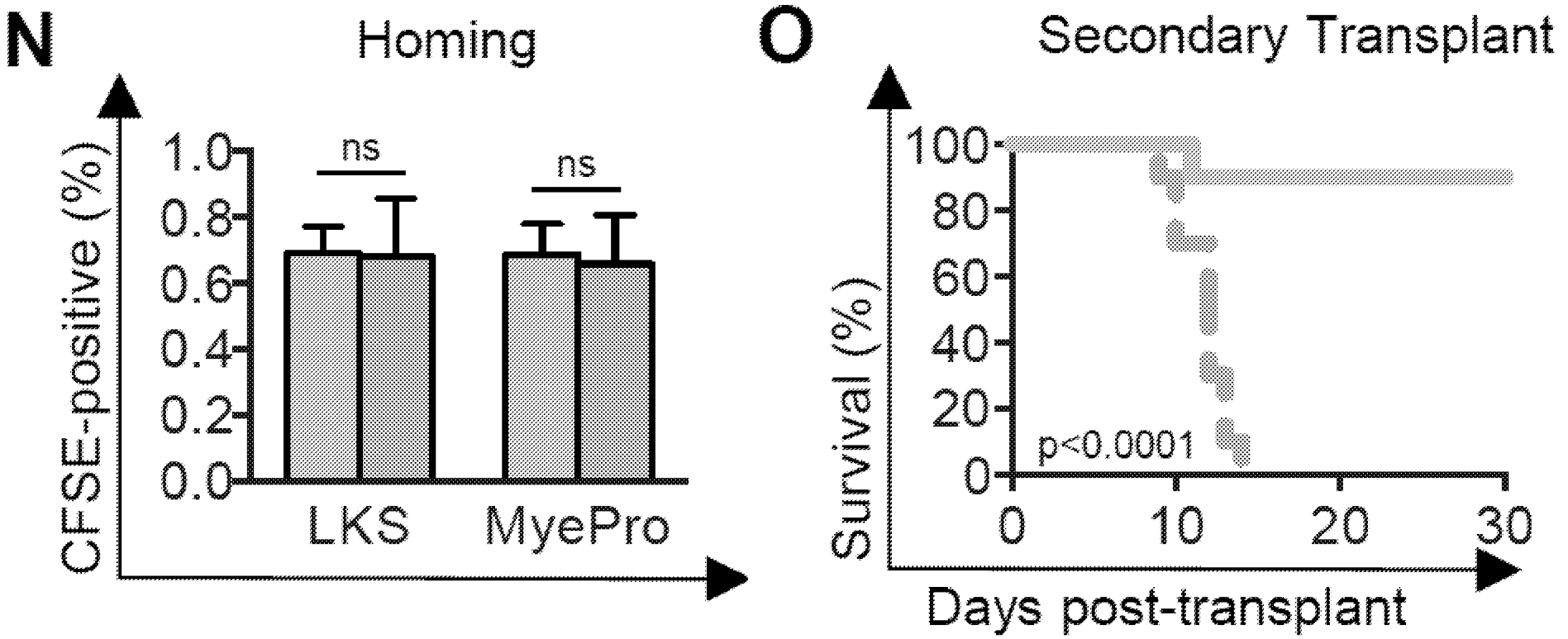

N
Homing
CFSE-positive (%)
1.0
0.8
0.6
0.4
0.2
0.0
ns
ns
LKS
MyePro
O
Secondary Transplant
Survival (%)
100
80
60
40
20
0
p<0.0001
0
10
20
30
Days post-transplant

FIG. 14A

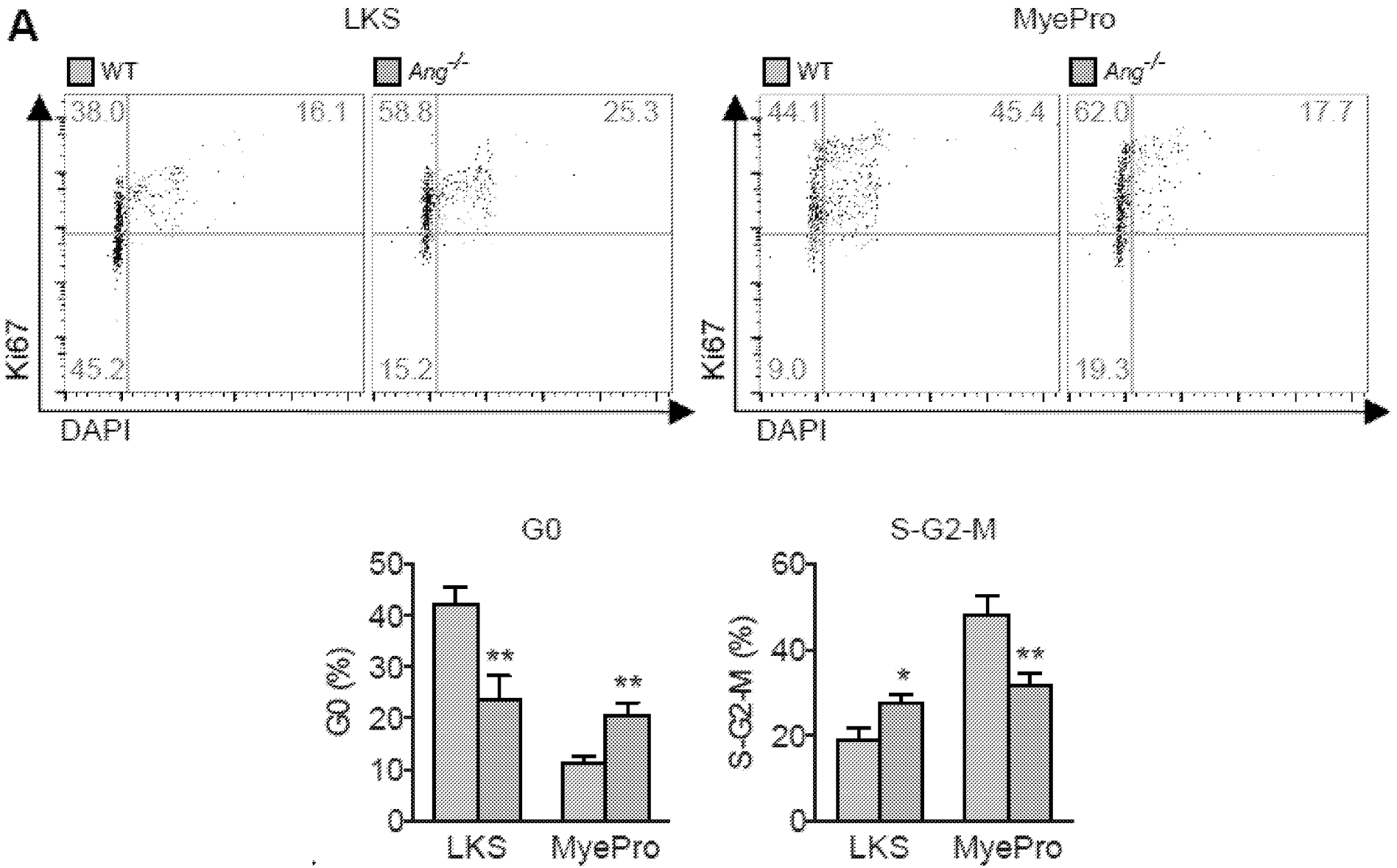

A
LKS
WT
Ang-/-
38.0
16.1
58.8
25.3
45.2
15.2
Ki67
DAPI
MyePro
WT
Ang-/-
44.1
45.4
62.0
17.7
9.0
19.3
Ki67
DAPI
G0
G0 (%)
50
40
30
20
10
0
**
**
LKS
MyePro
S-G2-M
S-G2-M (%)
60
40
20
0
*
**
LKS
MyePro

FIG. 14B

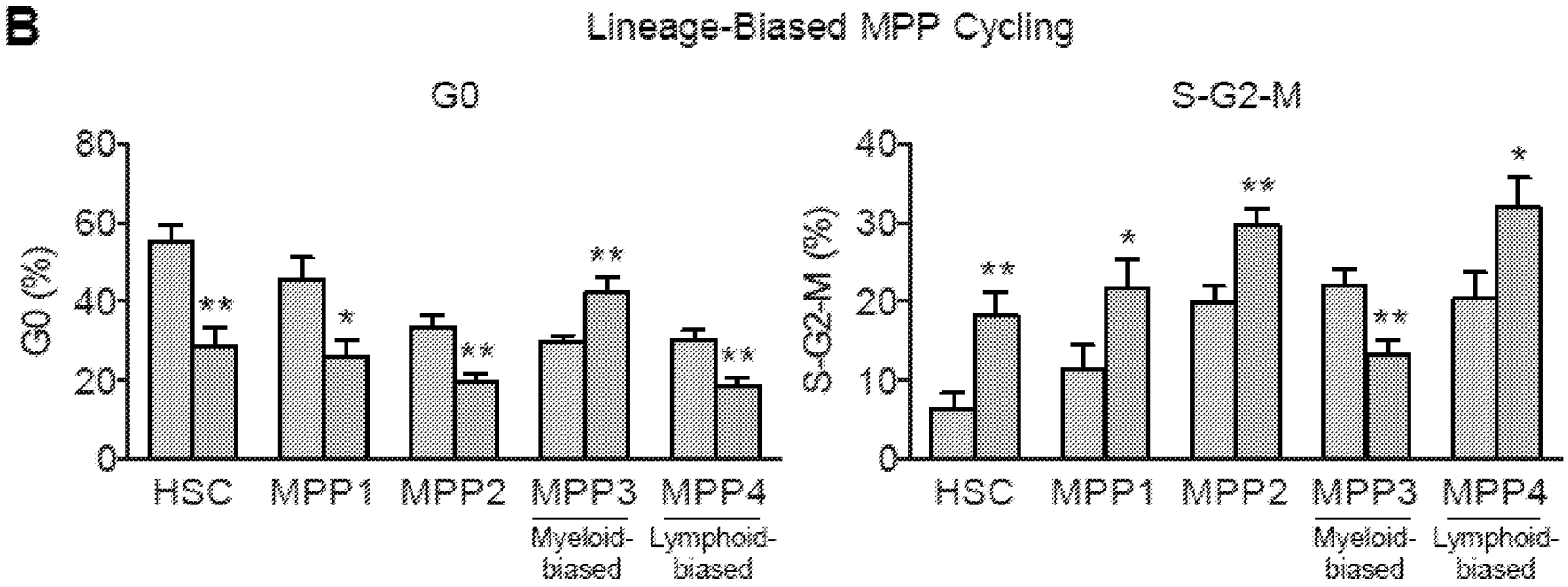

B
Lineage-Biased MPP Cycling
G0
G0 (%)
80
60
40
20
0
**
*
**
**
**
HSC
MPP1
MPP2
MPP3
MPP4
Myeloid-biased
Lymphoid-biased
S-G2-M
S-G2-M (%)
40
30
20
10
0
**
*
**
**
*
HSC
MPP1
MPP2
MPP3
MPP4
Myeloid-biased
Lymphoid-biased

FIG. 14C

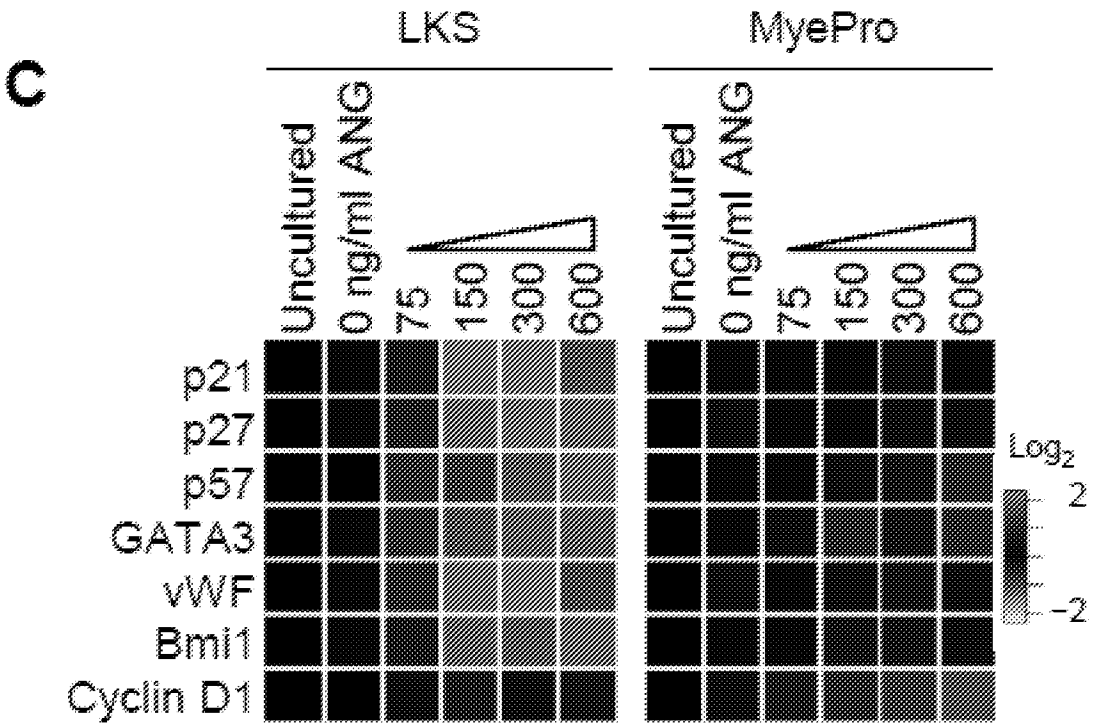
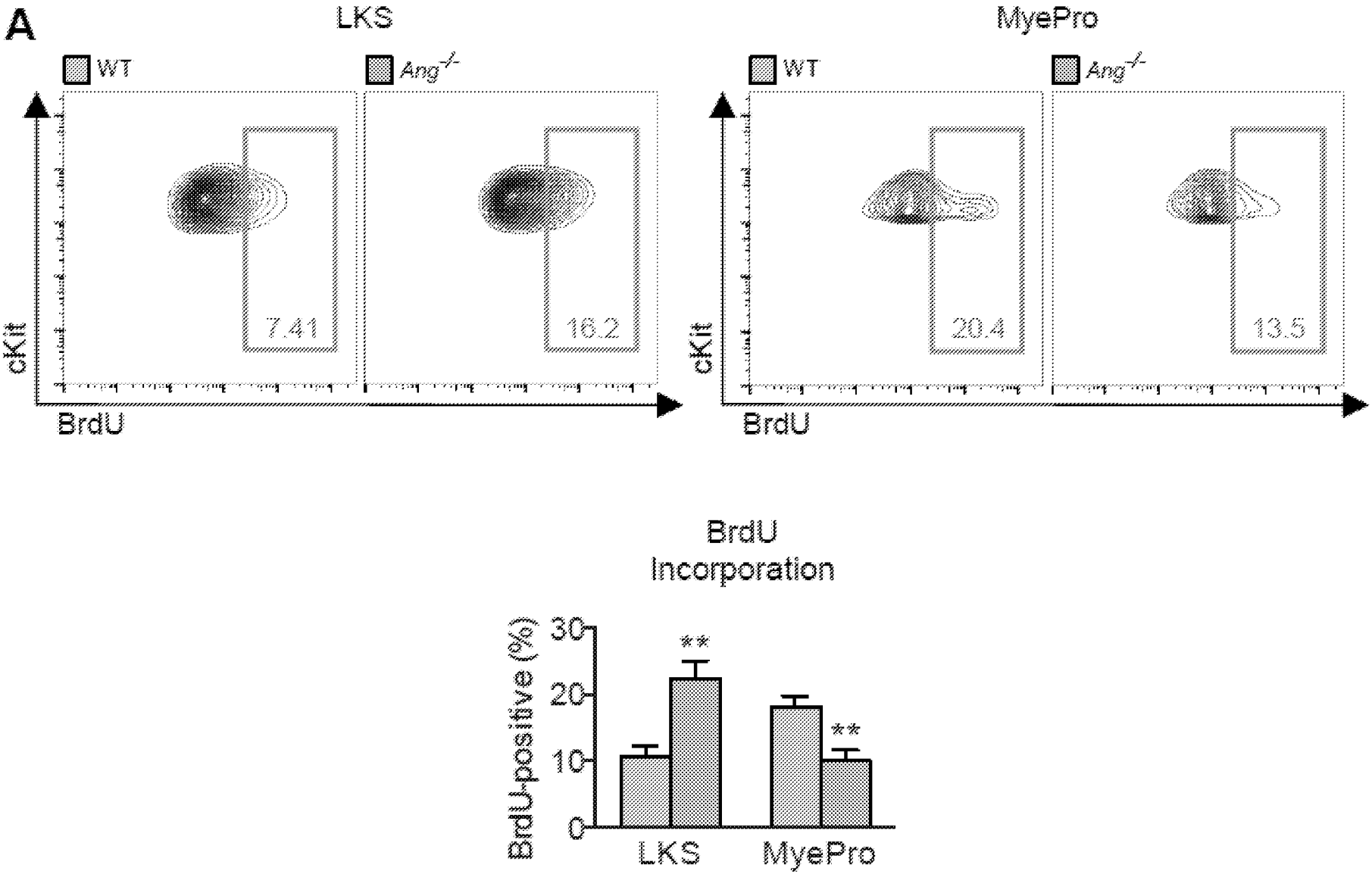
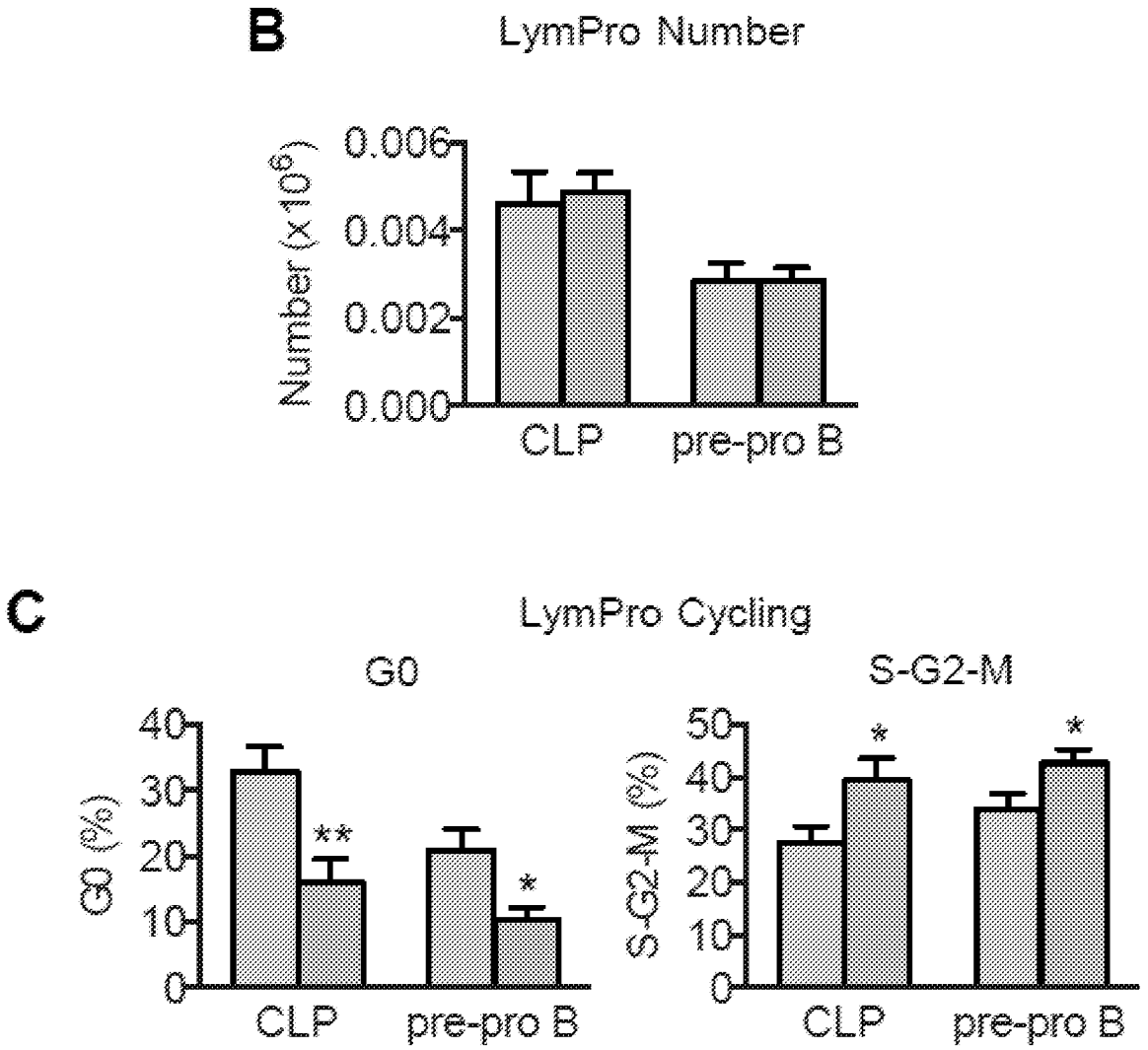

C
LKS
MyePro
Uncultured
0 ng/ml ANG
75
150
300
600
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
2
−2

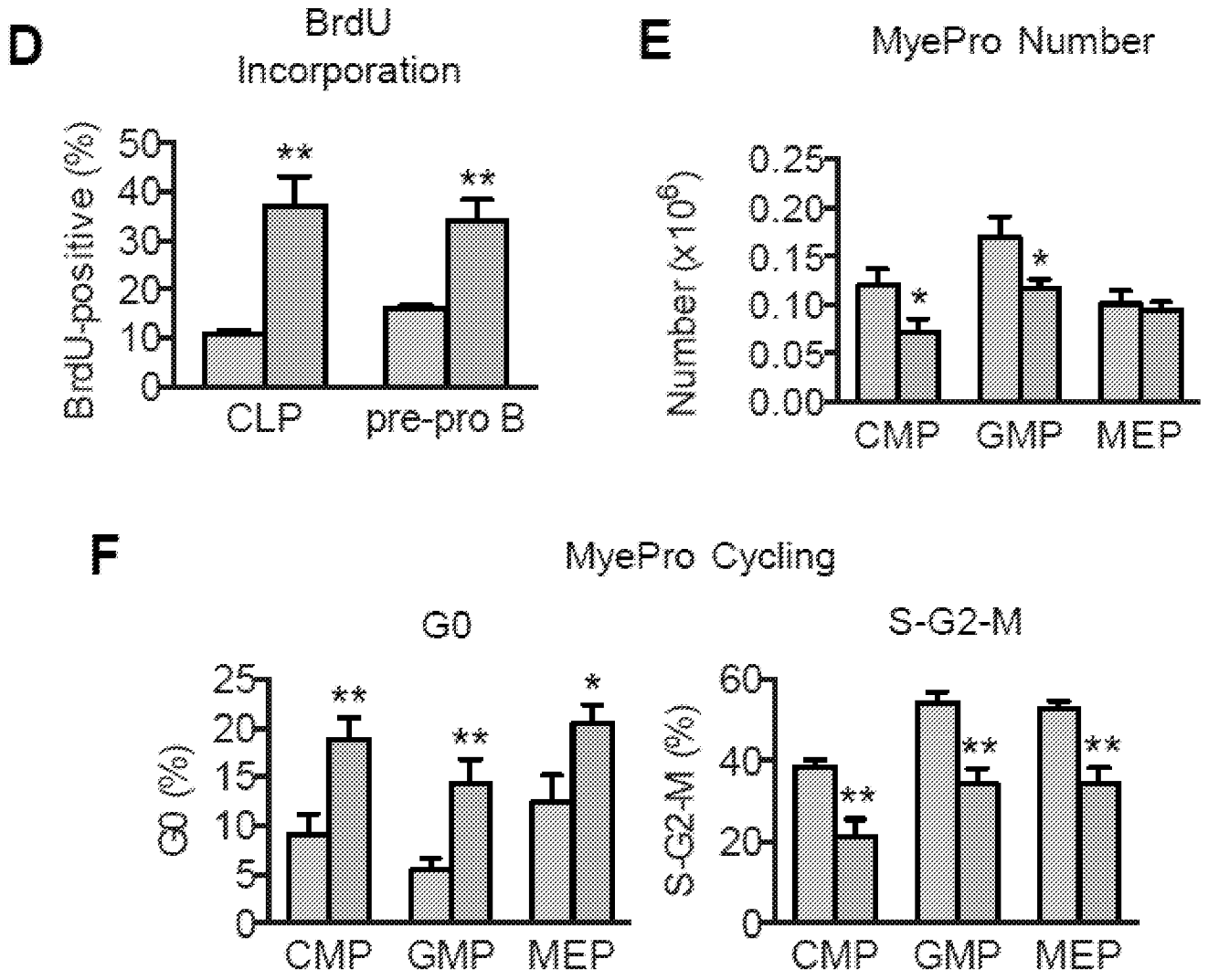
FIG. 15A


A
LKS
MyePro
WT
Ang-/-
WT
Ang-/-
cKit
BrdU
7.41
16.2
20.4
13.5
cKit
BrdU
BrdU
Incorporation
BrdU-positive (%)
30
20
10
0
**
**
LKS
MyePro

FIGs. 15B-15C


B
LymPro Number
Number (x10^6)
0.006
0.004
0.002
0.000
CLP
pre-pro B
C
LymPro Cycling
G0
G0 (%)
40
30
20
10
0
**
*
CLP
pre-pro B
S-G2-M
S-G2-M (%)
50
40
30
20
10
0
*
*
CLP
pre-pro B

FIGs. 15D-15F


D
BrdU Incorporation
BrdU-positive (%)
50
40
30
20
10
0
**
**
CLP
pre-pro B
E
MyePro Number
Number (x10^6)
0.25
0.20
0.15
0.10
0.05
0.00
*
*
CMP
GMP
MEP
F
MyePro Cycling
G0
S-G2-M
G0 (%)
25
20
15
10
5
0
**
**
*
CMP
GMP
MEP
S-G2-M (%)
60
40
20
0
**
**
**
CMP
GMP
MEP

FIGs. 15G-15H

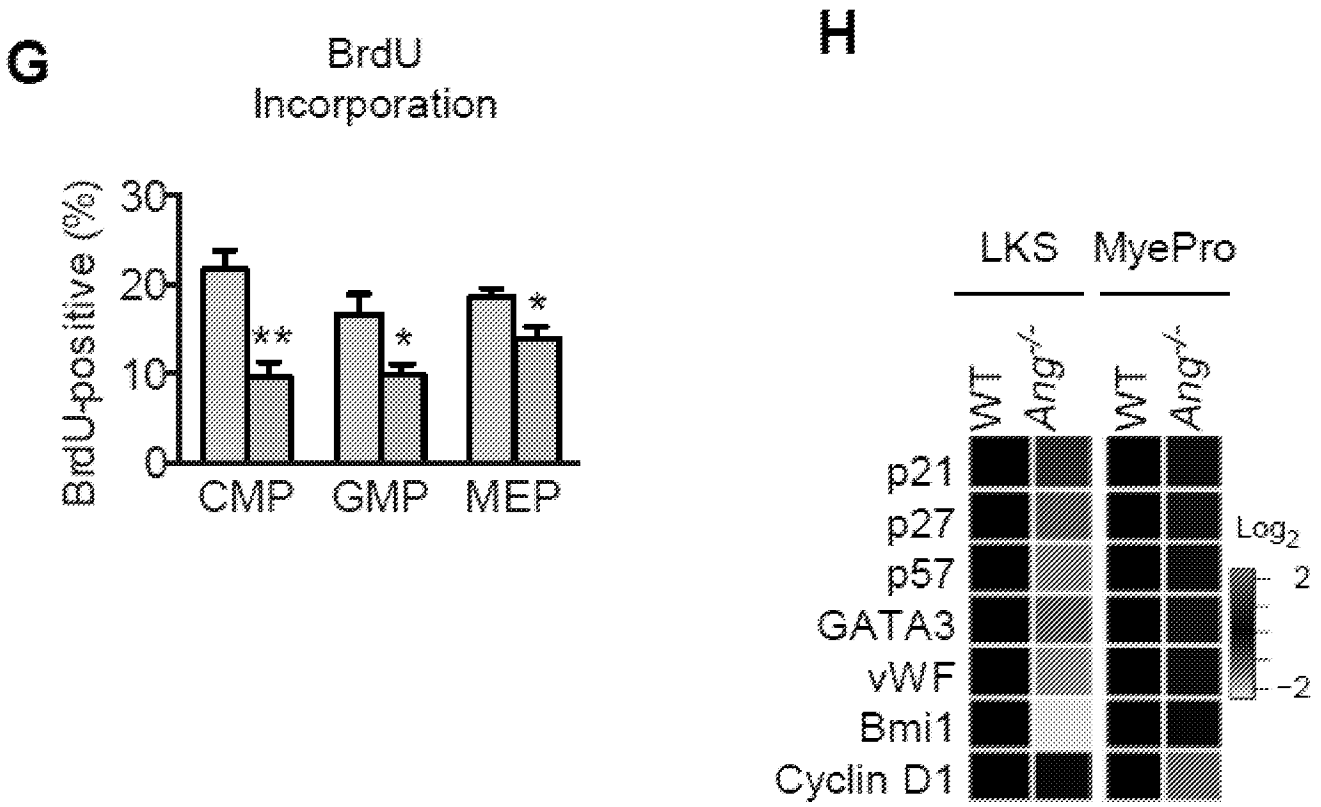

G
BrdU Incorporation
BrdU-positive (%)
30
20
10
0
**
*
*
CMP
GMP
MEP
H
LKS
MyePro
WT
Ang-/-
WT
Ang-/-
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
2
-2

FIG. 15I

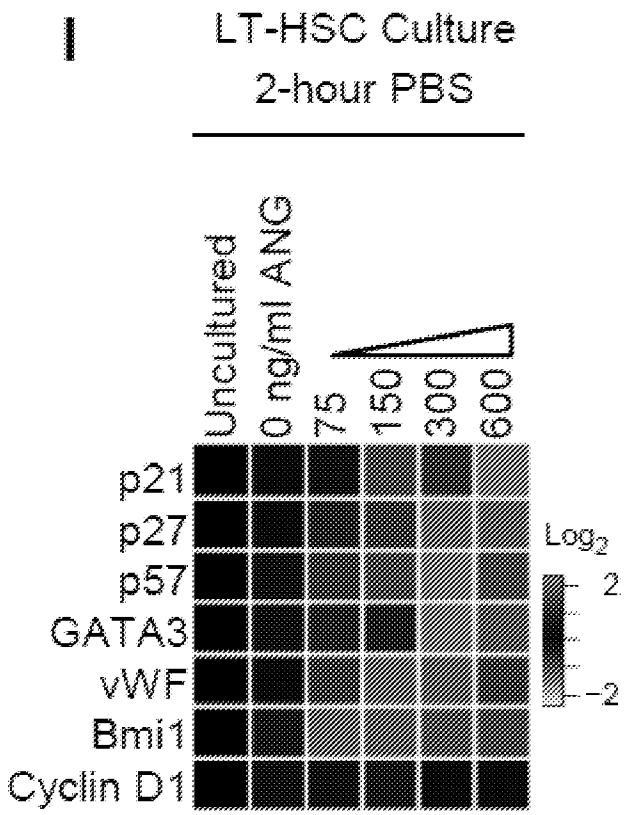

LT-HSC Culture
2-hour PBS
Uncultured
0 ng/ml ANG
75
150
300
600
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
2
-2

FIG. 15J

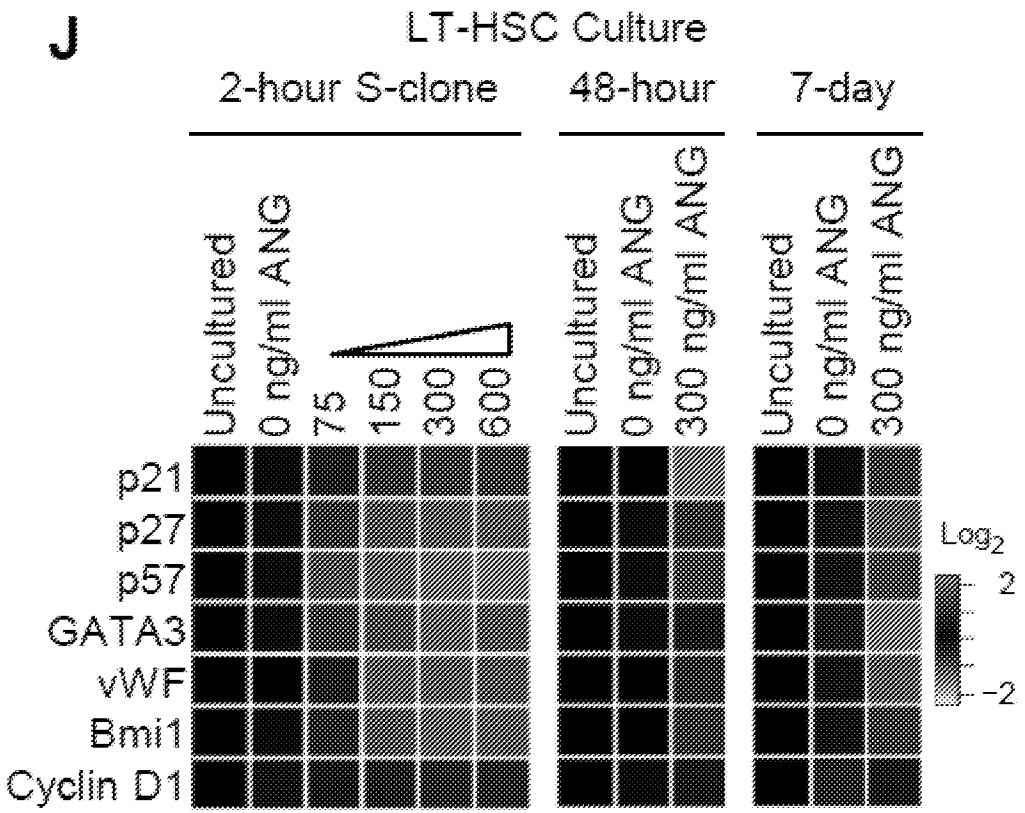

J
LT-HSC Culture
2-hour S-clone
48-hour
7-day
Uncultured
0 ng/ml ANG
75
150
300
600
Uncultured
0 ng/ml ANG
300 ng/ml ANG
Uncultured
0 ng/ml ANG
300 ng/ml ANG
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
2
−2

FIG. 15K

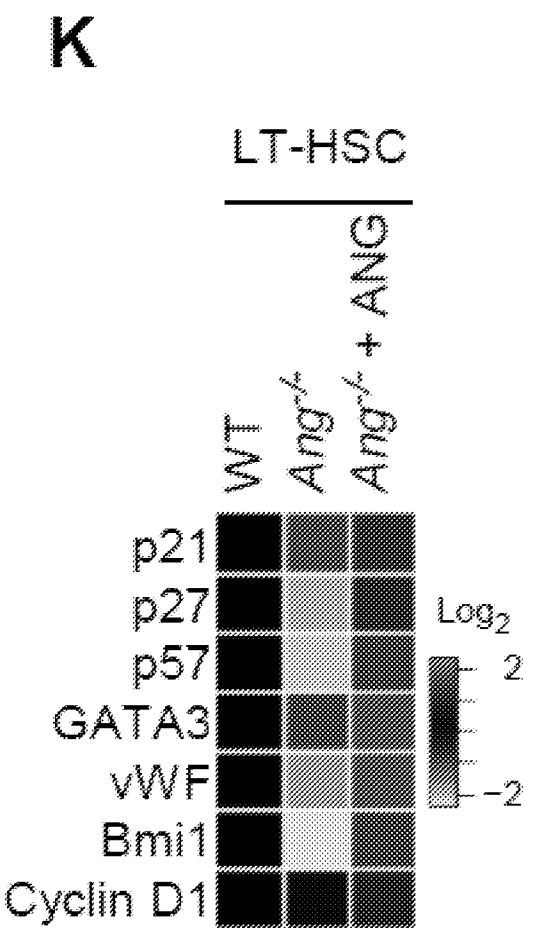

K
LT-HSC
WT
Ang-/-
Ang-/- + ANG
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
2
−2

FIG. 16A

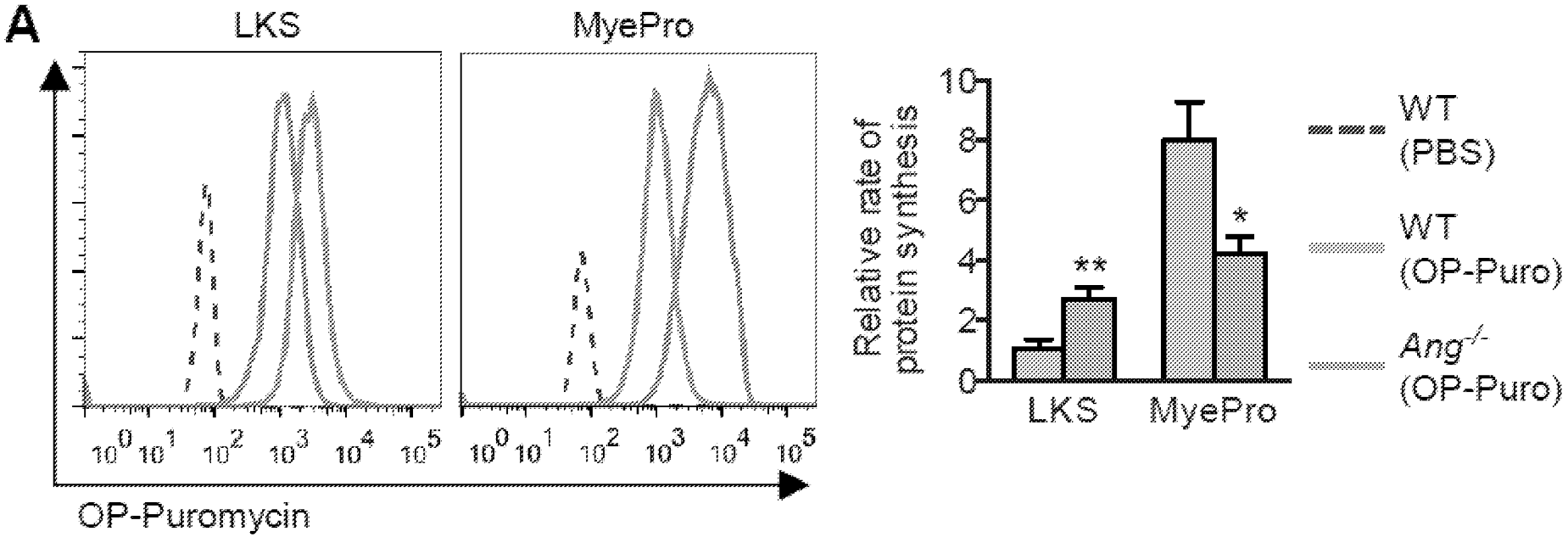

A
LKS
MyePro
OP-Puromycin
Relative rate of protein synthesis
WT (PBS)
WT (OP-Puro)
Ang-/- (OP-Puro)
LKS
MyePro
**
*

FIG. 16B

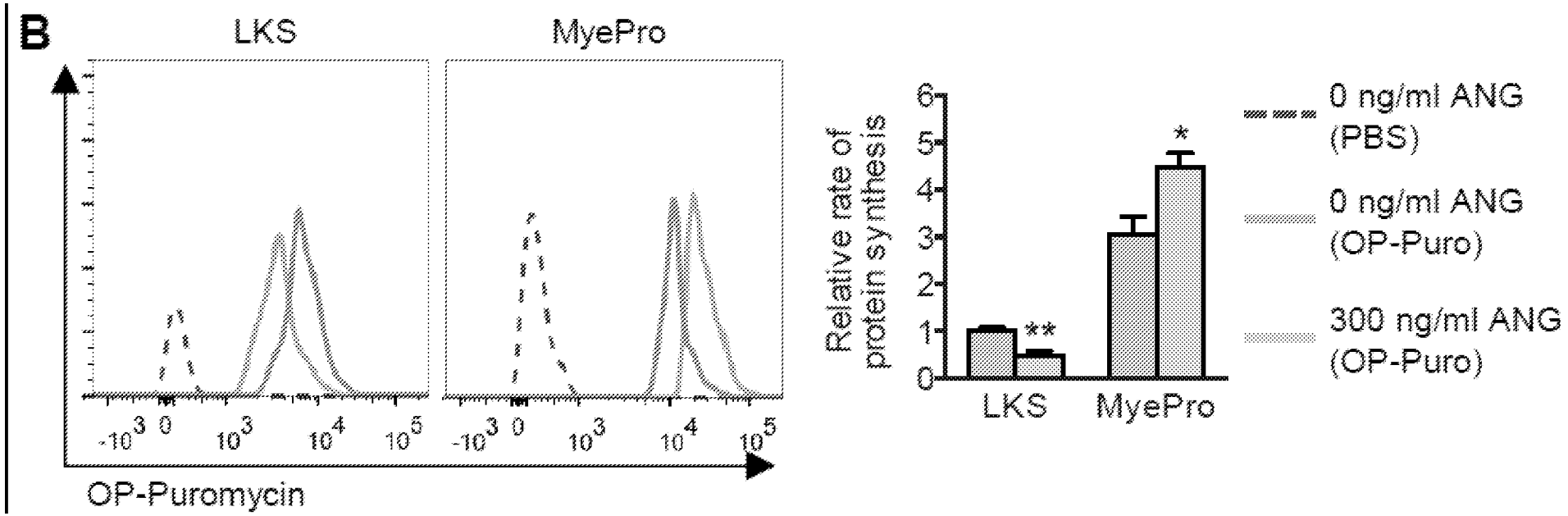
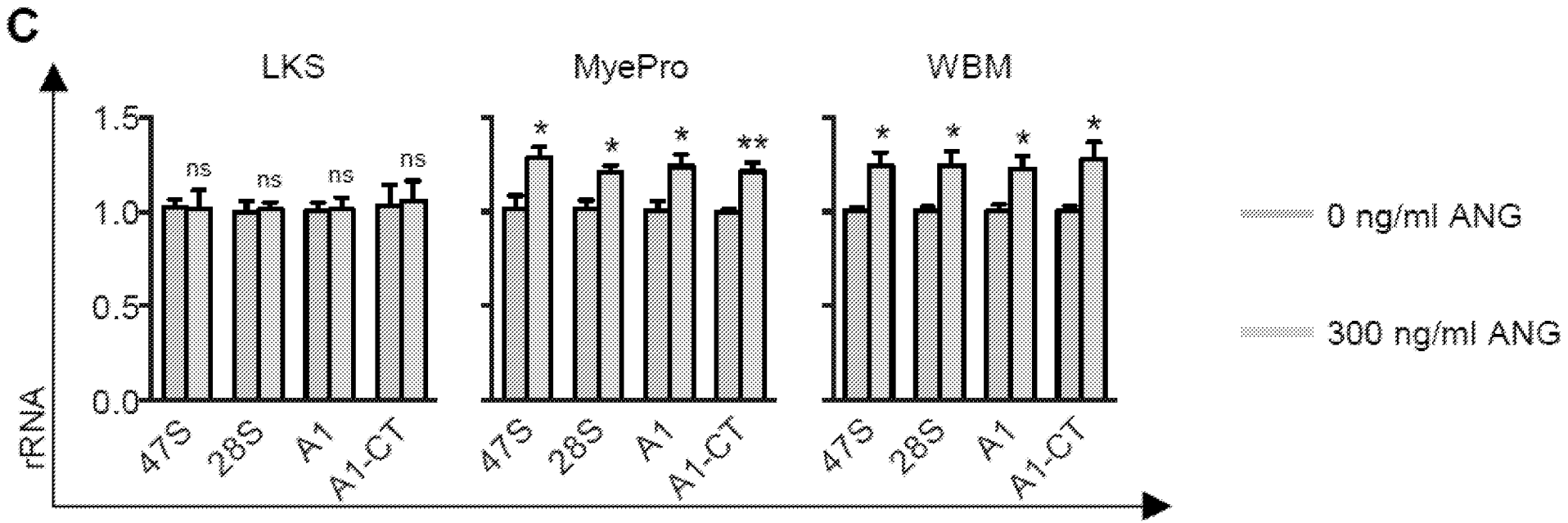
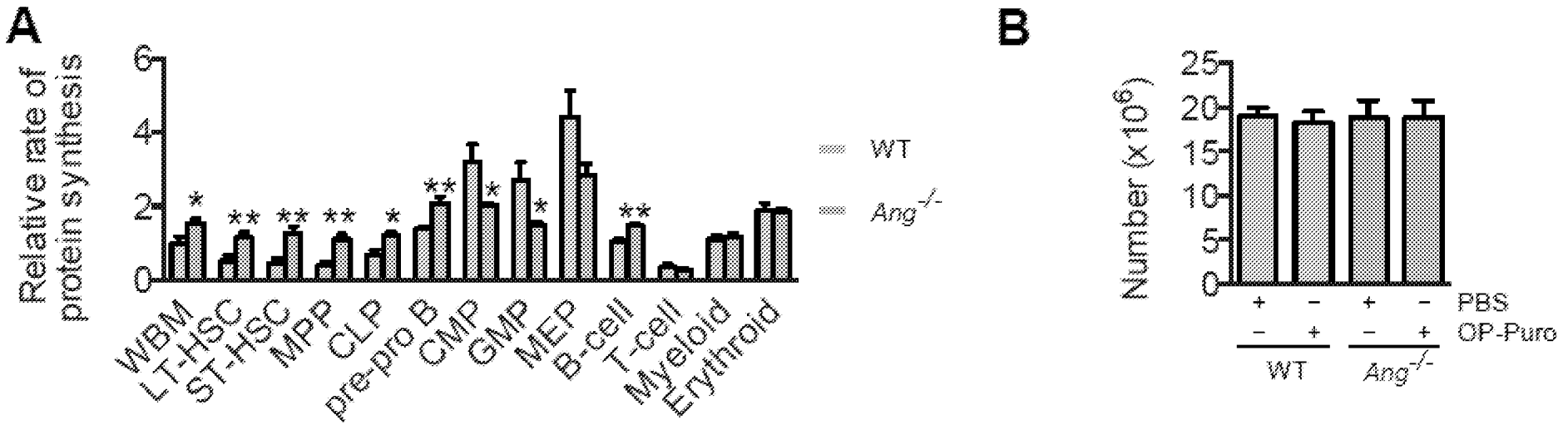

B
LKS
MyePro
-10^3 0 10^3 10^4 10^5
-10^3 0 10^3 10^4 10^5
OP-Puromycin
Relative rate of protein synthesis
0
1
2
3
4
5
6
**
*
LKS
MyePro
0 ng/ml ANG (PBS)
0 ng/ml ANG (OP-Puro)
300 ng/ml ANG (OP-Puro)

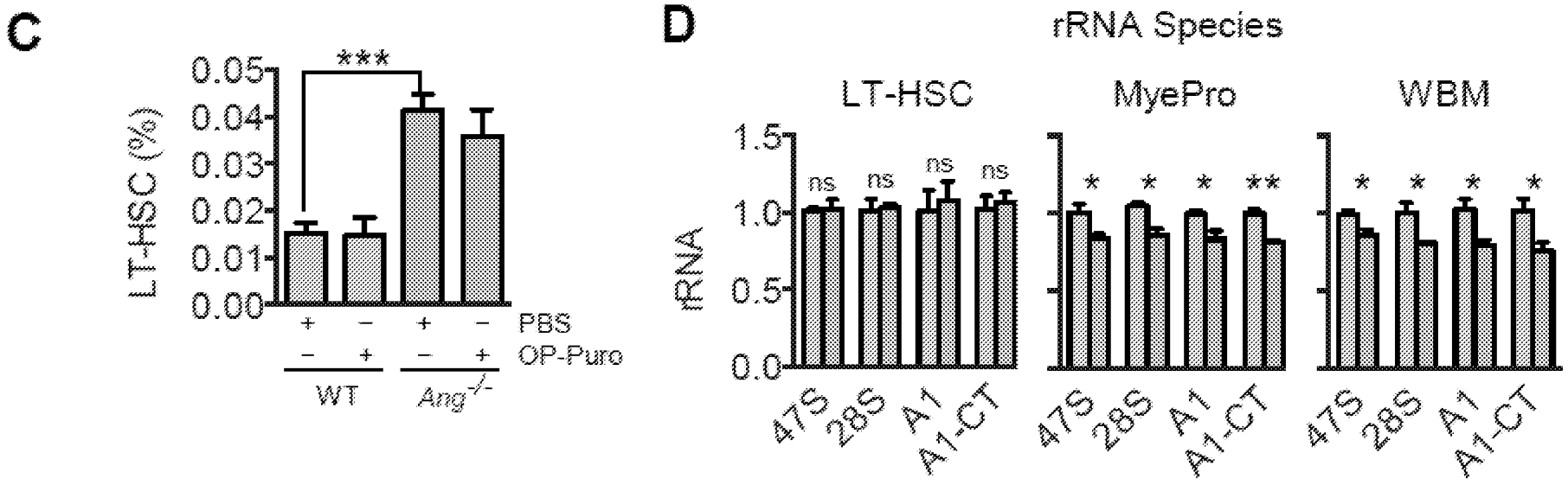
FIG. 16C


C
LKS
MyePro
WBM
1.5
1.0
0.5
0.0
rRNA
ns
ns
ns
ns
*
*
*
**
*
*
*
*
47S
28S
A1
A1-CT
0 ng/ml ANG
300 ng/ml ANG

FIGs. 17A-17B


A
Relative rate of protein synthesis
0
2
4
6
*
**
**
**
*
**
*
*
**
WBM
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP
B-cell
T-cell
Myeloid
Erythroid
WT
Ang-/-
B
Number (x10^6)
0
5
10
15
20
25
+ − + − PBS
− + − + OP-Puro
WT
Ang-/-

FIGs. 17C-17D


C
LT-HSC (%)
0.05
0.04
0.03
0.02
0.01
0.00
***
+ − + − PBS
− + − + OP-Puro
WT
Ang−/−
D
rRNA Species
LT-HSC
MyePro
WBM
rRNA
1.5
1.0
0.5
0.0
ns ns ns ns
* * * **
* * * *
47S 28S A1 A1-CT

FIGs. 17E-17DF

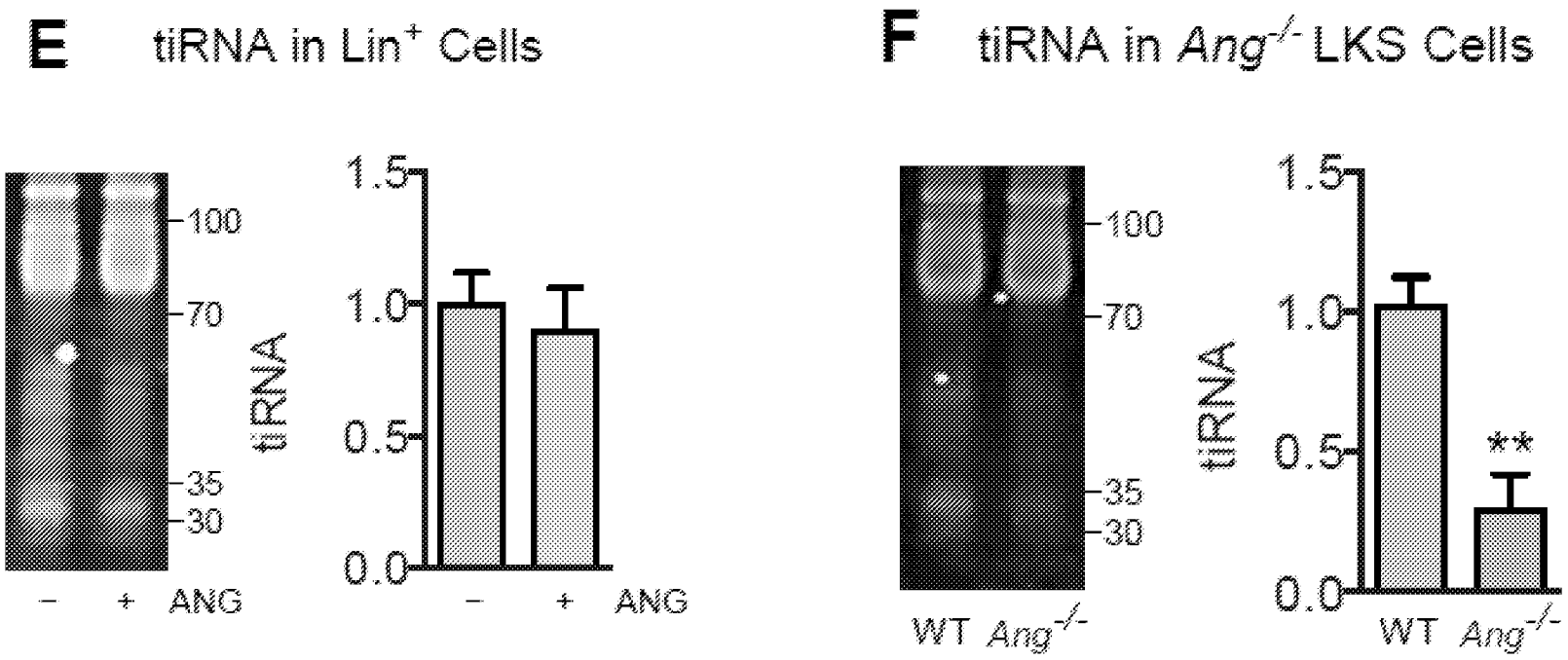


FIG. 17G

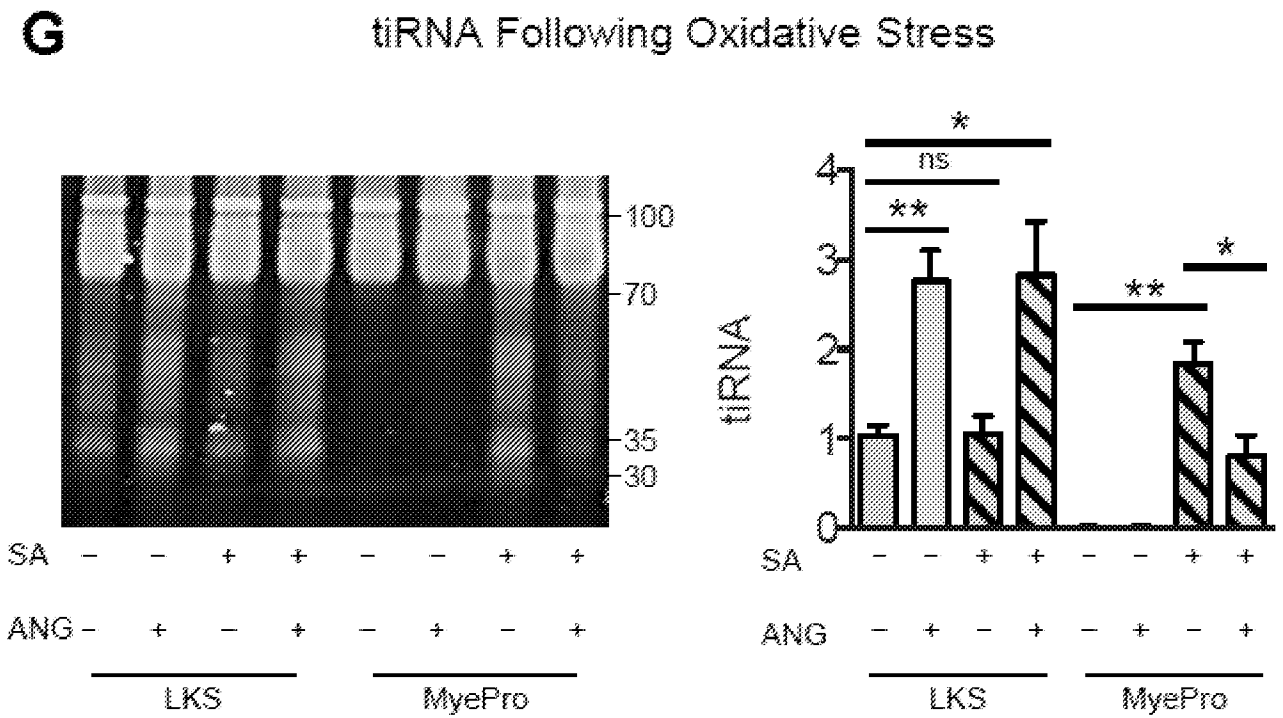
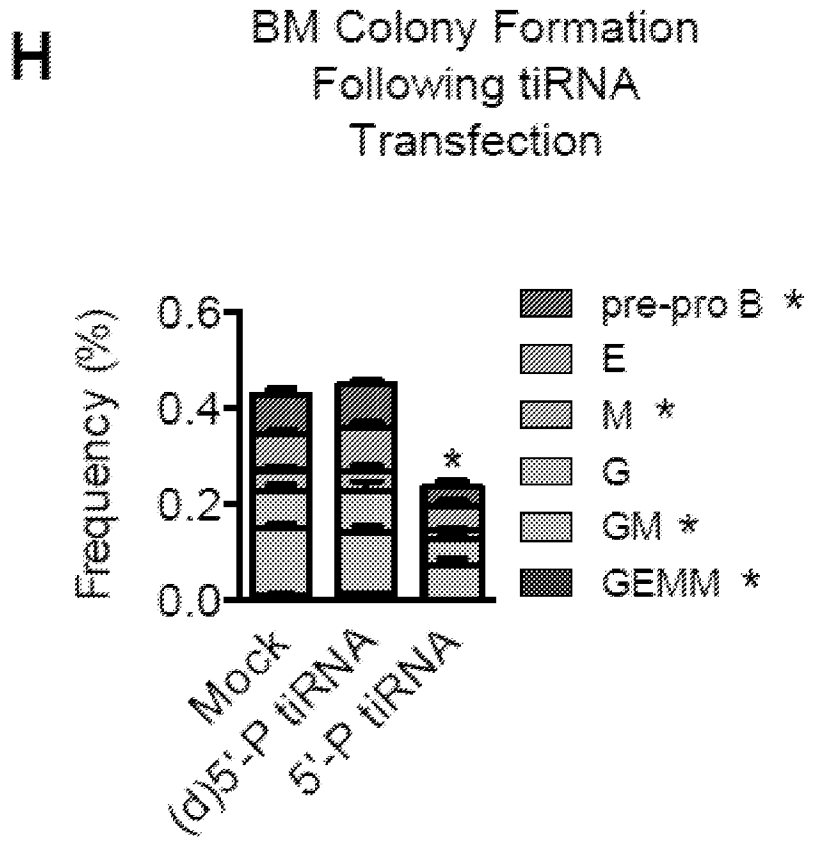

G
tiRNA Following Oxidative Stress
100
70
35
30
SA
ANG
LKS
MyePro
tiRNA
*
ns
**
**
*
0
1
2
3
4
SA
ANG
LKS
MyePro

FIG. 17H


H
BM Colony Formation
Following tiRNA
Transfection
Frequency (%)
0.6
0.4
0.2
0.0
*
pre-pro B *
E
M *
G
GM *
GEMM *
Mock
(d)5'-P tiRNA
5'-P tiRNA

FIGs. 18A-18B

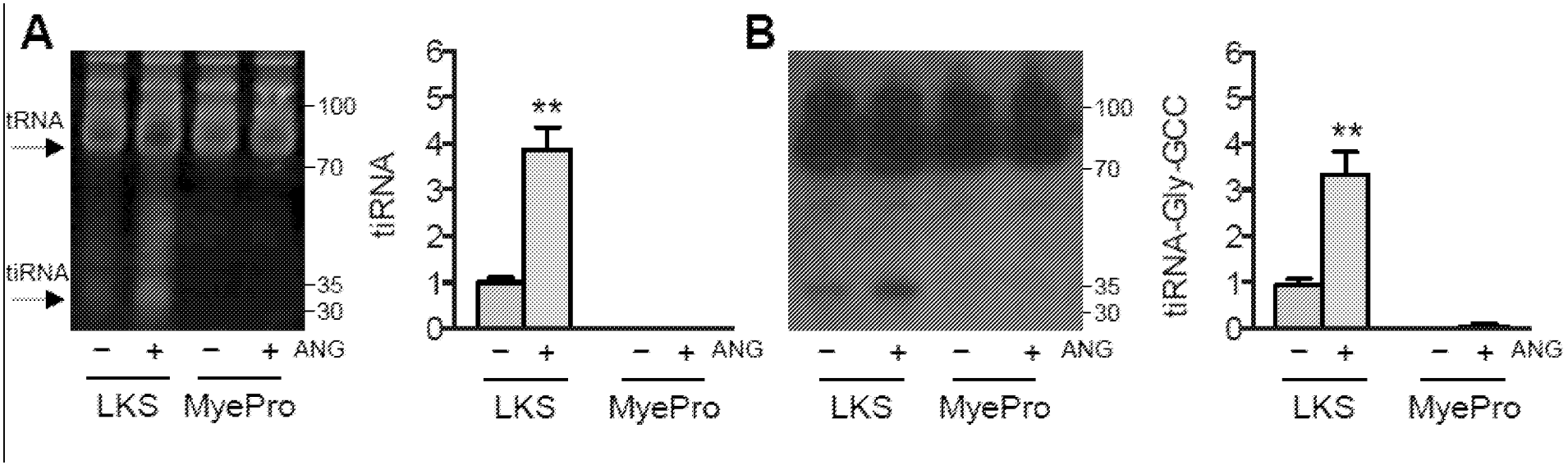

A
tiRNA
tiRNA
100
70
35
30
– + – + ANG
LKS
MyePro
tiRNA
6
5
4
3
2
1
0
**
– + – + ANG
LKS
MyePro
B
100
70
35
30
– + – + ANG
LKS
MyePro
tiRNA-Gly-GCC
6
5
4
3
2
1
0
**
– + – + ANG
LKS
MyePro

FIG. 18C

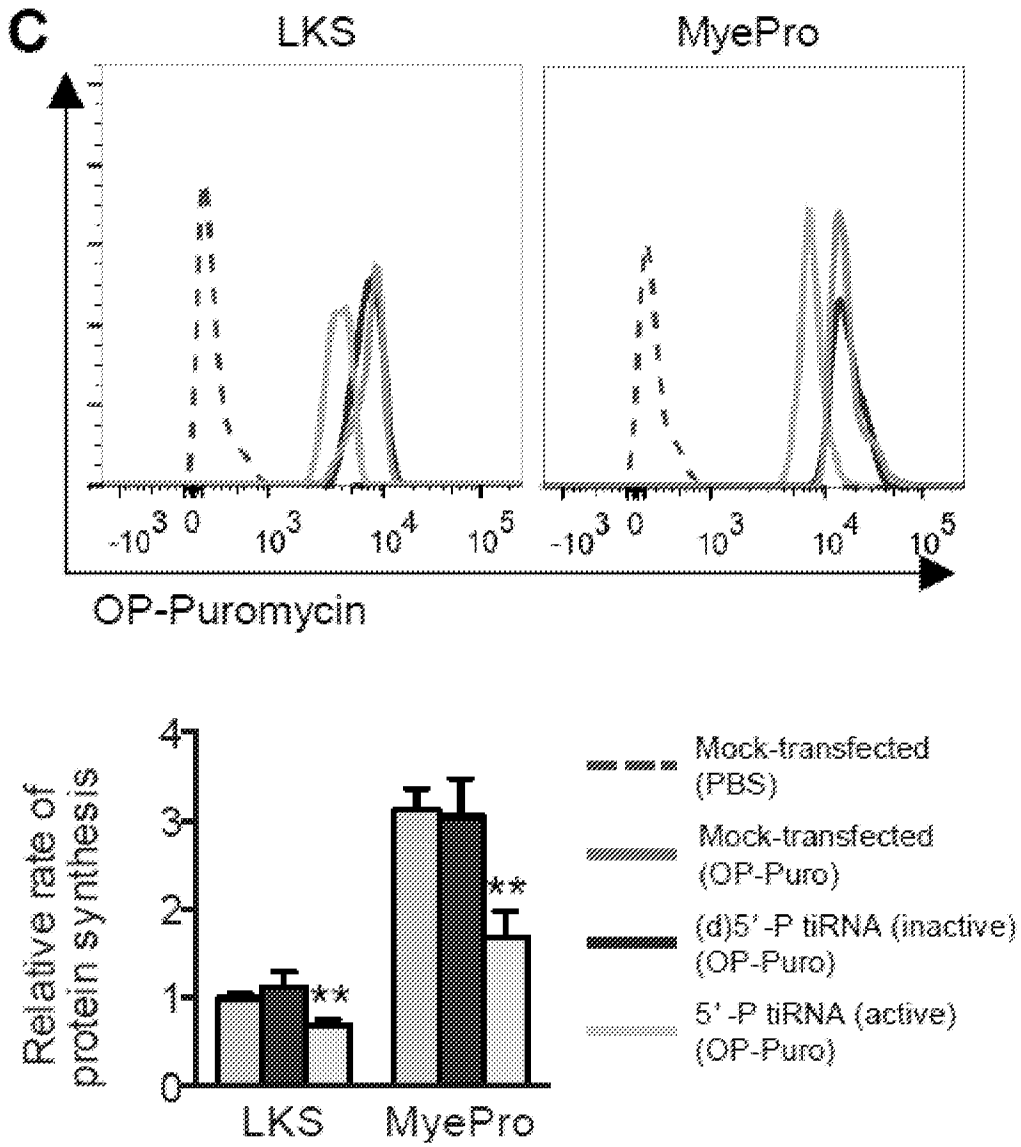

C
LKS
MyePro
-10^3 0 10^3 10^4 10^5
-10^3 0 10^3 10^4 10^5
OP-Puromycin
Relative rate of protein synthesis
0
1
2
3
4
**
**
LKS
MyePro
Mock-transfected (PBS)
Mock-transfected (OP-Puro)
(d)5'-P tiRNA (inactive) (OP-Puro)
5'-P tiRNA (active) (OP-Puro)

FIG. 18D

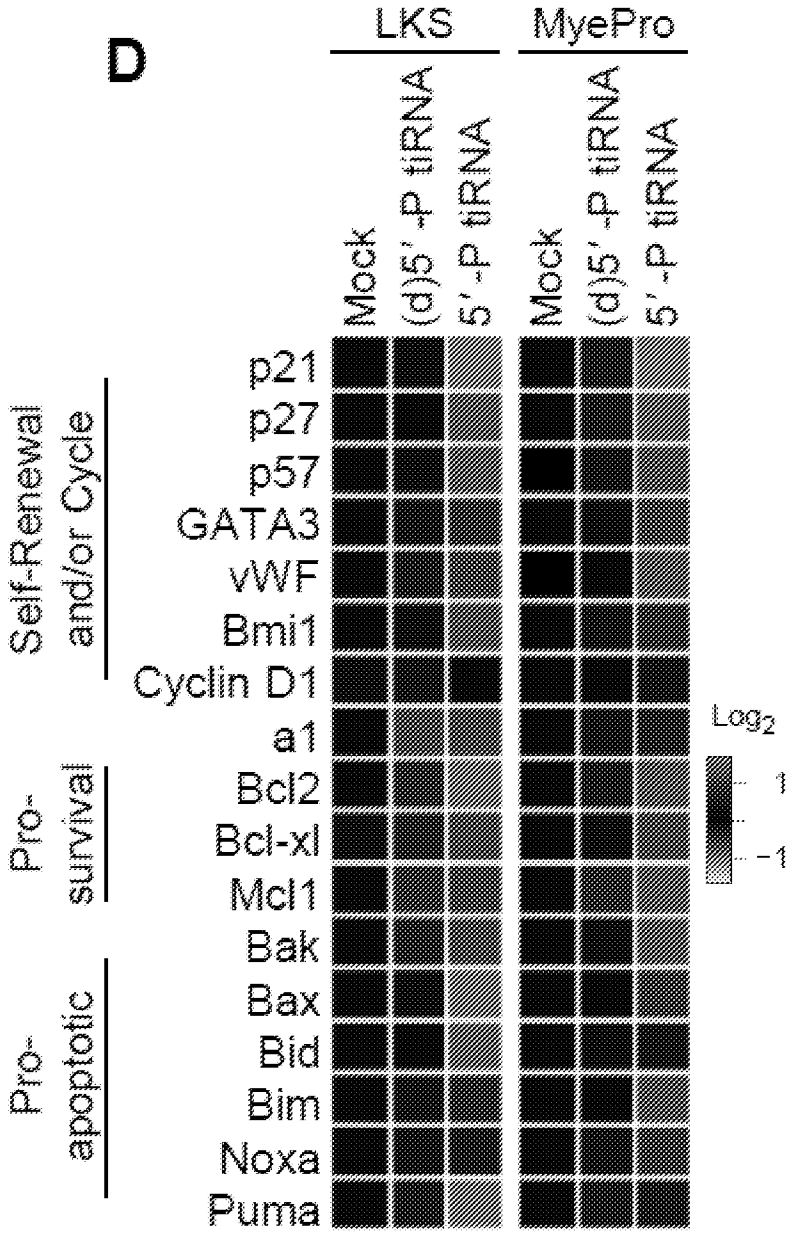

D
LKS
MyePro
Mock
(d)5'-P tiRNA
5'-P tiRNA
Mock
(d)5'-P tiRNA
5'-P tiRNA
Self-Renewal and/or Cycle
Pro-survival
Pro-apoptotic
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
a1
Bcl2
Bcl-xl
Mcl1
Bak
Bax
Bid
Bim
Noxa
Puma
Log2
1
-1

FIG. 18E

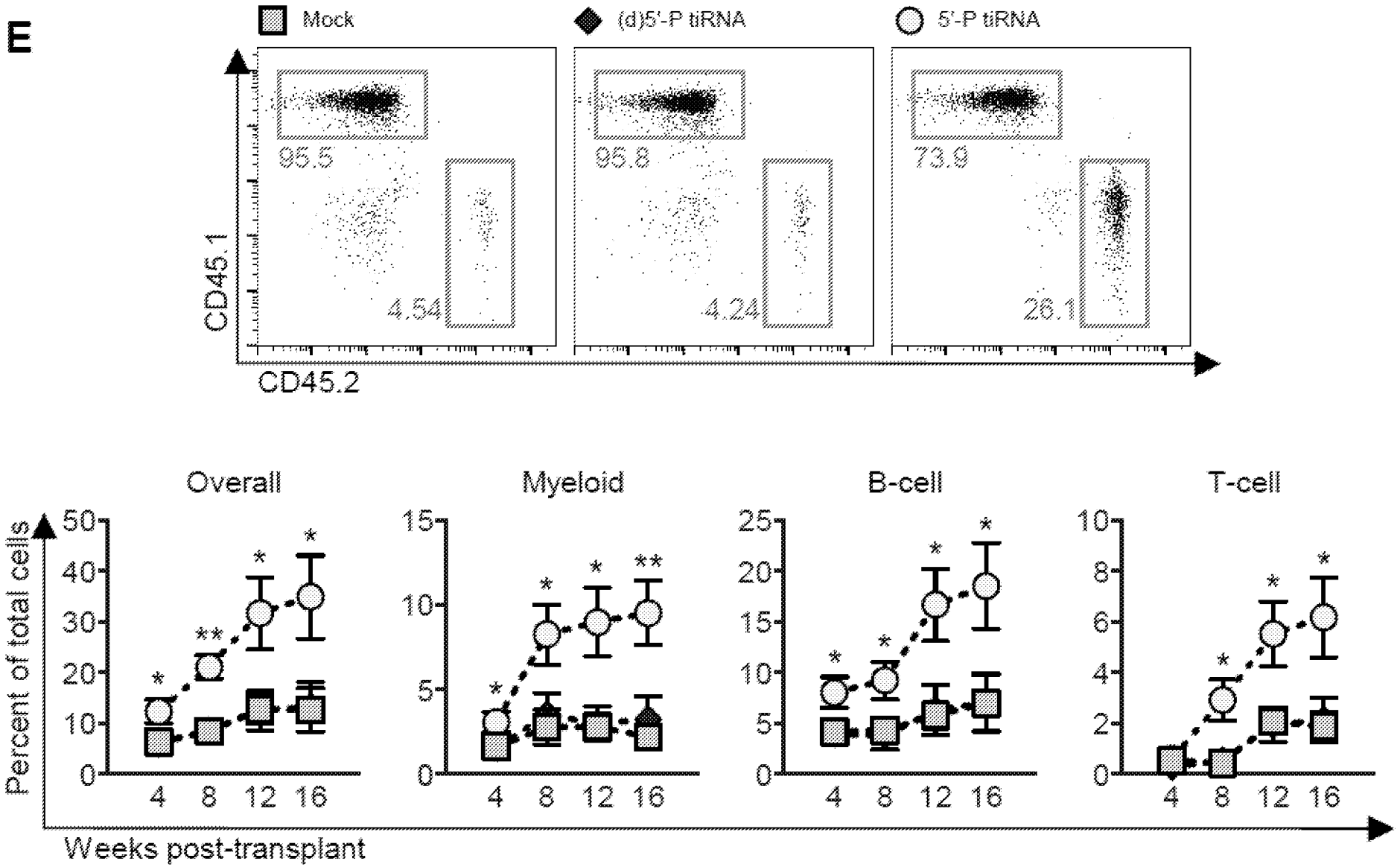

E
Mock
(d)5'-P tiRNA
5'-P tiRNA
95.5
4.54
95.8
4.24
73.9
26.1
CD45.1
CD45.2
Overall
Myeloid
B-cell
T-cell
Percent of total cells
Weeks post-transplant

FIG. 19A

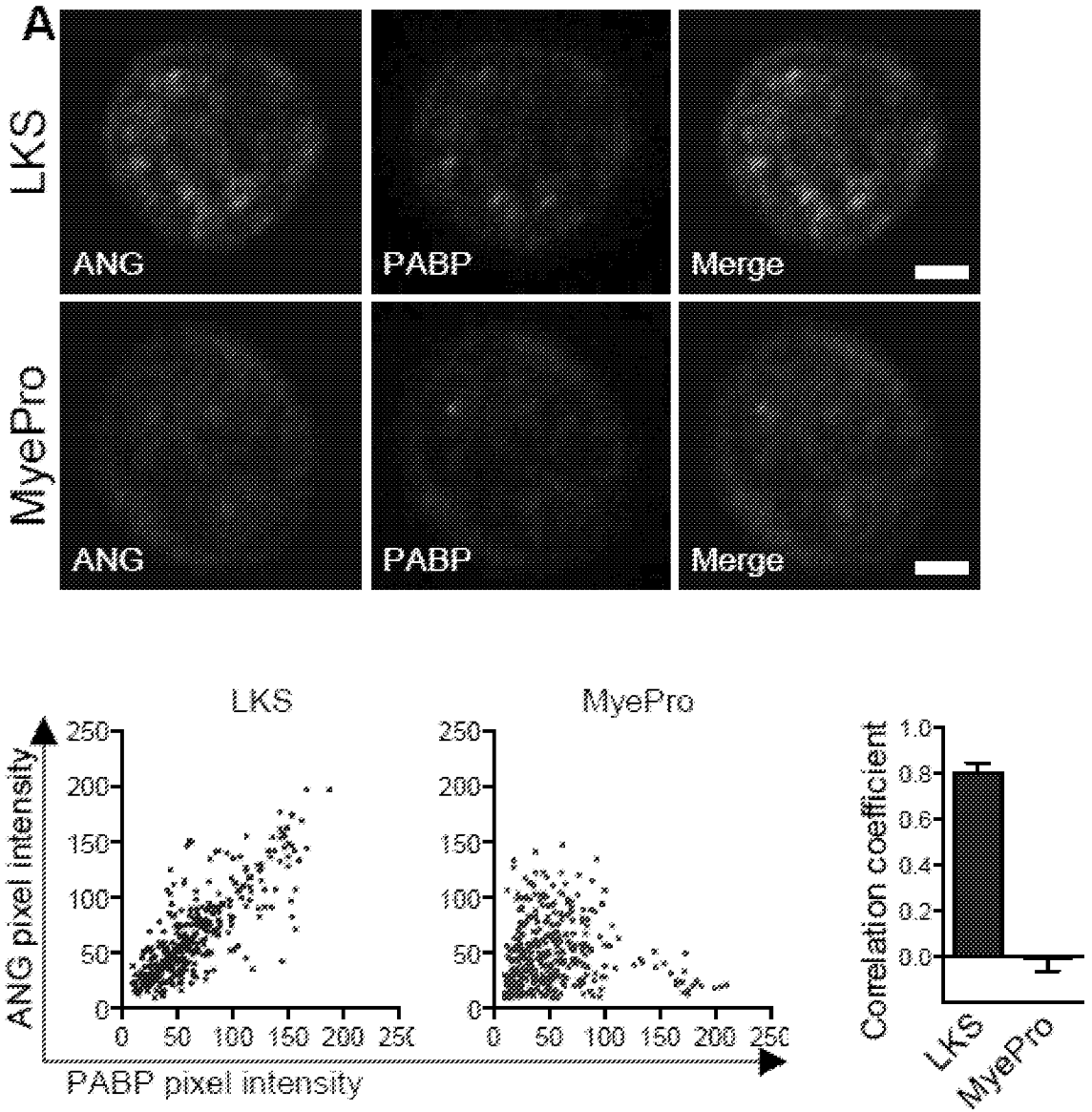

A
LKS
ANG
PABP
Merge
MyePro
ANG
PABP
Merge
LKS
MyePro
ANG pixel intensity
PABP pixel intensity
0
50
100
150
200
250
Correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
LKS
MyePro

FIG. 19B

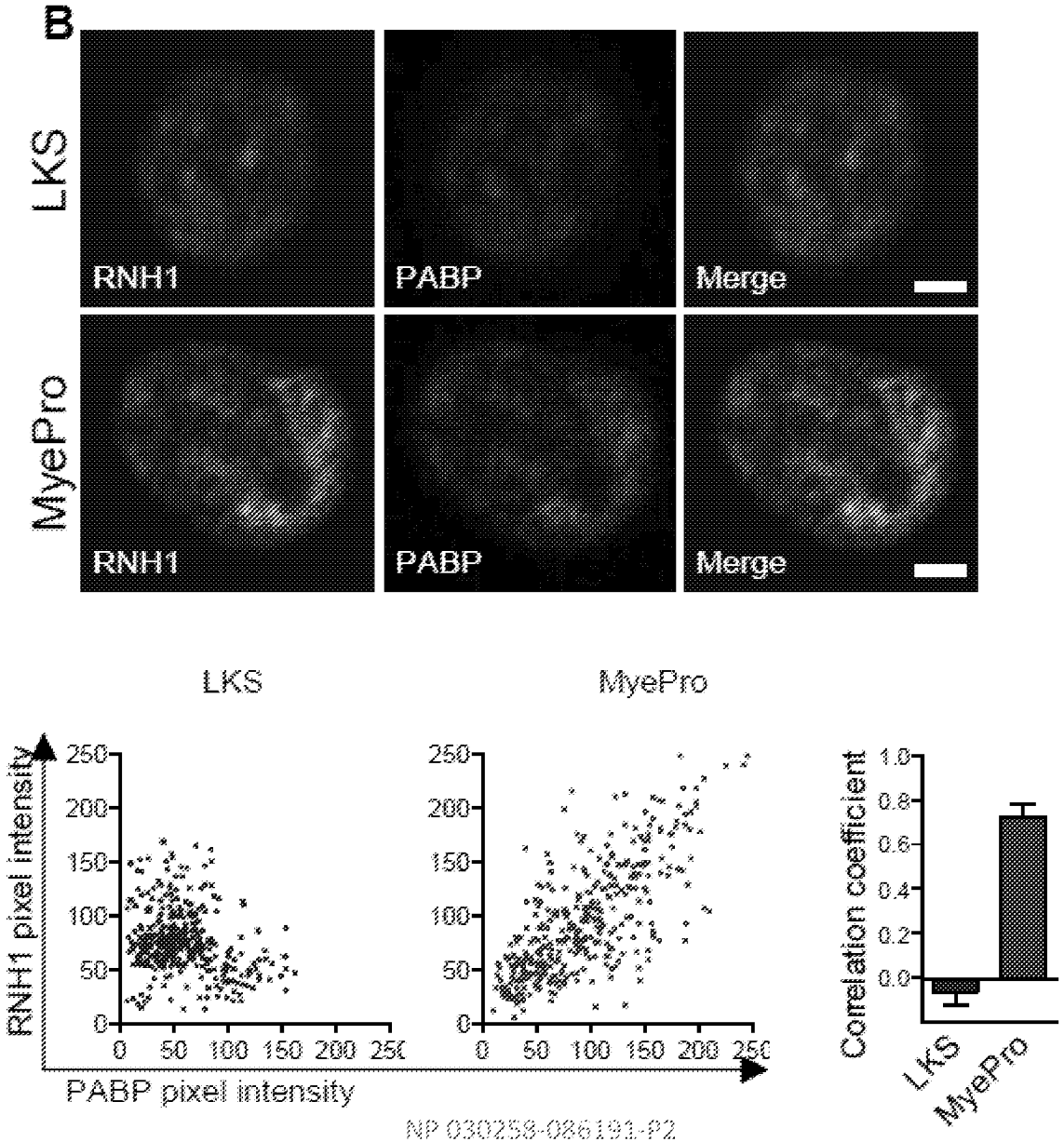

B
LKS
MyePro
RNH1
PABP
Merge
RNH1
PABP
Merge
LKS
MyePro
RNH1 pixel intensity
PABP pixel intensity
0
50
100
150
200
250
Correlation coefficient
0.0
0.2
0.4
0.6
0.8
1.0
LKS
MyePro

FIG. 19C

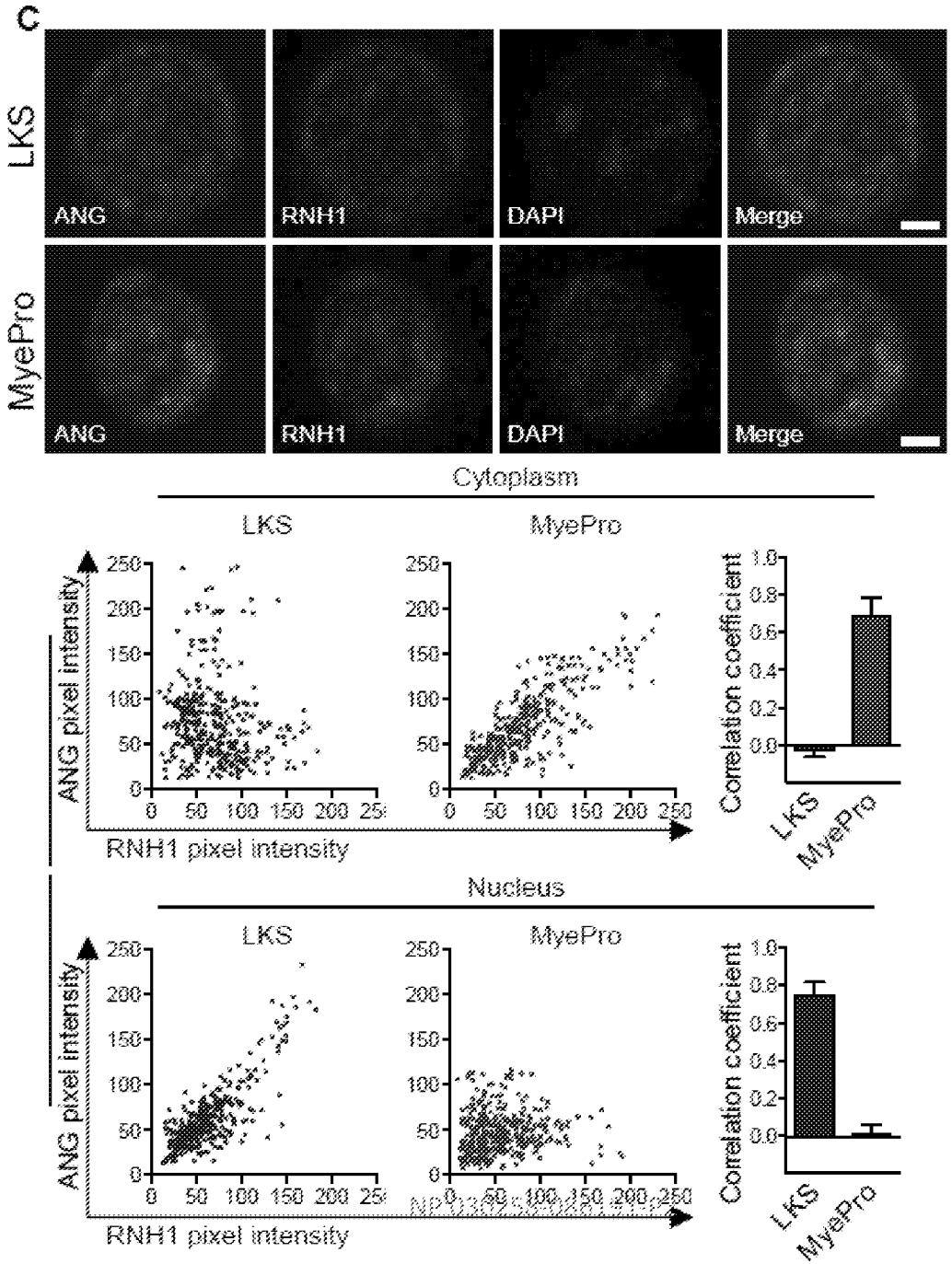
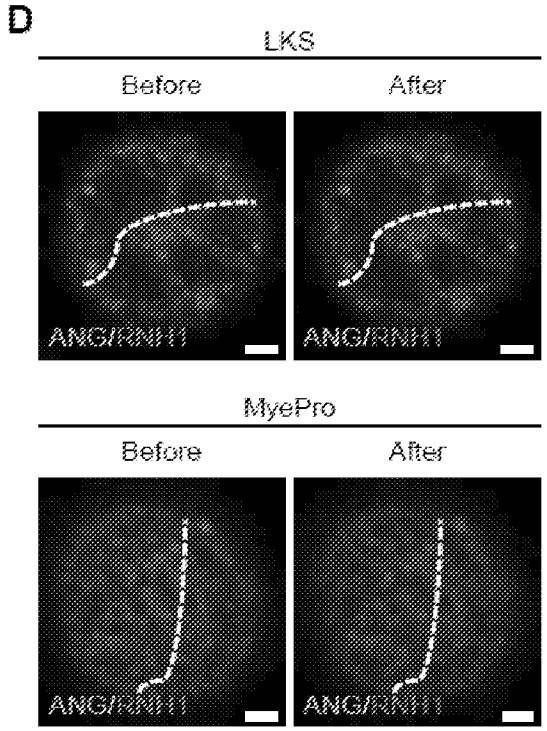
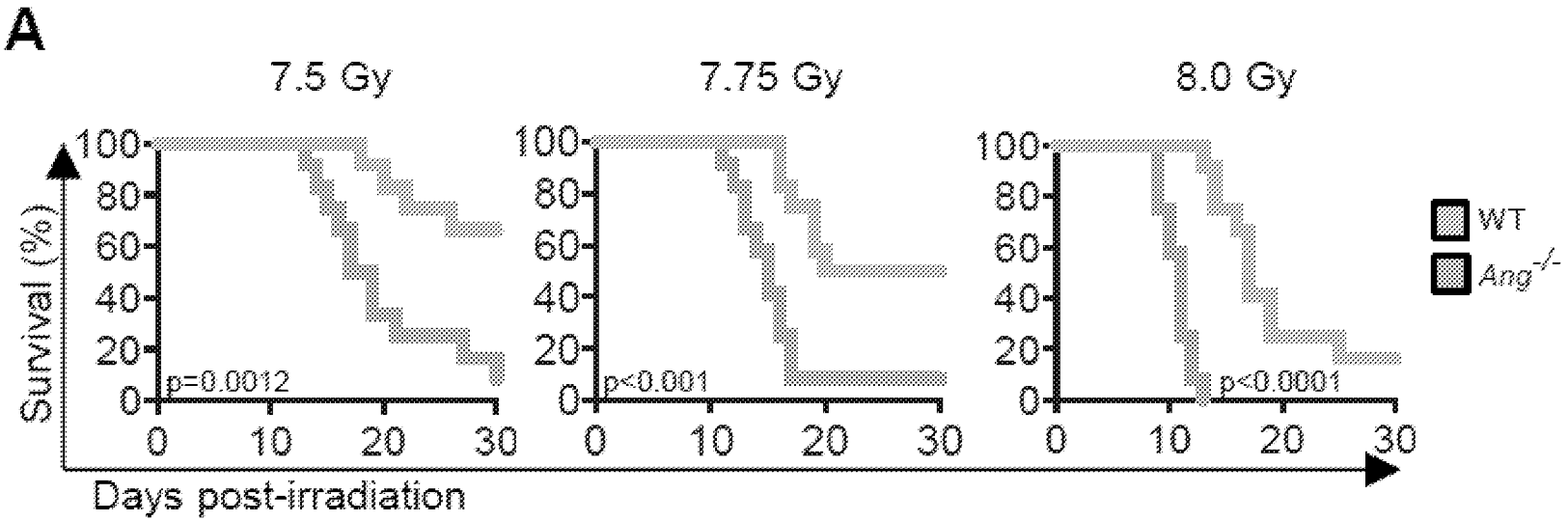

C
LKS
MyePro
ANG
RNH1
DAPI
Merge
Cytoplasm
LKS
MyePro
ANG pixel intensity
RNH1 pixel intensity
Correlation coefficient
Nucleus

FIG. 19D

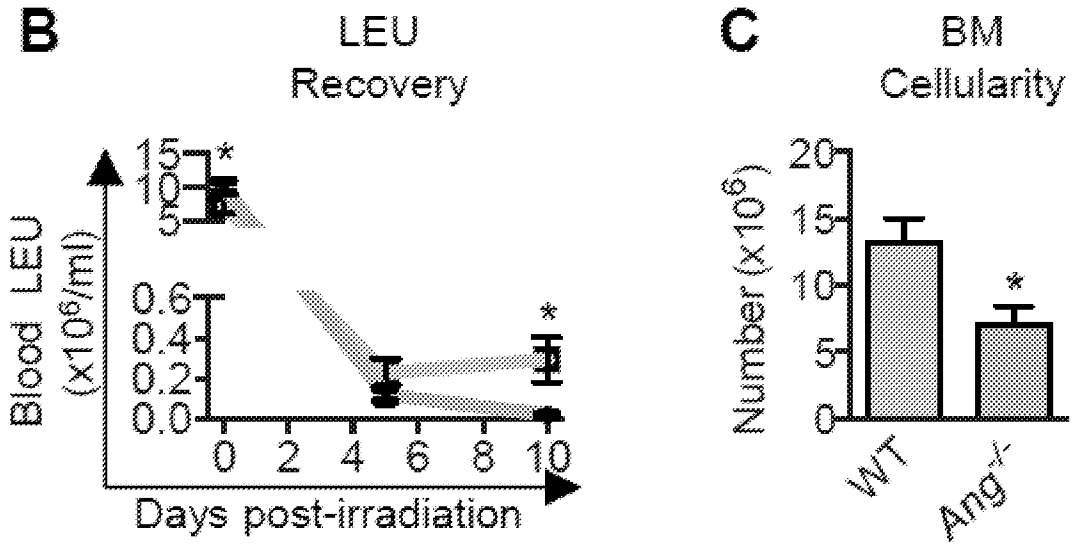


		Before Photobleaching		After Photobleaching		FRET Efficiency
		ANG (Donor)	RNH1 (Acceptor)	ANG (Donor)	RNH1 (Acceptor)	
Cytoplasm	LKS	116.7 ± 12.4	93.1 ± 17.3	117.5 ± 12.2	4.6 ± 2.6	**0.7 ± 0.5**
	MyePro	35.4 ± 14.3	77.8 ± 21.8	40.2 ± 15.6	4.1 ± 2.1	**14.4 ± 4.9**
Nucleus	LKS	104.4 ± 11.5	94.8 ± 31.2	117.1 ± 12.4	3.3 ± 1.2	**12.2 ± 2.7**
	MyePro	39.3 ± 10.2	83.9 ± 19.2	39.6 ± 10.3	3.6 ± 1.7	**0.8 ± 0.4**

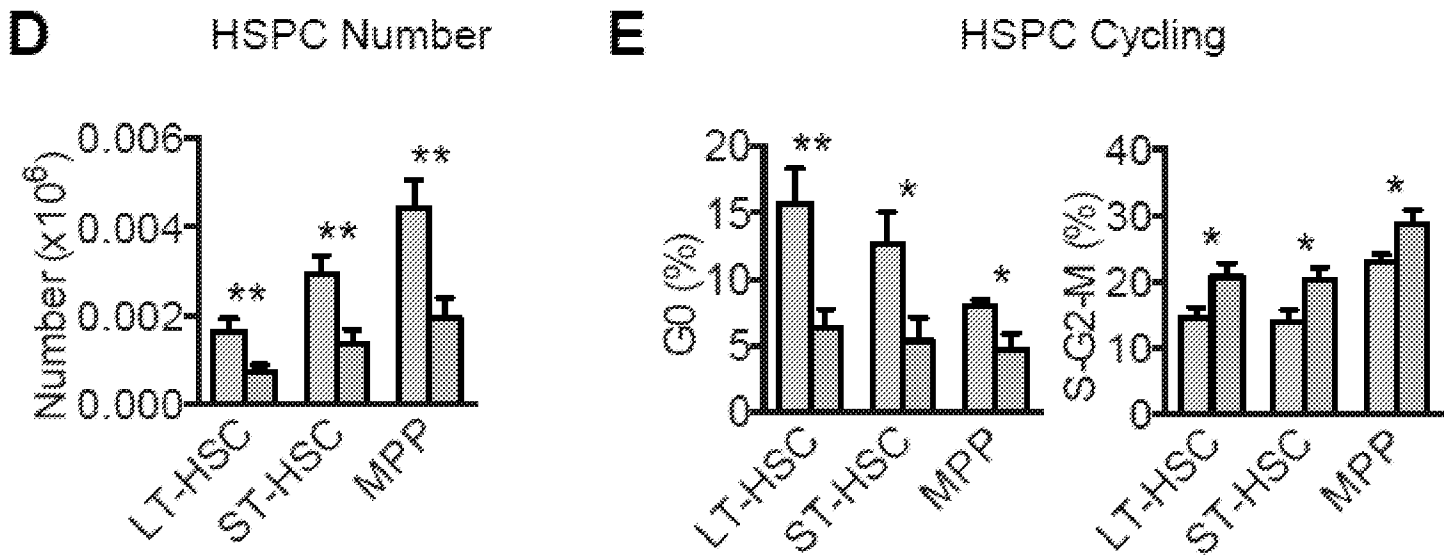
FIG. 20A


A
7.5 Gy
7.75 Gy
8.0 Gy
Survival (%)
100
80
60
40
20
0
p=0.0012
p<0.001
p<0.0001
0
10
20
30
Days post-irradiation
WT
Ang-/-

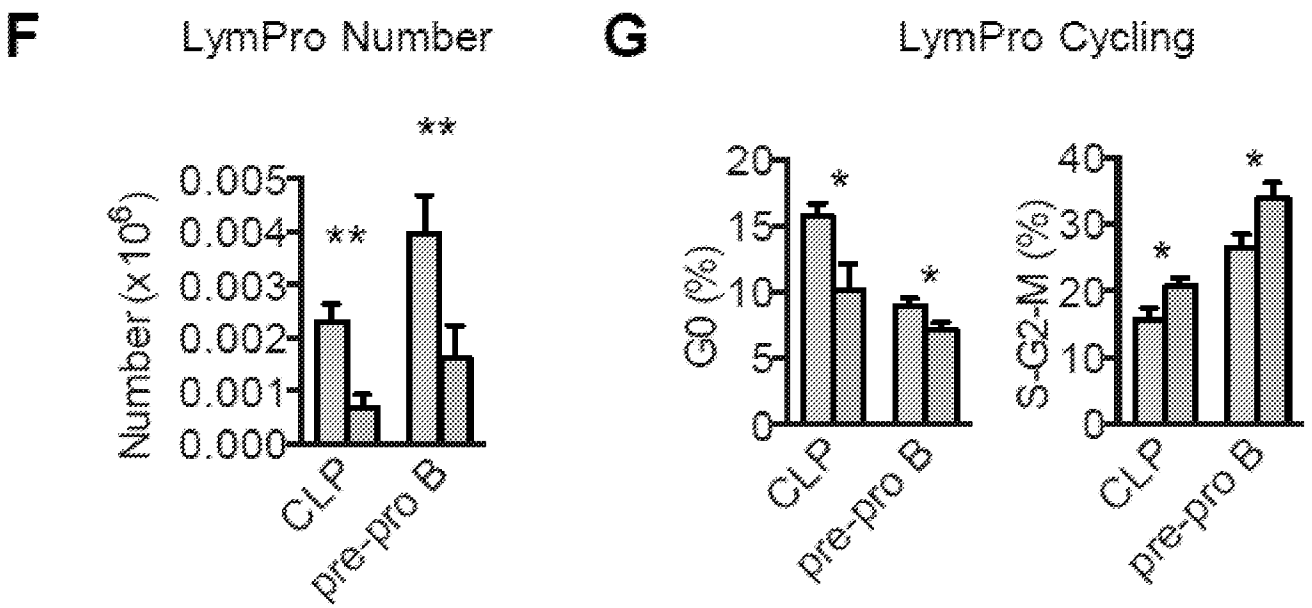
FIGs. 20B-20C


B
LEU
Recovery
Blood LEU
(x10^6/ml)
15
10
5
0.6
0.4
0.2
0.0
*
*
0 2 4 6 8 10
Days post-irradiation
C
BM
Cellularity
Number (x10^6)
20
15
10
5
0
*
WT
Ang-/-

FIGs. 20D-20E


D
HSPC Number
Number (x10^6)
0.006
0.004
0.002
0.000
**
**
**
LT-HSC
ST-HSC
MPP
E
HSPC Cycling
G0 (%)
20
15
10
5
0
**
*
*
LT-HSC
ST-HSC
MPP
S-G2-M (%)
40
30
20
10
0
*
*
*
LT-HSC
ST-HSC
MPP

FIGs. 20F-20G


F
LymPro Number
Number (x 10^6)
0.005
0.004
0.003
0.002
0.001
0.000
**
**
CLP
pre-pro B
G
LymPro Cycling
G0 (%)
20
15
10
5
0
*
*
CLP
pre-pro B
S-G2-M (%)
40
30
20
10
0
*
*
CLP
pre-pro B

FIGs. 20H-20I

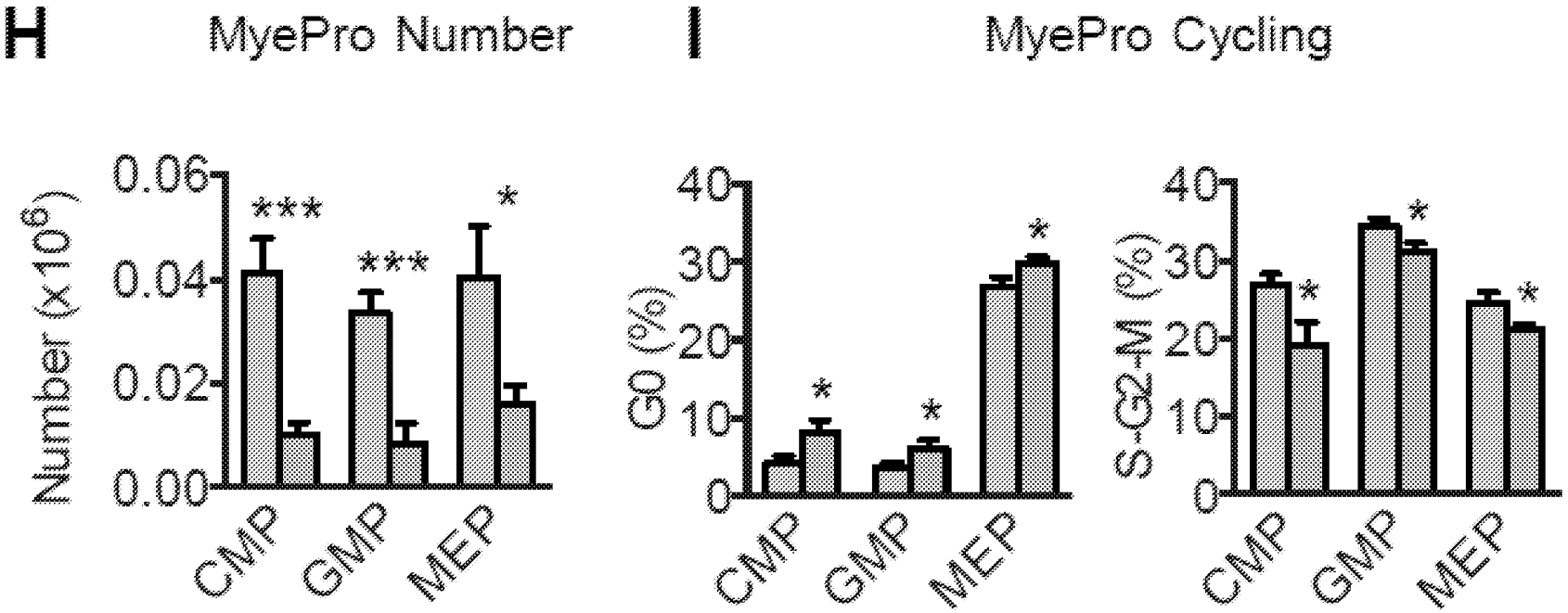

H
MyePro Number
Number (x10^6)
0.06
0.04
0.02
0.00
***
***
*
CMP
GMP
MEP
I
MyePro Cycling
G0 (%)
40
30
20
10
0
*
*
*
CMP
GMP
MEP
S-G2-M (%)
40
30
20
10
0
*
*
*
CMP
GMP
MEP

FIG. 20J

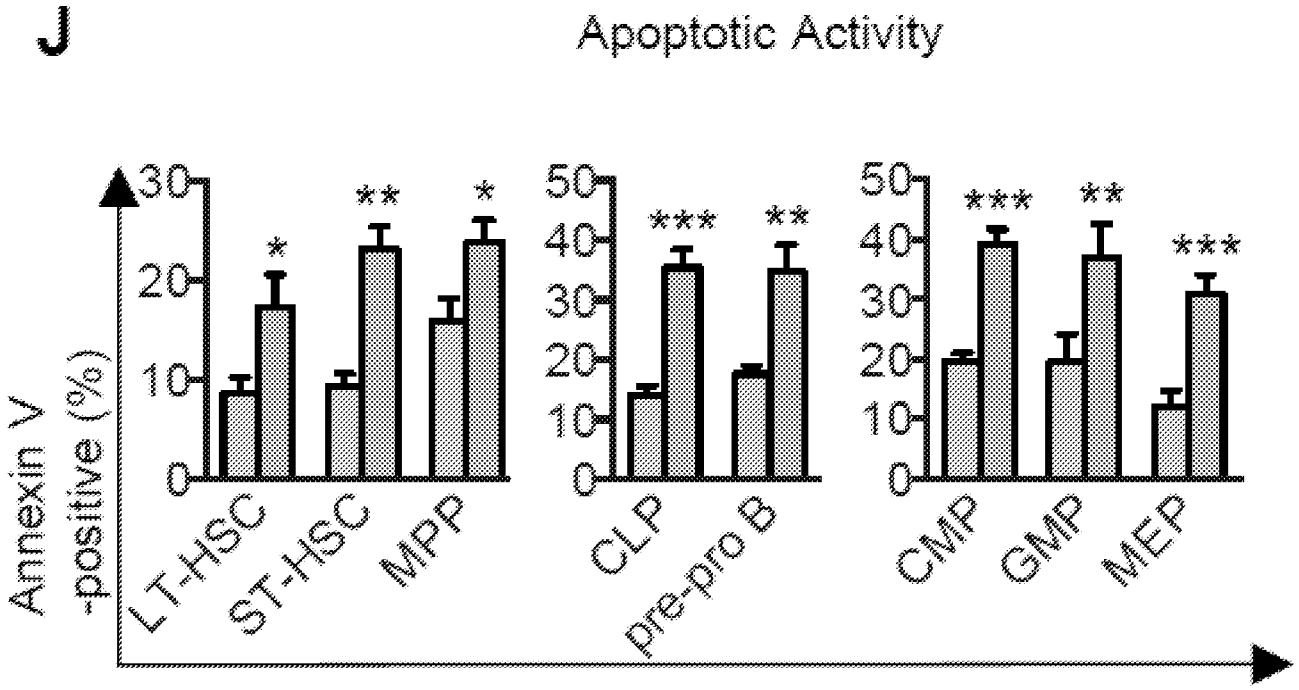
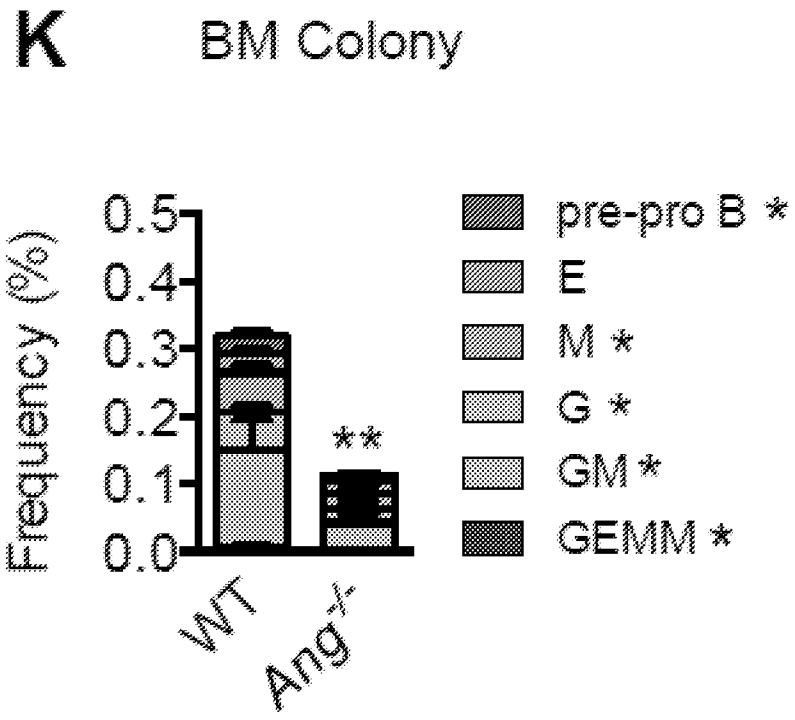

J
Apoptotic Activity
Annexin V
-positive (%)
0
10
20
30
40
50
*
**
*
***
**
***
**
***
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP

FIG. 20K


K
BM Colony
Frequency (%)
0.5
0.4
0.3
0.2
0.1
0.0
**
WT
Ang-/-
pre-pro B *
E
M *
G *
GM *
GEMM *

FIGs. 21A-21B

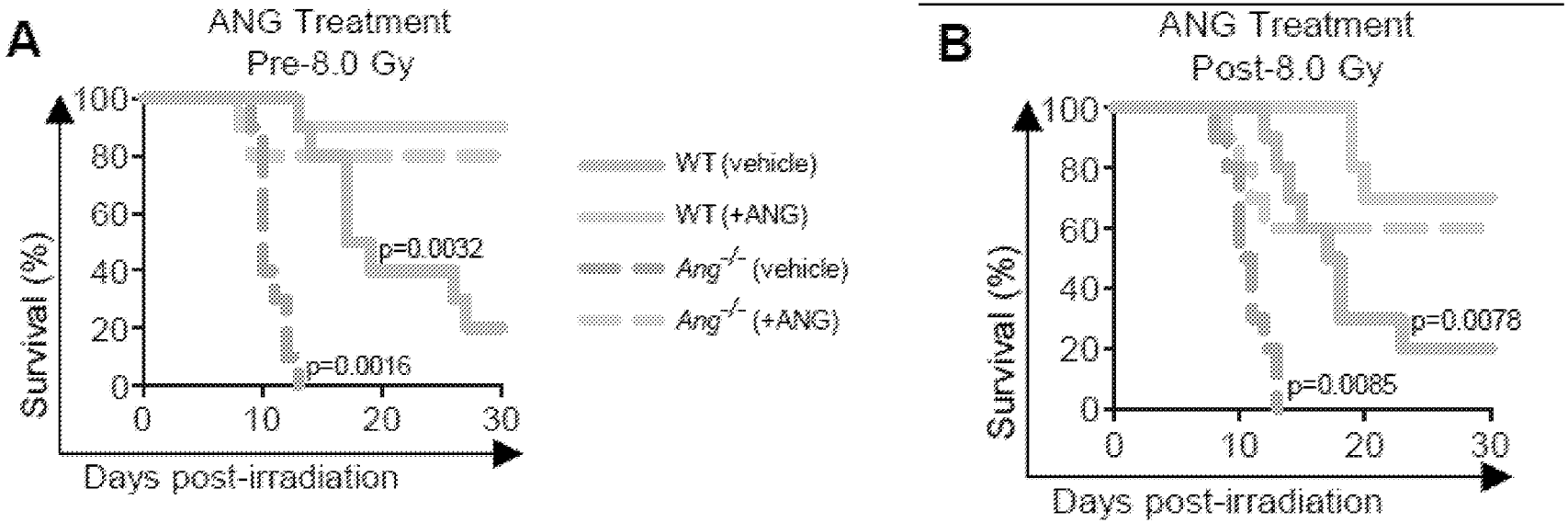

A
ANG Treatment
Pre-8.0 Gy
Survival (%)
100
80
60
40
20
0
0
10
20
30
Days post-irradiation
p=0.0032
p=0.0016
WT (vehicle)
WT (+ANG)
Ang-/- (vehicle)
Ang-/- (+ANG)
B
ANG Treatment
Post-8.0 Gy
Survival (%)
100
80
60
40
20
0
0
10
20
30
Days post-irradiation
p=0.0078
p=0.0085

FIG. 21C

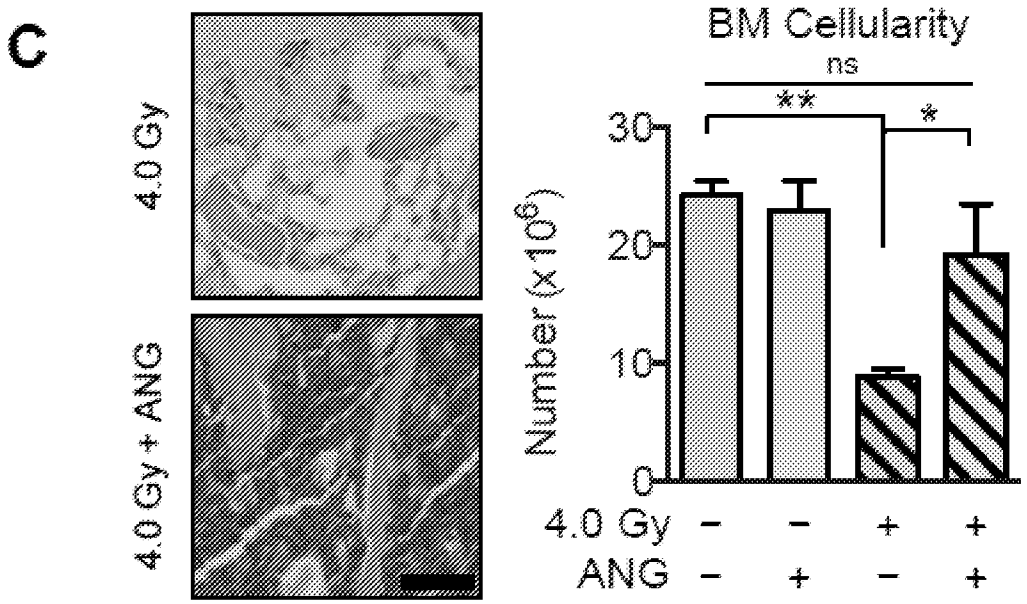

C
4.0 Gy
4.0 Gy + ANG
BM Cellularity
ns
**
*
Number (x10^6)
30
20
10
0
4.0 Gy − − + +
ANG − + − +

FIG. 21D

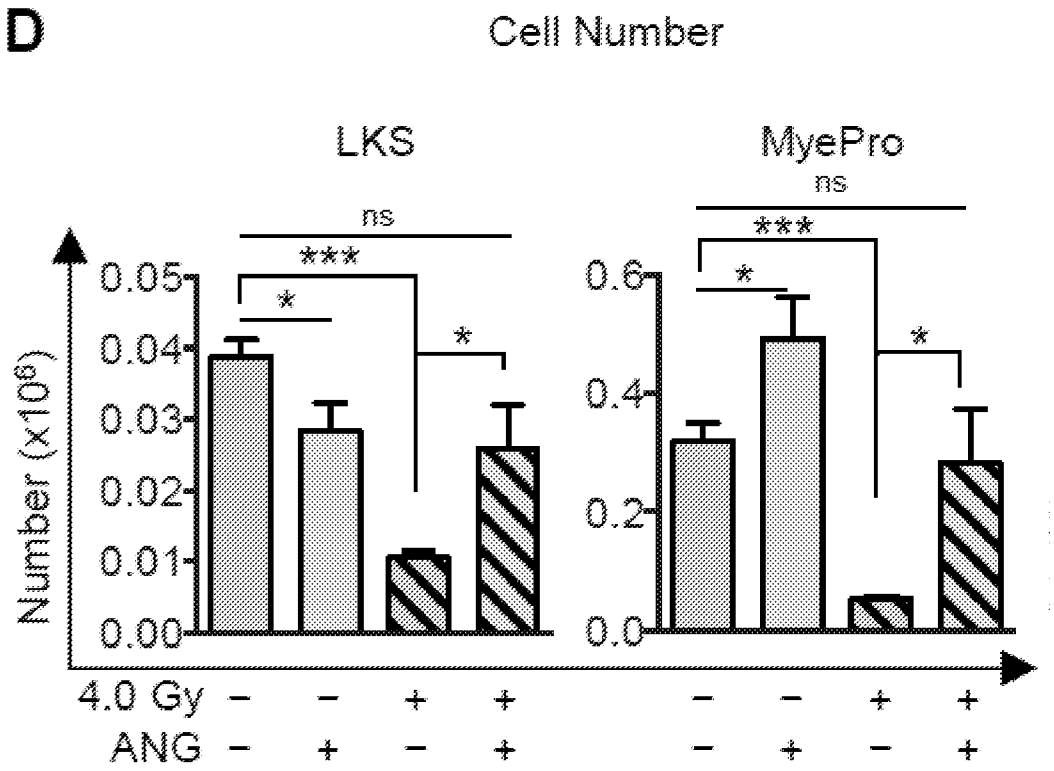

D
Cell Number
LKS
MyePro
ns
***
*
ns
***
*
*
Number (x10^6)
0.05
0.04
0.03
0.02
0.01
0.00
0.6
0.4
0.2
0.0
4.0 Gy – – + + – – + +
ANG – + – + – + – +

FIG. 21E

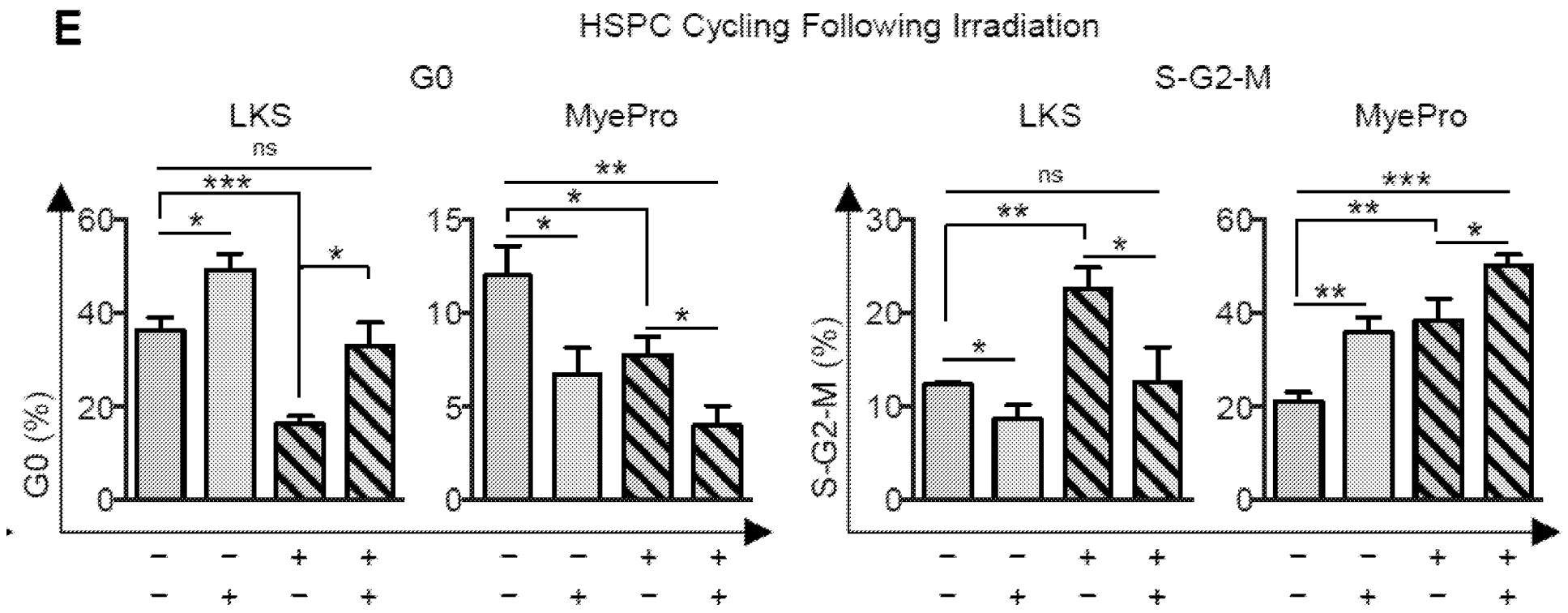
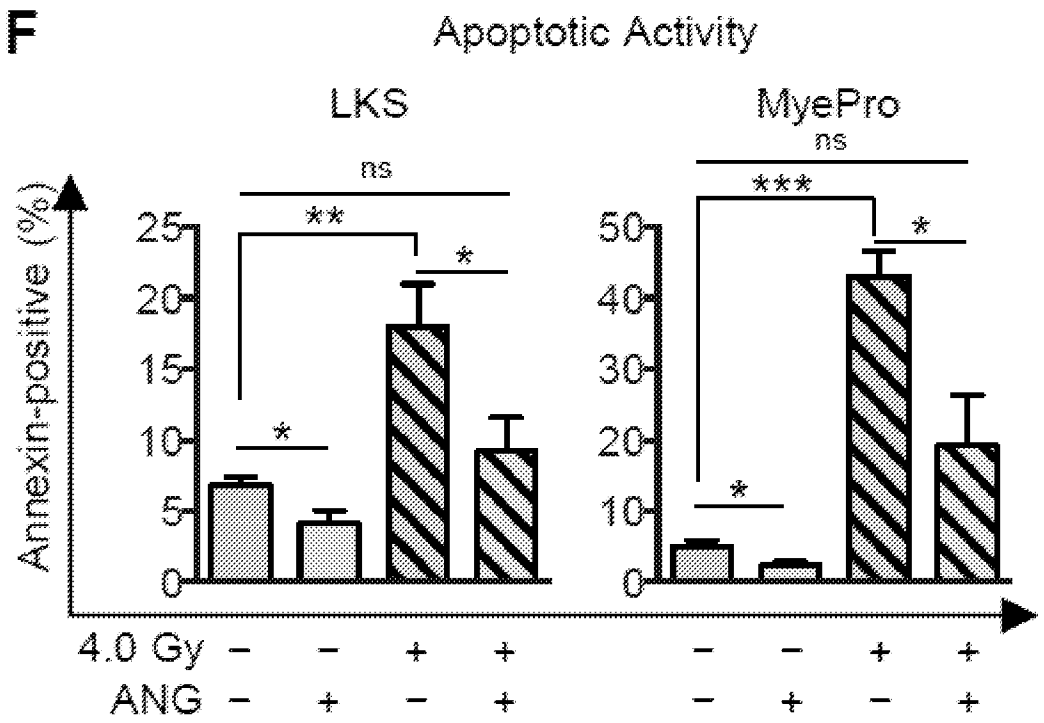
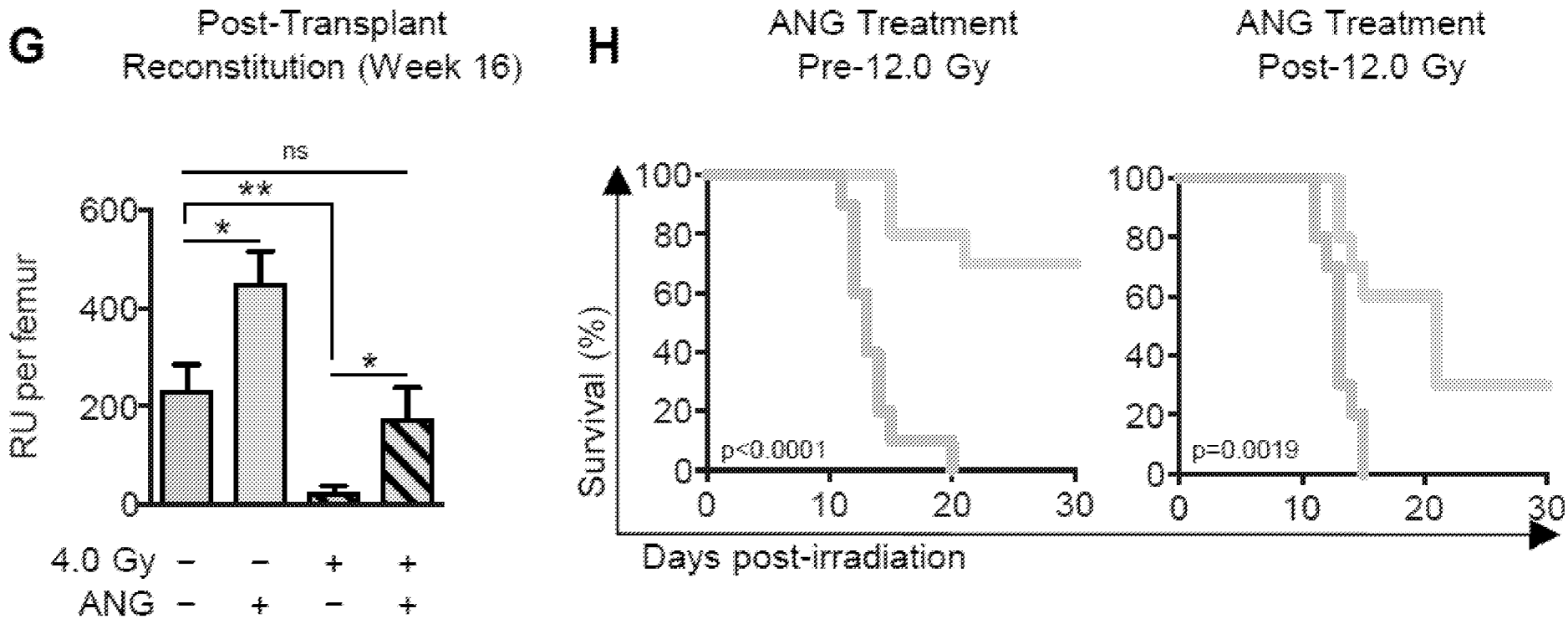

E
HSPC Cycling Following Irradiation
G0
S-G2-M
LKS
MyePro
LKS
MyePro
G0 (%)
S-G2-M (%)
ns
***
**
*
60
40
20
0
15
10
5
30
20
10
0
− − + +
− + − +

FIG. 21F


F
Apoptotic Activity
LKS
MyePro
Annexin-positive (%)
ns
**
*
*
ns
***
*
25
20
15
10
5
0
50
40
30
20
10
0
4.0 Gy – – + + – – + +
ANG – + – + – + – +

FIGs. 21G-21H


G
Post-Transplant
Reconstitution (Week 16)
ns
**
*
*
600
400
200
0
RU per femur
4.0 Gy – – + +
ANG – + – +
H
ANG Treatment
Pre-12.0 Gy
ANG Treatment
Post-12.0 Gy
100
80
60
40
20
0
Survival (%)
p<0.0001
p=0.0019
0
10
20
30
Days post-irradiation

FIGs. 21I-21J

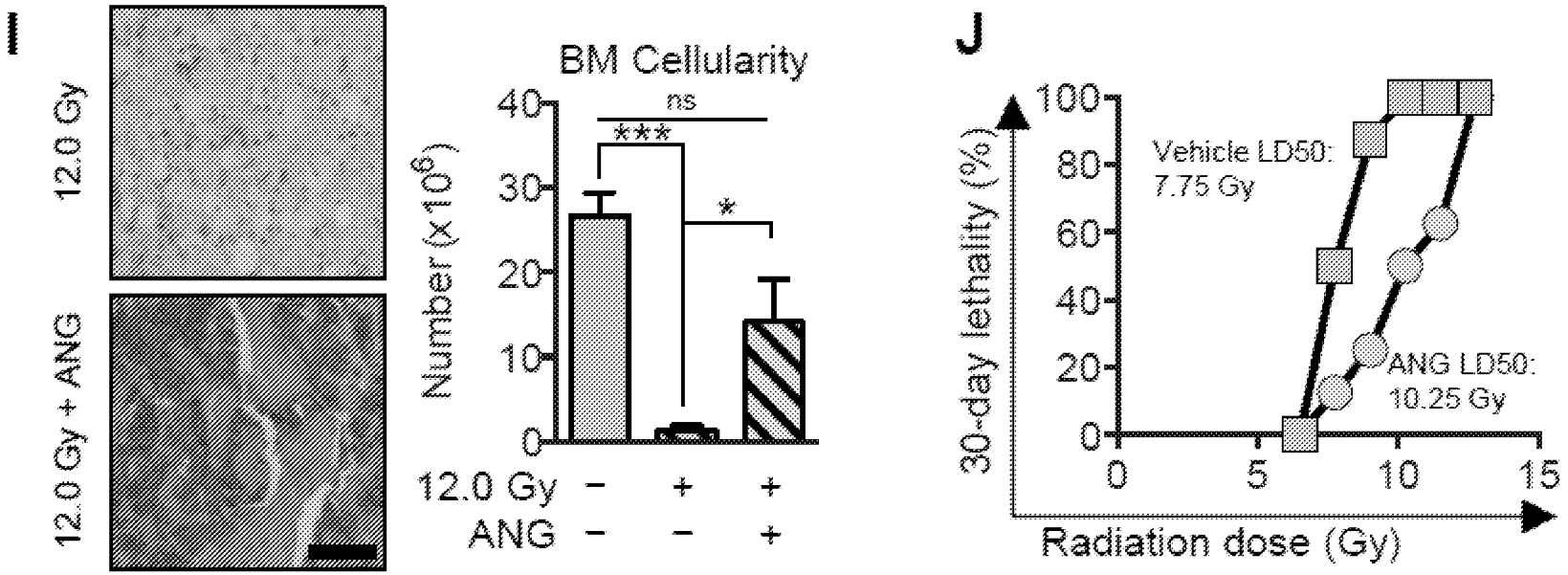

I
12.0 Gy
12.0 Gy + ANG
BM Cellularity
Number (x10^6)
ns
***
*
40
30
20
10
0
12.0 Gy − + +
ANG − − +
J
30-day lethality (%)
100
80
60
40
20
0
Vehicle LD50:
7.75 Gy
ANG LD50:
10.25 Gy
0
5
10
15
Radiation dose (Gy)

FIG. 21K

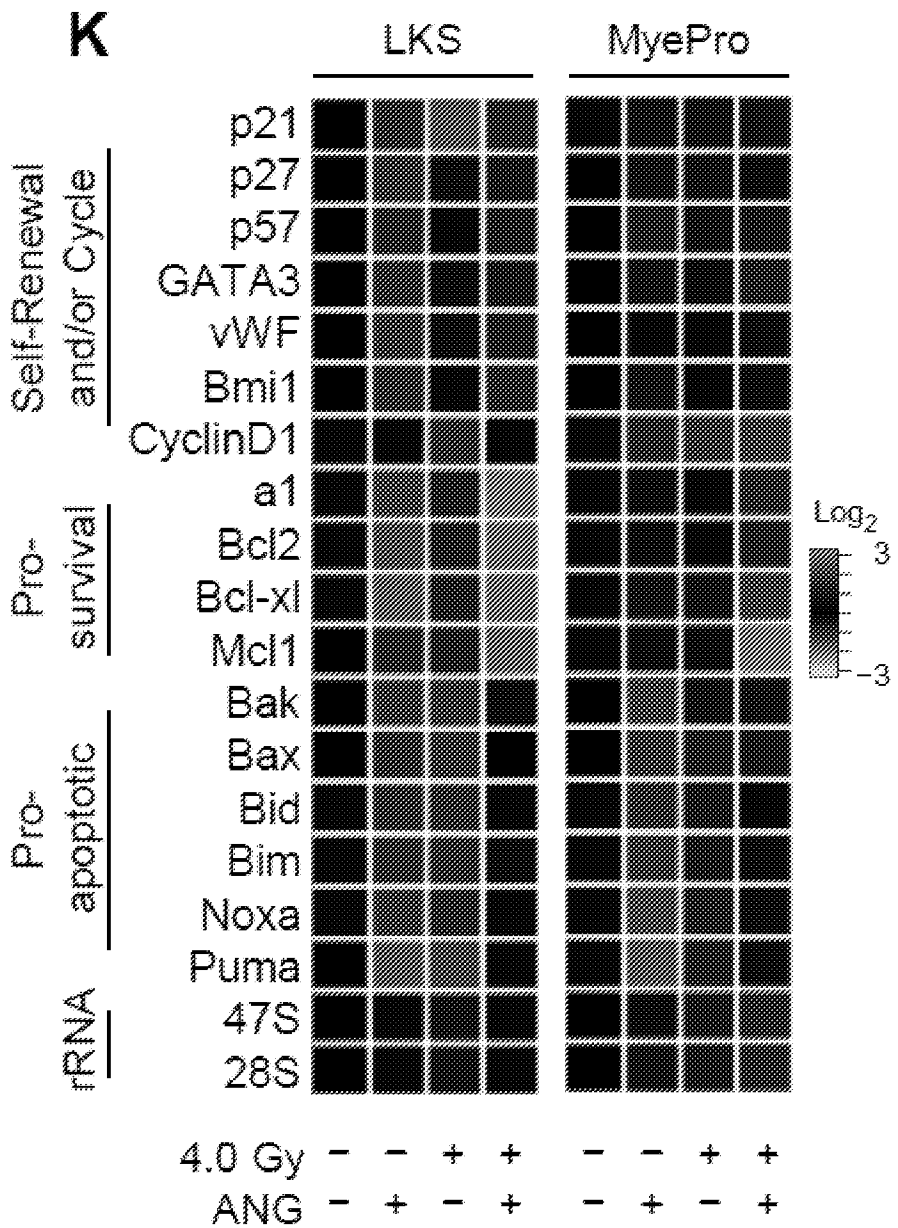
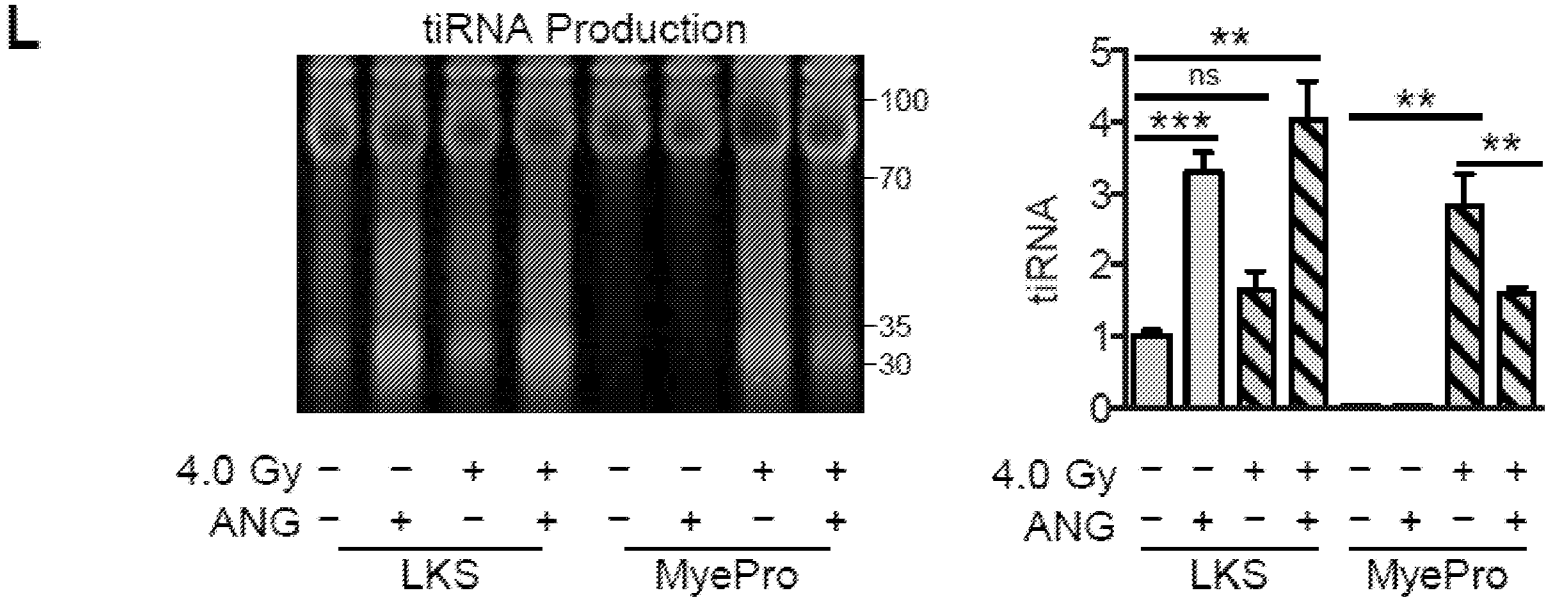

K
LKS
MyePro
p21
p27
p57
GATA3
vWF
Bmi1
CyclinD1
a1
Bcl2
Bcl-xl
Mcl1
Bak
Bax
Bid
Bim
Noxa
Puma
47S
28S
Self-Renewal and/or Cycle
Pro-survival
Pro-apoptotic
rRNA
Log2
3
−3
4.0 Gy − − + + − − + +
ANG − + − + − + − +

FIG. 21L


L
tiRNA Production
100
70
35
30
4.0 Gy − − + + − − + +
ANG − + − + − + − +
LKS
MyePro
tiRNA
5
4
3
2
1
0
**
ns
***
**
**
4.0 Gy − − + + − − + +
ANG − + − + − + − +
LKS
MyePro

FIGs. 22A-22B

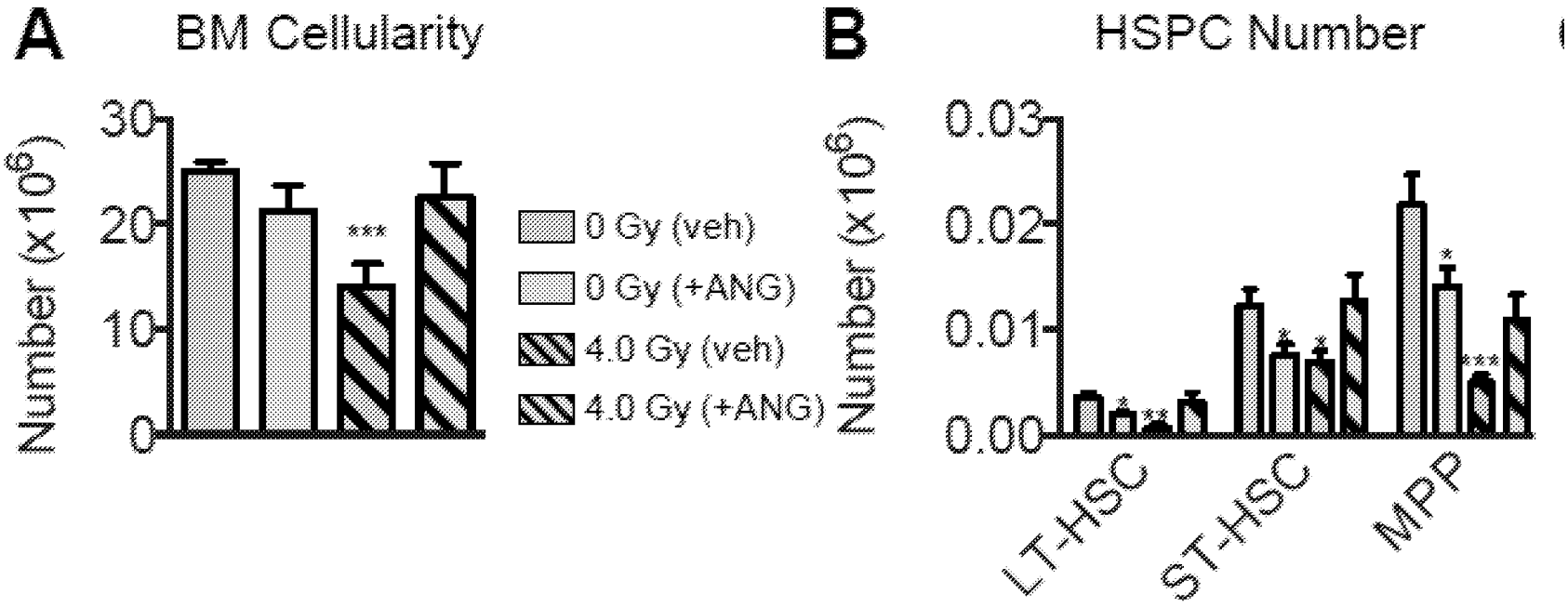
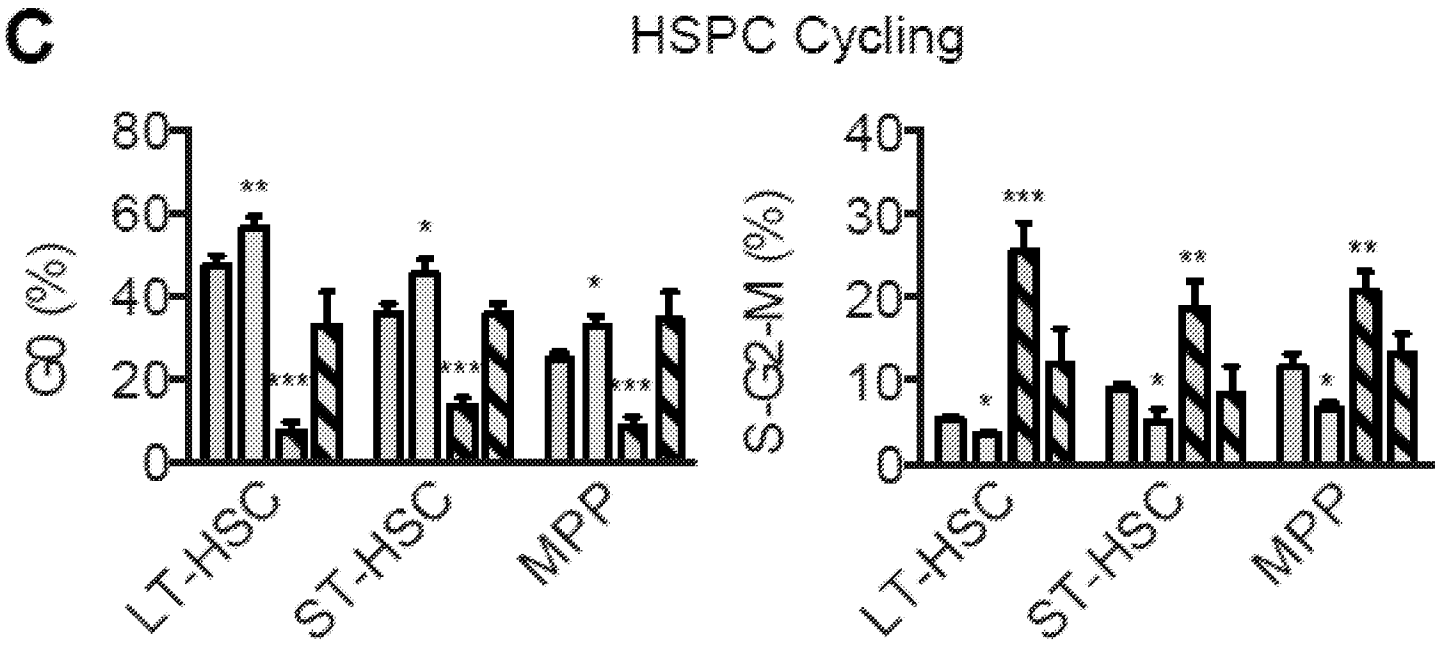

A
BM Cellularity
Number (x10^6)
30
20
10
0
***
0 Gy (veh)
0 Gy (+ANG)
4.0 Gy (veh)
4.0 Gy (+ANG)
B
HSPC Number
Number (x10^6)
0.03
0.02
0.01
0.00
LT-HSC
ST-HSC
MPP

FIG. 22C



FIGs. 22D-22E

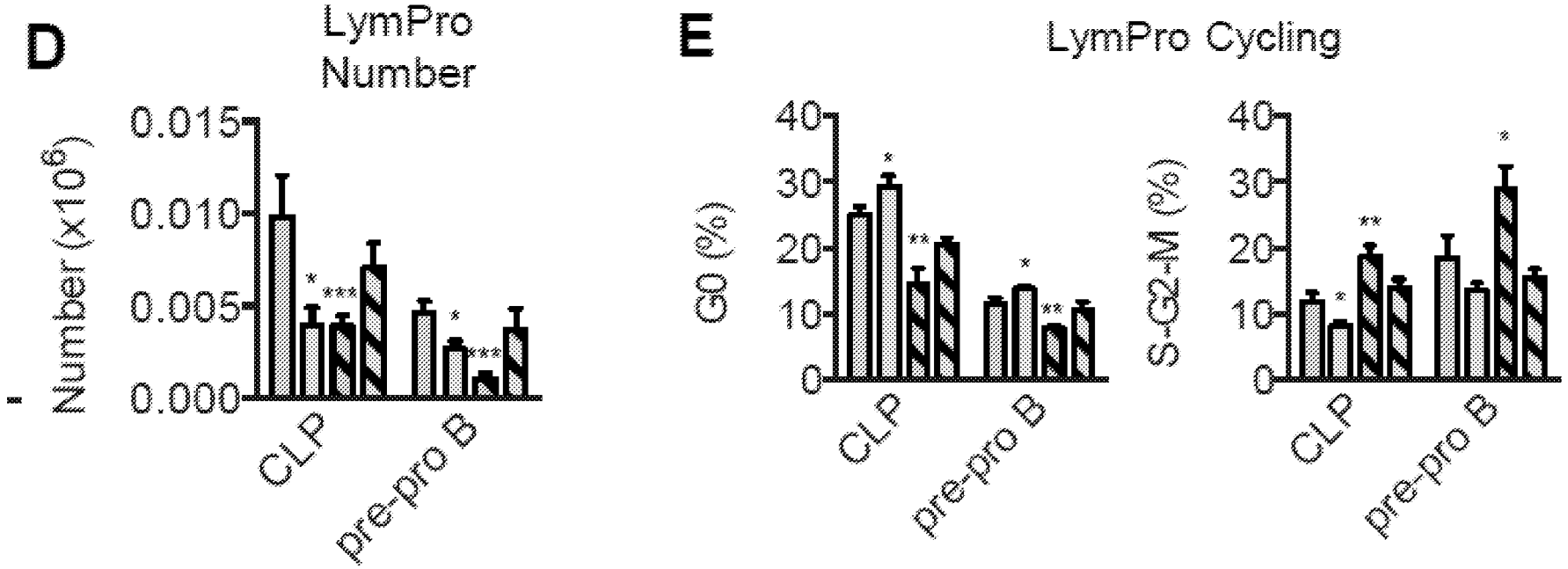

D
LymPro Number
Number (x10^6)
0.015
0.010
0.005
0.000
CLP
pre-pro B
E
LymPro Cycling
G0 (%)
S-G2-M (%)
40
30
20
10
0
CLP
pre-pro B

FIG. 22F

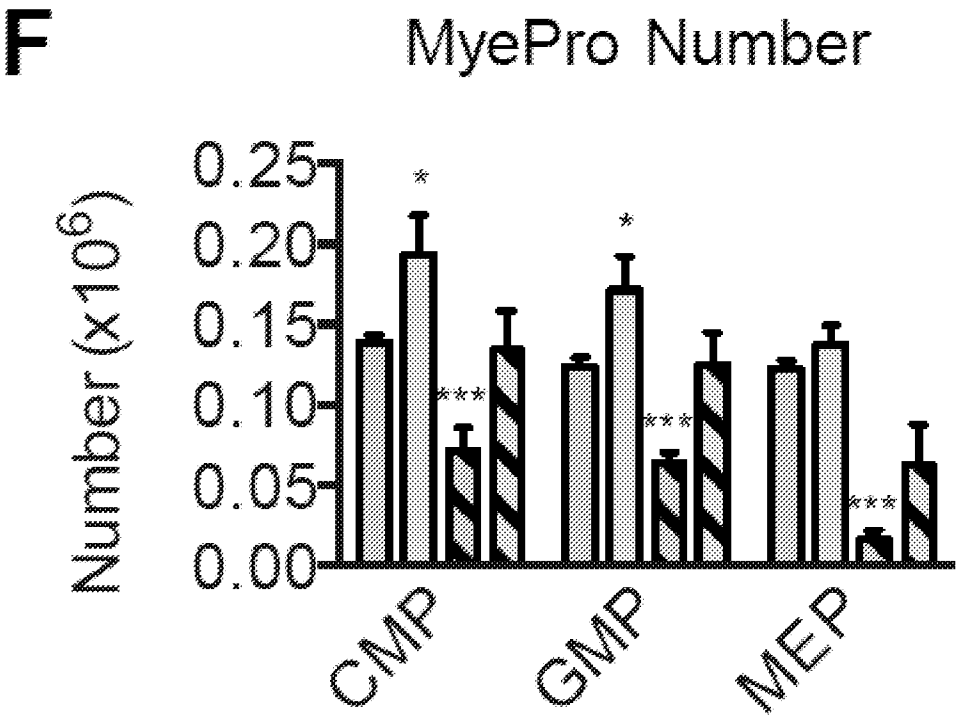

F
MyePro Number
Number (x10^6)
0.25
0.20
0.15
0.10
0.05
0.00
*
*
***
***
***
CMP
GMP
MEP

FIG. 22G

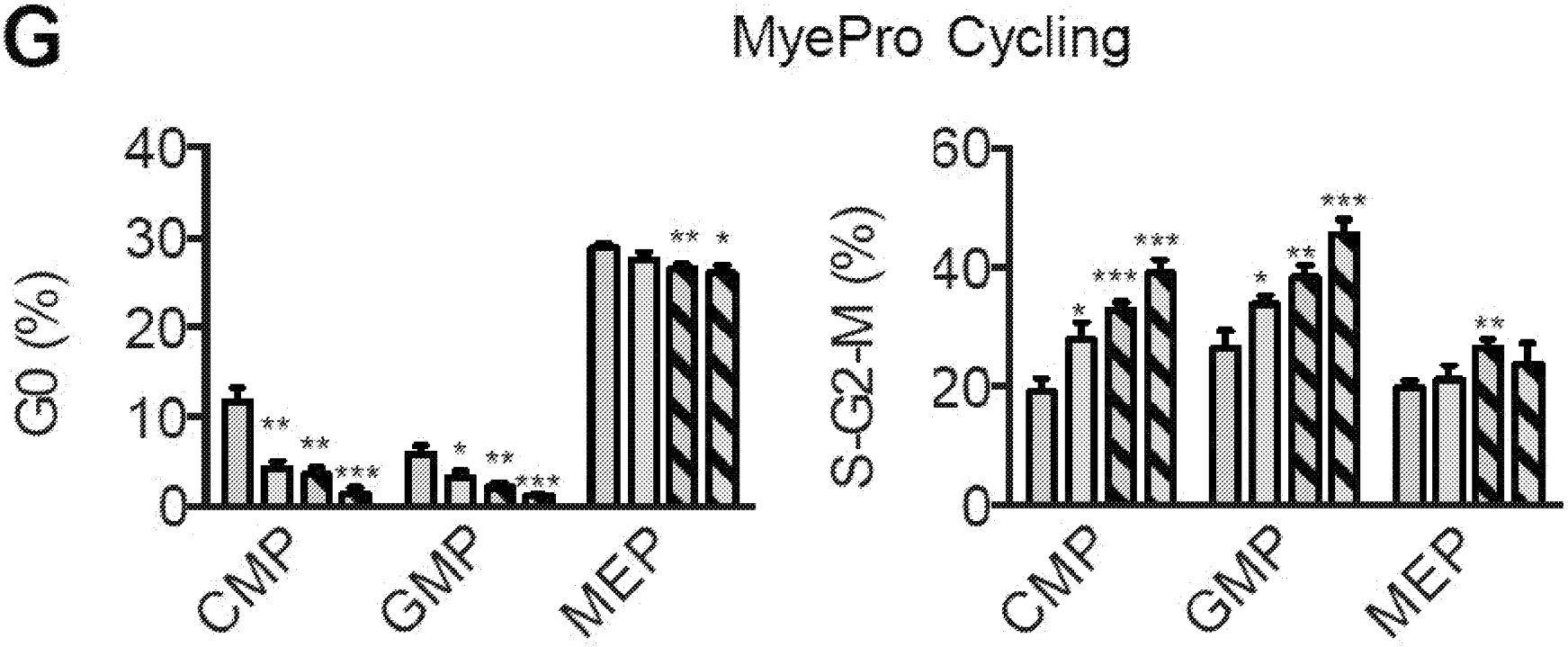
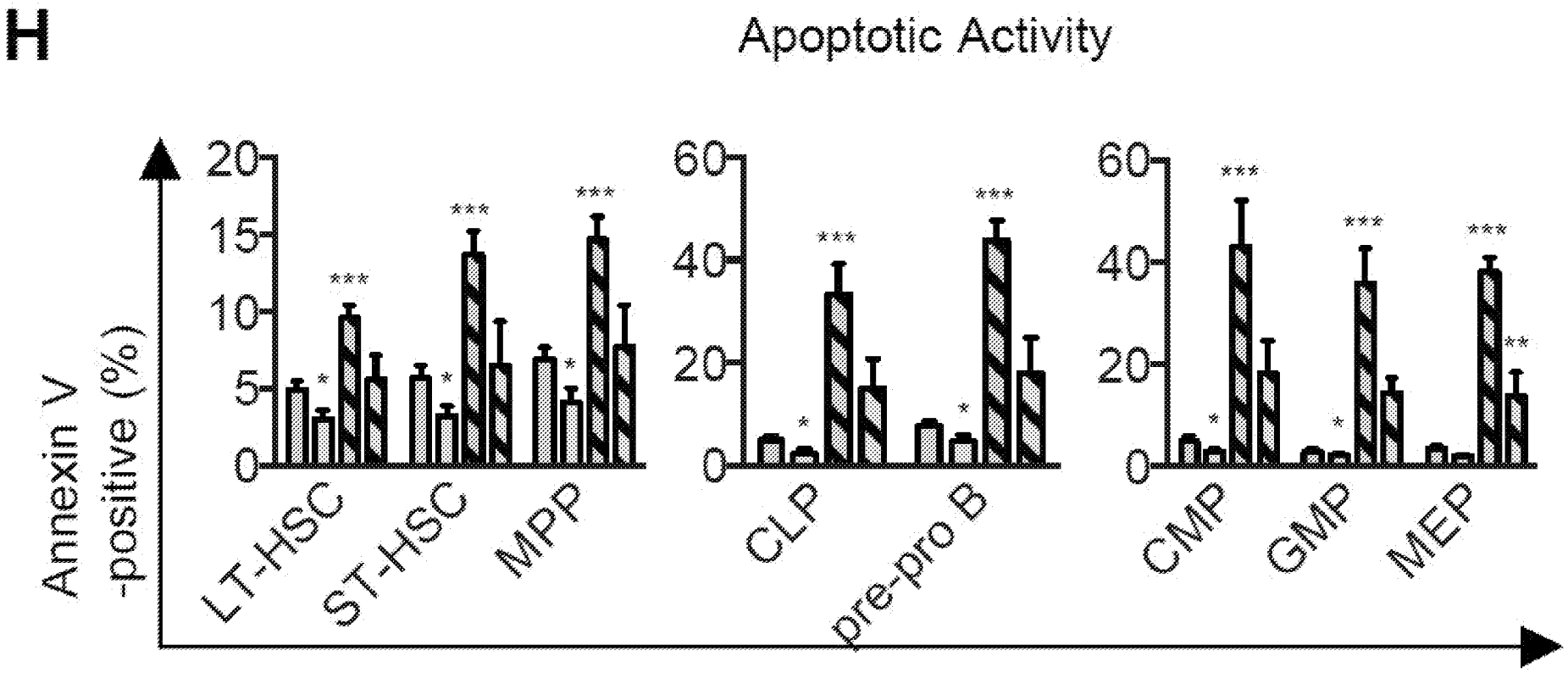
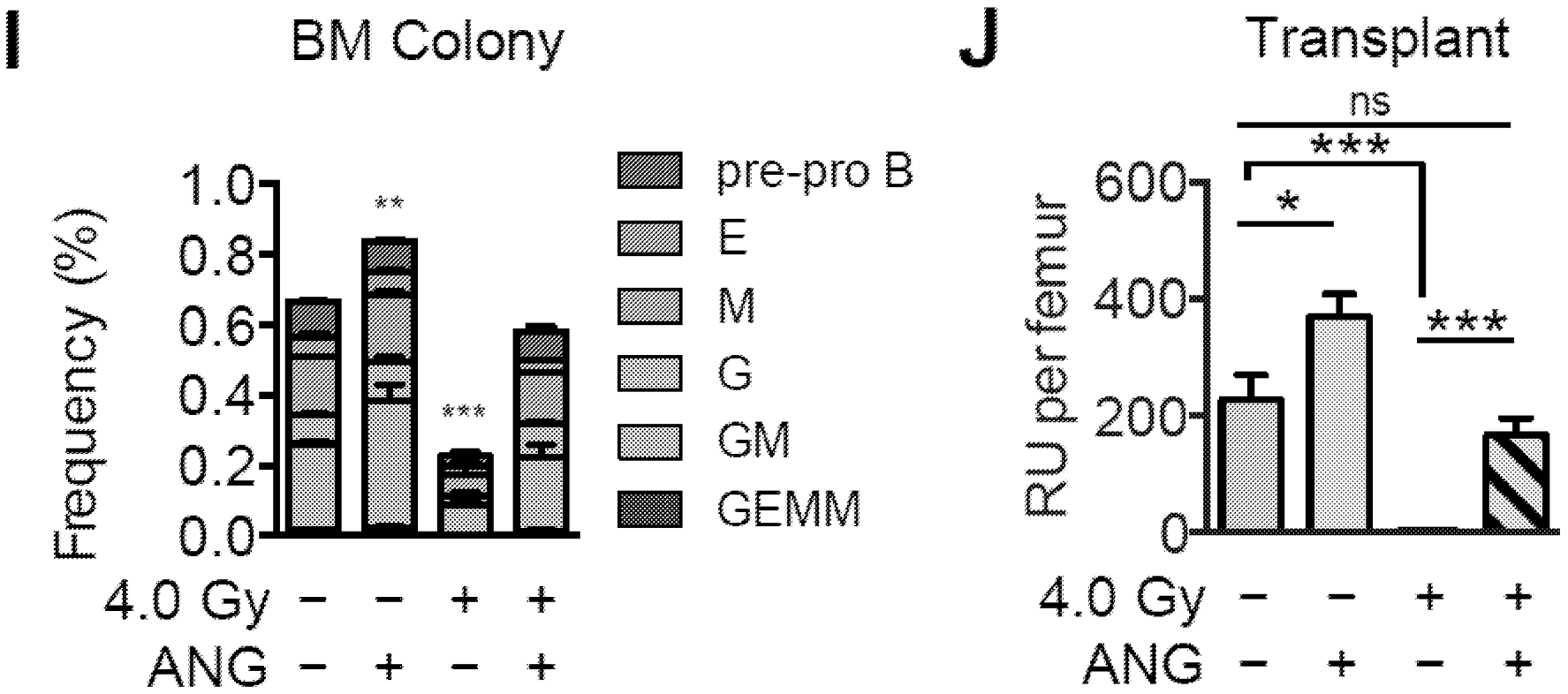

G
MyePro Cycling
G0 (%)
40
30
20
10
0
**
**
***
*
**
***
** *
CMP
GMP
MEP
S-G2-M (%)
60
40
20
0
*
***
***
*
**
***
**
CMP
GMP
MEP

FIG. 22H


H
Apoptotic Activity
Annexin V
-positive (%)
20
15
10
5
0
60
40
20
0
60
40
20
0
*
***
**
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP

FIGs. 22I-22J


I
BM Colony
Frequency (%)
1.0
0.8
0.6
0.4
0.2
0.0
**
***
pre-pro B
E
M
G
GM
GEMM
4.0 Gy − − + +
ANG − + − +
J
Transplant
RU per femur
600
400
200
0
ns
***
*
***
4.0 Gy − − + +
ANG − + − +

FIGs. 22K-22L

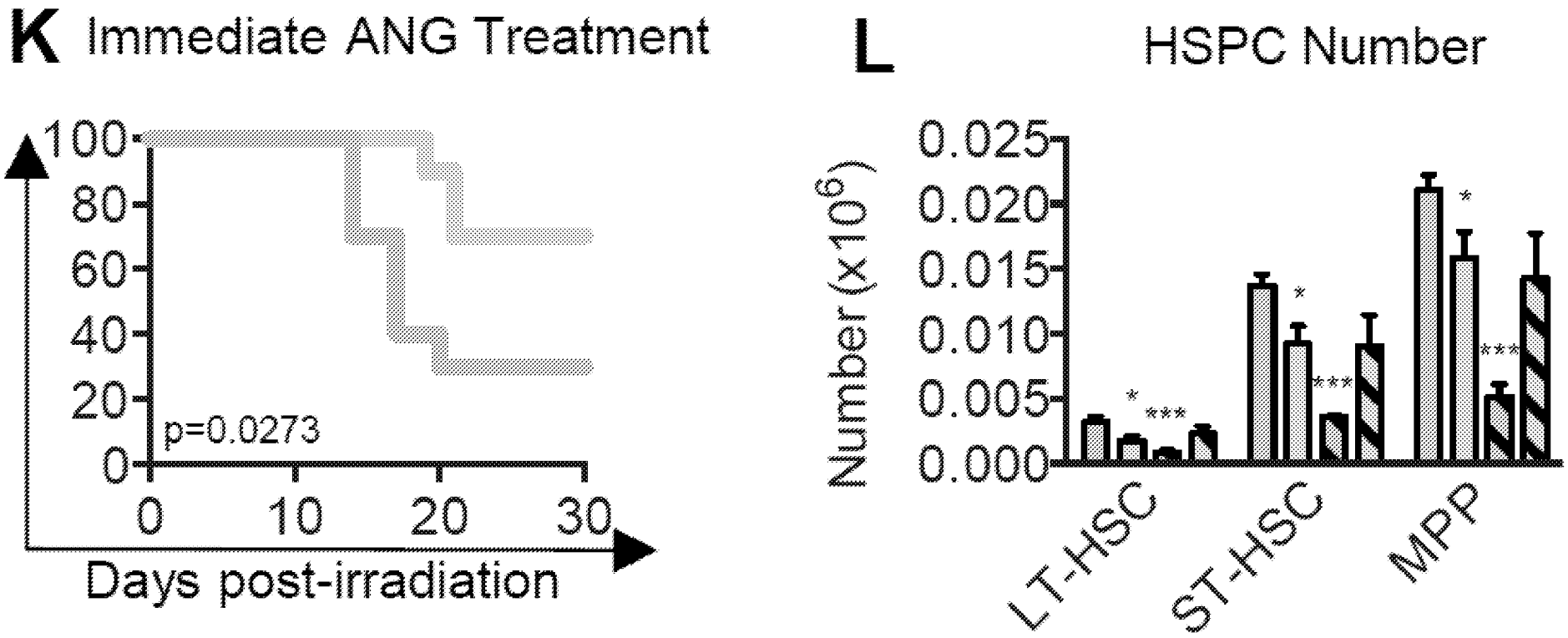

K
Immediate ANG Treatment
100
80
60
40
20
0
p=0.0273
0
10
20
30
Days post-irradiation
L
HSPC Number
Number (x10^6)
0.025
0.020
0.015
0.010
0.005
0.000
*
***
*
***
*
***
LT-HSC
ST-HSC
MPP

FIG. 22M

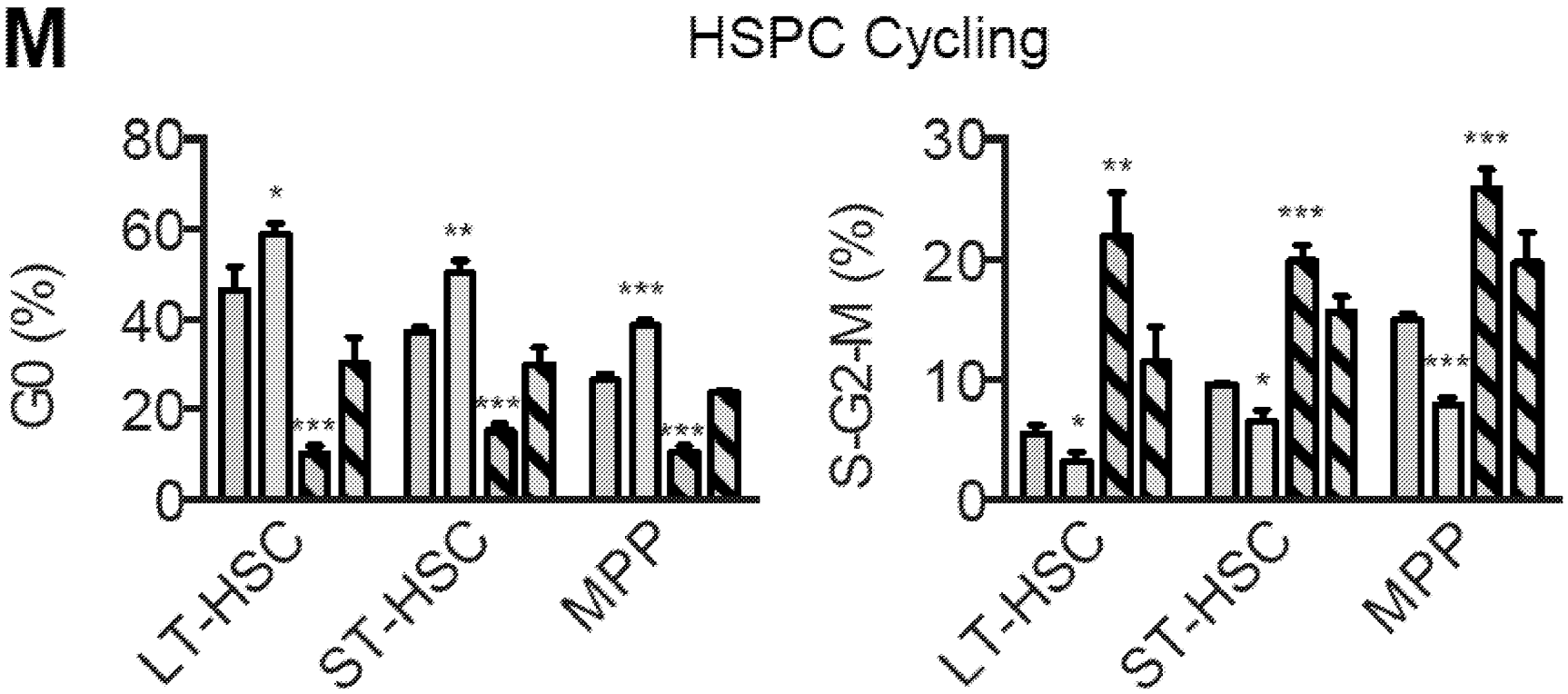

M
HSPC Cycling
G0 (%)
80
60
40
20
0
*
**
***
***
***
***
LT-HSC
ST-HSC
MPP
S-G2-M (%)
30
20
10
0
*
**
*
***
***
***
LT-HSC
ST-HSC
MPP

FIG. 22N

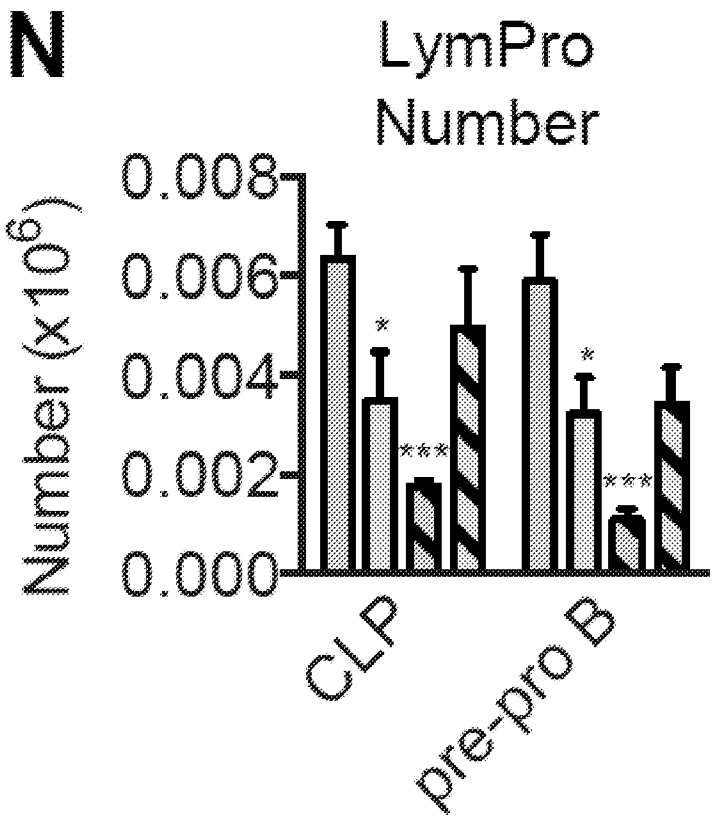

N
LymPro
Number
Number (x10^6)
0.008
0.006
0.004
0.002
0.000
*
***
*
***
CLP
pre-pro B

FIG. 22O

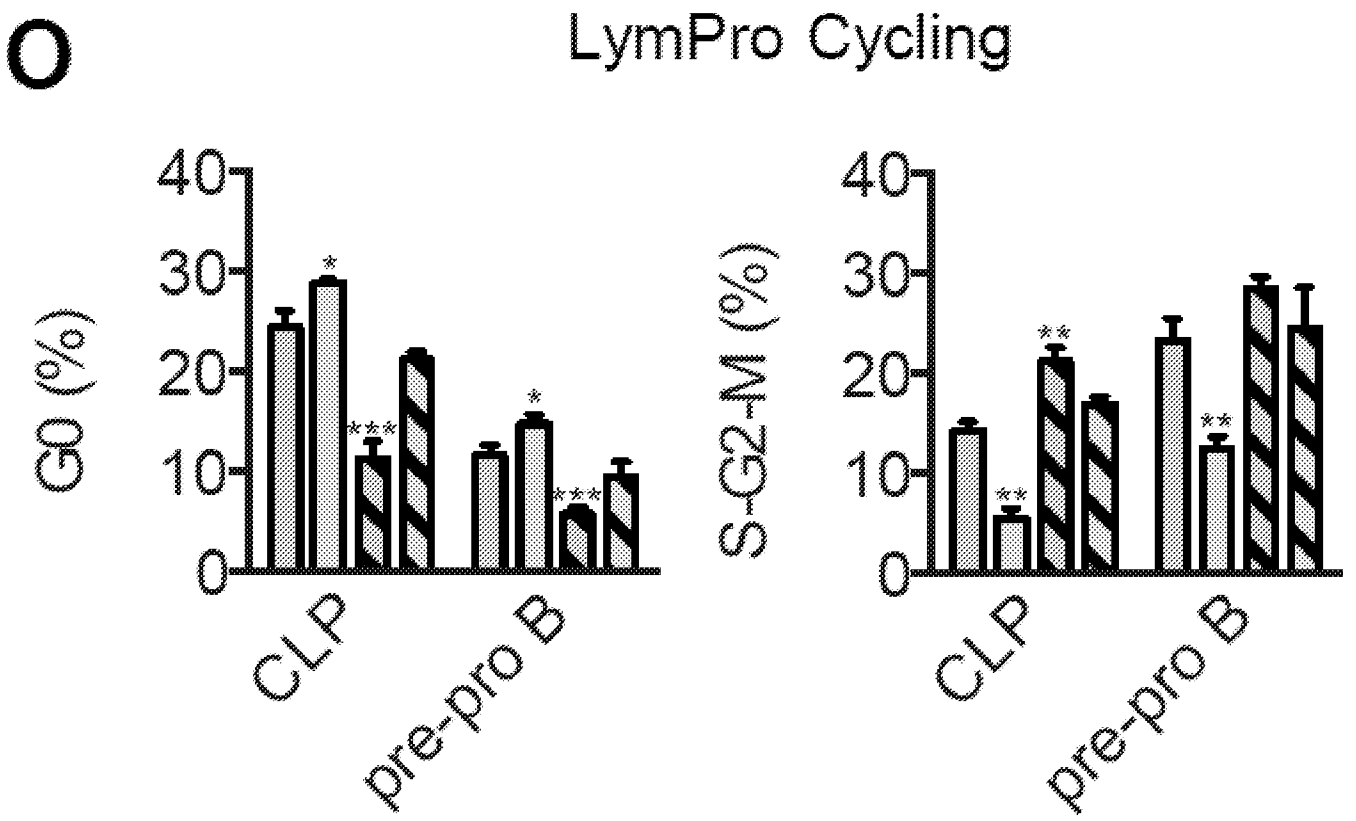


FIG. 22P

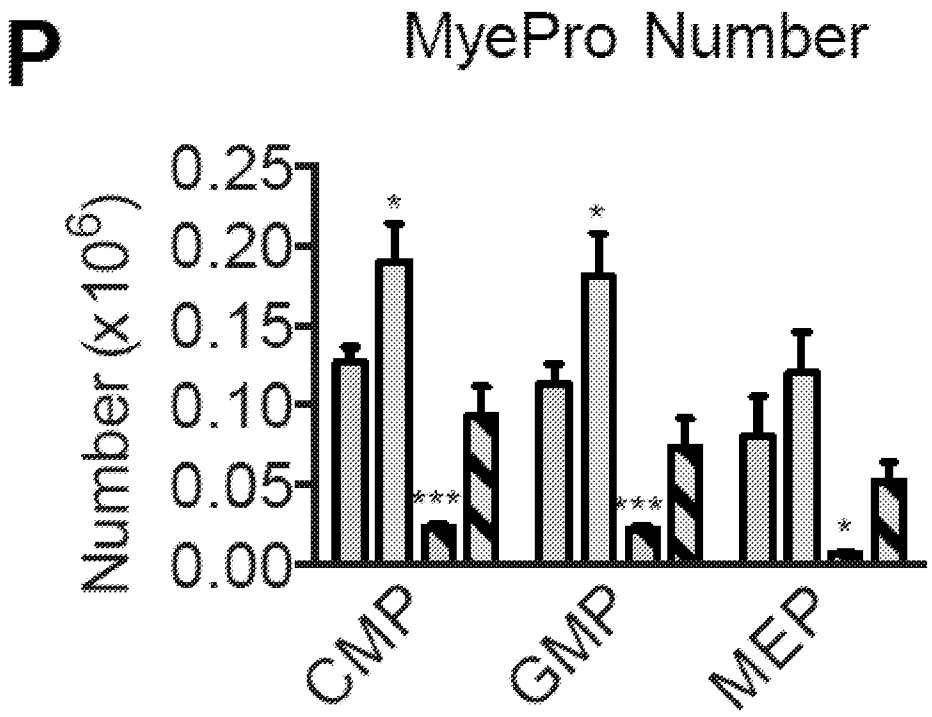

P
MyePro Number
Number (x10^6)
0.25
0.20
0.15
0.10
0.05
0.00
*
***
*
***
*
CMP
GMP
MEP

FIG. 22Q

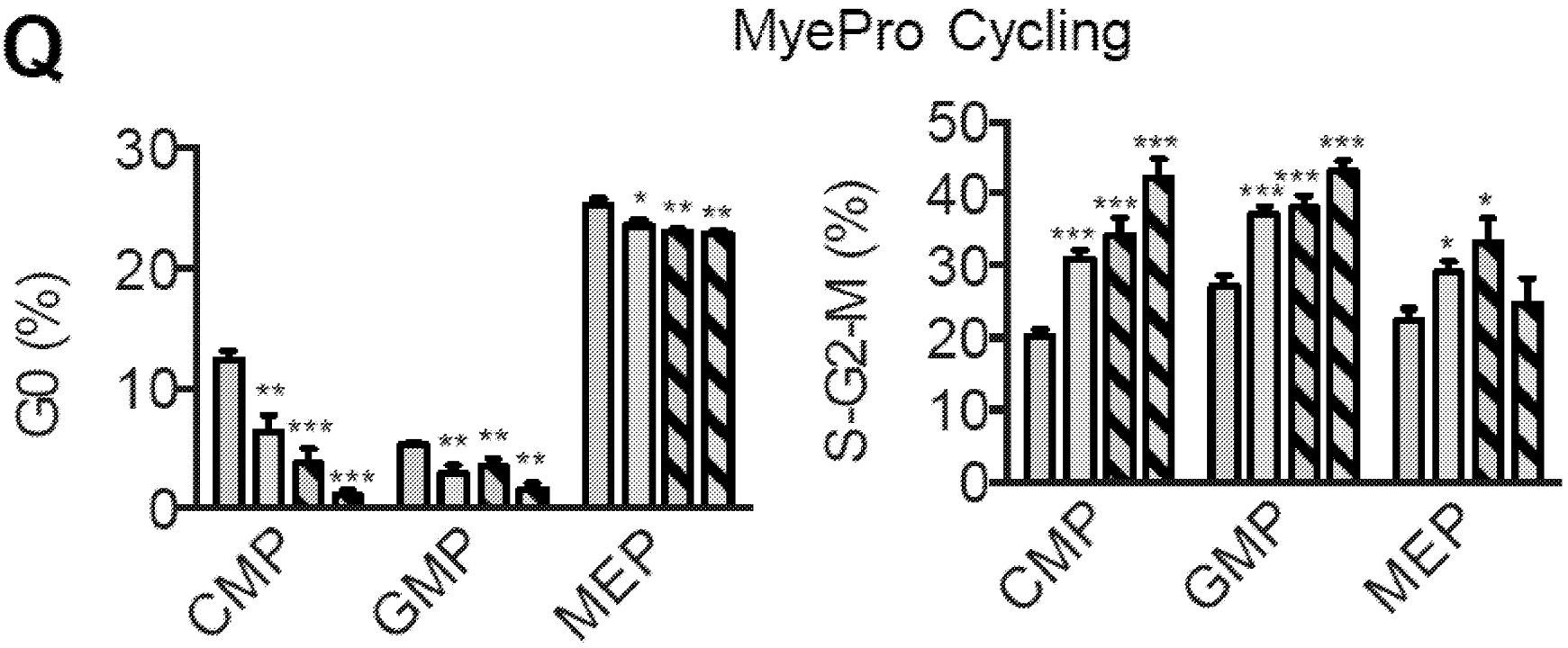

Q
MyePro Cycling
G0 (%)
30
20
10
0
**
***
***
**
**
**
*
**
**
CMP
GMP
MEP
S-G2-M (%)
50
40
30
20
10
0
***
***
***
***
***
***
*
*
*
CMP
GMP
MEP

FIG. 22R

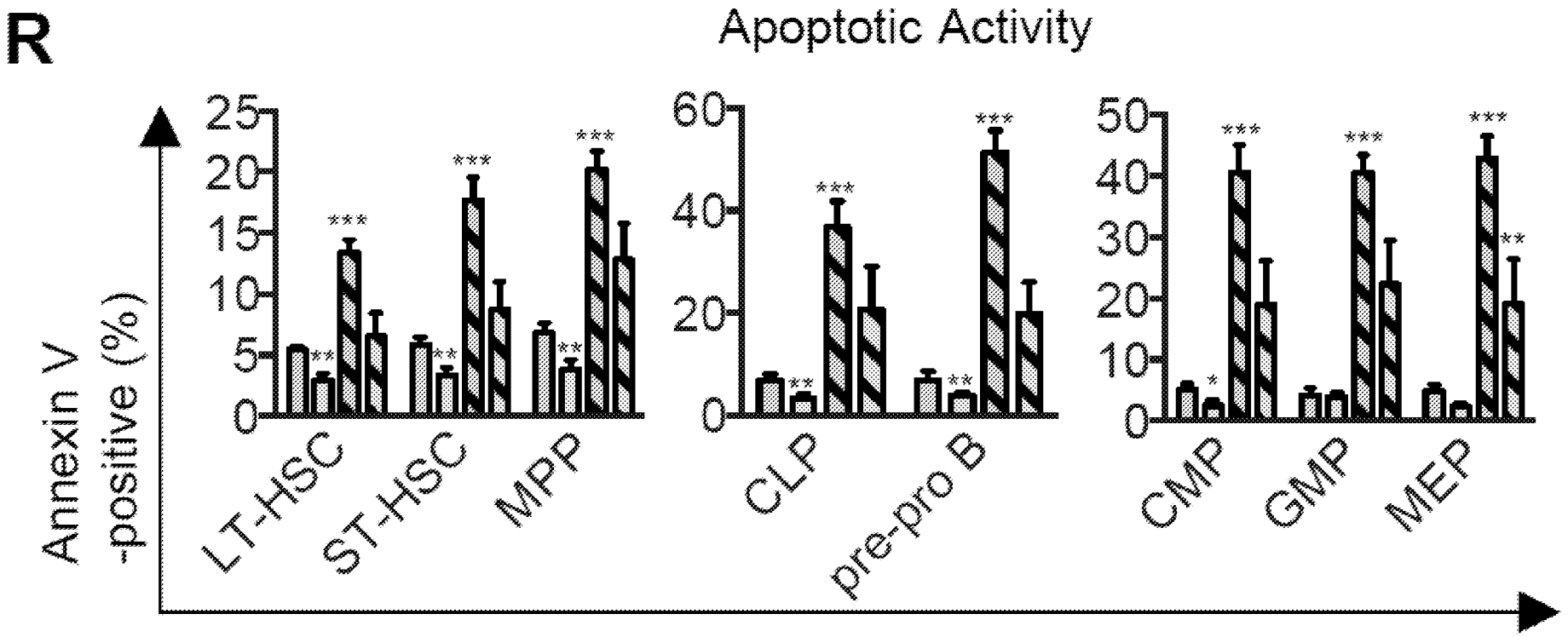

R
Apoptotic Activity
Annexin V
-positive (%)
25
20
15
10
5
0
60
40
20
0
50
40
30
20
10
0
LT-HSC
ST-HSC
MPP
CLP
pre-pro B
CMP
GMP
MEP

FIG. 22S

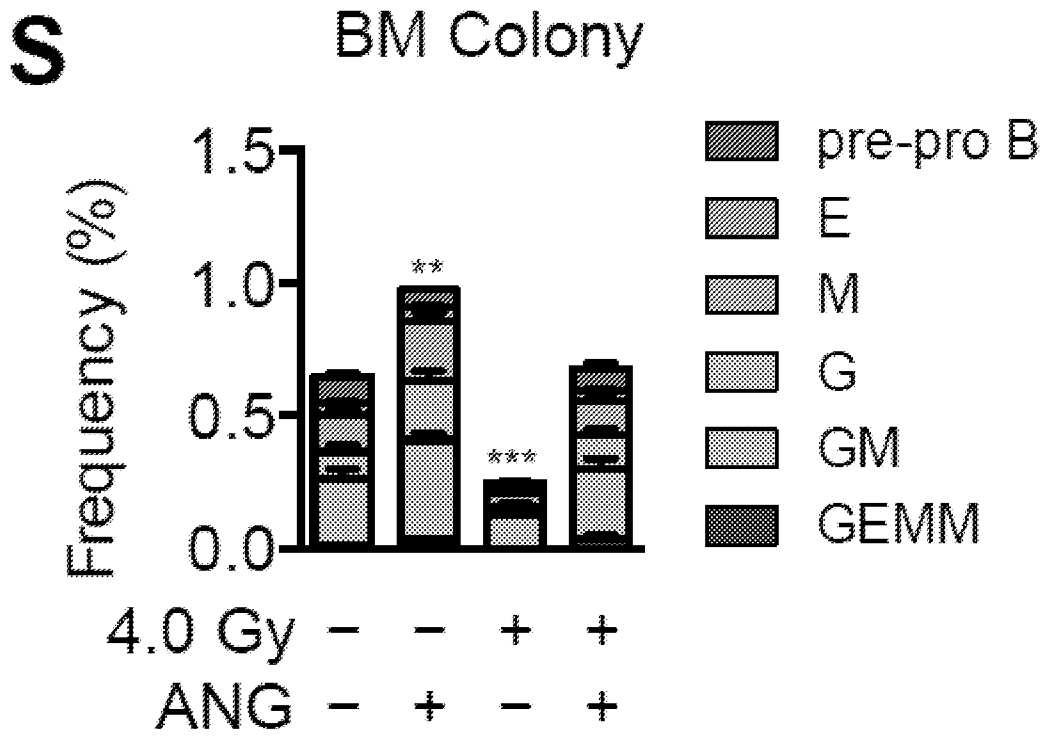


FIG. 23A

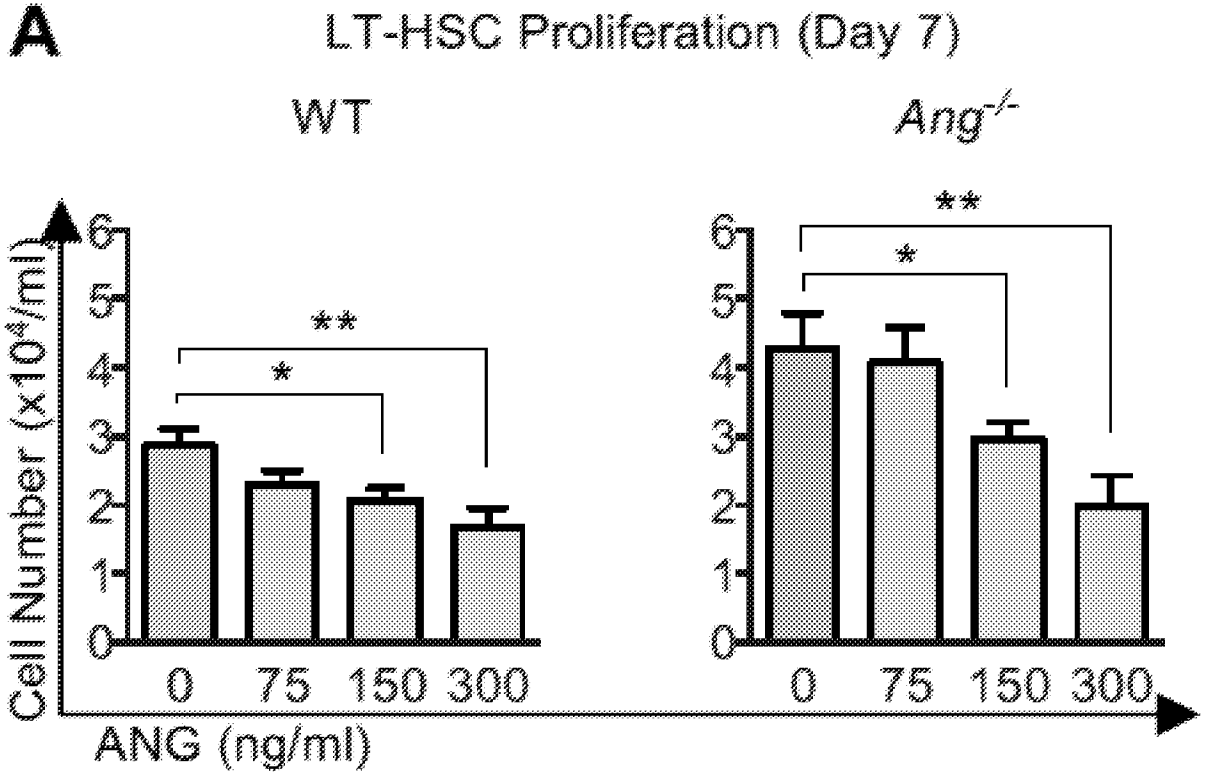

A
LT-HSC Proliferation (Day 7)
WT
Ang-/-
Cell Number (x10⁴/ml)
0
1
2
3
4
5
6
**
*
0
75
150
300
ANG (ng/ml)

FIG. 23B

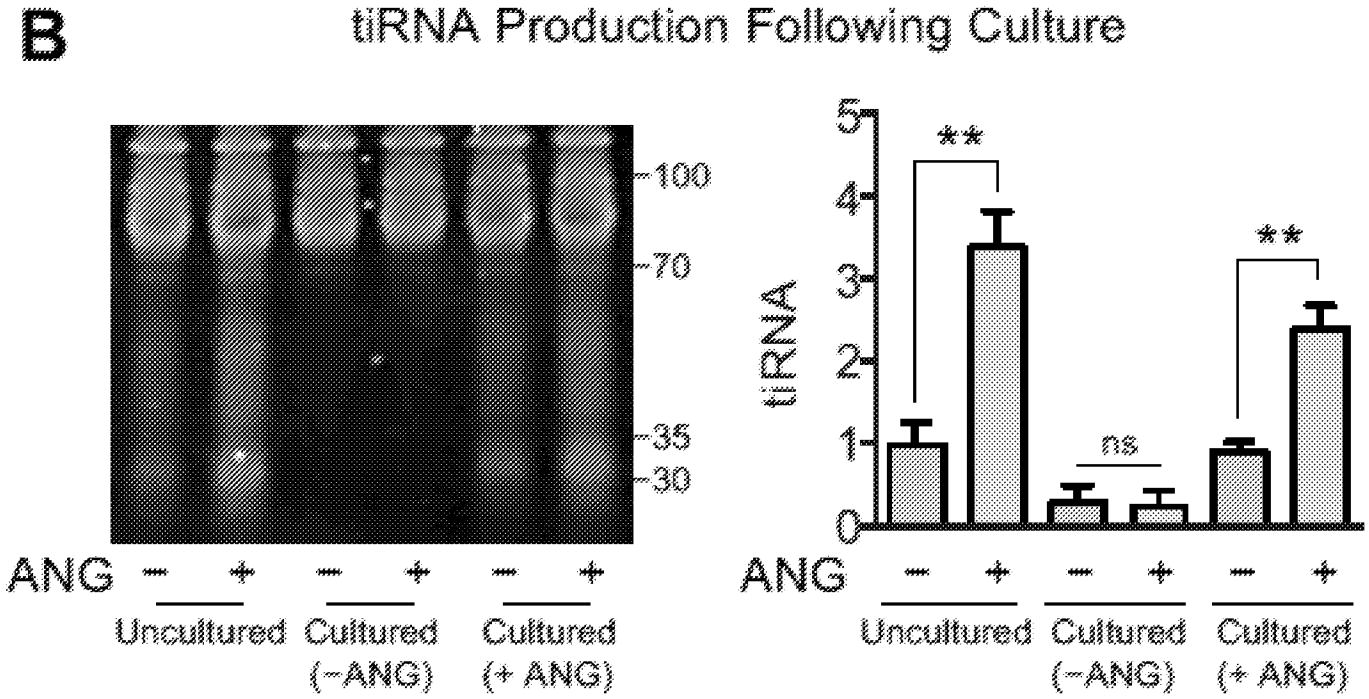

B
tiRNA Production Following Culture
100
70
35
30
ANG
Uncultured
Cultured (−ANG)
Cultured (+ ANG)
tiRNA
**
ns
**
0
1
2
3
4
5

FIG. 23C

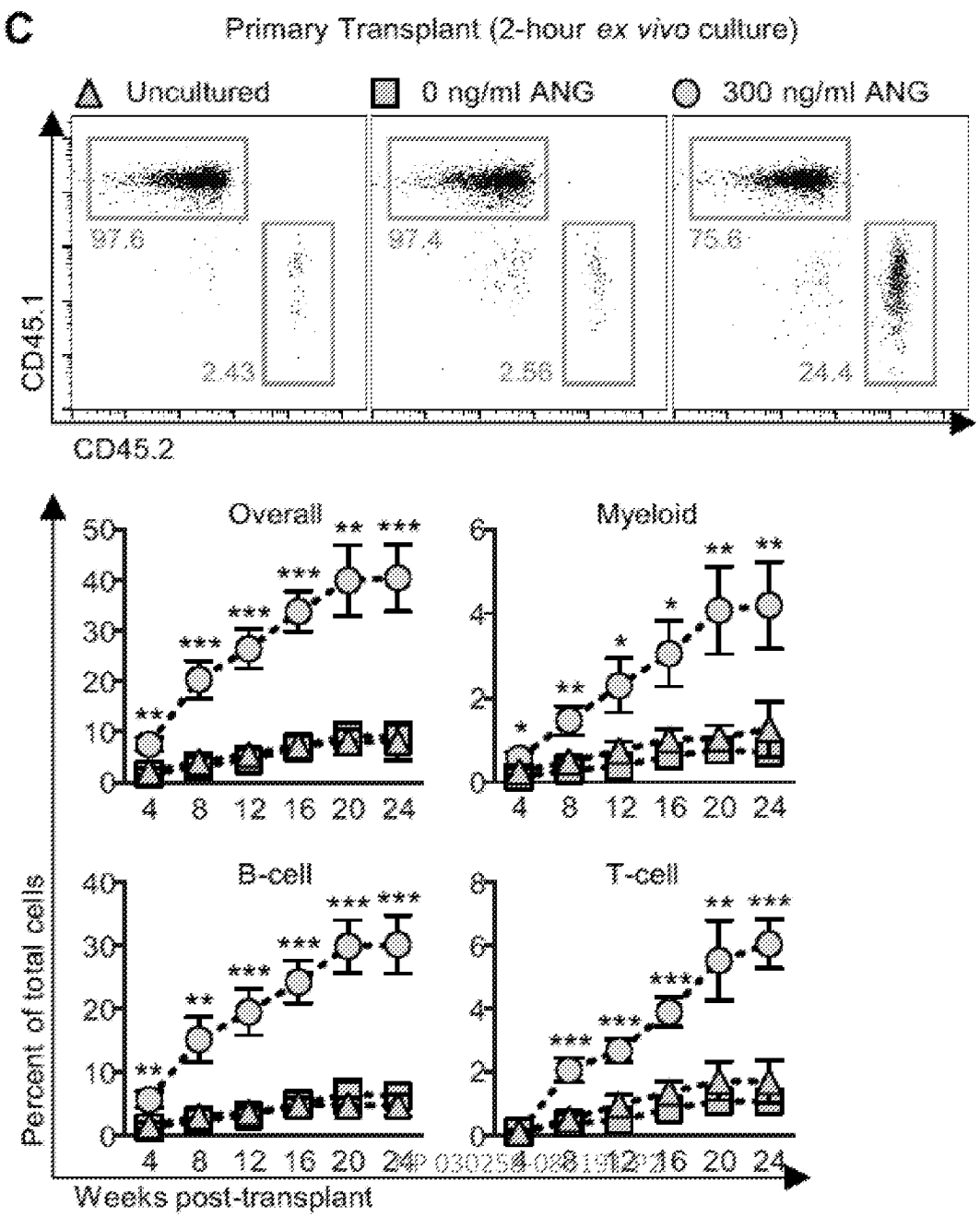

C
Primary Transplant (2-hour ex vivo culture)
Uncultured
0 ng/ml ANG
300 ng/ml ANG
97.6
2.43
97.4
2.56
75.6
24.4
CD45.1
CD45.2
Overall
Myeloid
B-cell
T-cell
Percent of total cells
Weeks post-transplant

FIG. 23D

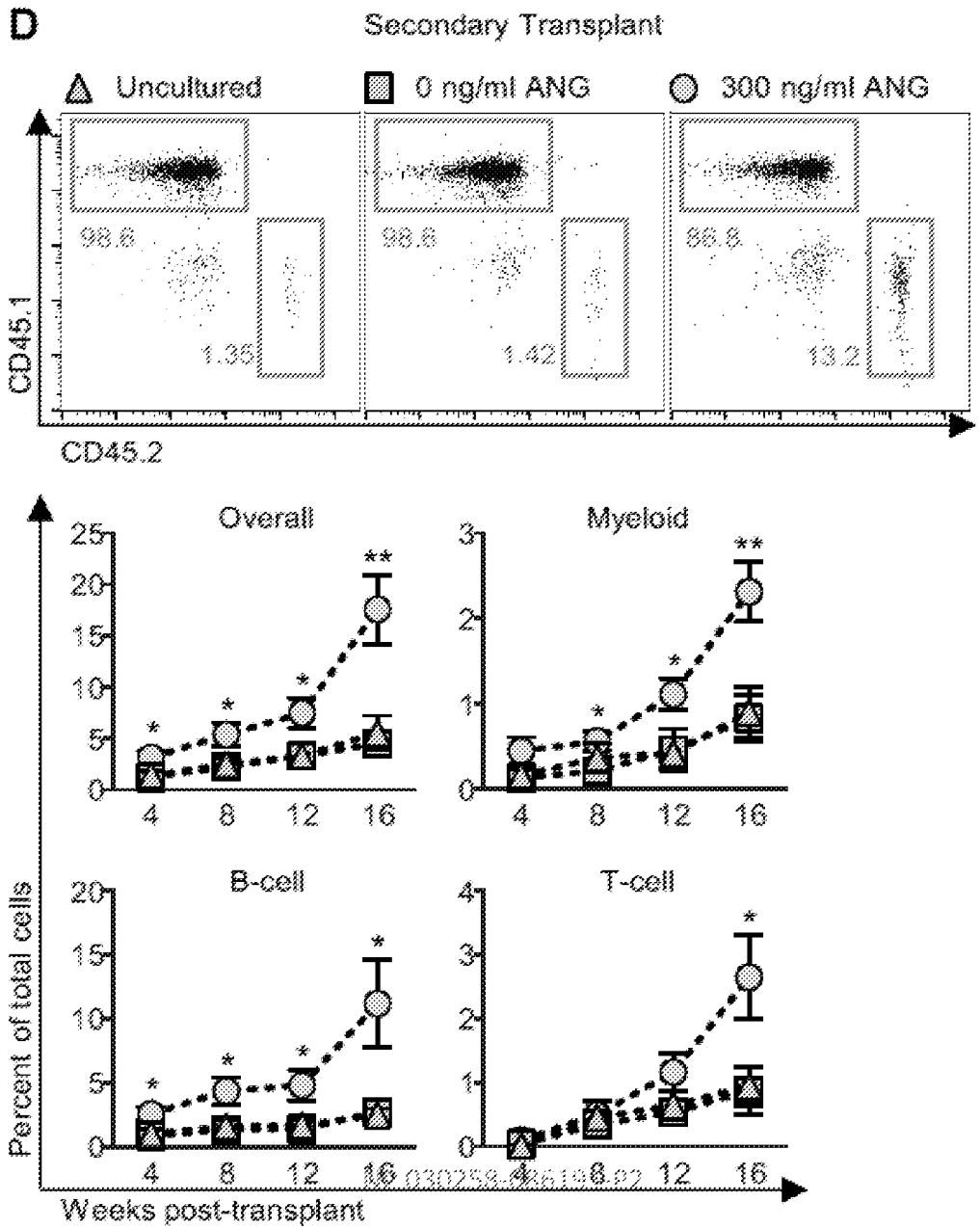

D
Secondary Transplant
Uncultured
0 ng/ml ANG
300 ng/ml ANG
98.6
1.35
98.6
1.42
66.8
13.2
CD45.1
CD45.2
Overall
Myeloid
B-cell
T-cell
Percent of total cells
Weeks post-transplant

FIG. 23E

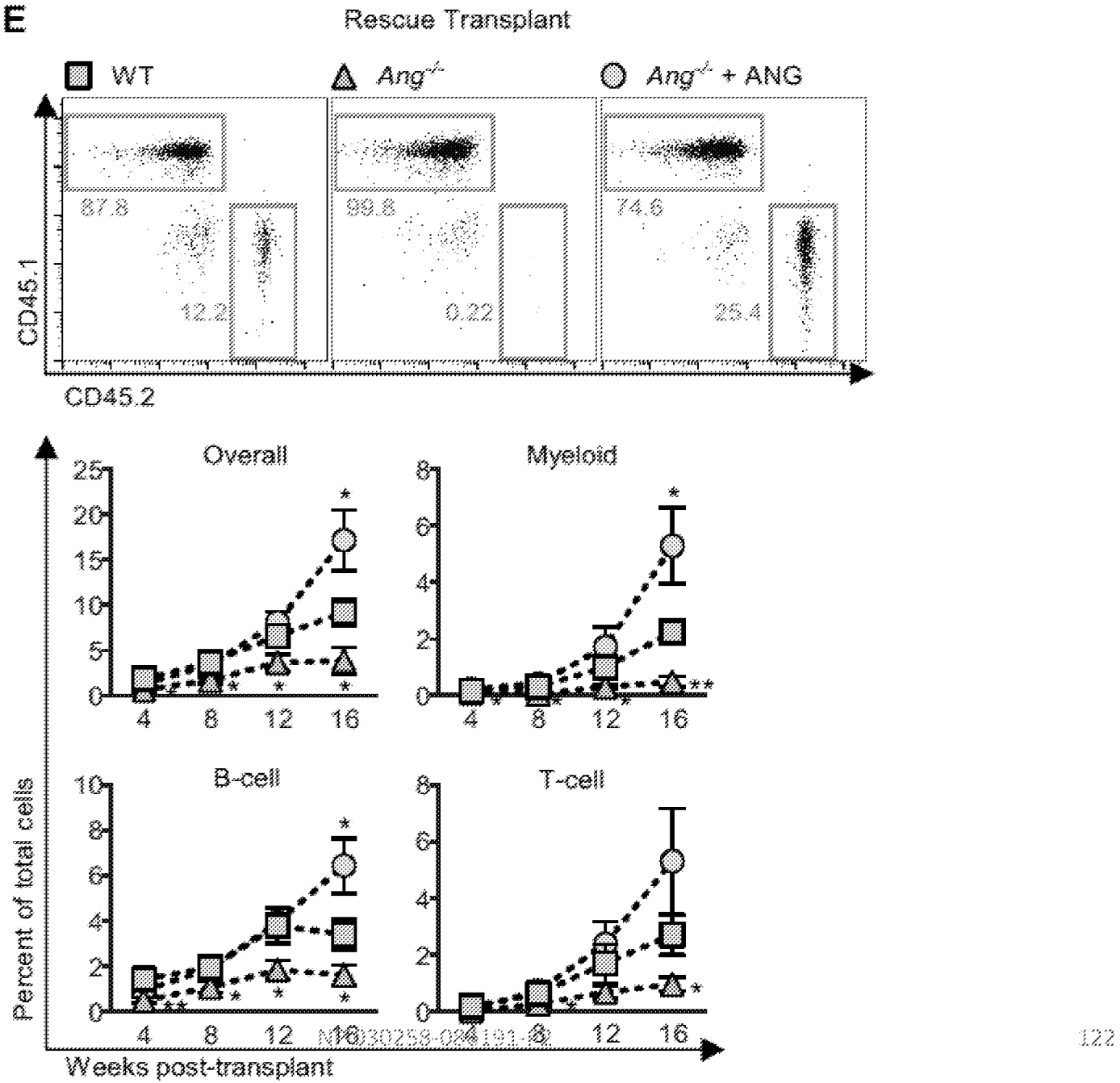
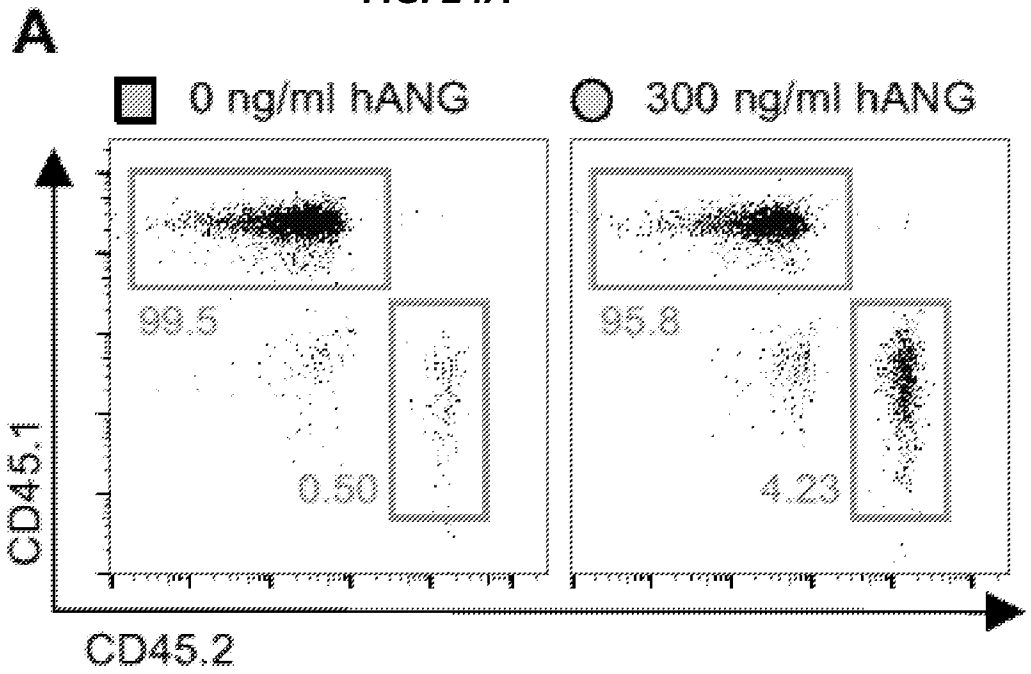
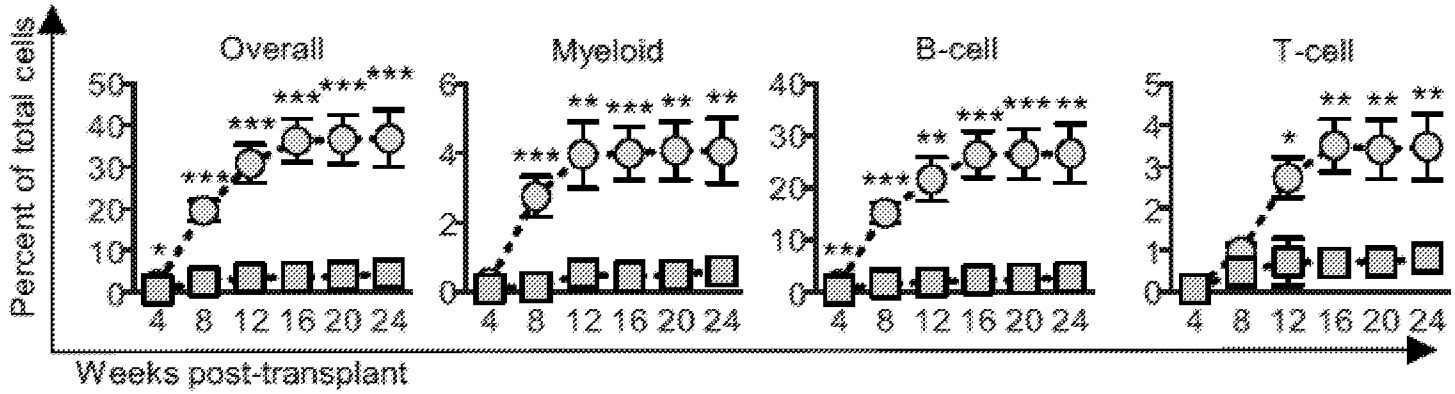

E
Rescue Transplant
WT
Ang-/-
Ang-/- + ANG
87.8
12.2
99.8
0.22
74.6
25.4
CD45.1
CD45.2
Overall
Myeloid
B-cell
T-cell
Percent of total cells
Weeks post-transplant

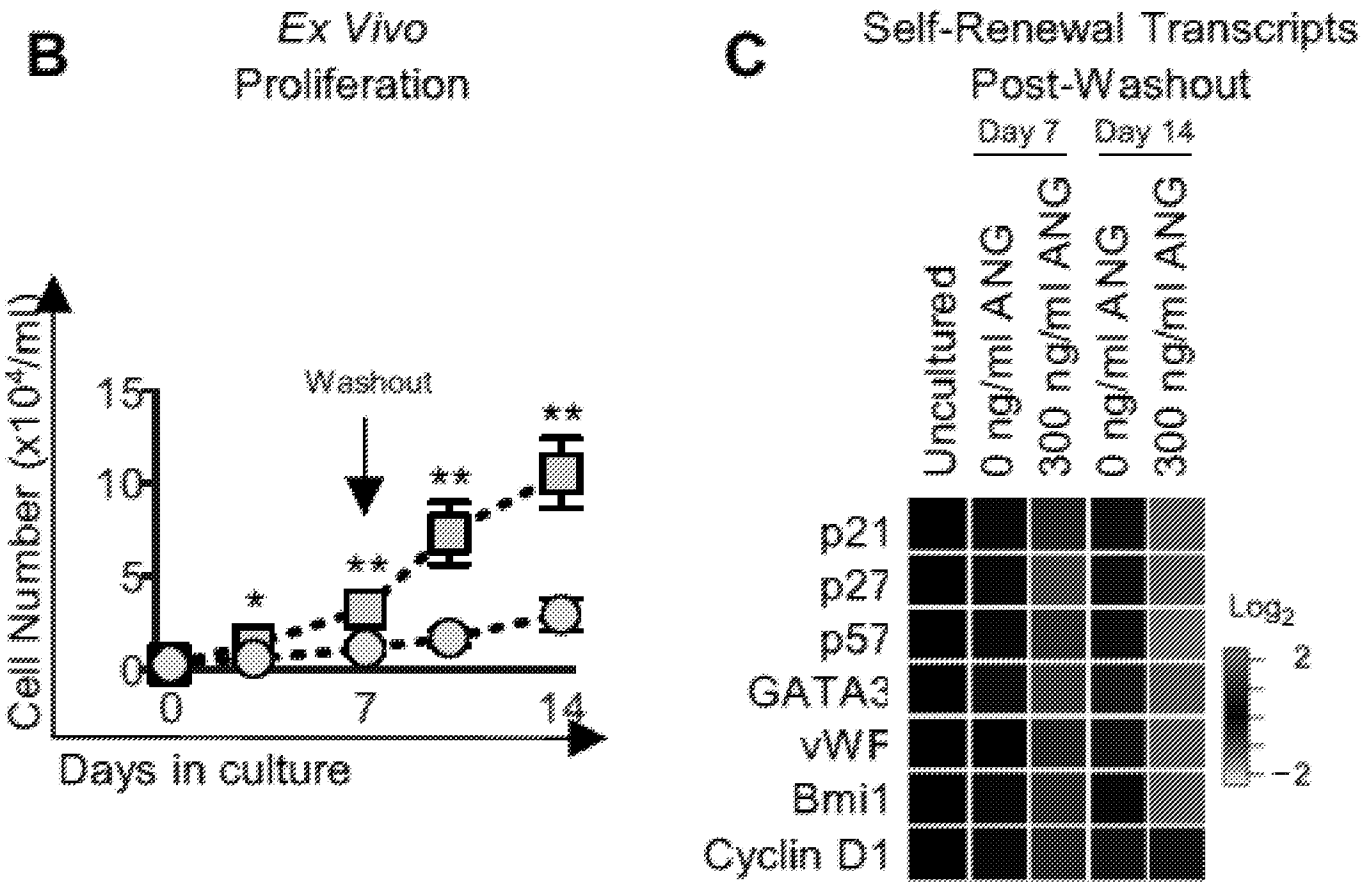
FIG. 24A



Primary Transplant (7-day *ex vivo* culture with ANG)



FIGs. 24B-24C


B
Ex Vivo
Proliferation
Cell Number (x10⁴/ml)
15
10
5
0
Washout
*
**
**
**
0
7
14
Days in culture
C
Self-Renewal Transcripts
Post-Washout
Day 7
Day 14
Uncultured
0 ng/ml ANG
300 ng/ml ANG
0 ng/ml ANG
300 ng/ml ANG
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
2
−2

FIGs. 24D-24E

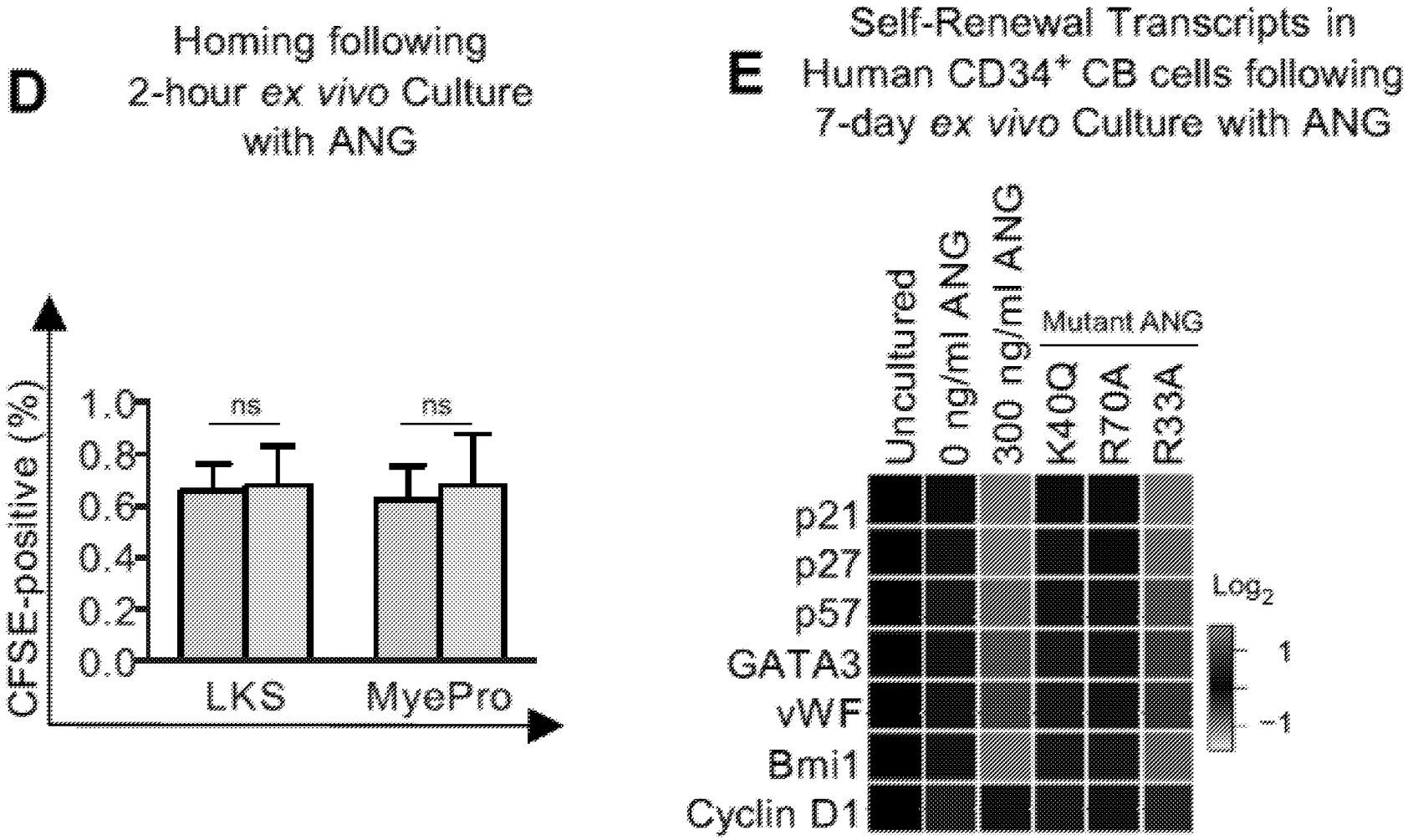

D
Homing following
2-hour ex vivo Culture
with ANG
CFSE-positive (%)
1.0
0.8
0.6
0.4
0.2
0.0
ns
ns
LKS
MyePro
E
Self-Renewal Transcripts in
Human CD34+ CB cells following
7-day ex vivo Culture with ANG
Uncultured
0 ng/ml ANG
300 ng/ml ANG
Mutant ANG
K40Q
R70A
R33A
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
1
−1

FIG. 24F

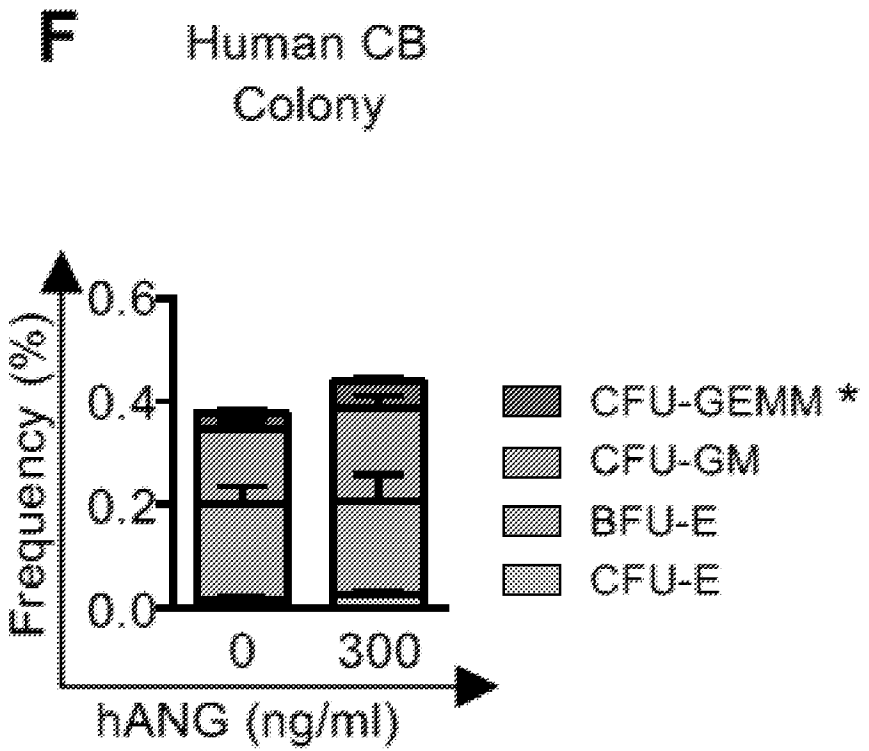

F
Human CB
Colony
Frequency (%)
0.6
0.4
0.2
0.0
0
300
hANG (ng/ml)
CFU-GEMM *
CFU-GM
BFU-E
CFU-E

FIG. 24G

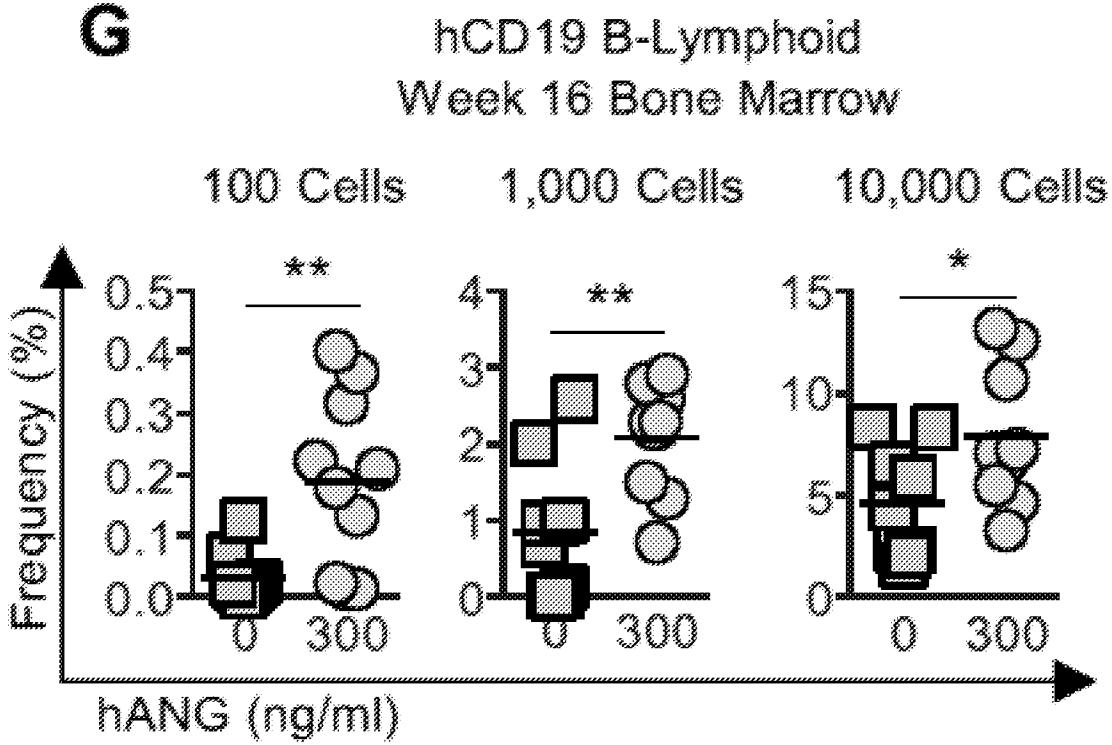

G
hCD19 B-Lymphoid
Week 16 Bone Marrow
100 Cells
1,000 Cells
10,000 Cells
Frequency (%)
0.5
0.4
0.3
0.2
0.1
0.0
4
3
2
1
0
15
10
5
0
**
**
*
0
300
0
300
0
300
hANG (ng/ml)

FIG. 24H

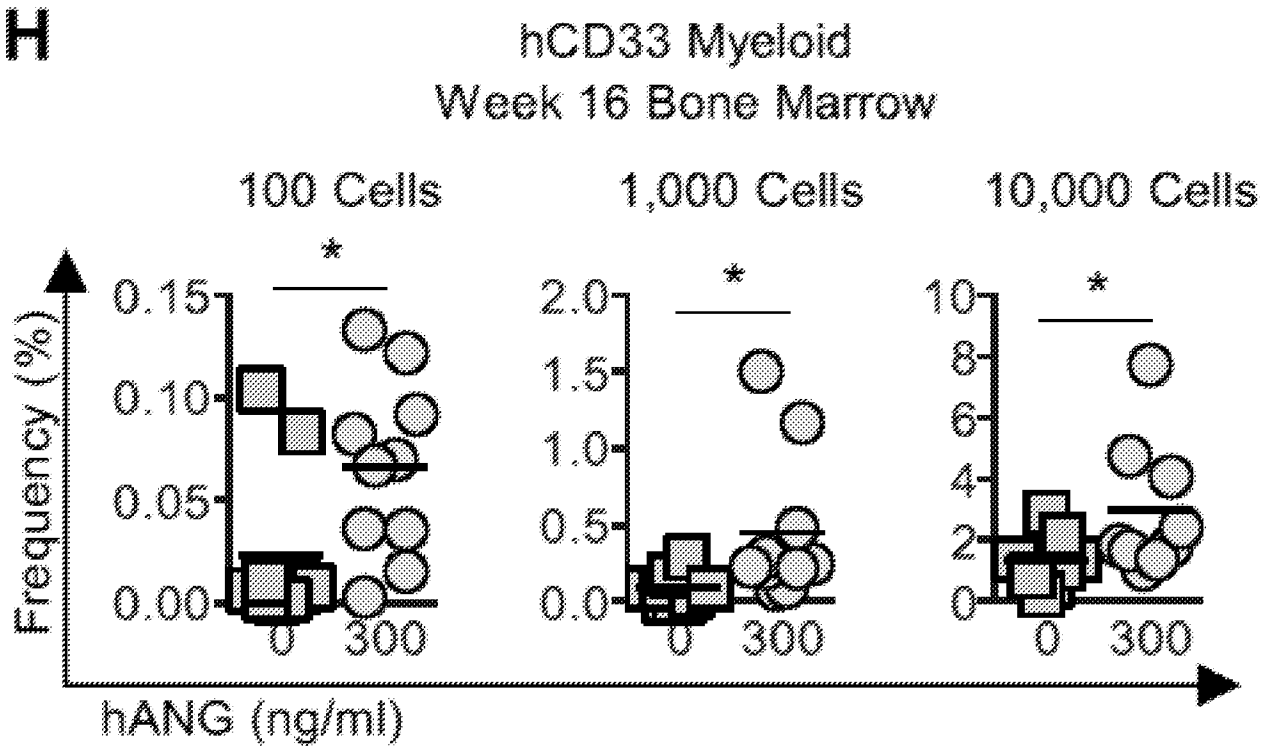

H
hCD33 Myeloid
Week 16 Bone Marrow
100 Cells
1,000 Cells
10,000 Cells
*
*
*
Frequency (%)
0.15
0.10
0.05
0.00
2.0
1.5
1.0
0.5
0.0
10
8
6
4
2
0
0
300
0
300
0
300
hANG (ng/ml)

FIG. 25A

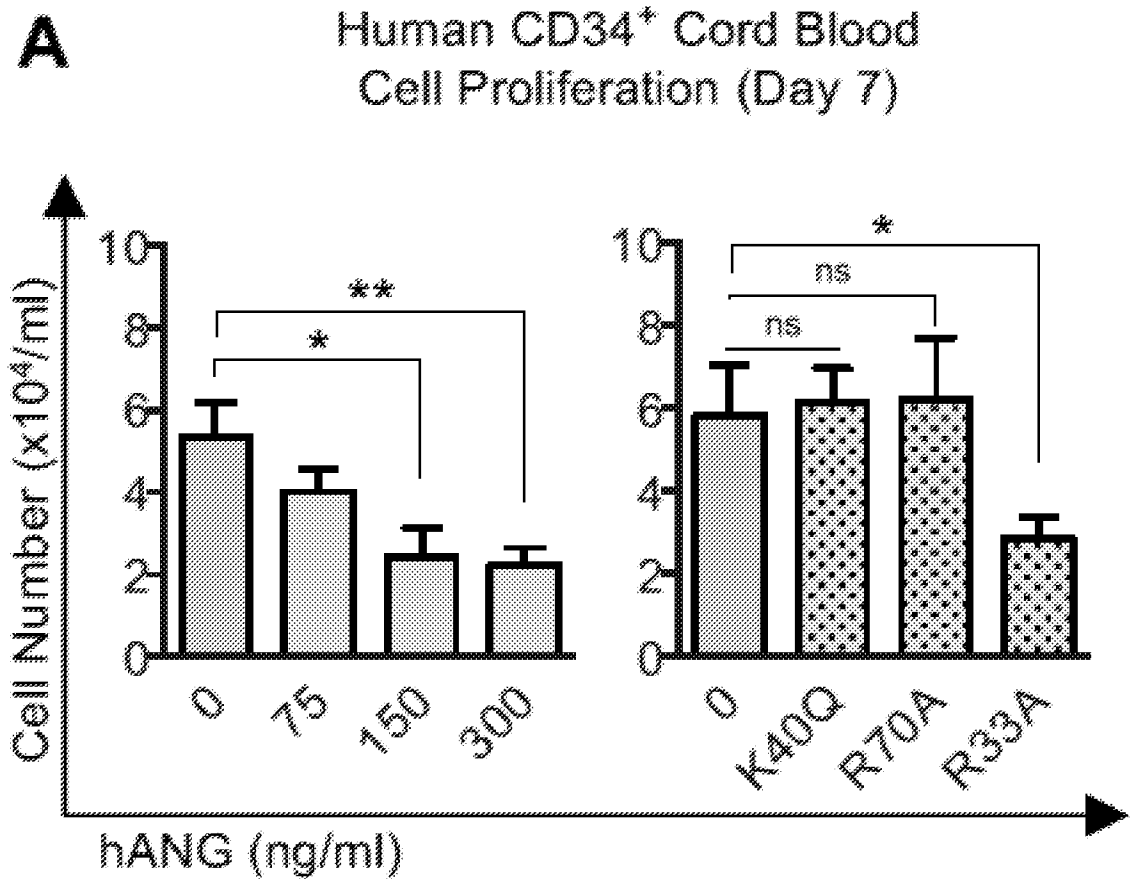

A
Human CD34+ Cord Blood
Cell Proliferation (Day 7)
Cell Number (x10⁴/ml)
10
8
6
4
2
0
**
*
ns
ns
*
0
75
150
300
0
K40Q
R70A
R33A
hANG (ng/ml)

FIG. 25B

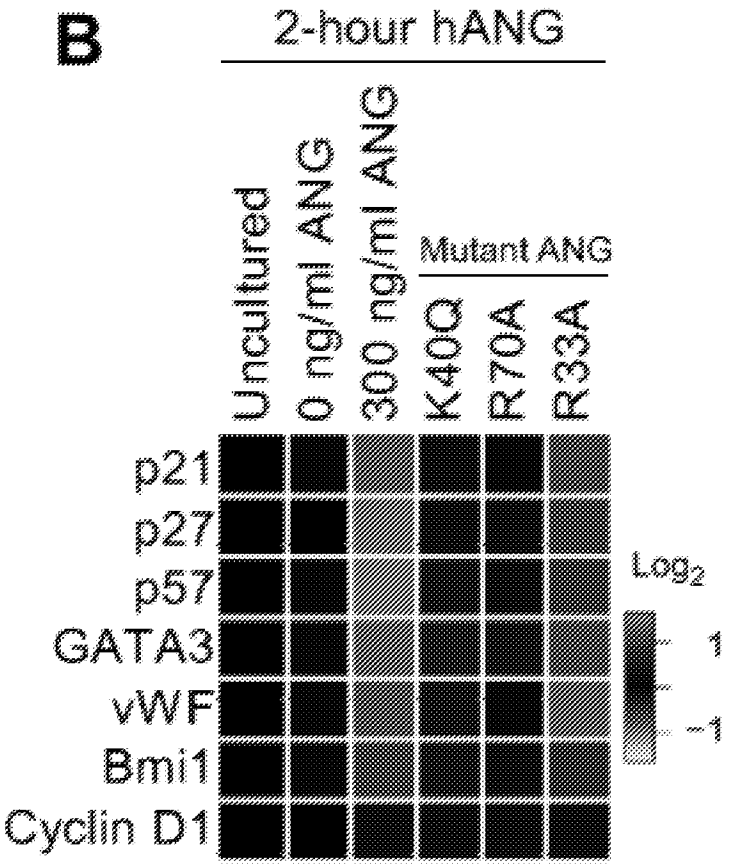

B
2-hour hANG
Uncultured
0 ng/ml ANG
300 ng/ml ANG
Mutant ANG
K40Q
R70A
R33A
p21
p27
p57
GATA3
vWF
Bmi1
Cyclin D1
Log2
1
−1

FIG. 25C

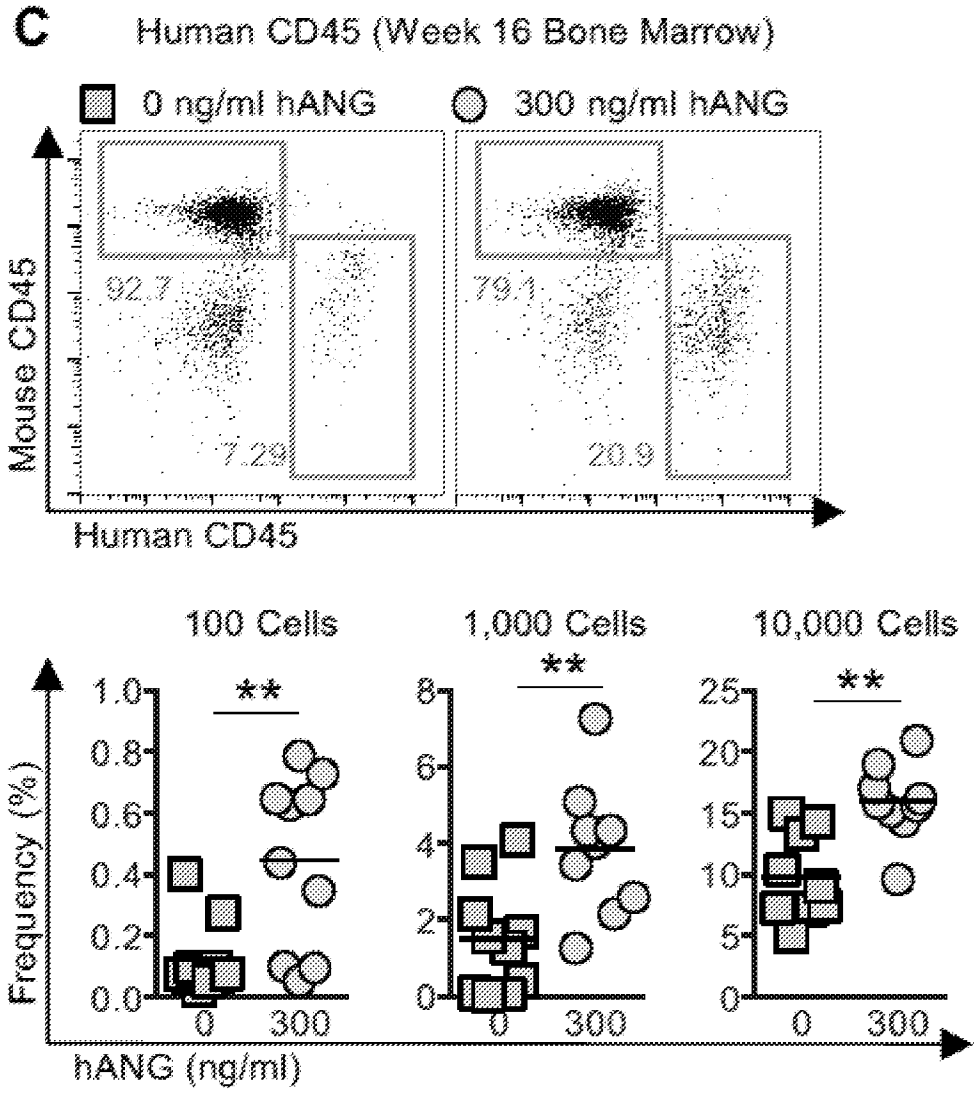
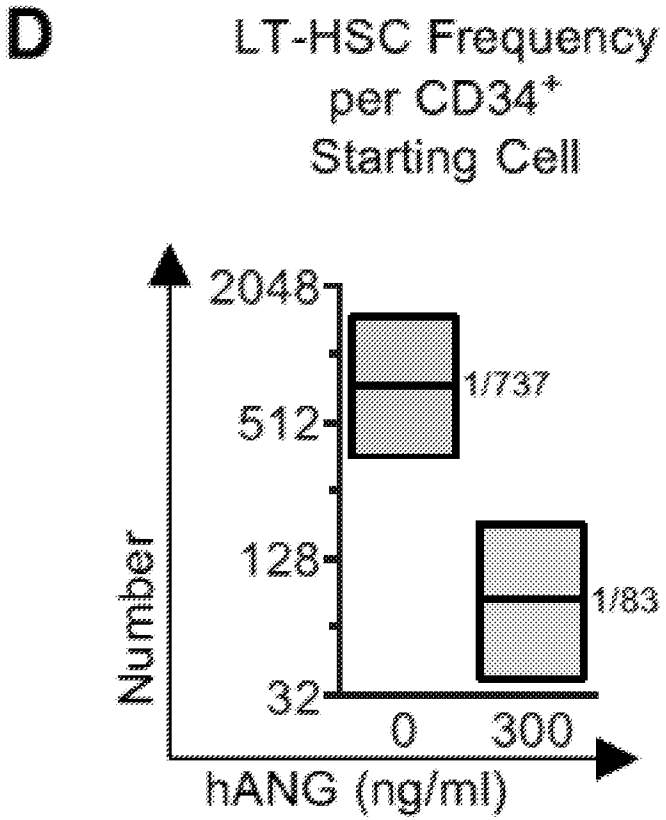

C
Human CD45 (Week 16 Bone Marrow)
0 ng/ml hANG
300 ng/ml hANG
92.7
7.29
79.1
20.9
Mouse CD45
Human CD45
100 Cells
1,000 Cells
10,000 Cells
**
**
**
Frequency (%)
1.0
0.8
0.6
0.4
0.2
0.0
8
6
4
2
0
25
20
15
10
5
0
0
300
0
300
0
300
hANG (ng/ml)

FIG. 25D


D
LT-HSC Frequency
per CD34+
Starting Cell
2048
512
128
32
Number
1/737
1/83
0
300
hANG (ng/ml)

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	TREATMENT WITH ANGIOGENIN TO ENHANCE HEMATOPOIETIC RECONSTITUTION
First Named Inventor/Applicant Name:	David SCADDEN
Filer:	Susanna Clare Benn
Attorney Docket Number:	030258-086191-P2

Filed as Small Entity

Filing Fees for Provisional

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Provisional Application Filing Fee	2005	1	130	130
Pages:				
Provis. Appl Size fee per 50 sheets >100	2085	2	200	400
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**530**

Electronic Acknowledgement Receipt

EFS ID:	25345784
Application Number:	62315281
International Application Number:	
Confirmation Number:	7025
Title of Invention:	TREATMENT WITH ANGIOGENIN TO ENHANCE HEMATOPOIETIC RECONSTITUTION
First Named Inventor/Applicant Name:	David SCADDEN
Customer Number:	50828
Filer:	Susanna Clare Benn/Payal Vyas
Filer Authorized By:	Susanna Clare Benn
Attorney Docket Number:	030258-086191-P2
Receipt Date:	30-MAR-2016
Filing Date:	
Time Stamp:	14:50:37
Application Type:	Provisional

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Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$530
RAM confirmation Number	1309
Deposit Account	500850
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	20160330_EFS_Transmittal_030258-086191-P2.pdf	115091 3a477913d706c7837b8734bc5f9caf9bc18839ea	no	1
Warnings:					
Information:					
2	Provisional Cover Sheet (SB16)	20160330_CoverSheet_030258-086191-P2.pdf	1477474 fc76843d1683af42b7087ed60db08a3c311144e6	no	3
Warnings:					
Information:					
3		20160330_Specification_030258-086191-P2.pdf	671094 cbf612f17344fe93269d49c16c6f9315ed0df1c5	yes	115

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	107
Claims	108	114
Abstract	115	115

Warnings:					
Information:					
4	Drawings-only black and white line drawings	20160330_Figures_030258-086191-P2.pdf	5775088 85b7b7b01f22f0635055e11cc704f2aeae98b50b	no	132
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	32008 698e82d085c657bda781748c71756693f2f2114c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	8070755		

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New International Application Filed with the USPTO as a Receiving Office

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