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Increased Hematopoiesis in Long-term Bone Marrow Cultures and Reduced Irradiation-induced Pulmonary Fibrosis in Von Willebrand Factor Homologous Deletion Recombinant Mice

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Abstract. *Aim: We investigated whether homologous recombinant deletion of the endothelial cell-specific protein Von Willebrand factor (vWF) affected hematopoiesis in long-term bone marrow cultures, and irradiation induction of pulmonary fibrosis/organizing alveolitis. Materials and Methods: We established long-term bone marrow cultures from $vWF^{-/-}$ (C57BL/6 background) and $vWF^{+/+}$ littermate mice. Non-adherent cells removed weekly were tested for formation of multi-lineage hematopoietic stem cells forming colonies at 7 and 14 days in secondary semi-solid medium cultures. Irradiation fibrosis in the lungs of 20-Gy thoracic irradiated mice was quantitated and scored. Results: Hematopoiesis was increased over 20 weeks in $vWF^{-/-}$ compared to $vWF^{+/+}$ cultures in production of non-adherent cells, and cells forming colonies at 7 or 14 days in secondary semi-solid medium culture. The irradiated lungs showed no increased fibrosis. Conclusion: Absence of vWF increases hematopoiesis in long-term bone marrow cultures and has a protective effect in irradiated lungs.*

The endothelial cell-specific protein Von Willebrand factor (vWF) is involved in the intrinsic pathway of blood clotting through its interaction with factor IX and blood platelets (1-3). Furthermore, vWF has been shown to be an adhesion molecule expressed on the surface of endothelial cells during their interaction with pathogenic microbes, as well as in migrating normal and metastasized malignant cells (1, 4).

Recent studies have demonstrated up-regulation of expression of RNA for vWF in the pulmonary endothelial cells of the irradiated C57BL/6J mouse lung (5-7). Stabilization of vWF and other endothelial cell-specific molecules in C57BL/6J mice between the acute radiation pneumonitis phase and late fibrosis phase suggests that stable vWF expression might be a biomarker of continuous oxidative stress of the lung, leading to accumulation of biochemical alterations and the initiation of fibrosis. Migration into the irradiated lungs of bone marrow stromal cell progenitors of the cells forming lung fibrosis, and recent demonstration of their migration through the circulation (5), suggests that pulmonary endothelial cells might be the first target for entry into the lung microenvironment of circulating mesenchymal stem cells, potentially hematopoietic stem cells, and cancer-initiating cells.

A biological organ culture system by which to test the effects of endogenous or exogenous oxidative stress is the mouse long-term bone marrow culture (LTBMC) system (8-10). The absence of intrinsic mediators of oxidative stress such as manganese superoxide dismutase (11) or senescent prone mice with ROS handling defects (12) have been shown to limit hematopoiesis in long-term culture. In contrast, genetic modifications leading to decreased production of ROS-generating proteins, including nitroxide synthase-1 (13), and Mothers Against Decapentaplegic homolog 3 (SMAD3) (14), a mediator of transforming growth factor-β signaling, have been shown to increase hematopoiesis in LTBMC.

The oxidative stress of continuous bone marrow culture is associated with the use of a high oxygen incubator and the continuous production of byproducts of metabolism in the culture medium such that acidosis is detected prior to weekly medium changes. The adherent microenvironment of long-term bone marrow cultures is known to contain fibroblasts (mesenchymal stem cells), endothelial cells, macrophages, and the progenitors of osteoblasts. We sought to determine whether absence of vWF in the adherent layer of long-term cultures adversely affected hematopoiesis. A defective interaction between adherent layer stromal cells and adherent hematopoietic colony-forming cells (recognized as cobblestone

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islands) would suggest a deleterious role of deletion of vWF in long-term culture. In addition lungs of *vWF*$^{-/-}$ mice might not attract circulating bone marrow stromal cells if vWF was missing from their endothelial cells.

Materials and Methods

Mice. C57BL/6J *vWF*$^{-/-}$ and control littermate *vWF*$^{+/+}$ mice were obtained from Denisa D. Wagner, Ph.D., Professor of Pediatrics, Harvard Medical School, Boston, MA, USA. Animals were housed four per cage according to the University of Pittsburgh Institutional Animal Care and Use Committee regulations and fed standard laboratory chow with hyperchlorinated drinking water.

Continuous bone marrow cultures. LTBMCs were established according to published methods (8-10). Briefly the bone marrow of a femur and tibia of an adult C57BL/6J mice were flushed into a T-25 Corning plastic tissue culuture flask, in 12 ml Fisher's medium supplemented with 25% horse serum and 10^{-5} M hydrocortisone hemi-succinate. Cultures were maintained in a high humidity incubator at 37˚C with weekly replacement of 50% of the volume with fresh medium. After week 5, the horse serum was replaced with 25% fetal bovine serum (FBS).

Measurements of hematopoiesis. The adherent layer of LTBMCs was scored for the percentage of the surface area covered by adherent cells (percentage confluence), and the number of flattened adherent colonies of hematopoietic cells *i.e.* 'cobblestone islands' per flask. Half of the non-adherent cells were removed weekly, counted and transferred to semi-solid methylcellulose supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin 3 (IL3), and other growth factors (Stem Cell Technology, Vancouver, BC, Canada), as published elsewhere (10). Subcultures were maintained in a high-humidity incubator for seven days and scored for the number of colonies greater than or equal to 50 cells per colony, then returned to the incubator, and scored again on day 14 for the number of more primitive cells which from colonies at later timepoints according to published methods (15).

Hematopoietic cell colony-forming assays: Non-adherent cells were removed from the LTBMC flasks and suspended at 1×10^6 cells/ml with 5×10^4 cells/dish were plated in triplicate in semi-solid medium consisting of methylcellulose in Iscove's modified Dulbecco's medium (IMDM) 10% FBS, 10% bovine serum albumin (BSA), L-glutamine, 3 U/ml erythropoietin, and 2-mercaptoethanol (Stem Cell Technology, Vancouver, BC, CA). WEHI-3 conditioned medium was added as a source of Il-3 (15). Colony-forming unit granulocyte-macrophage (CFU-GM) of 50 cells or greater were counted on days 7 and 14 after plating. Fresh marrow colonies were subdivided for CFU-GM, burst forming unit erythroid (BFUe) and colony-forming unit granulocyte-erythroid-megakaryocyte-macrophage (CFU-GEMM)

Quantitation of pulmonary fibrosis in lungs of irradiated mice. vWF$^{+/+}$ and vWF$^{-/-}$ mice were irradiated to 20 Gy to the pulmonary cavity with the remaining portion of the body shielded from the irradiation. The mice were sacrificed 200 days after irradiation at which time the lungs were expanded in Optimal Cutting Temperature compound (OCT), excised, frozen in OCT, sectioned and stained with hematoxylin and eosin (H&E) and Masson's Trichrome Stain. The sections were examined microscopically and the percent of the lung exhibiting fibrosis calculated as published elsewhere (15). The percentage of the lung displaying fibrosis was scored in 100 high-powered fields in 10 slides per lobe (five lobes) for each of five vWF$^{+/+}$ and five vWF$^{-/-}$ mice.

Statistical analysis. For LTBMC data, weekly cobblestone island numbers, non-adherent cell numbers ($\times10^5$), percentage confluence of adherent cells, number of day 7 colonies and day 14 colonies at each weekly harvest were counted as described elsewhere (15). Data are summarized as the mean±standard deviation, n is the number of mice used, and *p*-values were calculated with the two-sided two-sample *t*-test comparing vWF$^{+/+}$ to vWF$^{-/-}$ mice. A *p*-value of less than 0.05 was considered as significant. As this was an exploratory study, *p*-values were not adjusted for multiple comparisons (13).

Results

Continuous hematopoiesis in vWF$^{-/-}$ LTBMCs is equivalent to that in cultures from wild-type mice. As shown in Figure 1A, the adherent layer of LTBMCs from *vWF*$^{-/-}$ mice reached confluence and maintained steady confluence for 20 weeks, indistinguishable from that of wild-type culture. These results established that the absence of vWF did not adversely affect the attachment and adherence of bone marrow stromal cells, nor did it result in detectable toxicity such as sloughing of the adherent layer in marrow cultures. Such sloughing has been detected in the presence of toxins in some serum lots used for LTBMCs. As shown in Figure 1B, cobblestone islands were scored weekly, and the numbers were detected at a frequency comparable to that in LTBMCs from wild-type *vWF*$^{+/+}$ mice. Cumulative confluence and production of cobblestone islands was equivalent between genotypes (Figure 1A-1C).

Non-adherent cell production by LTBMCs of *vWF*$^{-/-}$ mice was measured weekly. As shown in Figure 1D, the release of non-adherent cells into the non-adherent layer of marrow cultures was comparable in *vWF*$^{-/-}$ LTBMCs on a weekly basis compared to that detected in wild-type marrow cultures. Fluctuation in cell numbers has been reported with LTBMCs, and the results were consistent with that previously published for C57BL/6J mouse marrow cultures compared to other mouse strains and stocks (10). Cumulative production of non-adherent cells was equivalent between mouse genotypes (Figure 1E).

Colony-forming progenitor cells detected as those forming colonies of 50 or more cells at day 7, and at day 14 (Table II) were compared between LTBMCs of each genotype. As shown in Figure 1F and Table I, weekly and cumulative production (Figure 1G) of non-adherent cells forming colonies at day 7 was increased in *vWF*$^{-/-}$ LTBMCs. The results for cells forming more primitive colonies at day 14 was also similar, showing increases in *vWF*$^{-/-}$ cultures, when

Table I. *Analysis of hematopoiesis in long-term bone marrow cultures (LTBMCs) for Von Willebrand factor (vWF*$^{-/-}$*) mice: Day-7 colony-forming unit granulocyte-erythroid-megakaryocyte-macrophage (CFU-GEMM). The day-7 colony counts at each week are shown, where data are summarized as mean±standard deviation, n is the sample size, and the p-value is for comparison to C57BL/6 group using the two-sided two sample t-test. Significant p-values of less than 0.05 are shown in bold.*

Group	Week 1	Week 2	Week 3	Week 4	Week 5
vWF$^{-/-}$	266.0±11.1 (n=3)	47.3±5.0 (n=3)	118.0±3.0 (n=3)	311.0±13.5 (n=3)	194.0±9.2 (n=3)
C57BL/6	245.3±10.1 (n=3)	98.0±6.0 (n=3)	107.3±5.5 (n=3)	176.7±10.1 (n=3)	157.7±14.0 (n=3)
p-Value	0.0756	**0.0004**	**0.0421**	**0.0002**	**0.0199**
Group	Week 6	Week 7	Week 8	Week 9	Week 10
vWF$^{-/-}$	100.7±6.5 (n=3)	48.7±7.0 (n=3)	130.0±8.0 (n=3)	111.3±7.0 (n=3)	57.3±5.5 (n=3)
C57BL/6	116.0±11.1 (n=3)	39.0±6.0 (n=3)	79.7±7.1 (n=3)	66.0±6.0 (n=3)	59.7±7.5 (n=3)
p-Value	0.1085	0.1441	**0.0012**	**0.0011**	0.6866
Group	Week 11	Week 12	Week 13	Week14	Week 15
vWF$^{-/-}$	105.3±4.5 (n=3)	115.7±8.5 (n=3)	92.7±6.5 (n=3)	109.0±5.0 (n=3)	33.7±3.5 (n=3)
C57BL/6	79.3±4.5 (n=3)	96.7±5.0 (n=3)	76.3±12.0 (n=3)	29.7±4.0 (n=3)	17.0±3.0 (n=3)
p-Value	**0.0021**	**0.0291**	0.1072	**<0.0001**	**0.0033**
Group	Week 16	Week 17	Week 18	Week 19	Week 20
vWF$^{-/-}$	40.7±4.5 (n=3)	36.0±4.0 (n=3)	14.0±2.0 (n=3)	47.7±6.0 (n=3)	0.0±0.0 (n=3)
C57BL/6	29.3±4.2 (n=3)	23.0±5.0 (n=3)	6.7±3.1 (n=3)	2.7±2.5 (n=3)	0.0±0.0 (n=3)
p-Value	**0.0329**	**0.0245**	**0.0254**	**0.0003**	1.0000

Table II. *Analysis of hematopoiesis in long-term bone marrow cultures (LTBMCs) from Von Willebrand factor (vWF*$^{-/-}$*) mice: Day-14 colony-forming unit granulocyte-erythroid-megakaryocyte-macrophage (CFU-GEMM). The day-14 colony counts at each week are shown, where data are summarized as mean±standard deviation, n is the sample size, and p-value is for comparison to C57BL/6 group using the two-sided two sample t-test. Significant p-values less than 0.05 are shown in bold.*

Group	Week 1	Week 2	Week 3	Week 4	Week 5
vWF$^{-/-}$	399.3±14.0 (n=3)	482.7±15.6 (n=3)	245.0±12.1 (n=3)	483.3±22.6 (n=3)	547.7±25.5 (n=3)
C57BL/6	374.7±10.1 (n=3)	377.7±13.5 (n=3)	194.3±8.0 (n=3)	274.7±9.7 (n=3)	394.0±19.1 (n=3)
p-Value	0.0688	**0.0009**	**0.0038**	**0.0001**	**0.0011**
Group	Week 6	Week 7	Week 8	Week 9	Week 10
vWF$^{-/-}$	367.7±9.1 (n=3)	314.7±11.4 (n=3)	402.7±16.0 (n=3)	364.7±12.2 (n=3)	275.0±9.0 (n=3)
C57BL/6	271.0±14.1 (n=3)	190.7±7.5 (n=3)	274.7±13.5 (n=3)	184.0±12.0 (n=3)	164.7±6.0 (n=3)
p-Value	**0.0006**	**0.0001**	**0.0005**	**0.0001**	**0.0001**
Group	Week 11	Week 12	Week 13	Week14	Week 15
vWF$^{-/-}$	427.0±9.0 (n=3)	335.3±7.0 (n=3)	278.3±12.5 (n=3)	321.7±6.7 (n=3)	203.3±9.5 (n=3)
C57BL/6	198.7±9.5 (n=3)	146.7±8.0 (n=3)	128.3±8.1 (n=3)	54.0±5.6 (n=3)	52.7±6.0 (n=3)
p-Value	**<0.0001**	**<0.0001**	**0.0001**	**<0.0001**	**<0.0001**
Group	Week 16	Week 17	Week 18	Week 19	Week 20
vWF$^{-/-}$	220.3±6.5 (n=3)	211.7±9.1 (n=3)	149.7±7.1 (n=3)	255.7±14.6 (n=3)	68.0±6.6 (n=3)
C57BL/6	133.3±12.0 (n=3)	109.7±4.0 (n=3)	25.7±5.5 (n=3)	57.0±7.5 (n=3)	11.7±2.1 (n=3)
p-Value	**0.0004**	**0.0001**	**<0.0001**	**<0.0001**	**0.0001**

scoring for weekly (Figure 1H and Table II) and cumulative production (Figure 1I). These results established that multiple parameters of hematopoiesis were not adversely affected with respect to longevity of *in vitro* LBTMCs by absence of the vWF protein.

The present results establish that homologous recombinant deletion of the gene for vWF, which would result in lack of production of vWF, does not adversely affect continuous hematopoiesis in LBTMCs.

Radiation fibrosis is reduced in lungs of vWF$^{-/-}$ *mice irradiated with 20 Gy to the thorax*. Mice were irradiated to the thoracic cavity as published elsewhere (6) and subgroups sacrificed at day 200 following irradiation. The percentage lung replaced by fibrosis in vWF$^{-/-}$ was compared to that in *vWF*$^{+/+}$ mice. The results showed no evidence of fibrosis in *vWF*$^{-/-}$ mouse lungs. Representative photographs of fibrotic areas in the lungs of two unirradiated and two irradiated *vWF*$^{-/-}$ mice at day 200 are

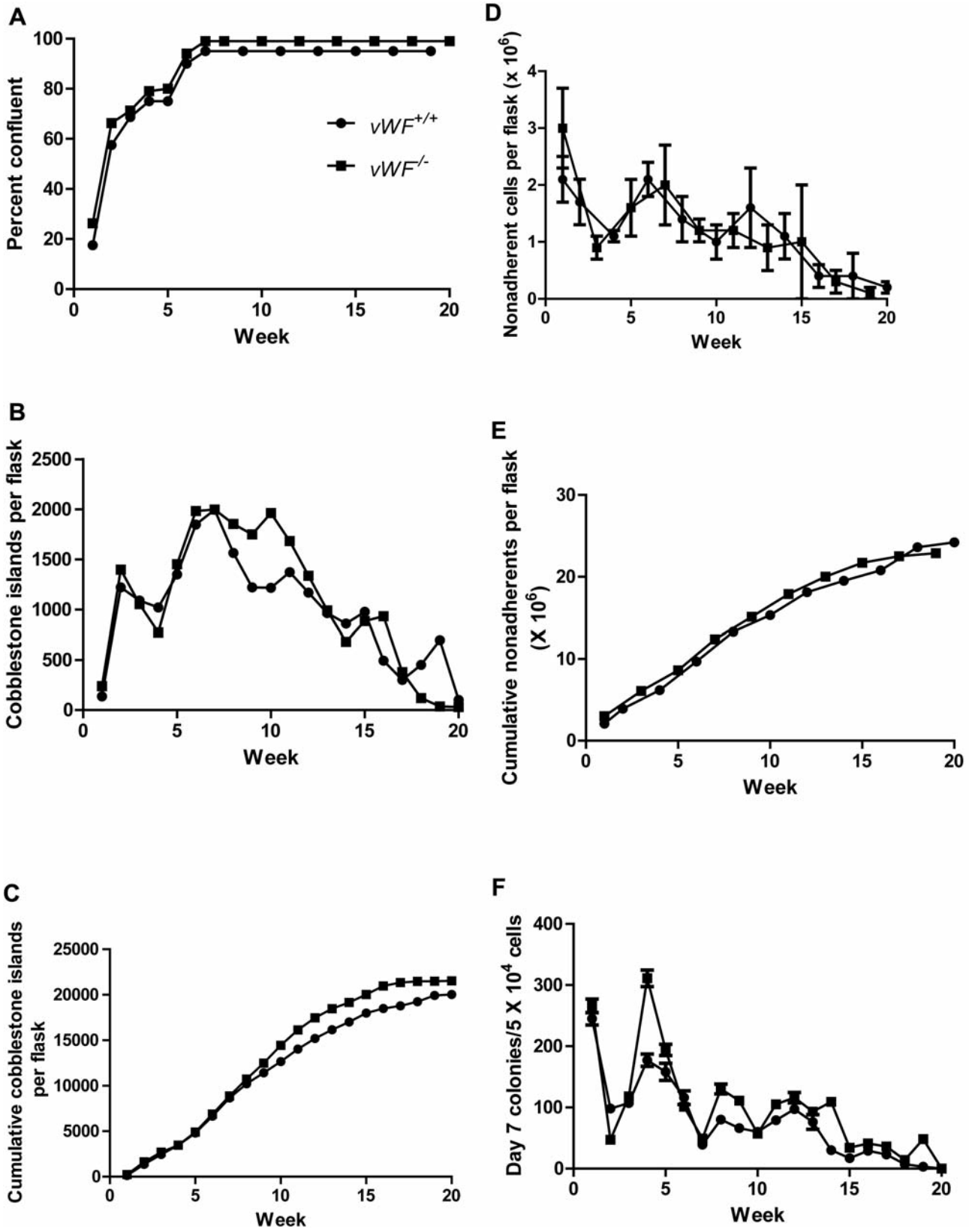

Figure 1. *Continued*

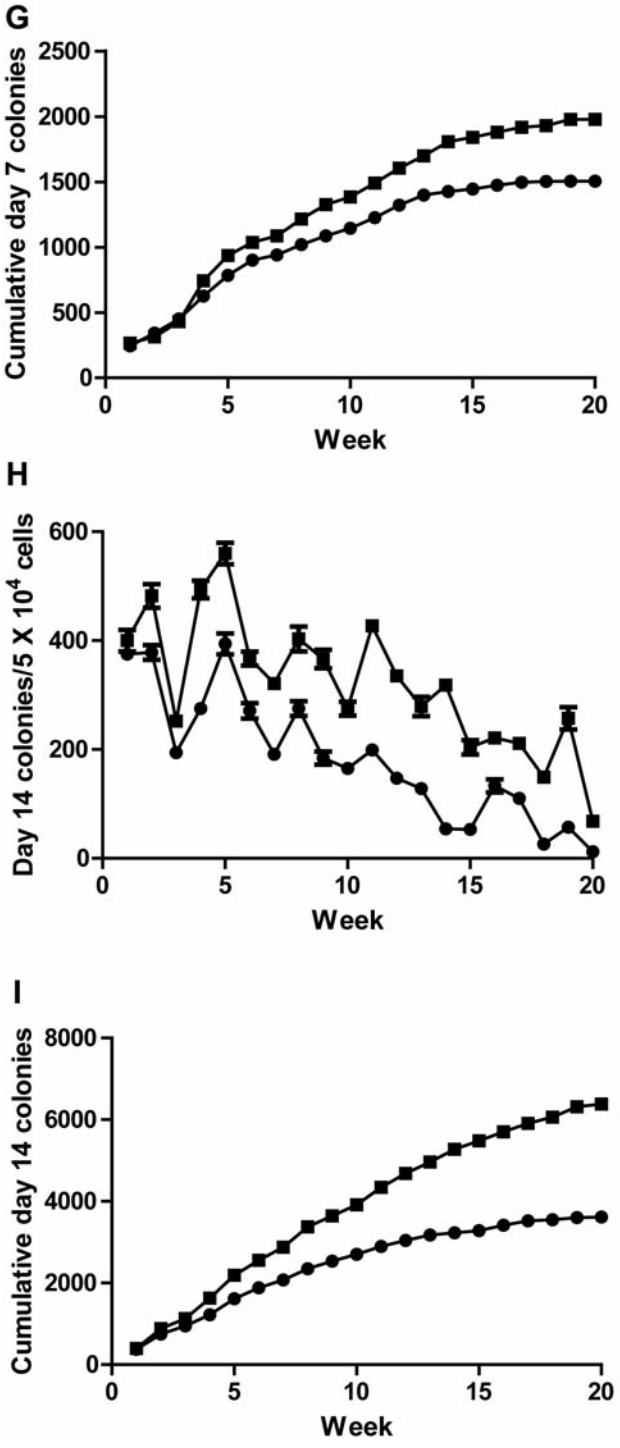

Figure 1. *Long-term marrow cultures from Von Willebrand Factor (vWF*$^{-/-}$*) compared to vWF*$^{+/+}$ *mice: A: percentage confluence; B: cobblestone islands produced; C: cumulative cobblestone islands; D: weekly non-adherent cells produced; E: cumulative nonadherent cells produced; F: weekly day-7 colony-forming unit granulocyte-erythroid-megakaryocyte-macrophage (CFU-GEMM); G: cumulative day-7 CFU-GEMM; H: weekly day-14 CFU-GEMM; I: cumulative day-14 CFU-GEMM.*

shown in Figure 2. These data are presented in quantitative form in Figure 3.

Discussion

The present results establish that homologous recombinant deletion of vWF does not adversely affect hematopoiesis in long-term culture and increases several parameters of robustness of hematopoiesis. Several parameters reflecting the stability of the hematopoietic microenvironment were shown to be indistinguishable in cultures from *vWF*$^{-/-}$ compared to wild-type mice. These included confluence of the adherent layer and maintenance of cobblestone islands; however, longevity of the production of non-adherent cells was not increased in LTBMCs from *vWF*$^{-/-}$ mouse marrow. These data establish that absence of vWF, a major adhesion molecule of the hematopoietic microenvironment, specifically in endothelial cells, does not adversely affect the interaction between hematopoietic stem cells and the bone marrow microenvironment *in vitro*.

Thoracic irradiation of C57BL/6J mice produces radiation-induced lung fibrosis after a radiation pneumonitis phase and latent period during which vWF and other endothelial-specific gene transcripts have been shown to be elevated (6). In contrast, C3H/HeNHsd mice, which do not develop radiation-induced fibrosis showed continuous maintenance of transcripts for acute radiation pneumonitis and no specific down-regulation or latent period (7). Endothelial cells have been shown to respond *in vitro* and *in vivo* to irradiation by up-regulation of vWF (1-3). The endothelial cell is known to be critically-involved in the pathophysiology of radiation damage to multiple organs, including the intestine (16, 17), lung (4-6), and central nervous system. A common component of endothelial responses to irradiation has been shown to be up-regulation of gene transcripts associated with the stress response, as well as those associated with adhesion of circulating inflammatory cells (4, 18-21).

In the present studies, we utilized a culture system in which interaction and attachment of hematopoietic cells with stromal cells can be studied for months *in vitro*. Interaction of hematopoietic and stromal cells is known to be critical for hematopoiesis. Long-term marrow culture from mice with defects in management of oxidative stress found in a high oxygen incubator has been shown to reduce the longevity of hematopoiesis (11). Similarly, genetic defects associated with senescence (12), and cultures from other mice with deletion of negative regulatory critical components 14) have been shown to modulate the longevity of hematopoiesis. Finally, intrinsic genetic variation between mouse strains has been shown to increase longevity of hematopoiesis (10).

The absence of a deleterious effect of homologous deletion of vWF with respect to hematopoiesis in LBTMCs was compared to the effect of this *vWF*$^{-/-}$ genotype on induction

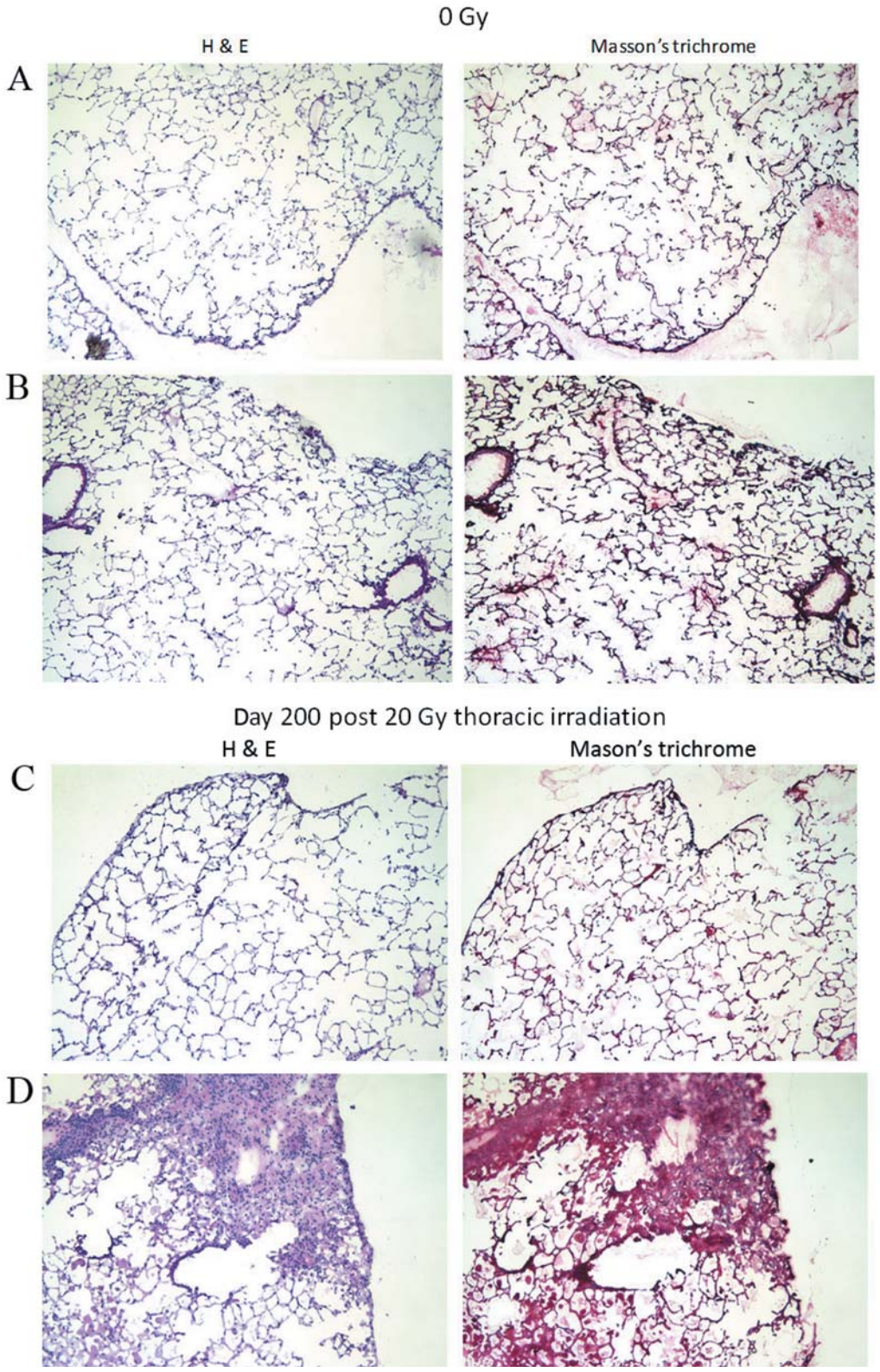

Figure 2. *Histopathological analysis of lungs from Von Willebrand Factor ($vWF^{-/-}$) mice irradiated with 20 Gy to the thorax. Histopathology of lungs removed at 200 days after 20 Gy thoracic irradiation of $vWF^{-/-}$ mice (×100). A-D: Hematoxylin and eosin (H&E) staining on the left, and Masson's trichrome stains for collagen on the right for each of four mice. Sections from two nonirradiated $vWF^{-/-}$mice are shown in A and B, while sections from two 20 Gy irradiated $vWF^{-/-}$mice are shown in C and D. Inflammatory cell infiltrates can be seen on the right in D. No mouse lung exhibited collagen deposition.*

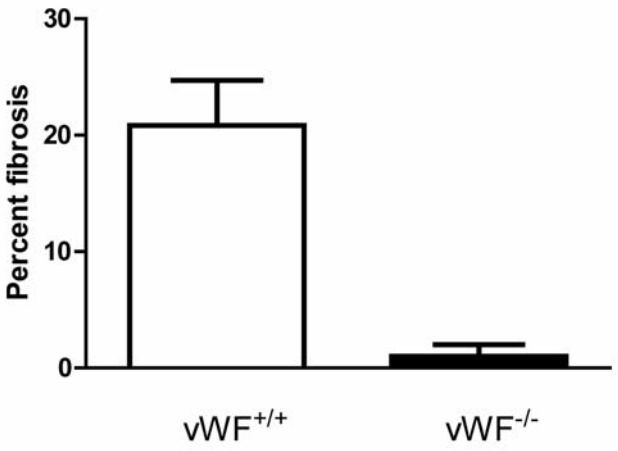

Figure 3. *Quantitation of pulmonary fibrosis in the lungs of Von Willebrand Factor (vWF*$^{-/-}$*) and wild-type mice irradiated with 20 Gy to the thorax. vWF*$^{+/+}$ *and vWF*$^{-/-}$ *mice were irradiated to 20 Gy to the pulmonary cavity. The mice were sacrificed 200 days after irradiation. The lungs were removed, frozen in Optimal Cutting Temperature compound, sectioned, stained with hematoxylin and eosin, and the percentage fibrosis determined in 100 high-powered fields examined in each of 10 slides per lung lobe (five lobes per mouse), five mice per group.*

of radiation fibrosis in C57BL/6J mice. The results demonstrated no evidence of fibrosis or collagen deposition in lungs after irradiation of *vWF*$^{-/-}$ C57BL/6J with 20 Gy to the thorax. Distinct foci of increased number of macrophages were found throughout the lung tissues of both C57BL/6J controls and *vWF*$^{-/-}$ mice. Increased numbers of heterogenous populations of other inflammatory cells, including granulocytes and lymphocytes, were present throughout the lungs of both strains. Further studies are required to determine the effect of the lack of the vWF gene product on the function of other organ systems *in vivo* and *in vitro*.

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