Pharmacological targeting of the thrombomodulin–protein C pathway mitigates radiation toxicity

Hartmut Geiger[1,2,*], **Snehalata A Pawar**[3,*], **Edward J. Kerschen**[4,*], **Kalpana J. Nattamai**[1,*], **Irene Hernandez**[4], **Hai Po Liang**[4], **Jose Á Fernández**[5], **Jose A. Cancelas**[1], **Marnie A. Ryan**[1], **Olga Kustikova**[6], **Axel Schambach**[6], **Qiang Fu**[3], **Junru Wang**[3], **Louis M. Fink**[7], **Karl-Uwe**

Correspondence: Hartmut Geiger, Division of Experimental Hematology, Department of Pediatrics Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, OH 45229, hartmut.geiger@cchmc.org, Phone: 513-636-1338 or +49 731 500 57650; Fax: 513-636-3768 or +49 731 500 57652.

*equal contribution

AUTHOR CONTRIBUTION

H. Geiger, designed and performed experiments, wrote the paper

S.A. Pawar, collected, analyzed, and summarized data

E.J. Kerschen, performed experiments

K. Nattamai, performed experiments, wrote parts of the paper

I. Hernandez, performed experiments

H.P. Liang, performed experiments

J. A. Fernandez, provided reagents and advised on experimental design

J.A. Cancelas, performed experiments

M.A. Ryan, performed experiments

O. Kustikova, advised on experimental design and provided experimental expertise

A. Schambach, advised on experimental design and provided reagents

Q. Fu, performed in vivo studies

J. Wang, performed in vitro studies

L.M. Fink, advised on experimental design and participated in writing the manuscript

K-U. Petersen, performed PK studies with solulin and advised on experimental details

D. Zhou, advised on experimental design and provided experimental expertise

J. H. Griffin, provided reagents and advised on experimental design

C. Baum, advised on experimental design and provided experimental expertise

H. Weiler, designed and performed experiments, wrote the paper

M. Hauer-Jensen, designed experiments, wrote the paper

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Petersen[8], **Daohong Zhou**[3], **John H. Griffin**[5], **Christopher Baum**[6], **Hartmut Weiler**[4,*], and **Martin Hauer-Jensen**[3,9,*]

[1]Division of Experimental Hematology and Cancer Biology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH, USA

[2]Department of Dermatology and Allergic Diseases, University of Ulm, Ulm, Germany

[3]Division of Radiation Health, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, AR, USA

[4]Blood Research Institute, Medical College of Wisconsin, Milwaukee, WI, USA

[5]Scripps Research Institute, La Jolla, CA, USA

[6]Department of Experimental Hematology, Hannover Medical School, Hannover, Germany

[7]Desert Research Institute, Las Vegas, NV, USA

[8]PAION Deutschland GmbH, Aachen, Germany

[9]Surgical Service, Central Arkansas Veterans Healthcare System, Little Rock, AR, USA

INTRODUCTORY PARAGRAPH

Tissue damage induced by ionizing radiation in hematopoietic and gastrointestinal/epithelial systems is the major cause of lethality in radiological emergency scenarios, and underlies deleterious side effects in patients undergoing radiation therapy [1,2]. Identifications of target-specific interventions that confer radio-mitigating activity are largely unmet challenges. Here we identify the thrombomodulin (Thbd)–activated protein C (aPC) pathway as a novel mechanism for mitigation of total body irradiation (TBI)-induced mortality. Therapeutic administration of recombinant, soluble Thbd or aPC to lethally irradiated wild type mice resulted in accelerated recovery of hematopoietic progenitor activity in bone marrow and in the mitigation of lethal TBI. Starting infusion of aPC as late as 24 hours after exposure to radiation was still sufficient for mitigating radiation-induced mortality. Our data also demonstrates a previously unrecognized role of the endogenous Thbd-aPC pathway in radiation mitigation. These findings suggest that pharmacologic augmentation of the protein C pathway activity by recombinant Thbd or aPC might offer a rational approach towards the mitigation of tissue injury and lethality caused by ionizing radiation.

RESULTS/DISCUSSION

Total body irradiation (TBI) is associated with dysfunction of radiosensitive organs [2–5]. To identify novel genes and pathways protecting hematopoietic stem and progenitor cells (HSPCs) against radiation injury we performed retroviral insertional mutagenesis screens with a replication deficient virus bearing a strong internal promoter expressing enhanced green fluorescent protein (EGFP) [6] (Supplementary Fig. 1a). At week 4, 7 and 10 following BM transfer, recipients were exposed to a single dose of 3 Gy TBI, resulting in three consecutive cycles of radiation-induced contraction and subsequent re-expansion of the hematopoietic system. Viral integration sites in genomic DNA in BM cells from animals in which post-transplant TBI had resulted in a significantly augmented relative abundance of

EGFP-positive cells in PB or BM were determined by ligation mediated (LM)-PCR [6] (Supplementary Fig. 1b-e, Supplementary file 1). Loci targeted by integration included genes known to play a role in radioprotection of either hematopoietic or neuronal cells [2,7,8], such as *PUMA* (Supplementary Fig. 2b-d) and *c-Jun* (data not shown). In animal 9 (Fig. 1a and Supplementary Fig. 1b) LM-PCR revealed integration of the virus 31.6 kb upstream of the Thrombomodulin (*Thbd*) gene (Fig. 1b), which was associated with increased abundance of endogenous Thbd transcript and protein in radio-selected EGFP-positive cells, while the integration had little effect on expression of other neighboring genes like the somatostatin receptor 4 (*SSTR4*) or the C-type lectin transmembrane receptor *CD93* (Fig. 1c, d).

To ascertain whether augmented Thbd expression in HSPCs was sufficient for conferring a competitive selection advantage to hematopoietic cells in response to TBI, HSPCs were transduced with lentiviral Thbd-expression constructs, and Thbd over-expressing cells were subsequently transplanted into pre-conditioned C57BL/6-CD45.1 recipients (Fig. 1e and Supplementary Fig. 3), followed by one 3 Gy TBI administered 4 weeks post-transplant and analysis of EGFP chimerism in PB at 3 weeks post-TBI. Cells over-expressing Thbd were 1.5-fold enriched in PB as compared to vector-only controls (Fig. 1f,g), demonstrating that elevated expression of Thbd in hematopoietic cells was sufficient to confer a selective advantage after radiation injury. However, Thbd over-expressing HSPCs were not protected from the effects of ionizing radiation *in vitro*, as determined by survival, apoptosis, and proliferation of progenitor cells in response to irradiation (Supplementary Fig. 4), indicating that the beneficial effects of Thbd on HSPCs *in vivo* required additional cells or molecules.

Endogenous Thbd is a multifunctional cell surface-associated receptor that regulates the activities of several physiological protease systems, including complement, fibrinolysis, and blood coagulation [9]. Biochemically, Thbd functions as a high-affinity receptor for thrombin. The Thbd/thrombin complex activates thrombin activatable fibrinolysis inhibitor (TAFI) and also converts the plasma zymogen protein C (PC) into the natural anticoagulant, activated protein C (aPC)[10–12]. aPC inhibits blood coagulation via proteolysis of blood coagulation factors V and VIII, promotes indirectly the activity of the fibrinolytic system and exerts potent anti-inflammatory and cytoprotective effects on endothelial cells, neurons and various innate immune cell populations [13] that are mediated through the interaction of aPC with signaling-competent receptors, such as Par1, Par2, and Par3, integrins, and the endothelial protein C receptor (Epcr)[13,14].

As the beneficial effects of Thbd *in vivo* could not be attributed to functions of Thbd intrinsic to HPCs, we hypothesized that extrinsically and thus systemically administered Thbd might promote systemic beneficial effects in response to radiation injury. Administration of recombinant soluble forms of THBD to baboons and humans is safe and exhibits anticoagulant and antithrombotic activities[15–17].

Administration of an oxidation-resistant form of soluble, recombinant human THBD (solulin, INN sothrombomodulin alpha, Supplementary Fig. 5) up to 30 minutes post-TBI at 8.5 or 9.5 Gy resulted in significant radioprotection of wild type mice, compared to vehicle-treated controls, with a 40%-80% survival benefit (Fig. 2a,b). Solulin has been shown to serve as the cofactor for conversion of the plasma zymogen protein C (PC) into the natural

anticoagulant, activated protein C (aPC) [10,16,18]. To determine whether the protective effects of soluble THBD could be related to the activation of protein C, we investigated whether infusion of recombinant aPC could reproduce the radio-protective effect of soluble THBD. In independent experiments conducted in three different laboratories, administration of recombinant murine aPC to C57BL/6 mice (at 5 μg/mouse i.v., equal to 0.4 mg kg^{-1}) conferred a significant survival benefit compared to vehicle-treated controls (Fig. 2c,d). Similar data were obtained with genetically distinct CD2F1 mice (at 0.35 mg kg^{-1} i.v., 30 minutes post-TBI, data not shown), indicating that the aPC effect was not dependent on genetic background. At 10 Gy, still 40% of animals survived after multiple injections of aPC (at 30 minutes, 1 hour, and 2 hours post-TBI) (Fig. 2d). Remarkably, even when the first injection of aPC was delayed until 24 hours post-TBI, with a second injection at 48 hours post-TBI, significant radiation mitigation was observed (Fig. 2e,f).

Given that the radiation doses used in our experiments result in death occurring 12–20 days post radiation exposure primarily due to failure of hematopoietic cells within the bone marrow, likely candidate cellular targets of solulin as well as aPC in radiation mitigation include epithelial and/or endothelial structures of the gut or bone marrow or bone marrow hematopoietic cells. Administration of solulin or aPC to lethally irradiated mice had no detectable effect on basic blood cell parameters at day 3 or 10 after radiation exposure (Supplementary Fig. 6a and data not shown), except for marginally elevated numbers of white blood cells in BM at day 10 in aPC treated animals (Fig. 3a,b). Hematopoietic progenitor cells in BM, determined by flow cytometry (Lin$^-$, c-Kit$^+$ cells, Fig. 3c) or functionally via CFU-C assays (Fig. 3d), were almost undetectable at 3 days post irradiation (data not shown), but at 10 days the were significantly increased in aPC-treated mice compared to controls. The frequency of animals presenting with more than 10 CFU-C colonies per 5×10^4 cells in BM at day 10 post TBI was likewise significantly higher in the aPC-treated group (data not shown), and correlated with the frequencies of aPC-treated animals surviving exposure to lethal radiation doses (see Fig. 2d,f). Similar observations were made in lethally irradiated mice receiving solulin (Fig. 3e,f), consistent with the notion that activation of protein C constitutes a relevant downstream effector of soluble THBD. Infusion of aPC did not alter biomarkers of radiation-induced gut injury, such as plasma citrulline levels and integrity of the gut epithelial surface (Supplementary Fig. 6c,d,e), indicating that mitigation of radiation damage to the intestine was unlikely to significantly contribute to the efficacy of aPC.

To gain more insight on molecular mechanisms of aPC action, we compared distinct recombinant variants of aPC with respect to their radio-mitigating activity. The mouse 5A-aPC variant, which exhibits full Epcr- and Par1-mediated cytoprotective function, but only residual (~8%) anticoagulant activity [19,20], did not prevent radiation-induced mortality. In contrast, infusion of E149A-aPC with augmented anticoagulant activity but with deficient signaling activities, (e.g., only ~5% of normal anti-apoptotic activity) [21] conferred a significant survival benefit that was comparable to that of wild type aPC (Fig. 3g). The biological activity of aPC that mediates radiomitigation is thus preserved in the E149A-aPC variant, but compromised in the 5A-aPC variant.

Both Par1 and Epcr are expressed on primitive BM cells [22–25]. HSPC lacking Par1 [26] or expressing greatly diminished levels of Epcr [27] were not impaired but rather slightly favored compared to wild type controls in competitive transplantation/radiation-injury experiments (Supplementary Fig. 7a,b,c), providing additional support that the radio-mitigation activity of aPC does not involve Epcr and Par1-dependent signaling on HSPCs which is consistent with the failure of the signaling-selective 5A-aPC variant to afford radio-protection.

Functions preserved by the E149A-aPC variant, but deficient in the 5A-aPC molecule potentially include the anticoagulant effect of aPC and potentially coagulation-independent aPC effects, such as the degradation of cytotoxic histone-DNA complexes released from damaged cells [28]. However, inhibition of cytotoxic histones 3 and 4 with the function-blocking BWA3 antibody, using conditions shown previously to reduce mortality in sepsis [28,29], did not result in radioprotection (Fig. 3h). Similarly, inhibition of the intrinsic coagulation pathway with anti-fXI antibody 14E11 [30] (Fig. 3i) or with low molecular weight heparin were both ineffective in radioprotection. Biomarkers indicative of the activation state of the blood coagulation system in PB (plasma thrombin-antithrombin complexes and fibrin D-dimer) were unaltered over a 24h time window following exposure to lethal radiation doses (Supplementary Fig. 8), suggesting that the radioprotective effect of the E149A-aPC variant is not due to its antithrombotic actions. While it is possible that optimized dosing with antibodies blocking thrombosis or histone-induced inflammation might reveal some beneficial effect of these reagents on radiation injury, it seems unlikely that the pathological mechanism inhibited by these antibodies are the critical targets of aPC that mediate the accelerated recovery of HPC activity and survival of radiation injury. The targets of aPC mediating accelerated recovery of HPC activity and survival of radiation injury therefore remain presently unknown and likely involve novel functions associated with wild type aPC and the E149A-aPC variant [20,31]

The above observations raised the question whether the endogenous Thbd-PC pathway might play a previously unrecognized role in mitigating the lethal consequences of radiation-induced bone marrow failure. In adult mice and humans, Thbd is expressed ubiquitously in endothelial cells of small blood vessels except for low levels in certain brain microvascular beds[32]. Within the human hematopoietic system, THBD is expressed in a subpopulation of human dendritic cells, in monocytes, and in a small subset of neutrophils[33,34]. Western Blot analysis of BM from radiation-exposed animals indicated the presence of Thbd protein in BM cells (Fig. 1d, GFP- control) and Thbd transcript was detected in differentiated BM cells, in HPCs, in eHPCs (Lin$^-$,c-Kit$^+$, Sca-1$^+$ cells) of BM (Fig. 4a), in BM derived CD45$^-$ Ter111$^-$ CD31$^+$ endothelial cells and in the CD45$^-$ Ter111$^-$ CD31$^-$ stroma cell compartment in BM (Fig. 4a,b). An in situ survey of β-galactoside expression in the femur of Thbd^{lacZ} knock-in mice indicated abundant Thbd expression within the endosteal region, as well as in femoral blood vessel endothelial cells supplying the marrow (Fig. 4c). Flow cytometry confirmed Thbd expression in Ly-6G^{NEG}Gr1^{POS}CD115^{POS} BM macrophages as well as in B220/CD19 positive B-cell precursors (Supplementary Fig. 9a and data not shown). Thbd-expressing macrophages are distinct from the two previously described populations of BM resident macrophage-like cells involved in maintenance of the hematopoietic niche in BM, i.e. cells with the surface phenotype CD169^{POS} CD115^{INT} F4/80^{POS} Gr1^{NEG} or CD11b^{POS} Ly-6G^{POS} F4/80^{POS}

(Supplementary Fig. 9b). Within the $CD45^{NEG}Pecam-1/CD31^{POS}$ endothelial population in BM, Thbd expression is detected in Sca-1 ^{NEG} sinusoidal endothelium, but is absent from Sca-1^{POS} arterial endothelium (Supplementary Fig. 9c). This combined analysis of Thbd-mRNA, Thbd-antigen, and lacZ-reporter gene expression is consistent with the presence of Thbd on hematopoietic cells and non-hematopoietic cells within the BM.

We next investigated whether the selective genetic disruption of protein C activation by endogenous Thbd which results in minimal residual Thbd function modified TBI survival[35,36,37]. Mice (Thbd$^{Pro/LacZ}$) [36] carrying only one functional Thbd-allele that encodes a Thbd variant (Thbd^{Pro}) with severely reduced ability to activate protein C showed increased sensitivity to TBI, with the dose of radiation eliciting 50% lethality shifted from ~8.75 Gy in wild type mice to ~ 7.5 Gy in Thbd-deficient mice (Fig. 4d), while Thbd$^{Pro/Pro}$ mice, which show a less severe Thbd deficiency than Thbd$^{Pro/LacZ}$ mice still presented with elevated sensitivity to TBI (Fig. 4e). In contrast, aPC^{HI} transgenic mice with constitutively elevated plasma aPC levels due to expression of a variant human protein C that is efficiently activated by thrombin even in the absence of Thbd [38], were protected against radiation-induced BM failure to a similar extent as wild type mice treated with recombinant aPC (Fig. 4f). Expression of the aPC^{HI} transgene also rescued the increased radiation sensitivity of Thbd-deficient Thbd$^{Pro/Pro}$ mice (Fig. 4f), providing direct genetic evidence that the increased radiation sensitivity of Thbd-deficient mice is caused by inadequate activation of endogenous PC.

Competitive hematopoietic reconstitution of lethally irradiated wild type recipients with BM from Thbd^{ProPro} and wild type mice, followed by exposure to 3 Gy TBI given 8 weeks after transplantation (Fig. 4g), showed a significantly reduced recovery of Thbd^{ProPro} cells when compared to wild type cells (Fig. 4h). The initially lower contribution of Thbd^{ProPro} BM cells to chimerism before irradiation might owe to not yet investigated additional functions of Thbd in HSC biology. Non-competitive reconstitution of irradiated Thbd^{ProPro} recipients with wild type BM (Fig. 4i), followed by a second exposure to a LD50-dose of TBI, resulted in significantly increased 30-day mortality compared to wild type animals reconstituted with wild type BM (Fig. 4j). Hence, endogenous Thbd expression on hematopoietic cells as well as on non-hematopoietic stroma cells affords protection against radiation mirroring the protection conferred by forced Thbd over-expression in HSPC or by therapeutic administration of soluble Thbd/aPC.

In summary, the current work identified the Thbd-protein C pathway as a physiologically relevant mechanism that accelerates HSPC recovery in response to lethal TBI to an extent that results in significant radio-mitigation. Our data are consistent with a mechanism in which endogenous Thbd expressed on stromal endothelial cells promotes protein C activation and release of aPC into the BM microenvironment, followed by aPC-stimulated recovery from radiation-induced hematopoietic suppression. Judged by the effect of Thbd-deficiency on 30-day mortality (Fig. 4j), Thbd expression on stromal endothelial cells appears to make a more important contribution to overall survival than hematopoietically expressed Thbd. Nevertheless, Thbd expression on HSPC supports hematopoietic recovery upon TBI in an apparent cell-autonomous manner, with as yet unknown effects on whole animal survival of radiation injury. While the cellular and molecular mechanism underlying

the effect of soluble THBD or aPC on the recovery of HPC activity still need to be studied in more detail, they are likely distinct from previously explored pathways, including those mediated by agonists of toll-like receptor 5 [39], inhibitors for CDK4/6, or various antioxidant compounds [40]. While the effect of endogenous aPC may largely remain confined to it's site of formation, i.e. the local microenvironment of Thbd-expressing cells, systemic administration of soluble THBD or of aPC can reproduce and augment the radio-protective effect of the endogenous protein C pathway.

Recombinant human aPC has undergone extensive clinical testing in patients, and recombinant soluble human THBD is currently being investigated for efficacy in antithrombotic therapy in man. Our data encourage the further evaluation of these proteins for their radio-mitigating activities.. Moreover, these agents, possibly in combinations with other compounds targeting other pathways, may provide novel medical countermeasures against radiation induced pathologies that may arise in environmental or therapeutic settings that involve exposure to high levels of radiation.

MATERIAL AND METHODS

Animals

Animals were housed under standardized conditions with controlled temperature and humidity and a 12/12-hour day/night light cycle. C57BL/6, C57BL/6CD45.1, and CD2F1 animals were obtained from commercial vendors (Charles River, The Jackson Laboratories, Harlan Sprague Dawley). Thbd$^{Pro/Pro-}$, Thbd$^{lacZ\ -}$, Epcr$^{low/-}$, and Par1$^{-/-}$ mice have been described previously [1–6] and were on a C57BL/6 background (>14 backcrosses). Because of the early embryonic lethality of Thbd-null mice we generated mice with minimal residual Thbd function (Thbd$^{Pro/lacZ}$ carrying one Thbd-Pro allele encoding a Thbd variant (ThbdPro) with greatly diminished ability to support the activation of protein C[1,2], and one Thbd-null allele, in which the *Thbd* gene is disrupted by insertion of a lacZ reporter[7]). The Thbd Pro mouse carries a point mutation resulting in a Glu-Pro exchange in the EGF3-4 inter-domain loop of Thbd. The mutation results in a substantial suppression of Thbd-dependent aPC generation, while additional functions associated with Thbd (such as the lectin-domain effects on complement regulation and leukocyte-endothelial interaction) are retained. Thbd antigen levels in the lung are reduced 2-3-fold in Thbd$^{Pro/Pro}$ mice. In all animal experiment male and female mice were used in an unspecified ratio. Animal experiments were approved by the IACUCs at Cincinnati Children's Hospital Medical Center (CCHMC), the University of Arkansas for Medical Sciences (UAMS)/Central Arkansas Veterans Healthcare System (CAVHS), or the Medical College of Wisconsin.

Reagents

Recombinant human Thbd (CD141) (Cat no.2374) and PC (Cat.no. 239F) for *in vitro* experiments (protein C activation assay) were purchased from American Diagnostica and thrombin was purchased from Sigma. Recombinant human Thbd (CD141) (10mg) was purified from the Chinese Hamster Ovary (CHO) cell line and provided as a lyophilized powder that was reconstituted with deionized water. aPC (100 μg/ml) was provided in a 50% (vol/vol) glycerol/water mixture. Recombinant mouse wild-type aPC, a "signaling-selective"

aPC variant (5A-aPC), and a "signaling-defective" aPC variant (E149A-aPC, a hyperantithrombotic, non-cytoprotective Glu149Ala mutant version of aPC) have been described previously [8,9]. Solulin (soluble human recombinant thrombomodulin, ZK 158 266, INN sothrombomodulin alfa), the modified recombinant human Thbd molecule (Paion Deutschland GmbH) is comprised of the extracellular portion of Thbd (the N-terminal lectin-binding domain, the 6 EGF-like repeats, and the serine/threonine-rich domain), but lacks the transmembrane and intracellular domains, as well as the chondroitin sulfate moiety. It derives from the molecule originally described by Glaser et al. and referred to as TMLEO [10], being distinguished by the following mutations: deletion of the first three amino acids of the amino terminus, Met388Leu, Arg456Gly, His457Gln, Ser474Ala, and deletion of the last seven amino acids of the carboxy terminus [11]. Solulin (4.6 mg/ml) was provided as a sterile liquid solution for injection containing Solulin in 10 mM sodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, and 5% mannitol at pH 7.0.

Hybridoma cells secreting the rat–anti mouse Thbd monoclonal AB 273-34A and 411-201B [12] were generously provided by Dr. Kennel (University of TN Graduate School of Medicine, Knoxville, TN, USA). The AB was used at a 1:200 dilution. For flow cytometric analyses, a polyclonal secondary anti-rat antibody labeled with APC was used for detection of Thbd expressing cells. Thbd (M-17) an affinity purified goat polyclonal antibody (sc-7097, Santa Cruz Biotechnology) was used for Western blotting according to the dilutions indicated by the manufacterer.. The BWA3 antibody was provided by Dr. Marc Monestier (Temple University School of Medicine, Philadelphia, PA). This antibody exerts anticoagulation without causing bleeding, because it only targets the excessive amplification of thrombin generation through the intrinsic pathway. The 14E11 antibody was provided by Drs. David Gailani and Andras Gruber (Vanderbilt University, Nashville, TN).

Total body irradiation (TBI) of mice

Irradiation was performed at the CAVHS with a Shepherd Mark I, model 25, Cs-137 irradiator (J.L. Shepherd & Associates) as described [11]. The average dose rate was 1.37 Gy per minute. Irradiation was performed with a Shepherd Mark I-68 Cs-137 irradiator with an average dose rate of 0.52 Gy per minute (located at CCHMC), or a Gammacell 40 Extractor Cs-137 (Best Theratronics; average dose rate 0.97 Gy-min^{-1}, Blood Research Institute). All irradiators were calibrated at least once a year. For TBI mitigation experiments, mice were monitored for up to 30 days post-TBI. The number of dead/moribund mice was recorded twice daily. Kaplan-Meier survival curves, median survival times, and lethality at 30 days were recorded.

Retroviral and lentiviral transduction of hematopoietic cells

Hematopoietic stem and progenitor cells (HSPCs) derived from BM were transduced with the SFbeta virus containing an IRES EGFP sequence or a Thbd IRES EGFP construct at a multiplicity of infection of about 2–4, which results on average in 1 or 2 integration sites per single genome. These cells were subsequently transplanted into recipient animals preconditioned with 11.75 Gy (7 Gy + 4.75 Gy, 4 hours apart), as previously published [13–15]. LM-PCR and sequencing of integration sites was performed as published [14].

Blood parameters, flow cytometry, isolation of stroma cells, and quantitative real-time RT-PCR

Blood parameters were determined by a Hemavet Instrument (Drew Scientific Inc). Immunostaining and flow cytometry analyses were performed according to standard procedures and analyzed on a FacsCanto flow cytometer (BD Biosciences). Anti-Ly5.2 (clone 104, BD Biosciences, FITC conjugated) and anti-Ly5.1 (clone A20, BD Biosciences, PE conjugated) monoclonal antibodies were used to distinguish donor from recipient and competitor cells. For lineage analysis in hematopoietic tissues, anti-CD3ε (clone 145-2C11, PE-Cy7 conjugated), anti-B220 (clone RA3-6B2, APC conjugated), anti-CD11b (clone M1/70, APC-Cy7 conjugated) and anti-Gr-1 (clone RB6-8C5, APC-Cy7 conjugated, all from BD Biosciences) were used. Antibodies were used at a 1:100 dilution. The level of RNA expression was determined by real-time RT-PCR using, Taqman Universal PCR and RT reagents from ABI (Life Technologies Corporation). RNA was purified with the Qiagen RNeasy micro kit (Qiagen Inc). The following Taqman probes were used to determine expression of selected genes in the neighborhood of the viral integration site in experimental animals.

Taqman Gene Expression Probes

Mouse ID	Gene name	Ref Seq #	Assay ID
Mouse 2	Bbc3 (Puma)	NM_133234.1	Mm00519268_m1
	Ccdc9	NM_172297.1	Mm00552307_m1
	Ube11a	NM_019748.1	Mm00502282_m1
Mouse 7	Obfc1	NM_175360.2	Mm00614838_m1
	Junb	NM_008416.1	Mm00492781_s1
	Hook2	NM_133255.1	Mm00466969_m1
	Ly86	NM_010745.1	Mm00440240_m1
	Rreb1	NM_026830.1	Mm01325346_m1
Mouse 9	Sla	NM_001029841.1	Mm00447294_m1
	Thbd	NM_009378.1	Mm00437014_S1
	CD93	NM_010740.2	Mm00440239_g1

Studies to assess the effect of TBI on Thbd+/+, Thbd*Pro/lacZ*, EPCR low, and PAR-1 animals or BM cells

To analyze the effect of TBI on Thbd*Pro/lacZ* animals, mice were subjected to TBI at 7 Gy or 11 Gy. For competitive transplants followed by TBI for radioselection, BM cells harvested from tibia and femur of 6- to 8-week-old Thbd*Pro/Pro* mice (donor) as well as B6.SJL(BoyJ) (competitor) mice (2×10^6 cells from each) were transplanted into BoyJ recipient mice that were lethally irradiated with a total dosage of 11.75 Gy (7 Gy + 4.75 Gy, 4 hours apart). Cells were transplanted into the retro-orbital sinus or via tail vein in a volume of 200 µl in IMDM/2% FCS. Three to four weeks post-transplantation, chimerism of the peripheral blood was analyzed by flow cytometry using a panel consisting of CD45.2, B220 for B cells, CD3ε for T cells, and Mac-1/Gr-1 combined for the myeloid lineage. Animals

were subsequently irradiated with 3 Gy TBI for radioselection, and peripheral blood was analyzed for chimerism at the timepoints post–3 Gy indicated in the experiments.

Effects of recombinant aPC, aPC variants, and Solulin on survival in response to TBI

Mice were subjected to TBI at the doses indicated and subsequently randomly assigned to receive wild-type aPC, signaling-selective aPC (5A-aPC), signaling-defective aPC (E149A-aPC, a hyperantithrombotic, non-cytoprotective Glu149Ala aPC mutant), or vehicle. aPC or its variants were dissolved in vehicle buffer (50 mM Tris, 100 mM NaCl, pH 7.4). Mice received a single dose or, where indicated, multiple doses of either recombinant aPC, an aPC variant, or vehicle buffer at 0.35 to 0.4 mg/kg in a volume of 200 μL or less by tail vein (i.v.) injection at the time points indicated after TBI. Male mice were injected subcutaneously with Solulin (1 mg/kg or 3 mg/kg) or vehicle 30 min after TBI. The mice were returned to the cages with free access to food and water and monitored twice daily for 30 days for weight loss, apparent behavioral deficits, and survival.

Apoptosis/survival assay

Transduced cells sorted for EGFP were subjected to 1.5 Gy *in vitro* and cultured overnight in Iscove's modified Dulbecco's medium supplemented with 10% FBS, 2% penicillin-streptomycin, 100 ng/ml of mSCF, 100 ng/ml of TPO and 100 ng/ml of G-CSF. 18hrs post irradiation cells were harvested and washed twice with ice cold PBS and 1×10^5 cells were resuspended in PBS. Cells were stained with anti C-kit –APC antibody (BD Pharmingen) for 20 min on ice. Then cells were stained with anti-Annexin V PE antibody (BD Pharmingen) and 7AAD (1mg/ml) in 1x binding buffer and analyzed using a BD FACSCanto. Antibodies were used at a 1:100 dilution.

Proliferation assay

Triplicates of $2\text{-}5 \times 10^5$ transduced cells were resuspended in 2ml of IMDM supplemented with 10%FBS, 2% penicillin-streptomycin, 100 ng/ml of murine stem cell factor, 100 ng/ml of TPO and 100 ng/ml of granulocyte colony-stimulating factor. The cell counts were determined on day 3,5 and 7.

Colony-forming cell assay

CFC assays were performed using methocult (M3234 Stem Cell Technologies Inc) containing a final concentration of 50ng/ml mSCF, 10ng/ml mIL-3 and 10ng/ml mIL-6 (Peprotech). The cells were plated in triplicates in 6 well plates and irradiated with 1.5 and 3Gy using a Cs-137 source. Plates were incubated at 37°C in 5% CO_2 and colonies were counted between 7 and 10 days after plating.

In vitro irradiation

In vitro radiation experiments were performed in a cell-free system (i.e., in a system not confounded by transcriptional regulation or ectodomain shedding of Thbd) as described previously [16]. Briefly, all Thbd and Solulin samples were dissolved in buffer (described above) and irradiated in 1-mL polypropylene microcentrifuge tubes in a total volume of 500

μL. At least 3 independent samples for Thbd and Solulin were irradiated at each dose level for all experiments, not including optimization and validation studies.

Thbd activity assay

Recombinant Thbd and Solulin were dissolved to a final concentration of 50 nM in a buffer containing 10 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.1% polyethylene glycol, pH 8.0. The effect of single-dose irradiation on Thbd functional activity was assessed with the protein C activation assay after exposure to 0, 1.77 Gy (0.3 min radiation exposure), 10 Gy (1.7 min), 20 Gy (3.4 min), and 40 Gy (6.8 min). The PC activation assay was performed as follows. Irradiated and sham-irradiated samples were diluted to a final concentration of 2.5 nM Thbd and incubated with 200 nM PC and 1.4 nM thrombin for 60 minutes at 37°C in a 96-well plate to generate aPC. The amount of aPC generated was measured by monitoring hydrolysis of the chromogenic substrate, S-2366 (Diapharma), at 405 nm in a microplate reader (Bio-Tek Instruments) at 5-minute intervals for 60 minutes. The results were expressed as the mean optical density (OD) at 60 minutes. All assays were performed in triplicate, and the average was considered a single value for statistical purposes.

Solulin pharmacokinetics

As intravenous pharmacokinetics of Solulin in mice and rats are highly comparable (PAION Deutschland GmbH, data not shown), dose selection for subcutaneous administration of Solulin in mice was based on available rat data. Solulin, injected subcutaneously at 3 mg/kg in male rats, reached plasma concentrations of about 40 nM after 6 hours and about 100 nM after 26 hours (maximal observed value). As these levels are associated with pronounced aPC-mediated effects of intravenous Solulin in animal models of thrombotic vessel occlusion [17], doses of 1 and 3 mg/kg were selected for mouse radiation studies.

Determination of plasma citrulline levels

The plasma level of citrulline is a well-validated biomarker for functional enterocyte mass (Crenn 2008) and exhibits close correlation with other markers of intestinal radiation injury, including mucosal surface area and the crypt colony assay (Lutgens 2003, Berbee 2009). The plasma citrulline levels reflect enterocyte mass and correlate directly with radiation dose. Citrulline plasma concentration was determined using an LC-MS/MS recently described by Gupta, et al. (Gupta 2011). Briefly, plasma proteins were precipitated in a 96-well Strata Impact 2 ml filtration plate (Phenomenex, Torrance, CA). Each plasma sample (10 μL) was treated with 490 μL acetonitrile:water:formic acid (85:14.8:0.2 v/v) containing internal standard (2 μM). After mixing gently, the plate was covered, allowed to stand for 5 minutes, and the filtrate was collected under vacuum. Calibration was performed by using isotope dilution approach, with L-citrulline as calibration standard (0.125-200 μM) and L-Citrulline (5-13C, 99%; 4,4,5,5-D4, 93%+) as internal standard. The LC-MS/MS system was an Acquity UPLC system interfaced to a Quattro Premier triple quadrupole mass spectrometer (Waters Corp.). Chromatographic separation was achieved on a Phenomenex 1.7 μM Kinetex HILIC analytical column (50 × 2.1 mm (i.d.)). Mobile phase A was acetonitrile containing 0.1% formic acid, 0.2% acetic acid and 0.005% trifluoroacetic acid. Mobile phase B was water containing 0.1% formic acid, 0.2% acetic acid and 0.005% trifluoroacetic acid. The initial flow rate was 0.6 ml/min and then increased to 0.7 ml/min at

1.3 min. The gradient program was as follows: Initial 9% B; 0-1.2 min: 11% B; 1.2-1.3 min: 30% B and held for 0.6 min; 1.9-2.0 min:9% B. The total run time was 3.0 min. The sample injection volume was 3 μL. The sample loop volume was 10 μL. Positive ions for citrulline and citrulline+5 were generated using electrospray ionization at a capillary voltage of 3 kV and cone voltage of 18 V. Product ions were generated using argon collision induced disassociation at collision energy of 10 eV while maintaining a collision cell pressure of 4.8 × 10^{-3} torr. Mass spectrometric detection was performed in the multiple-reaction-monitoring (MRM) mode using the precursor→product ions, m/z 176→159 and m/z 181→164 for citrulline and citrulline+5, respectively. The lower limit of quantation was 0.125 μM while the upper limit of quantiation was 200 μM. Predicted values for all calibrators were within 90 – 110% of their nominal value.

Intestinal mucosal surface area (MSA) measurements

Intestinal mucosal surface area is a well-validated, sensitive parameter of intestinal radiation injury. The assay has greater sensitivity at the relatively low radiation doses used here (less than 10 Gy) than the traditional crypt colony assay. Mucosal surface area was measured in vertical sections of the jejunum stained with hematoxylin and eosin (H&E), using a projection/cycloid method as described by Baddeley et al.[18]. The method has previously been validated by us specifically for surface area determination of the intestinal mucosa after irradiation[19].

HSPC Staining post irradiation

2 million TBM cells were blocked with 2% mouse serum (M5905-5ml, Sigma) for 10 min followed by staining with a mixture of biotin conjugated lineage antibodies including CD5 (BD Biosciences Clone 53-7.3), CD45R (BD Biosciences, B220 Clone RA3-6B2), Mac1 (BD Biosciences, CD11b Clone M1/70), Gr1 (Biosciences, Ly-6G and Ly-6c BD Clone RB6-8C5), CD8a (BD Biosciences Clone 53-6.7) and Ter119 (BD Biosciences Clone TER-119) for 20 min. Then the cells were washed once and stained with anti-cKit (BD Biosciences CD117 Clone 2B8, FITC conjugated), anti-Sca-1 (e Biosciences Ly-6A/E PE-Cy7 Conjugated) and SA-APC-Cy7 (BD Biosciences 554063) for 30 min and analyzed using BD Facs Canto. Antibodies were used at a 1:100 dilution.

Statistical analysis

Sample size for the TBI experiments was estimated per the recent publication by Kodell et al. for radiation countermeasures studies [20]. Statistical analyses were performed using NCSS 2004 for Windows (NCSS). Data were presented as the mean ± the standard error of the mean (SEM). Two-sided tests were used throughout, and differences were considered statistically significant when the p-value was less than 0.05. Pairwise (univariate) comparisons were performed with the Student's t-test or the Mann-Whitney U test as appropriate. Mouse survival curves were constructed using the Kaplan-Meier method, and 30-day survival rates were compared with logistic regression analysis within GraphPad Prism (Graphpad Sofware). The LD50 values for comparison of mouse survival after radiation exposure were calculated with logit-transformed data, and standard error estimates were obtained by the delta-method.

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Supplementary Material

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Acknowledgments

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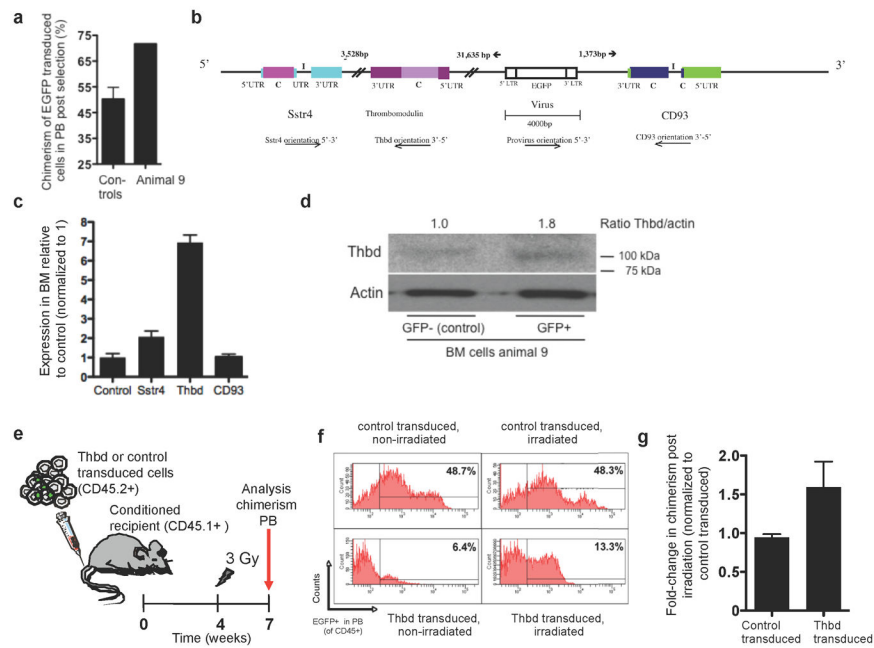
Figure 1. Elevated expression of Thbd selects for primitive hematopoietic cells upon irradiation *in vivo*

(**a**) EGFP chimerism in PB of animal 9 after exposure to 3 Gy TBI every week for 3 consecutive weeks versus controls (same experiment, non-selected). (**b**) Graphical representation of the provirus integration into chromosome 2 of animal 9 (BM) as determined by LM-PCR followed by sequencing of the dominant integron product (see Supplementary Table 1 for a list of all integration sites in animal 9). (**c**) Transcriptional level of expression of the genes surrounding 5′ and 3′ side of integrated provirus of animal 9 (BM cells). Determination by quantitative real-time RT-PCR compared to a pool of control samples (BM cells from C57BL/6CD45.1 mice, and animals 11 and 18, which were transplanted with transduced HSPCs but not irradiated). (**d**) Expression of Thbd in the BM of animal 9 compared to control animals (C57BL/6), determined by Western blot analysis. (**e**) Experimental setup: over-expression of Thbd in HSPCs by retroviral transduction, followed by transplantation and subsequent selection by irradiation. Recipient animals were pre-conditioned by irradiation with 11.75 Gy to accept the graft. Animals consistently presented with a graft chimerism (CD45.2+ cells in PB) of higher than 90% 3 weeks post transplantation. (**f**) Representative data depicting the level of EGFP expression/selection among CD45.2+ cells in PB of individual animals with and without irradiation in Thbd-transduced compared to control-transduced cells transplanted. (**g**) Quantification of the radioselection of Thbd-transduced hematopoietic cells in PB *in vivo* post-irradiation relative to control (GFP only)–transduced hematopoietic cells; n = 3 independent experiments with at least 3 recipients per single experiment. * p = < 0.05.
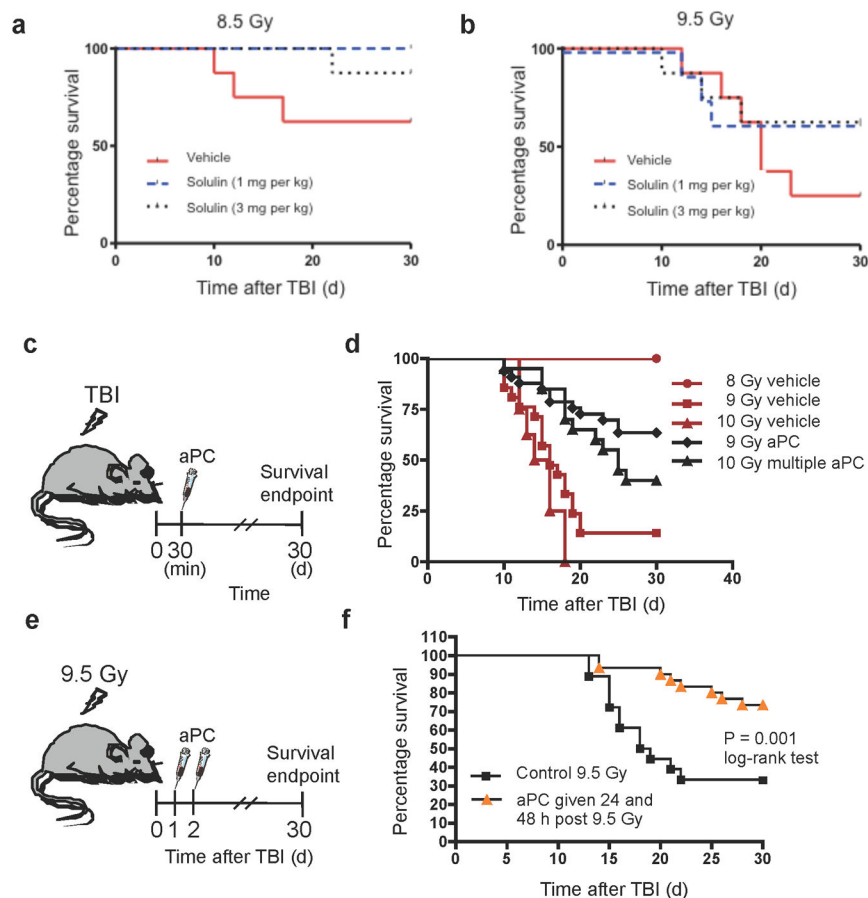
Figure 2. Solulin and recombinant aPC confer mitigation of radiation toxicity after TBI
(**a,b**) 30 d survival of mice injected subcutaneously (n = 8 per group) 30 min after exposure to (**a**) 8.5 Gy or (**b**) 9.5 Gy TBI, p<0.05 for both Solulin at 3 mg kg^{-1} and control. (**c**) Experimental setup for experiments depicted in (d). (**d**) 30 d survival of C57BL/6 animals injected with recombinant murine aPC (0.35 to 0.4 mg kg^{-1}) or vehicle (PBS) i.v. 30 min after single-dose TBI; n = 8 for 8 Gy vehicle, n = 33 for 9 Gy aPC, n = 23 for 9 Gy vehicle, n = 8 for 10 Gy vehicle, n = 20 for 10 Gy multiple aPC (30 min, 1 h and 2 h post-irradiation, 9 Gy vehicle vs. 9 Gy aPC, p < 0.0001; 10 Gy vehicle vs. 10 Gy multiple aPC, p<0.0001). (**e**) Experimental setup: mitigation of TBI toxicity when aPC is given no earlier than 24 hours post-TBI. (**f**) 30 d survival of C57BL/6 animals injected with vehicle or murine aPC at a dose of 5 μg/mouse through a single i.v. injection at 24 and 48 hours post TBI (9.5 Gy).
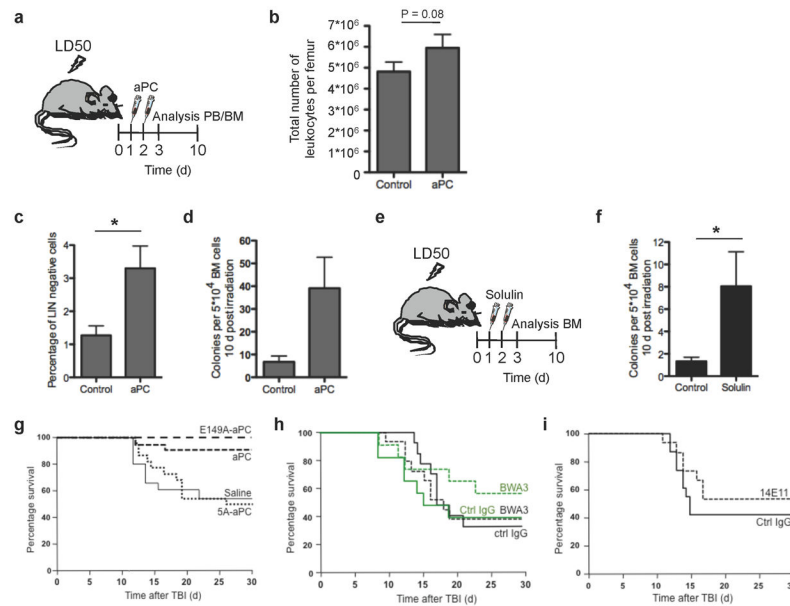
Figure 3. Mechanisms of action of mitigation by soluble Thbd and aPC

(**a**) Experimental design. (**b**) Total number of leukocytes per femur in control animals and animals treated with aPC 10 d post irradiation. (**c**) Frequency of Lin$^-$, c-Kit$^+$ cells in BM 10 d post irradiation. (**d**) Frequency of CFCs in BM cells 10 d post irradiation. (**e**) Experimental design for data presented in s(f). (**f**) Frequency of CFCs in BM cells 10 days post irradiation. (**g**) 30 d survival of mice (n = 15 per group) irradiated with an LD50 receiving either 5 μg/ mouse of the hyper-anticoagulant E149A mutant form of aPC or vehicle control through a single tail vein injection 30 min after TBI, p < 0.001 for E149A-aPC as well as for aPC versus saline and 5A-aPC. (**h**) 30 d survival of mice given a LD50/30 TBI and treated with the histone blocking antibody BWA3 (i.p. 30 mg kg^{-1}) 0 h (black lines) or 16 h (green lines) post irradiation, compared to control injections of IgG Ab, n   12 per experimental group. (**i**) 30 d survival of mice given a LD50/30 TBI and treated with an anti-factor XIa antibody (14E11) at 0 h post irradiation (i.p. 30 mg kg^{-1}) compared to control Ab treated animals, n 16 per experimental group.
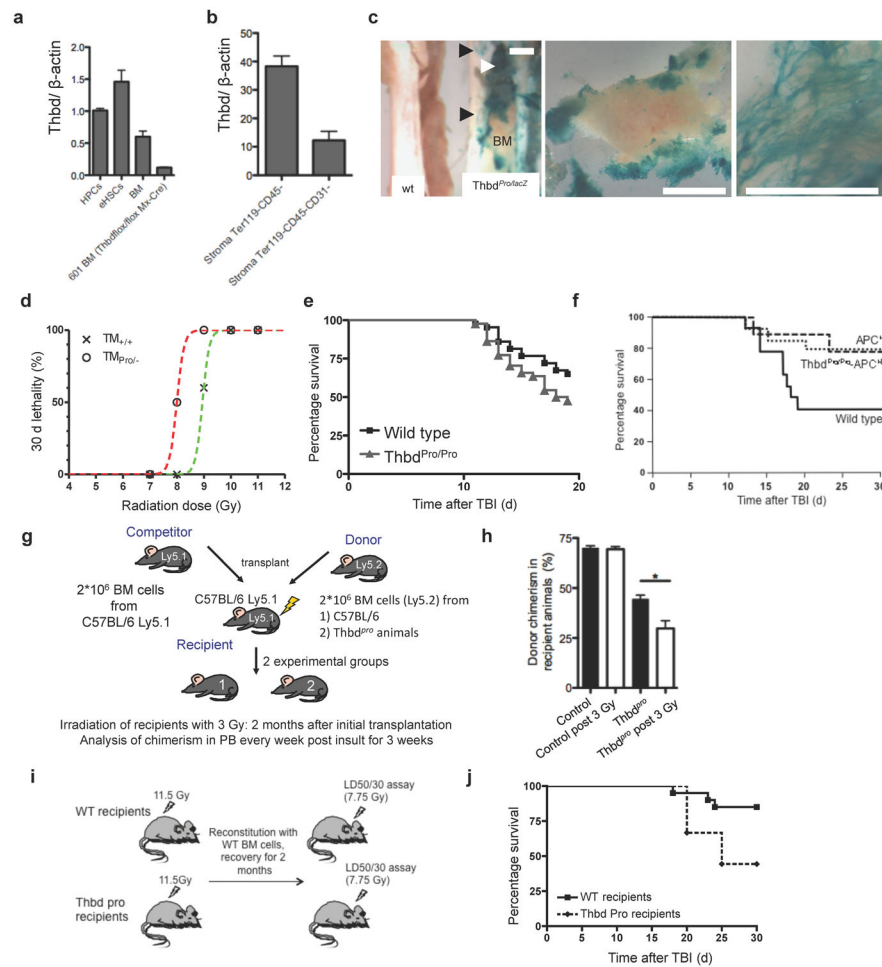
Figure 4. A role of endogenous Thbd in radiation protection
(**a**) Expression of Thbd relative to β-actin in hematopoietic cells. As an additional control, RNA from BM cells from an floxed Thbd allele crossed to an Mx-Cre animals treated with pIC to delete the allele in BM cells (601 BM, deletion of up to 80% confirmed by PCR) was used. (**b**) Expression of Thbd relative to β-actin in BM stroma cells containing endothelial cells (CD45⁻, Ter119⁻ cells) and in non-endothelial stroma cells (CD45⁻,Ter119⁻, CD31⁻ cells). (**c**) Femoral bone marrow of Thbd$^{Pro/lacZ}$ mice stained *in situ* with a substrate for β-galactosidase. Blue staining indicates expression of the LacZ reporter gene controlled by the endogenous Thbd-promoter. (left) Staining occurs in the endothelium of blood vessel on the outer surface of the bone or penetrating the bone (black arrowhead), and in some vessels within the bone marrow mass. Intense staining is seen in a loose meshwork of cells located between the entire inner surface of the bone and the central bone marrow (white arrow; BM: bone marrow partially exposed by removal of the lacZ-positive layer). No staining was noted in parallel-processed WT controls. (middle) LacZ-positive clusters of cells are predominantly associated with the periphery of the marrow extruded from the bone cavity. (right) lacZ-positive endothelial cells seen in small blood vessels supplying the outer bone surface. The expression of lacZ correlated well with the data obtained by real-time RT-PCR in (a,b). White bar represent 1 mm. (**d**) 30 d survival of Thbd$^{+/+}$ and Thbd$^{Pro/lacZ}$ mice (n =

8 per group) subjected to TBI with doses ranging between 7-11 Gy, p = 0.0001. (**e**) 20 d survival of Thbd$^{Pro/Pro}$ animals irradiated with an LD50/30 TBI, n   11 per group, p = 0.09. (**f**) 30 d survival of animals treated with an LD50/30 TBI, n = 10 for APC^{HI}, n = 15 for Thbd$^{Pro/Pro}$/APC^{HI} and WT control, p < 0.05 for Thbd$^{Pro/Pro}$/APC^{HI} over WT. (**g**) Experimental setup: competitive transplantation/radioselection experiments with BM cells from Thbd$^{Pro/Pro}$ mice. (**h**) Donor chimerism in PB of animals competitively transplanted according to (g) 3 weeks post–3 Gy irradiation of recipients compared to controls, n   4 recipients per group, * = p < 0.05. (**i**) Experimental setup: transplantation/radioselection experiments with Thbd$^{Pro/Pro}$ and WT mice reconstituted with WT BM cells. (**j**) 30 d survival of WT and Thbd$^{Pro/Pro}$ recipients treated with a second dosage of 7.75 Gy, n = 20 for WT and n = 9 for Thbd$^{Pro/Pro}$ recipients, p < 0.05 for Thbd$^{Pro/Pro}$ recipients compared to WT recipients.