Supplemental Data

EPCR deficiency or function-blocking antibody protects against joint bleeding-induced pathology in hemophilia mice

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Materials and methods

Reagents

Recombinant human FVIIa was provided the Late Walter Kisiel, University of New Mexico, Albuquerque (NM). rFVIIa was expressed and purified as described earlier[1,2] and essentially similar to that of commercially available rFVIIa (NovoSeven). FVIIa protein concentration was determined from the absorbance of protein at 280 nm and using the extinction coefficient ($E^{1\%}_{280}$) of FVIIa as 13.9.[2] Monoclonal antibodies against mouse EPCR (mAb 1560/blocking mAb and mAb 1567/non-blocking mAb) were prepared by immunizing rats with recombinant mouse soluble EPCR.[3] Rat anti-mouse F4/80 antibody was from BioLegend (San Diego, CA) and polyclonal rabbit anti-mouse CD31 antibody was from Abcam (Cambridge, MA). Blocking solution and other histochemical reagents were from Dako (Agilent; Santa Clara, CA). Iron staining kit was obtained from American Mastertech Scientific, Inc. (Lodi, CA). Alcian blue, Saranin O, and fast green stains were obtained from Sigma-Aldrich (St. Louis, MO). ELISA kit for measuring mouse IL-6 was from Affymetrix (ThermoFisher Scientific. Waltham, MA).

Animals

Wild-type mice (C57BL/6J) and $FVIII^{-/-}$ mice (B6/129S) were obtained from Jackson Laboratories (Bar Harbor, ME) or bred in-house. $FVIII^{-/-}$ mice in the B6/129S background were backcrossed with C57BL/6J mice for more than 10 generations to generate $FVIII^{-/-}$ mice in the C57BL/6J genetic background. $EPCR^{-/-}FVIII^{-/-}$ mice were generated first crossing $Procr^{flox/flox}$ and $Procr^{+/flox}Meox2^{+/cre}$ mice with $FVIII^{-/-}$ mice to generate $Procr^{flox/flox}FVIII^{-/-}$ and $Procr^{+/flox}Meox2^{+/cre}FVIII^{-/-}$ mice and then crossing female $Procr^{flox/flox}FVIII^{-/-}$ with male $Procr^{+/flox}Meox2^{+/cre}FVIII^{-/-}$ mice. $EPCR^{+/+}FVIII^{-/-}$ mice were generated by crossing Tie2-EPCR mice with $FVIII^{-/-}$ mice. Generation of $Procr^{flox/flox}$, $Procr^{+/flox}Meox2^{+/cre}$, and Tie2-EPCR mice was described earlier.[3,4] Briefly, the vector containing mutated EPCR allele flanked by two loxp sites was targeted into 129-derived ES cells to generate $Procr^{flox/flox}$ mice.[4] After extensively back-crossing with the C57BL/6 genotype, $Procr^{flox/flox}$ mice were crossed with $Meox2^{+/cre}$, which were in the C57BL/6 genetic background, to generate $Procr^{+/flox}Meox2^{+/cre}$ mice. Tie2-EPCR mice were generated by

microinjecting the T2PE-MESV transgene comprised of the Tie2 promoter, murine EPCR cDNA, SV40 polyA sequence, and Tie2 intron 1 enhancer into fertilized FVB/N oocytes.[3] A founder transgenic mouse with high EPCR expression was backcrossed into C57BL/6 mice to generate EPCR-overexpressing Tie2-EPCR mice. EPCR-deficient mice had no detectable expression of EPCR, whereas EPCR expression levels were markedly higher in Tie2-EPCR mice. Li et al. showed that EPCR expression in the lungs of EPCR-deficient mice (designated as *Procr*Lox mice) was undetectable by real-time PCR.[4] Plasma soluble EPCR and lung EPCR antigen levels were also below the level of detection by ELISA.[4] Immunohistochemistry showed no detectable expression of EPCR in blood vessels. In Tie2-EPCR mice, EPCR antigen levels were at least eightfold higher in transgenic than in wild-type mice.[3] We confirmed the above findings. In our studies, we found sEPCR levels in the plasma of wild-type mice were 88 ± 15.5 ng/ml, and Tie2-EPCR mice were 1027 ± 196 ng/ml, whereas sEPCR was not detectable in plasma of EPCR-deficient mice (detection limit, 0.2 ng/ml EPCR in ELISA). Twelve to 16-week old mice, both sexes weighing between 24 to 30 g, were used in our experiments. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee and conducted according to the animal welfare guidelines outlined in the Guide for the Care and Use of Laboratory Animals.

Induction of joint bleeding by needle puncture

Intraarticular bleeding into knee joints was induced by a needle puncture injury, as described earlier.[5,6] Briefly, a day before the injury, the hair over both knee joints was removed by using Nair hair removal cream. Mice were anesthetized with ketamine/xylazine, and the right knee joint capsule was punctured with a 30 x 0.5-g needle below the patella to induce bleeding in the joint. The left knee joint of the same animal served as the uninjured control. Mice were injected subcutaneously with analgesics Buprenorphine (0.1mg kg^{-1} body weight) 20 min before the injury and once a day for the next 3 days following the injury. After that, Buprenorphine was provided in the drinking water for 4 days at a concentration of 0.02 mg/ml for the pain relief. At specified times following the injury, mice were euthanized, the skin was removed from over the knee joints, the knee joints were photographed, and processed for histology, immunohistochemistry, or collecting the synovial fluid.

Assessment of bleeding into knee joints

Uninjured and injured knee, five h following the needle puncture injury, were excised from mice. After removing the skin, hind limbs (including the joint) were trimmed to equal lengths, cut into small pieces, and added to ABX lyse bio solution to extract hemoglobin. After overnight extraction, the supernatants were removed, and their absorbance was measured at 540 nm. The values were extrapolated to hemoglobin standards. In the same animals, a small amount of blood was also collected before the injury and five hours after the injury to measure hematocrit and hemoglobin levels in peripheral blood.

Treatments

Mice were treated with either a single dose or three doses of rFVIIa (1mg kg^{-1} body weight) by intravenous injection via the tail vein. The single dose or the first dose of 3 doses of rFVIIa was administered to mice 20 min following the injury. For the 3-dose regimen, the second dose of rFVIIa was given one day following the injury, and the third dose was given three days after the injury. In the case of EPCR antibody treatment, a single dose of EPCR antibodies (1 mg kg^{-1} body weight) was given one day before the injury, either i.v. or i.p. Since no significant differences were found in data obtained with these two routes of administration, the data were combined.

Evaluation of hemarthrosis

Knee diameter, before the injury and alternate days for two weeks following the injury, was measured using electronic calipers. The diameter of the knee joint of before injury was subtracted from the diameter following the injury, and the differences in the diameter was plotted as the percent change in joint diameter. Gross examination of knee joints was performed every alternate day to assess visually the extent of blood leaked into joints and knee mobility. A visual bleeding score was assigned to score the extent of injury (0, normal knee and absence of blood; 1, normal knee, presence of blood; 2, distended but not a tense knee, presence of blood; 3, tense and distended knee, presence of blood). The visual bleeding score was assigned in a blinded fashion where the person giving the score was unaware of the treatment or mouse genotype.

Histology of knee joints and synovitis scoring

Excised knee joints (the femur and tibia/fibula area, ~1 cm each direction from the joint) was fixed in EXCEL buffered formalin fixative. After 24-48 h of fixation, knee joints were decalcified for 16 to 20 h, and then decalcified knee joints are processed in graded alcohol and embedded in paraffin. Five µm thin sections were cut, and the sections were stained with hematoxylin and eosin (H&E). The stained sections were viewed under microscope and scored for hemophilic synovitis based on synovial hyperplasia (0-3 points), vascularity (0-3 points), and the presence of discoloration by hemosiderin, blood (erythrocytes), and presence of synovial villi (0 for absence and 1 for their presence in each category), and cartilage degeneration (0 to 2 points), resulting in a combined score of 0 to 11 points for increasing pathology (Supplemental Table 1). The above scoring system was formulated and validated by Valentino and Hakobyan.[5] Joint sections were also stained with Safranin O-fast green stain to proteoglycans and glycosaminoglycans to assess cartilage damage. The Safranin O staining was scored 0-3 scale (0, normal; slight reduction, 1; moderate reduction, 2; and severe reduction, 4). The presence of iron deposits in the joint tissue was evaluated by staining the sections with Prussian blue stain for tissue iron. The presence of iron is scored as no iron, 0; weakly positive, 1; positive, 2; and strongly positive, 3.

Immunohistochemistry

Knee joint tissue sections were stained for F4/80 antigen to evaluate infiltration of macrophages, and the sections were graded 0 to 4 scale (no macrophages, 0; scattered macrophages, 1, lines of macrophages, 2; clusters of

macrophages, 3, and sheets of macrophages, 4). The formation of new vessels in the knee joints was examined by staining sections with CD31 antibodies. The number of vessels seen in multiple high power fields (40X) covering the entire synovium were counted and then averaged to a number of vessels per high power field.

Imaging

Tissue sections were visualized in Olympus microscope equipped with 4x/0.13, 10x/0.30, 20x/0.50, 40x/0.75 objective lenses. Images were captured with an Olympus DP27 camera using Olympus Cellsens software. Images of knee joint sections that include portions of tibia and femur were captured at 4X and 40X magnification.

Collection of synovial fluid

Synovial fluid from joints was collected on day seven following the injury for measurement of IL-6 levels by ELISA. After removing the skin overlying knee joints, the muscle tissue around the joints was removed. The knee joint was trimmed carefully closer to femur and tibia to obtain the joint portion without damaging the synovial cavity and minimizing the surrounding tissue. The joint is placed in a small Petri dish, and an incision was made with a surgical scalpel to open the synovial cavity and expose the synovial fluid. The synovial tissue was carefully transferred to the Eppendorf tube containing the extraction buffer (100 µl, 20 mM TBS, 137 mM NaCl, 5mM EDTA, and 1% protease cocktail inhibitor). The samples were vortexed to elute the synovial fluid from the tissue. This method, in our hands, appeared to be more reliable and easier to collect the synovial fluid rather than absorbing the fluid onto a filter paper and eluting it from the filter paper as described in an earlier publication.[7] The samples were stored at -80^{0}c until they were used for measuring cytokine levels in ELISA.

IL-6 assay

IL-6 levels in the synovial fluid were estimated using the IL-6 ELISA kit (Affymetrix) according to the manufacturer's instructions.

Data analysis

Four to 10 animals were used in each group, and the data were shown as mean ± SEM. Survival studies data were derived from 12 to 31 animals/group. Statistical significance among the groups was analyzed by two-way analysis of variance, repeated measures followed by Tukey's post hoc multiple comparison test for the visual bleeding score, and percent change in the diameter and one-way analysis of variance followed by Dunnett's post hoc multiple comparison test for synovitis score. When comparing two groups, the Mann Whitney test was used to determine statistical significance. Survival curves were plotted using the Kaplan-Meier method, and statistical significance was determined by the log-rank (Mantel-Cox) test. GraphPad Prism 8.3 (San Diego, CA) was used to plot the data and to determine the statistical significance.

References

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2. Jurlander B, Thim L, Klausen NK, et al. Recombinant activated factor VII (rFVIIa): characterization, manufacturing, and clinical development. *Semin Thromb Hemost*. 2001;27(4):373-384.
3. Li W, Zheng X, Gu J, et al. Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin. *J Thromb Haemost*. 2005;3(7):1351-1359.
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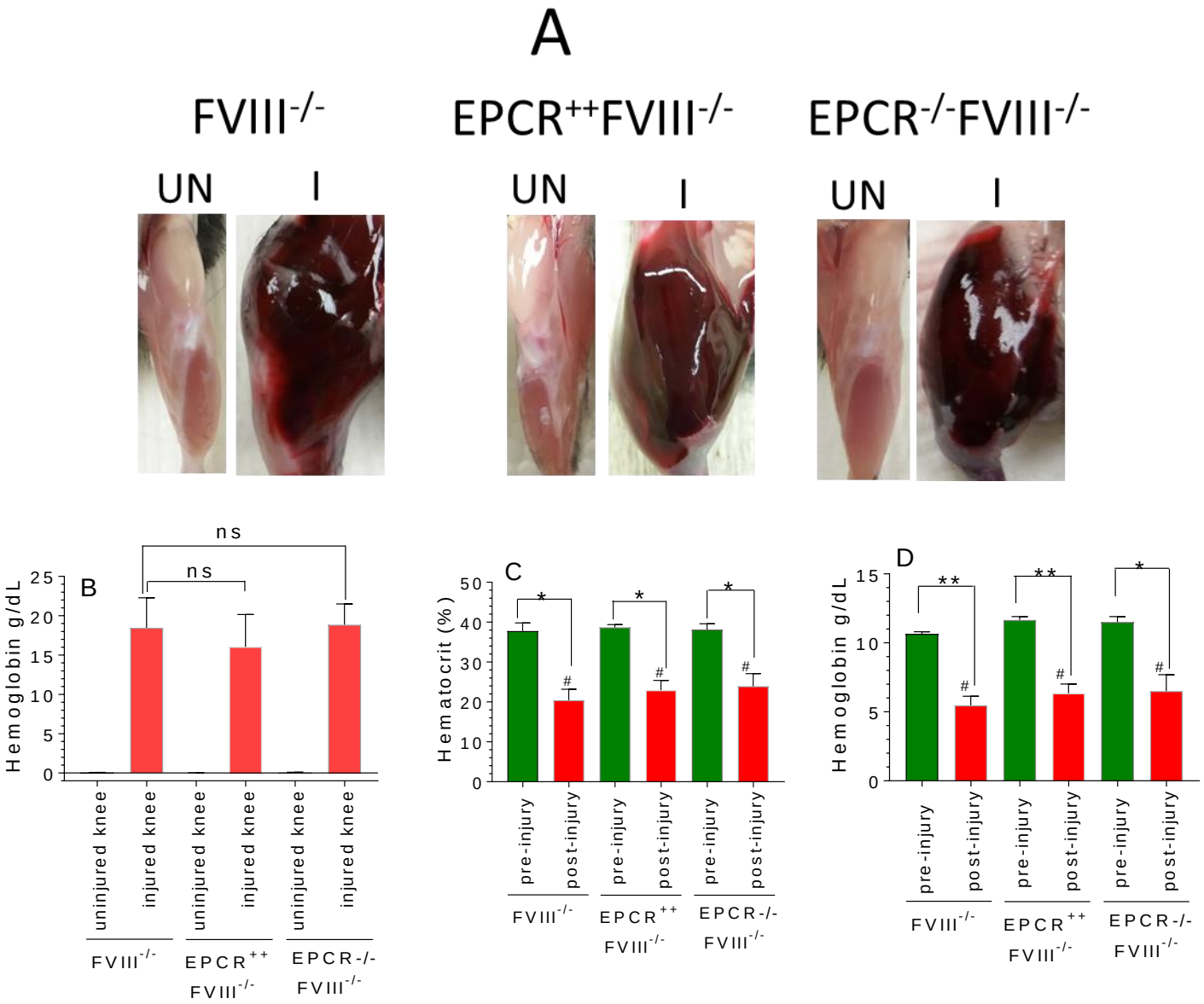
5. Hakobyan N, Enockson C, Cole AA, Sumner DR, Valentino LA. Experimental haemophilic arthropathy in a mouse model of a massive haemarthrosis: gross, radiological and histological changes. *Haemophilia*. 2008;14(4):804-809.
6. Sun J, Hakobyan N, Valentino LA, Feldman BL, Samulski RJ, Monahan PE. Intraarticular factor IX protein or gene replacement protects against development of hemophilic synovitis in the absence of circulating factor IX. *Blood*. 2008;112(12):4532-4541.
7. Ovlisen K, Kristensen AT, Jensen AL, Tranholm M. IL-1 beta, IL-6, KC and MCP-1 are elevated in synovial fluid from haemophilic mice with experimentally induced haemarthrosis. *Haemophilia*. 2009;15(3):802-810.

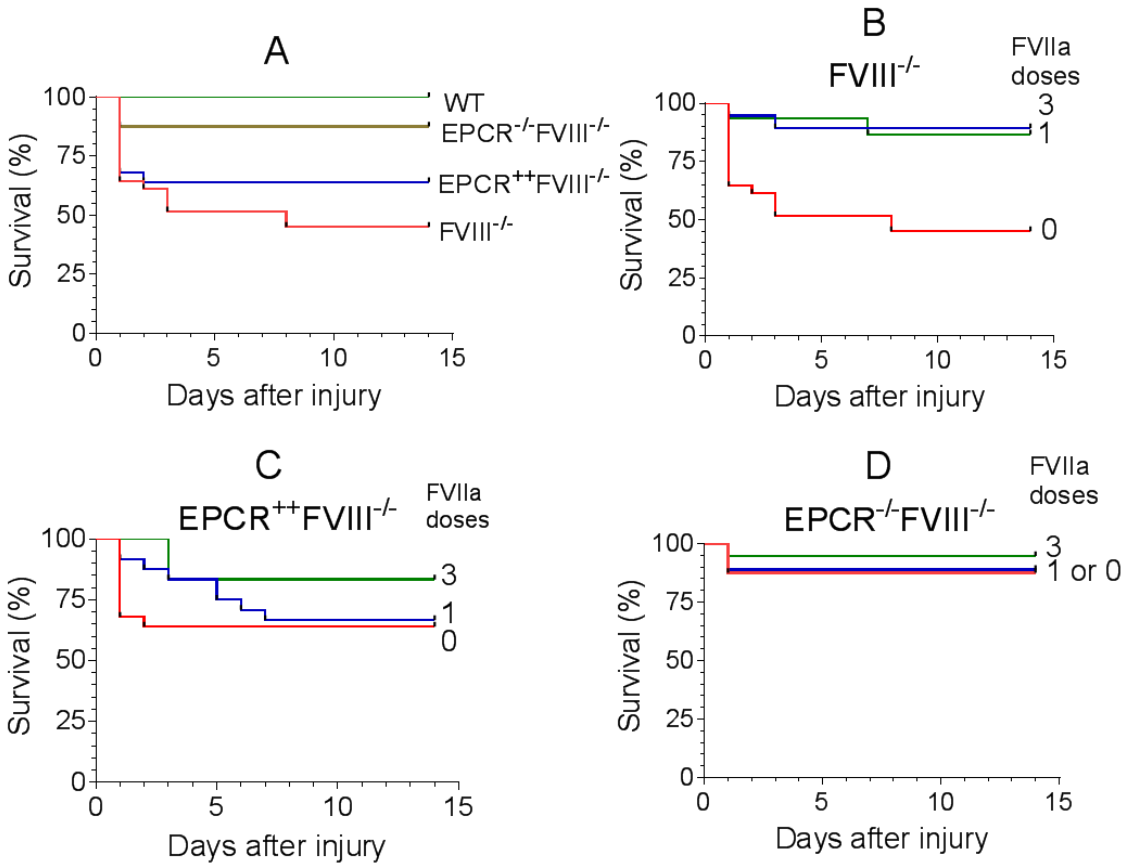
Table 1. Grading scheme for the visual bleeding score, hemophilic synovitis, macrophage invasion and iron deposition in the synovium following hemarthrosis

Characteristic	Score	Description
Visual blood score	0	Absent
	1	Presence of blood
	2	Distended but not tense, presence of blood
	3	Tense and distended, presence of blood
Synovitis score		
Synovial hyperplasia	0	Normal, less than four layers
	1	Four to five layers
	2	Six to seven layers
	3	More than seven layers
Neoangiogenesis	0	None
	1	Less than 10 vessels count
	2	10 to 25 vessels count
	3	More than 25 vessels
Discoloration by hemosiderin	0	Absent
	1	Present
Blood or erythrocytes	0	Absent
	1	Present
Villus formation	0	Absent
	1	Present
Cartilage degeneration	0	Absence of cartilage degeneration
	1	Cartilage degeneration by loss of proteoglycan content and pannus formation
	2	Cartilage degeneration by loss of proteoglycan content, pannus formation, and femur remodeling
Maximum total score	**11**	
Macrophages score	0	Absence of macrophage infiltration
	1	Scattered macrophages
	2	Line of macrophages
	3	Clusters of macrophages
	4	Sheets of macrophages
Iron score	0	No iron or Prussian blue color
	1	Weekly positive
	2	Positive
	3	Strongly positive

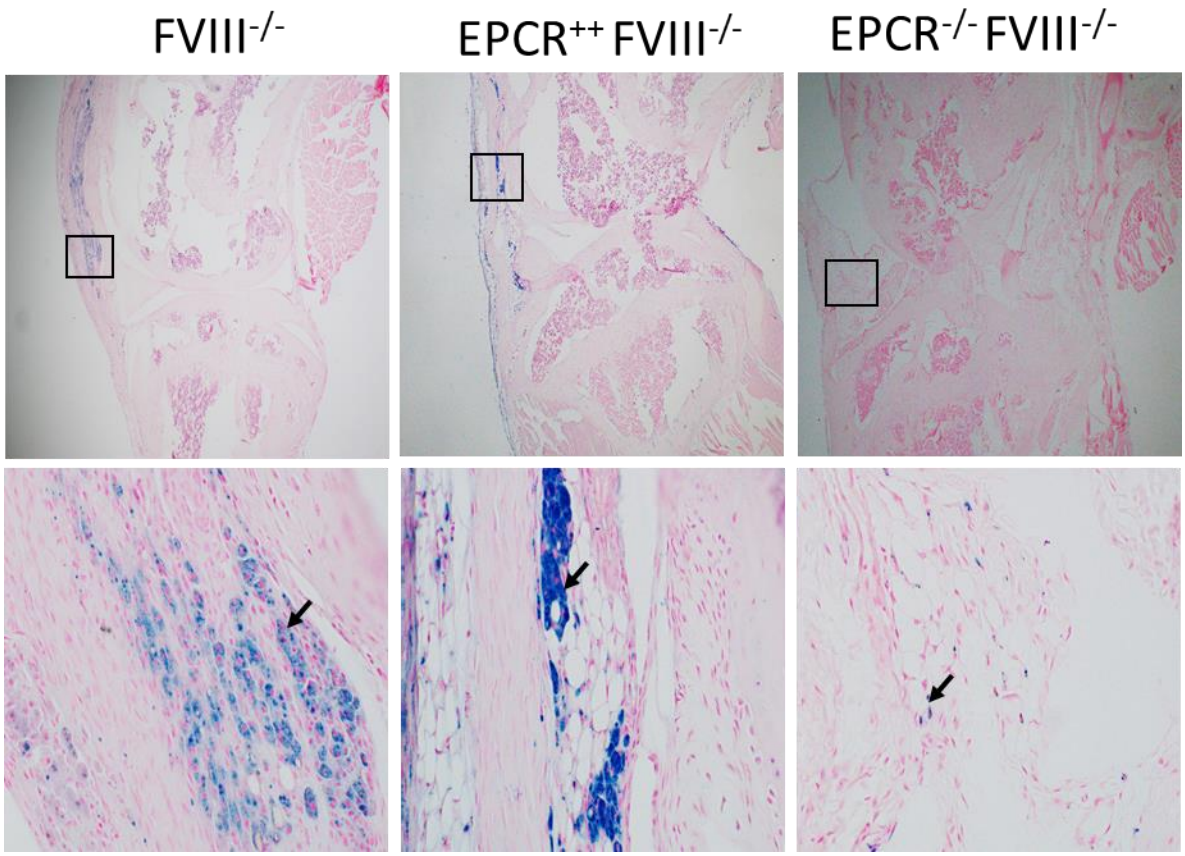
Supplemental Figure 1. Assessment of joint bleeding in FVIII$^{-/-}$, EPCR^{++}FVIII$^{-/-}$, and EPCR$^{-/-}$FVIII$^{-/-}$ mice following the needle puncture of hind limb joints (knee joints). Mice were anesthetized with ketamine/xylazine, and the right knee joint capsule was punctured with a 30 x 0.5-g needle below the patella to induce bleeding in the joint (I). The left knee joint of the same animal served as uninjured control (UN). Five hours following the injury, the mice were euthanized, and the skin was removed over the knee joints. The knee joints were photographed, and joint tissues were processed to extract hemoglobin. Prior to euthanization, peripheral blood was drawn to measure hematocrit and hemoglobin content. (A) Representative photographs of uninjured and injured knees; (B) Hemoglobin content extracted from joint tissues; (C) Hematocrit of peripheral blood; (D) Hemoglobin levels in peripheral blood. ns denotes not statistically significant difference; *, p<0.05; **, p<0.01; #, no statistical significant differences among injured groups (n = 3 to 7 mice/group).



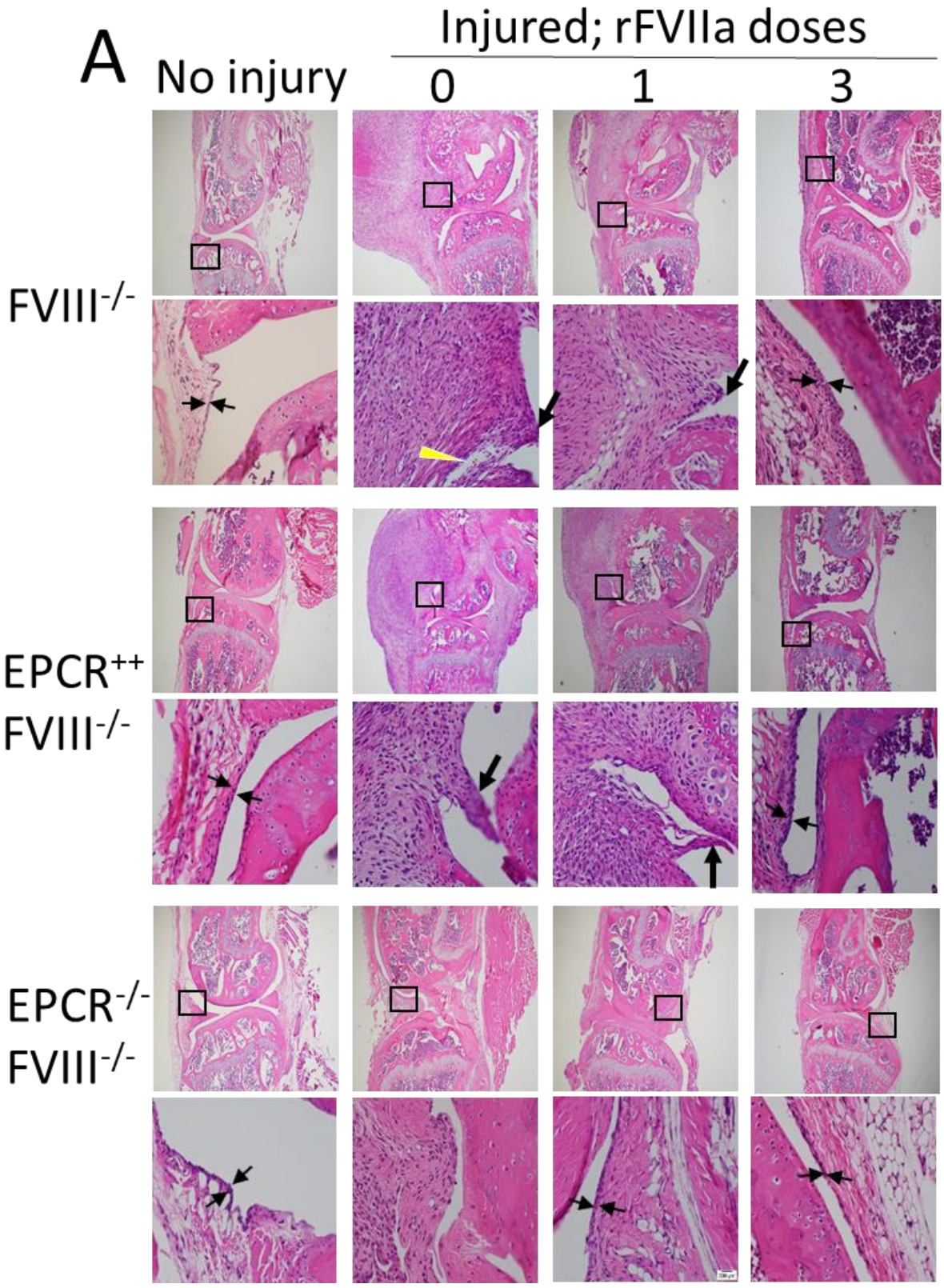
Supplemental Figure 2. EPCR deficiency protects against mortality following joint bleeding in hemophilia A mice. (A) Joint bleeding in $FVIII^{-/-}$, $EPCR^{++}FVIII^{-/-}$, and $EPCR^{-/-}FVIII^{-/-}$ was induced by needle puncture, and the survival of mice was monitored for 14 days. $FVIII^{-/-}$ (B), $EPCR^{++}FVIII^{-/-}$ (C), and $EPCR^{-/-}FVIII^{-/-}$ (D) mice were left untreated (0) or treated with a single dose (20 min after the injury, 1) or three doses (20 min, 1 day, and 3 days following the injury, 3) with rFVIIa (1 mg/Kg) mice. The survival was monitored for 14 days (n = 12 to 31 mice/group).

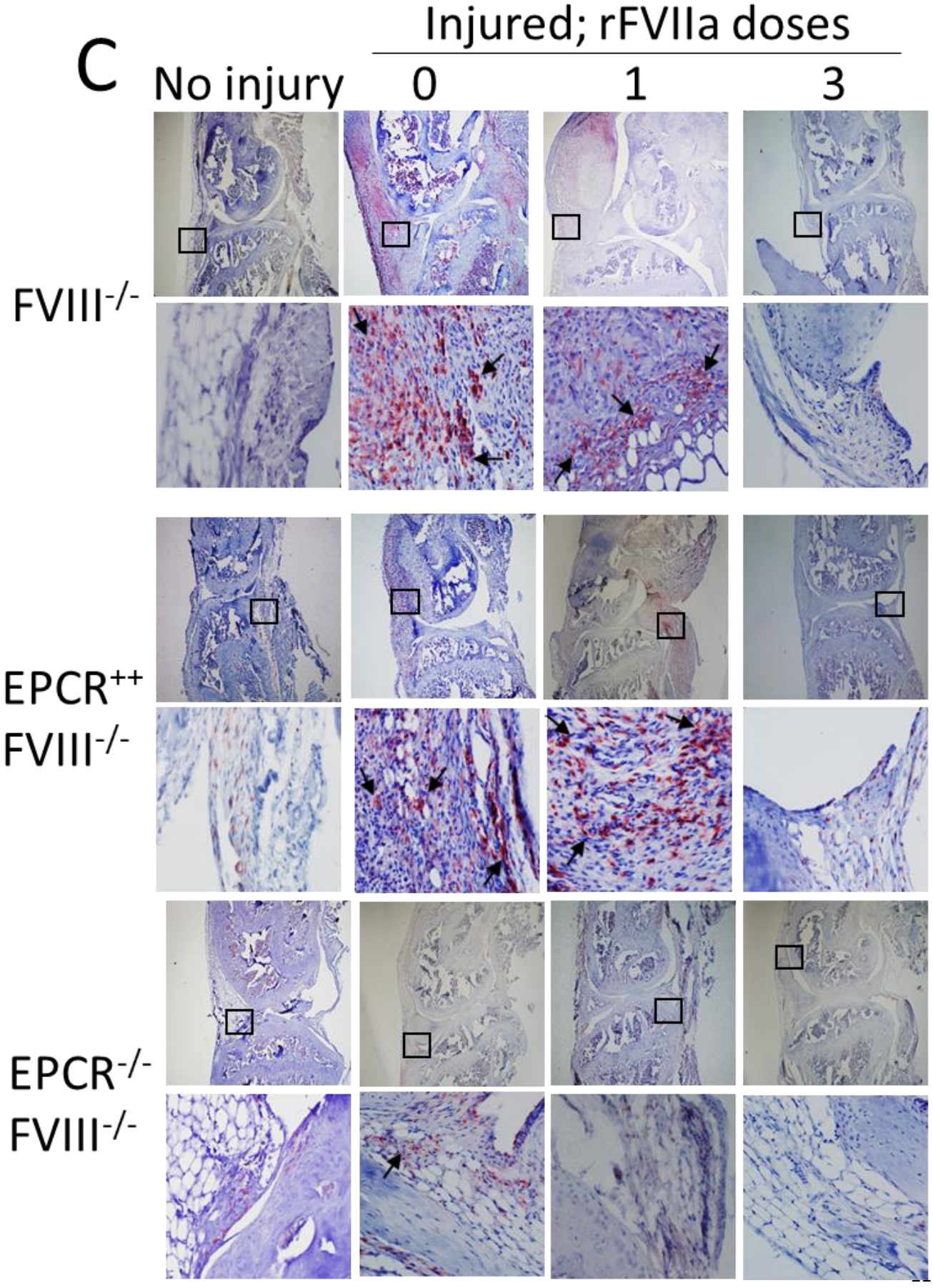


Supplemental Figure 3. Assessment of long-term joint bleeding in FVIII$^{-/-}$, EPCR^{++}FVIII$^{-/-}$, and EPCR$^{-/-}$FVIII$^{-/-}$ mice following the needle puncture injury. Joint tissues harvested 30 days following the injury were sectioned and stained with Prussian blue stain to detect iron deposits. Top panel sections show 4X magnification. The boxed area was imaged at 40X magnification (bottom panels). Arrow marks in bottom panels point out iron staining.

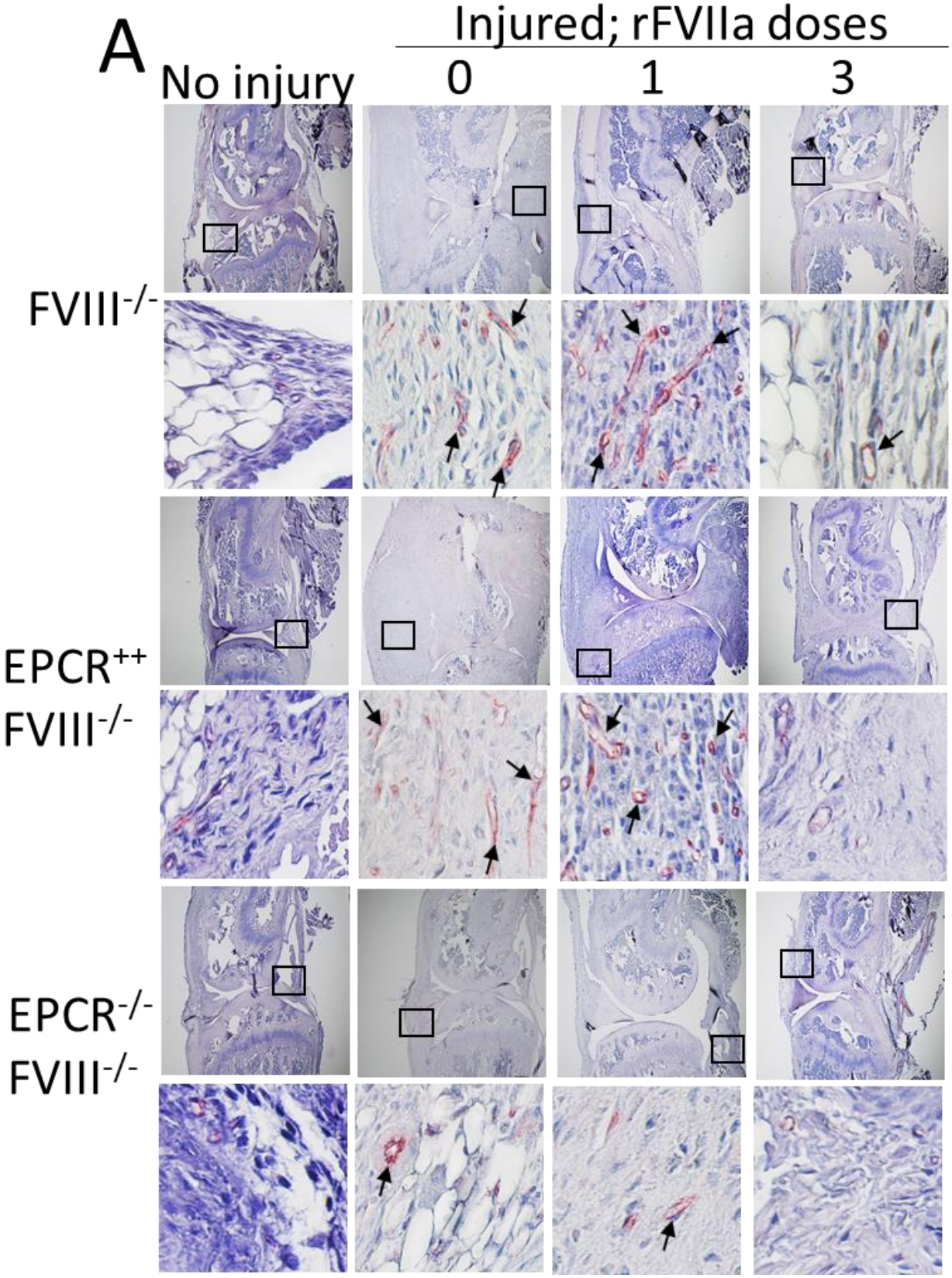


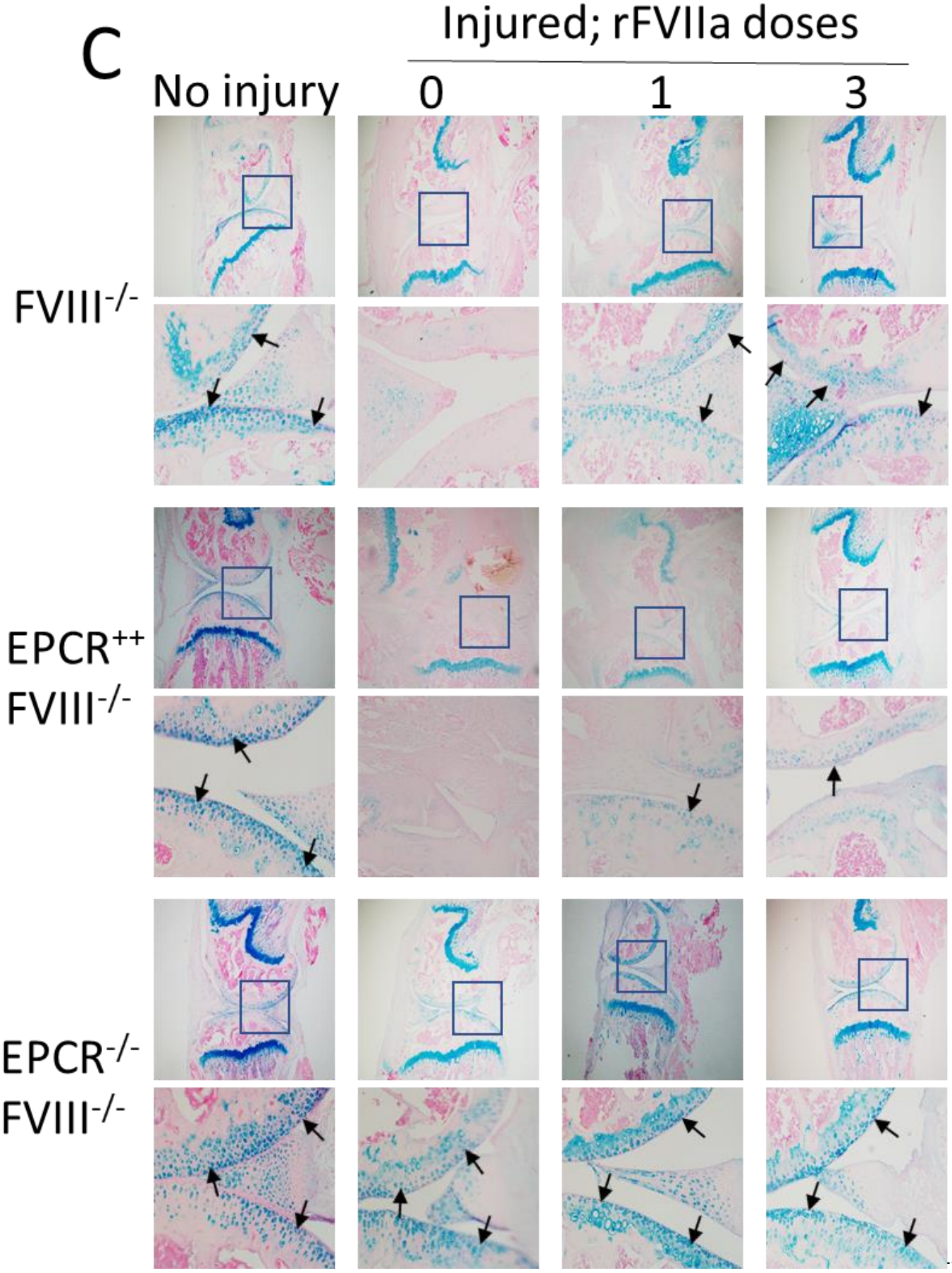
Supplemental Figure 4. Enlarged images of joint tissue sections stained with H&E or macrophage marker F4/80 (same as Figure 2A and 2C of the main article; see the main article for figure legend).



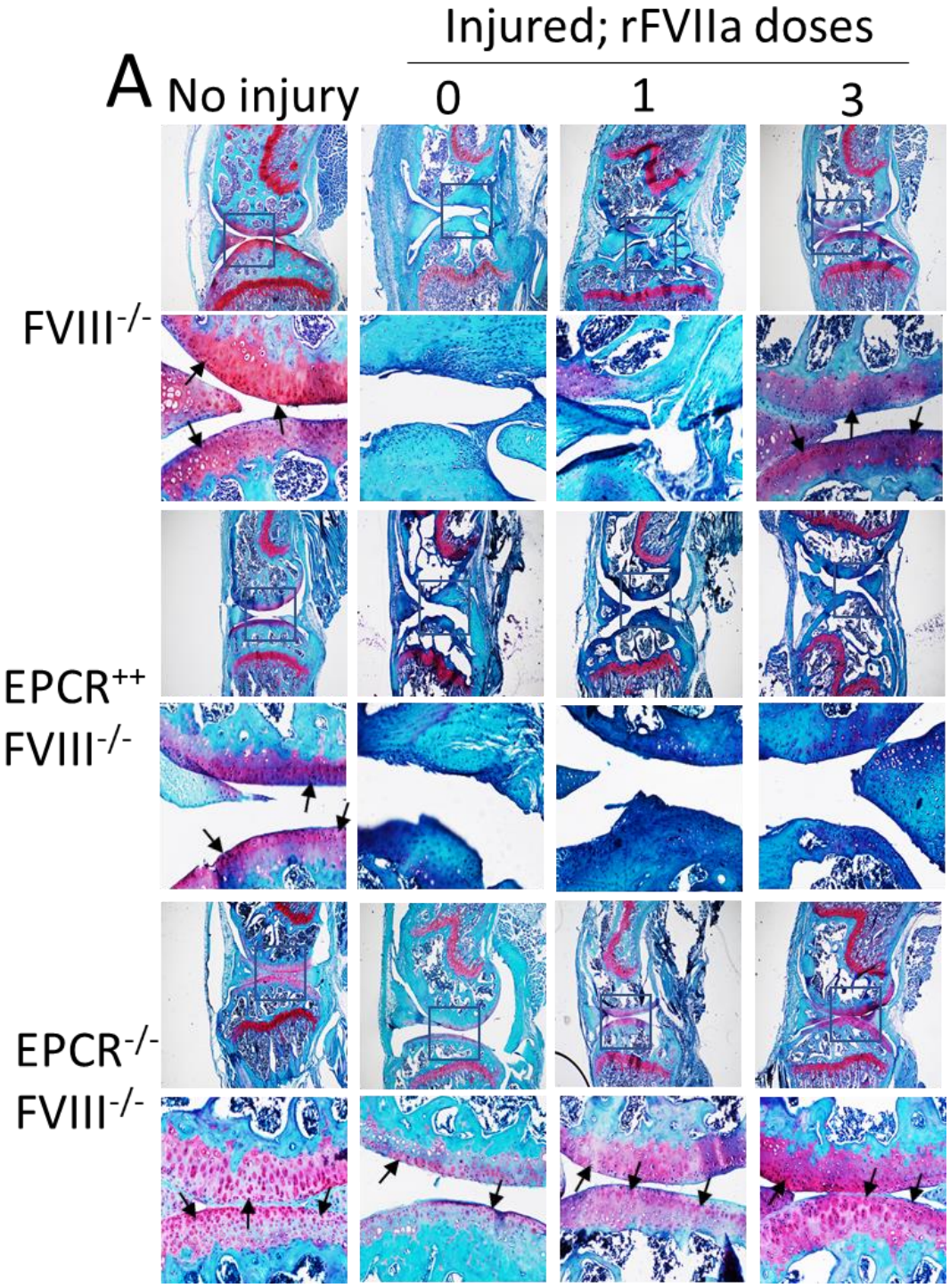

C
Injured; rFVIIa doses
No injury
0
1
3
FVIII-/-
EPCR++
FVIII-/-
EPCR-/-
FVIII-/-

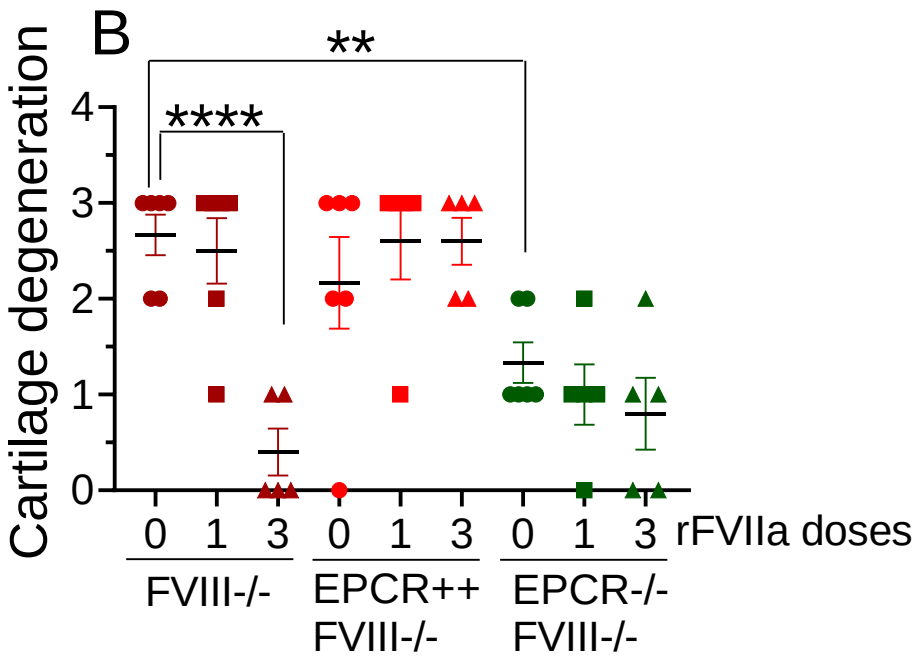
Supplemental Figure 5. Enlarged images of joint tissue sections stained with endothelial cell marker CD31 and Alcian blue (same as Figure 3A and 3C of the main article; see the main article for figure legend).




C
Injured; rFVIIa doses
No injury
0
1
3
FVIII-/-
EPCR++
FVIII-/-
EPCR-/-
FVIII-/-

Supplemental Figure 6. Evaluation of cartilage degeneration of joint tissue of FVIII$^{-/-}$, EPCR^{++}FVIII$^{-/-}$, and EPCR$^{-/-}$ FVIII$^{-/-}$ mice two weeks after needle puncture-induced joint bleeding by Safranin O/fast green staining. Injury and treatment modalities were the same as described in Supplemental Figure 1. (A) representative images. Top panel images, 4x magnification. The squared area was imaged at 40x magnification (bottom panel). Intact articular cartilage stains red. A loss or decrease in red staining indicates cartilage degeneration. Arrows point out the articular cartilage. (B) Quantification of cartilage denegation. The intensity of red staining in the articular cartilage was scored in 0-3 scale (0, normal/bright red staining of cartilage; 1, a slight reduction in staining; 2, a moderate reduction in staining; and 3, severe reduction/complete loss of staining).




B
**
****
Cartilage degeneration
4
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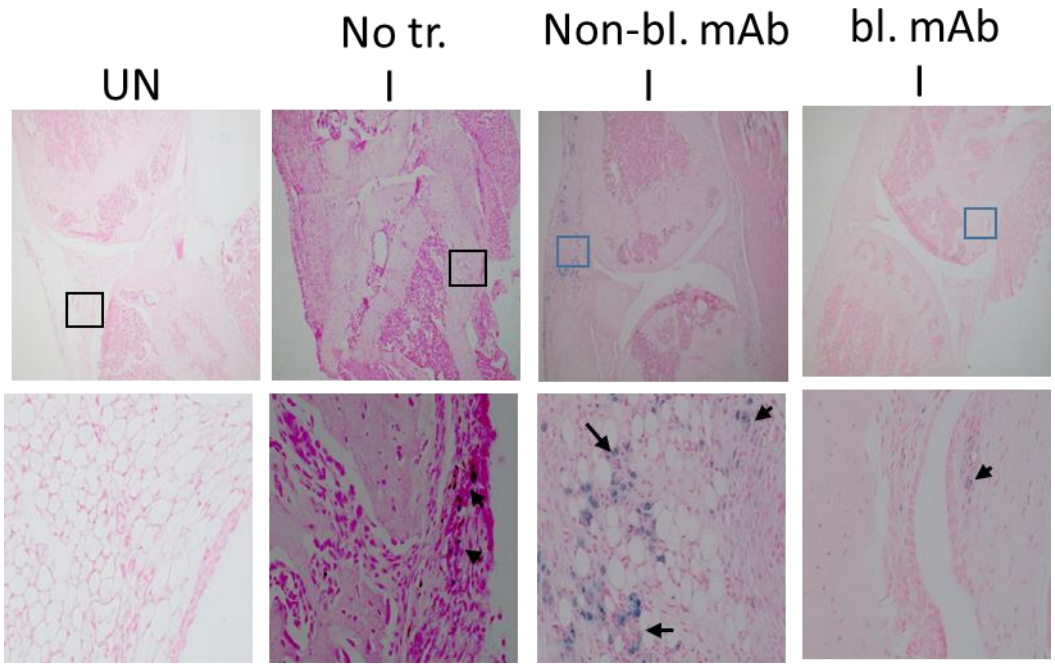
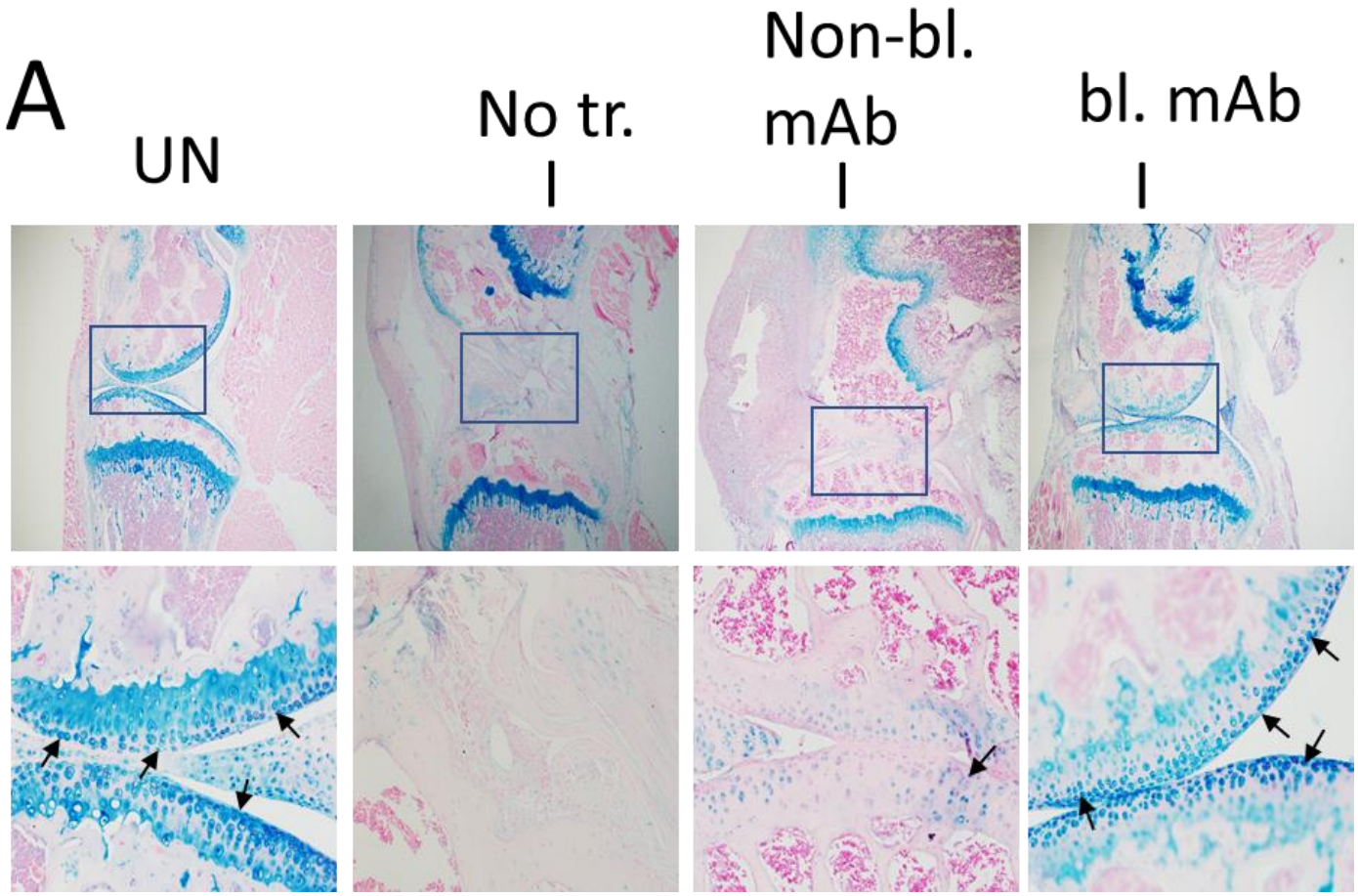
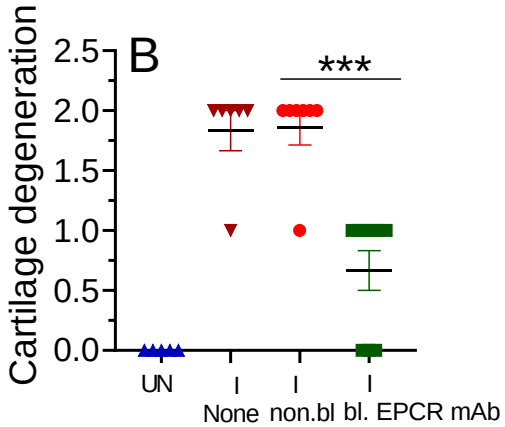
0 1 3 0 1 3 0 1 3
rFVIIa doses
FVIII-/-
EPCR++
FVIII-/-
EPCR-/-
FVIII-/-

Supplemental Figure 7. EPCR blocking mAb treatment reduces joint bleeding in FVIII$^{-/-}$ mice. FVIII$^{-/-}$mice were administered with EPCR non-blocking, EPCR blocking mAb (1 mg/kg), or none. Twenty-four hours later, joint bleeding was induced by needle puncture. Fourteen days following the injury, the knee joint was excised, sectioned, and stained with Prussian blue to detect iron. Arrow marks point iron deposits. Please note that iron deposition at 14 days following the injury was not as prominent as observed at 30 days following the injury, as shown in Supplemental Figure 3. UN, uninjured; I, injured; No tr., no treatment; Non-bl. mAb, EPCR non-blocking mAb; bl. mAb, EPCR blocking mAb.



Supplemental Figure 8. EPCR blocking prevents the degeneration of cartilage in FVIII$^{-/-}$ mice following joint bleeding. FVIII$^{-/-}$ mice were administered with EPCR blocking (bl.), EPCR non-blocking (non-bl.) mAb, or none. Twenty-four hours later, joint bleeding was induced with needle puncture injury (I). UN indicates an uninjured knee. Two weeks after the injury, mice were euthanized, knee joints were excised, and joint tissue sections were stained Alcian blue stain. (A) Representative images. Top panel, 4X magnification; bottom panel, 40X magnification of the squared area shown in the top panel. Arrows point out the articular cartilage. Loss of blue staining indicates cartilage degeneration (loss of glycosaminoglycans/proteoglycans). (B) Cartilage degeneration was quantified in a 0 to 2 scale based on the intensity of proteoglycan staining (blue color) in the articular cartilage region.





Supplemental Figure 9. Schematic representation of the pathogenesis of hemophilic arthropathy: A novel (EPCR blocking) treatment option. The deficiency of FVIII or IX protein or function in hemophilia prevents the generation of factor Xa that leads to the downregulation of prothrombinase and defective thrombin generation. However, it in itself may not lead to spontaneous joint bleeding as traces of FXa could be formed independently of FVIII and FIX, which might be sufficient to generate enough thrombin to sustain hemostasis and prevent spontaneous joint bleeding in hemophilia. But traces of thrombin formed could also drive EPCR-mediated activation of protein C, which leads to the inactivation of factor Va. This, in conjunction with the reduction in FXa generation in hemophilia, leads to severe defective thrombin generation that results in recurrent, spontaneous joint bleeding. Recurrent joint bleeding and the deposition of iron in the synovium induces many cellular processes, including synovial cell proliferation (hyperplasia), inflammation, neoangiogenesis, and breach in vascular integrity. These events together lead to cartilage destruction, bone remodeling, ultimately, joint destruction. Prophylactic treatment of hemophilia patients with clotting factor replacement (not shown in the figure) is a common mode of therapy to prevent the development of hemophilic arthropathy. rFVIIa, used as a bypassing agent in hemophiliacs with inhibitors, activates factor X, independent of FVIII and FIX, which results in increased prothrombinase activity that leads to the generation of sufficient thrombin to prevent spontaneous joint bleeding. FVIIa-EPCR-induced anti-inflammatory signaling and barrier stabilization may also inhibit the progression of hemophilic arthropathy. Alternatively, blocking the EPCR function could also enhance prothrombinase activity by curtailing APC generation and thus preventing APC inactivation of FVa. This is sufficient to generate enough thrombin to prevent joint bleeding and the development of hemophilic arthropathy. EPCR blockade could become a more attractive treatment option than current traditional options as it could work in all categories of hemophilia patients.