Supplemental Data

Mucosally-transplanted mesenchymal stem cells stimulate intestinal healing by promoting angiogenesis

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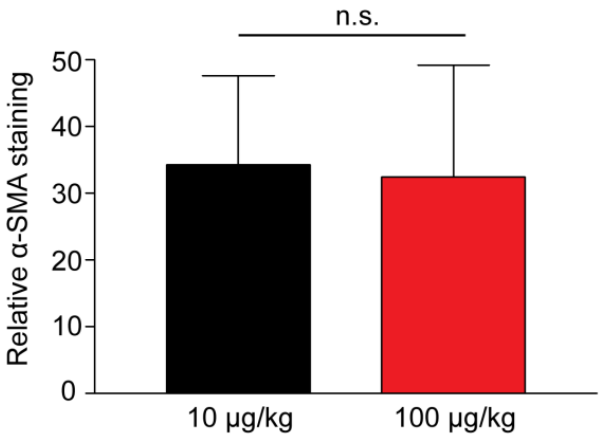
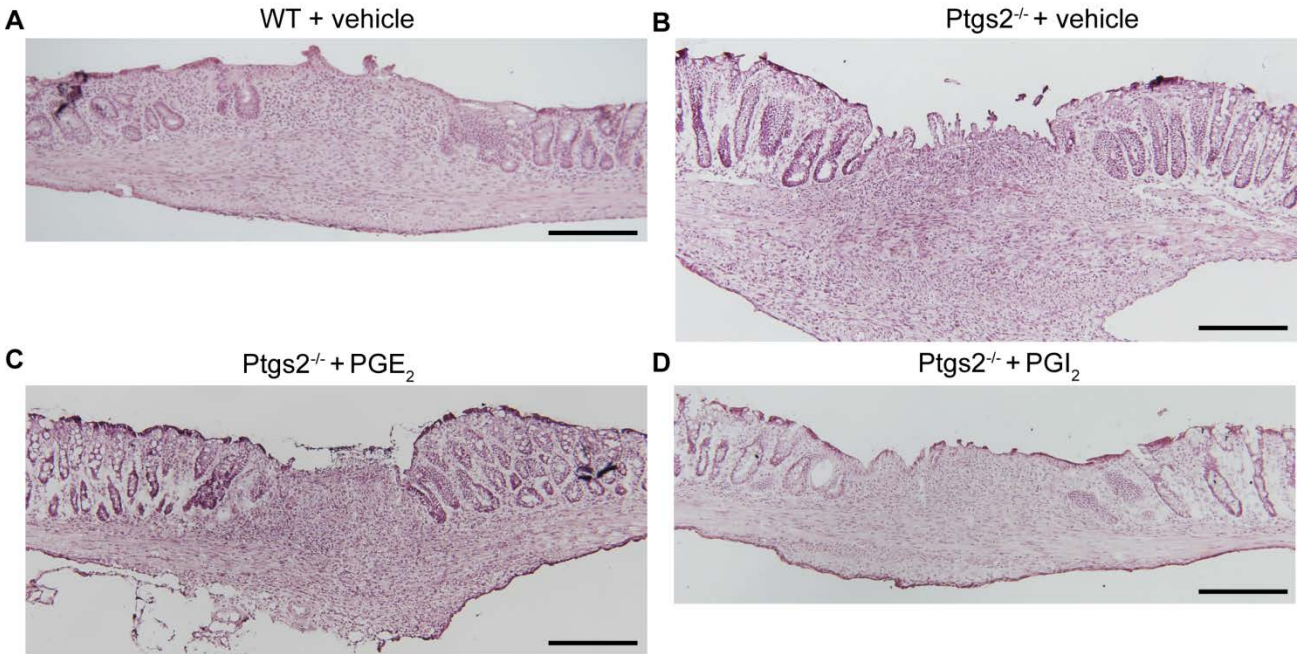
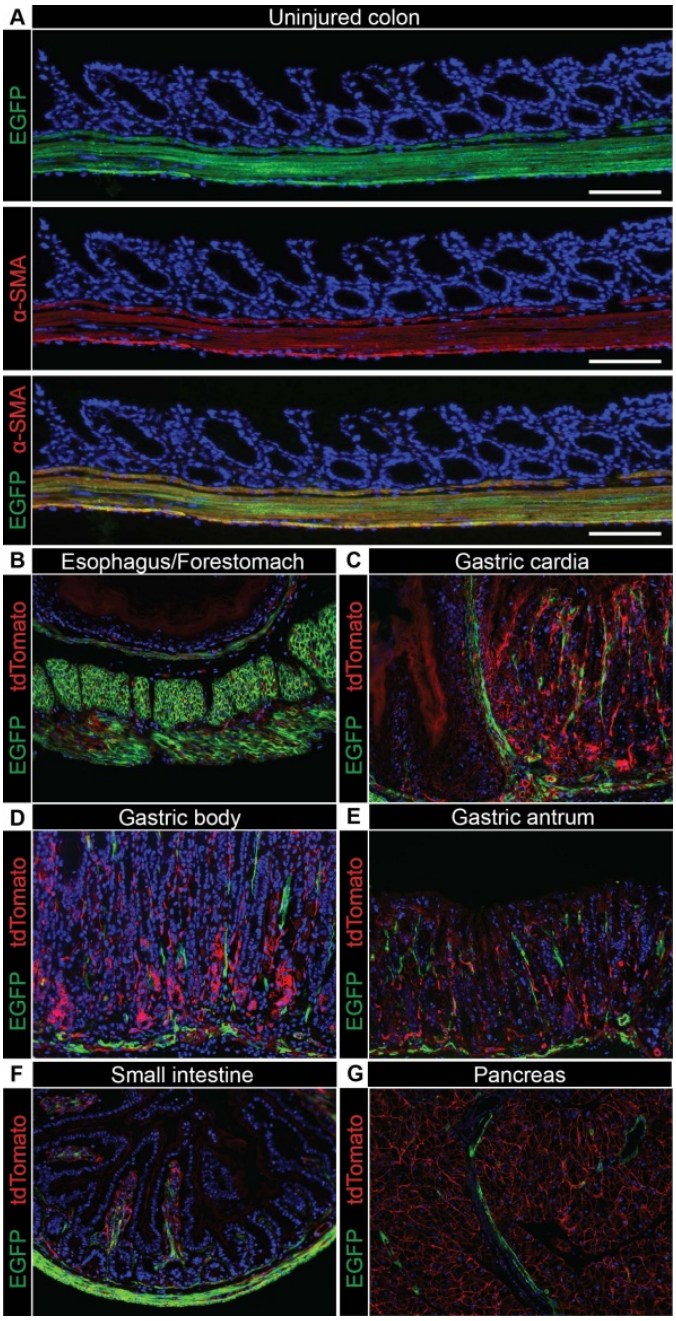
Supplementary Figures 1-18



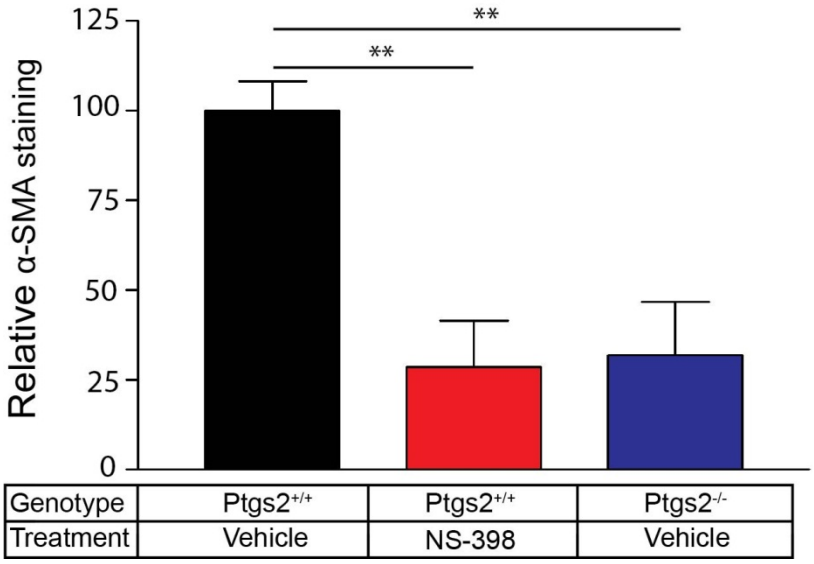
Supplemental Figure 1. Low and high dose PGE_2 analogs are insufficient to prevent loss of α-SMA staining after mucosal injury. Graph showing the relative α-SMA staining in the outer muscle layer underlying wounds from *Ptgs2*$^{-/-}$ mice given daily i.p. injections of the indicated dose of PGE_2 analogs. Values=mean ±SEM. *P* =0.93 by Student's *t*-test, n.s. indicated not significant. *n*=9 wounds per group from 3 mice per group in 3 experiments.



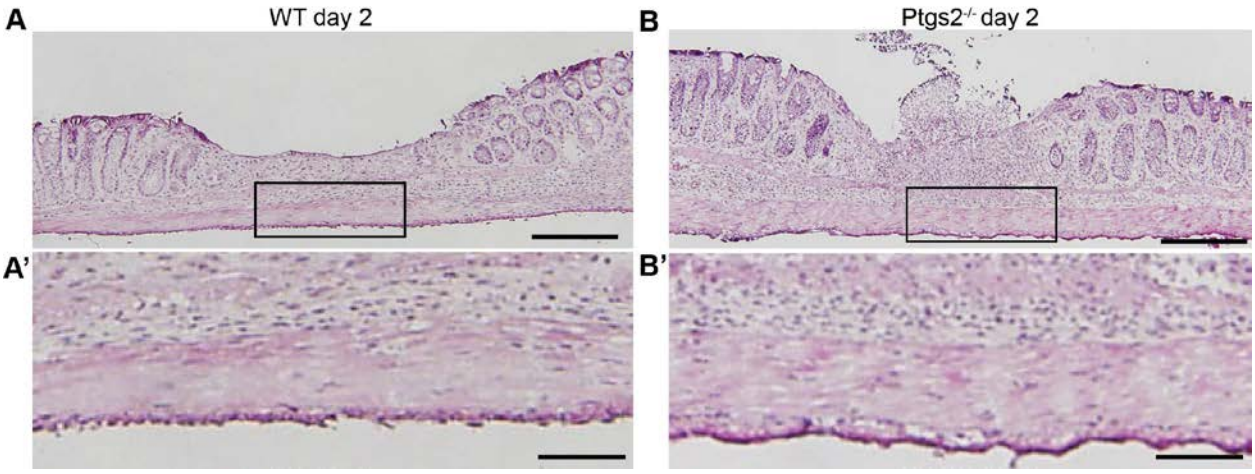
Supplemental Figure 2. PGI_2 stable analogs rescue the smooth muscle defect in injured *Ptgs2*$^{-/-}$ mice. (A-D) Representative H&E colonic sections (*n*=9 wounds per group from 3 mice per group in 3 experiments) from WT (**A**) or *Ptgs2*$^{-/-}$mice (**B-D**) treated with vehicle (**A-B**), PGE_2 analogs (**C**), or PGI_2 analogs (**D**) six days post-injury. Bars=250µm.



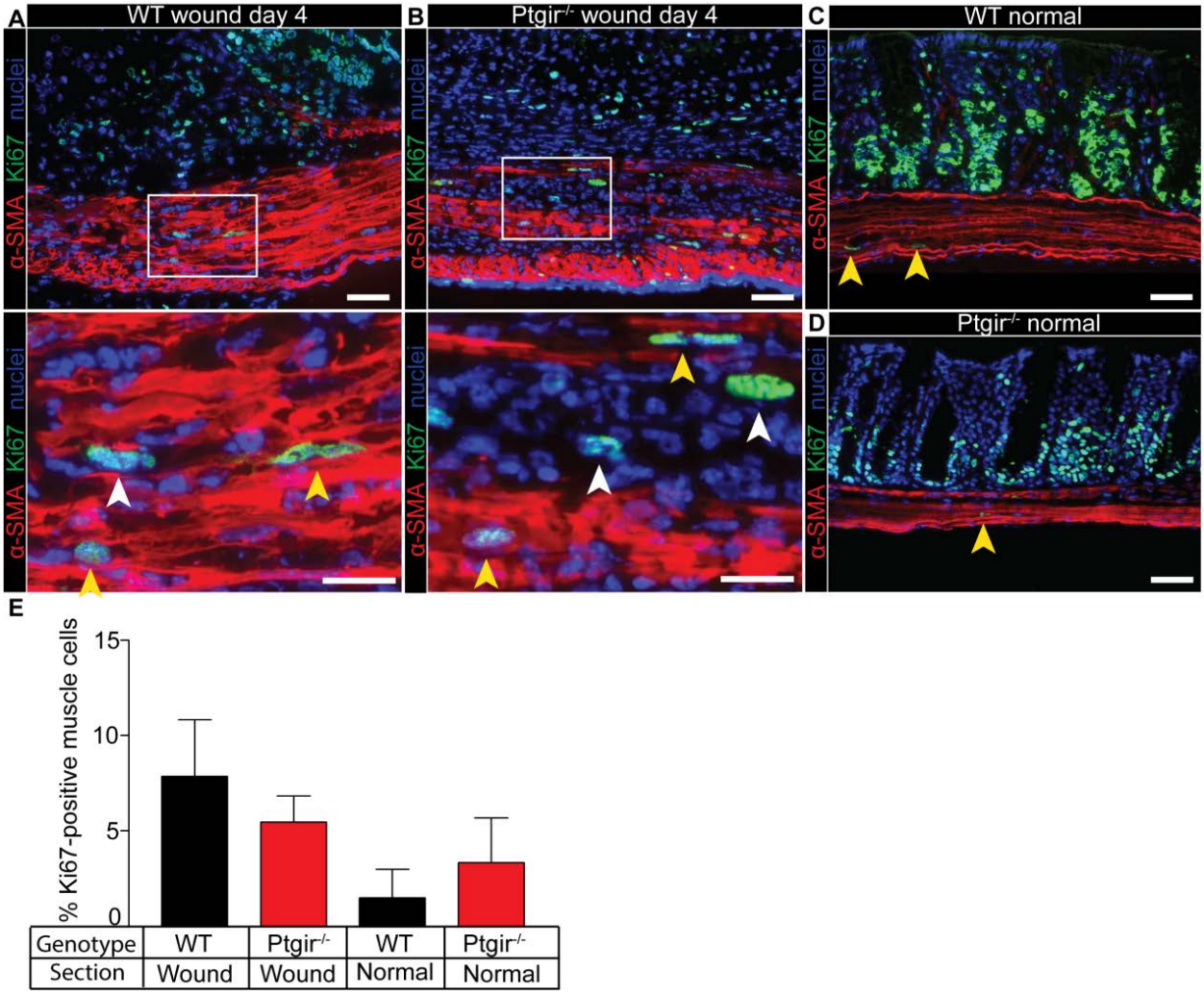
Supplemental Figure 3. **Smooth muscle cells are labeled throughout the gastrointestinal tract using *Acta2-CreER^{T};R26$^{mT/mG}$* mice.** (**A**) Representative section of uninjured colon (*n*=3 wounds from 3 mice in 3 experiments) from *Acta2-CreER^{T};R26$^{mT/mG}$* mice given one dose of tamoxifen two weeks before sacrifice stained with antisera against α-SMA (red) and bis-benzimide (blue, nuclei). Smooth muscle cells were permanently marked as green (top), and nearly all smooth muscle cells that were positive for α-SMA (red, middle) were also green and appeared yellow (bottom). Bars=100μm. (**B-G**) Representative sections of indicated uninjured tissues (*n*=3 mice in 3 experiments) from *Acta2-CreER^{T};R26$^{mT/mG}$* mice given one dose tamoxifen and stained with bis-benzimide (blue, nuclei). The tdTomato is expressed in non-muscle cells, while the EGFP is expressed in *Acta2*-expressing muscle cells.



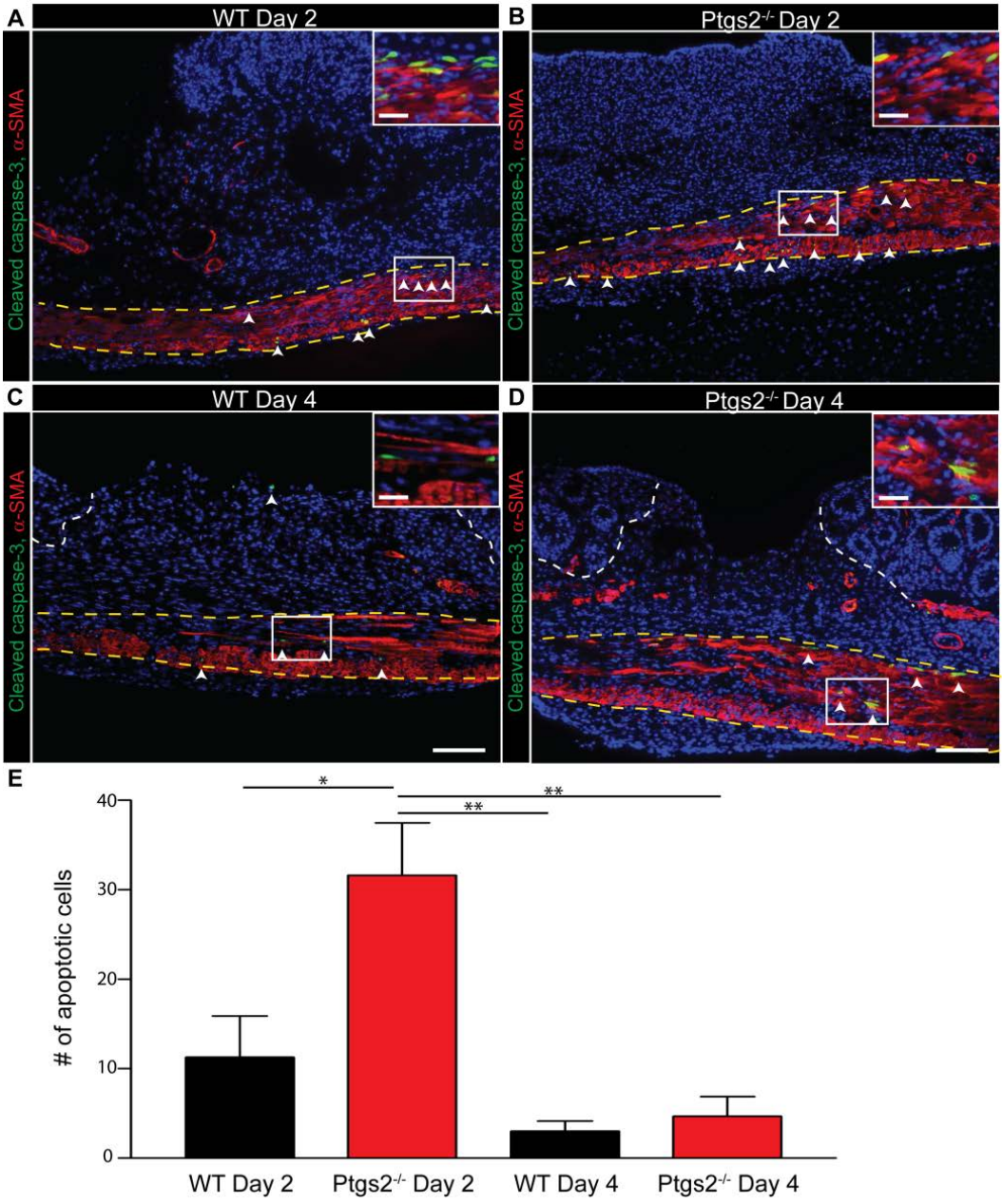
Supplemental Figure 4. **NS-398-treated mice have loss of α-SMA staining similar to *Ptgs2*$^{-/-}$ mice six days after colonic biopsy injury.** Graph of the relative α-SMA staining in the outer muscle layer underlying day six wounds from *Acta2-CreER*^{T};*R26*$^{mT/mG}$ mice (*Ptgs2*$^{+/+}$) treated with vehicle (*n*=6 wounds), *Acta2-CreER*^{T};*R26*$^{mT/mG}$ mice treated with NS-398 (*n*=6 wounds) or *Ptgs2*$^{-/-}$ mice treated with vehicle (*n*=8 wounds). Values=mean ±SEM. One-way ANOVA with *P*=0.0022, followed by Tukey's post-test, ***P*<0.01, *n*=3 mice per group in 3 experiments.



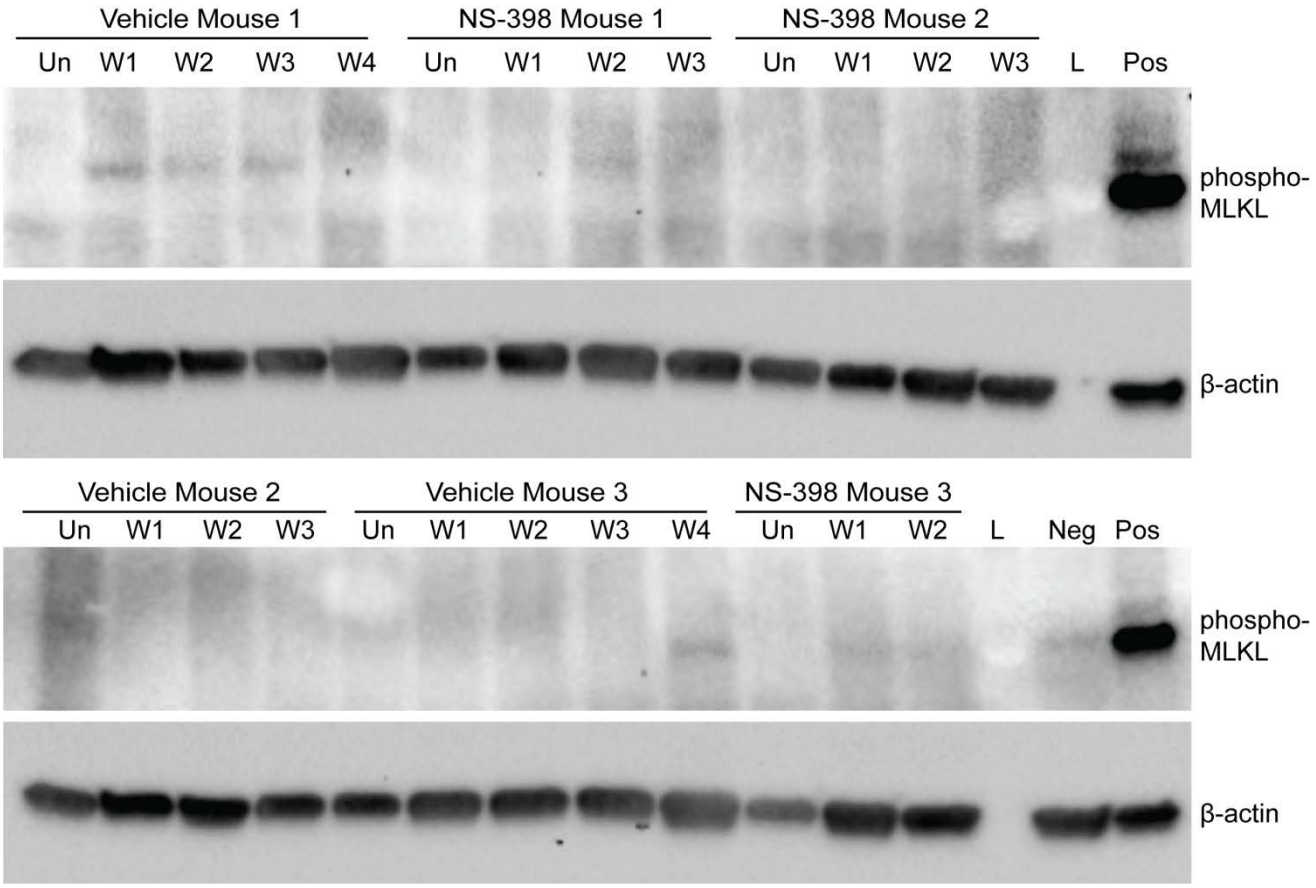
Supplemental Figure 5. **WT and *Ptgs2*$^{-/-}$ mice do not have necrotic smooth muscle layers two days after biopsy injury.** (**A**-**B**) Representative sections of colon (*n*=4 wounds per group from 2 mice per group in 2 experiments) from WT (**A**) and *Ptgs2*$^{-/-}$ mice (**B**) two days after colonic biopsy injury were stained with hematoxylin and eosin. No necrosis in the muscularis propria was observed in either genotype at this time point. Black box in (**A**) and (**B**) is the area for magnification in (**A'**) and (**B'**). Bars=200µm, inset bars=50µm.



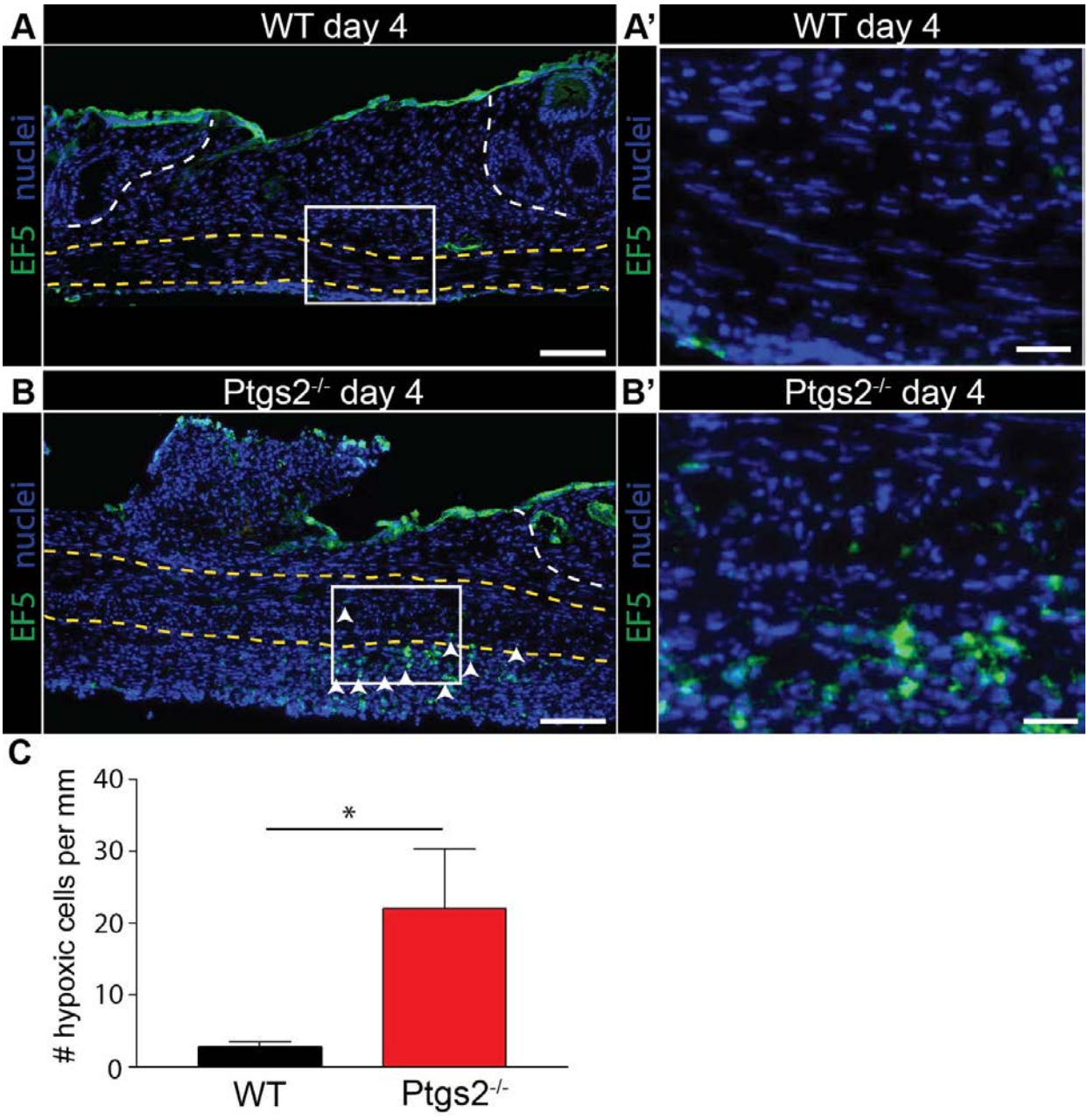
Supplemental Figure 6. WT and *Ptgir*$^{-/-}$ wounds have few proliferative smooth muscle cells four days post-injury. (**A-D**) Representative images of colonic sections (*n*=5 wounds per group from 2 mice per group in 2 experiments) from WT (**A** and **C**) and *Ptgir*$^{-/-}$ (**B** and **D**) mice four days post-injury in the area of the wound (**A** and **B**) and far from the wound (**C** and **D**). Sections were stained with anti-α-SMA antisera (red), anti-Ki67 antisera (green), and bis-benzimide (blue, nuclei). Solid white box indicates area for magnification below in **A** and **B**. Yellow arrowheads denote Ki67-positive smooth muscle cells, white arrowheads denote Ki67-positive, α-SMA-negative cells within the area of the muscularis propria. Bars=50μm and 100μm (insets). (**E**) Graph showing the percentage of α-SMA-positive smooth muscle cells in the muscularis propria that are Ki67-positive in wounded and normal regions in WT and *Ptgir*$^{-/-}$ mice. *P*=0.2298 by ANOVA. Values=mean ±SEM. *n*=5 wounds per group from 2 mice per group in 2 experiments.



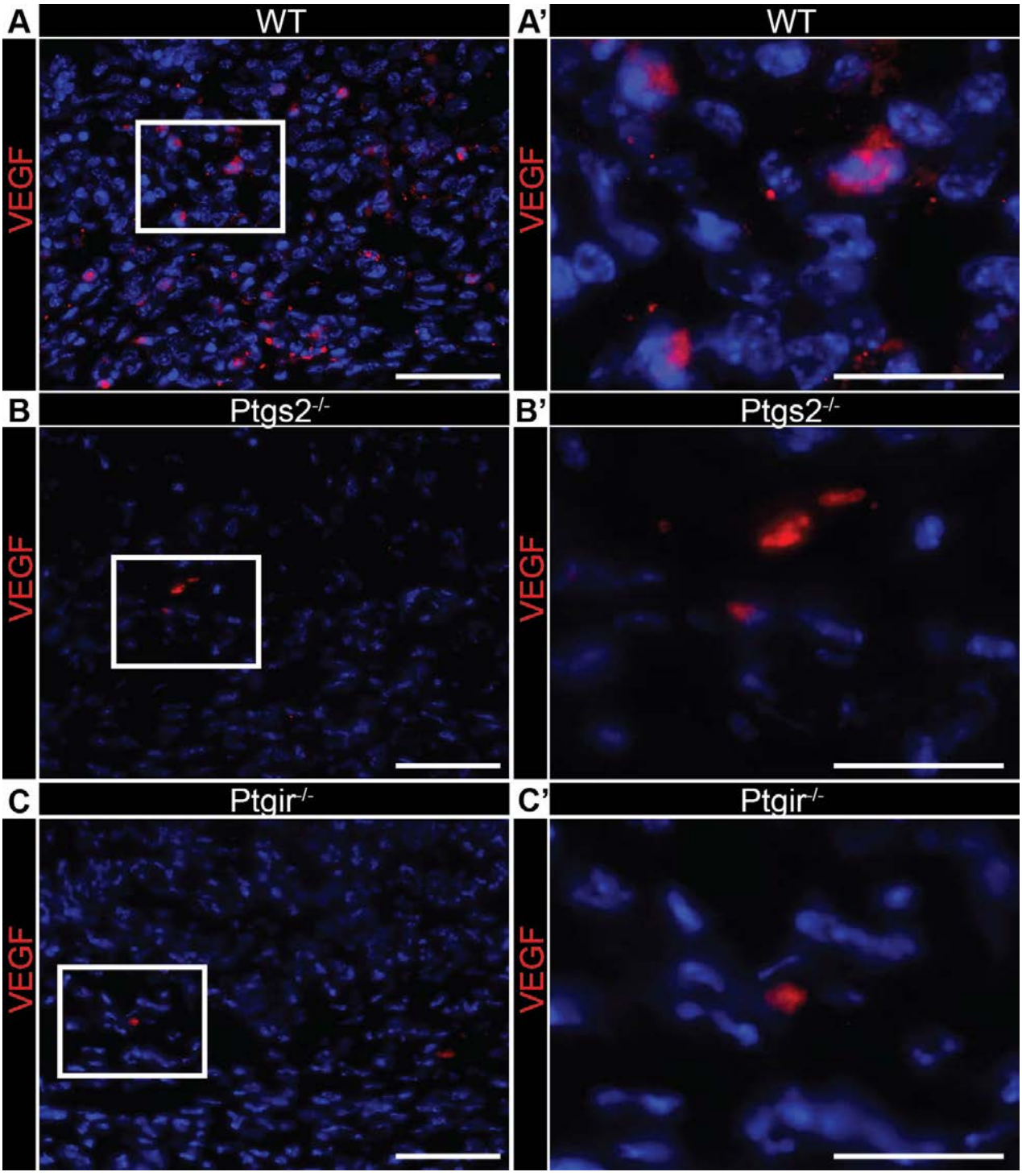
Supplemental Figure 7. WT and *Ptgs2*$^{-/-}$ mice have decreasing levels of caspase-3-positive cells in the wound bed over time. (**A**-**D**) Representative colonic sections (n=5-11 wounds per group from 3-4 mice per group in 3 experiments) from WT (**A** and **C**) and *Ptgs2*$^{-/-}$ mice (**B** and **D**) two (**A** and **B**) and four (**C** and **D**) days after colonic biopsy injury. Sections were stained with anti-cleaved caspase-3 antisera (green), anti-α-SMA antisera (red), and bis-benzimide (blue). Bars=100μm, inset bars=25μm. (**E**) Graph depicting the number of cleaved caspase-3-positive cells per wound two days post-injury in WT (n=9 wounds from 3 mice) and *Ptgs2*$^{-/-}$ (n=11 wounds from 4 mice) and four days post-injury in WT (n=5 wound from 3 mice) and *Ptgs2*$^{-/-}$ (n=6 wounds from 3 mice) mice. Values=mean ±SEM. P=0.0009 by one-way ANOVA, followed by Tukey's post-test, $^{**}P < 0.01$, $^{*}P<0.05$. n=3 experiments.



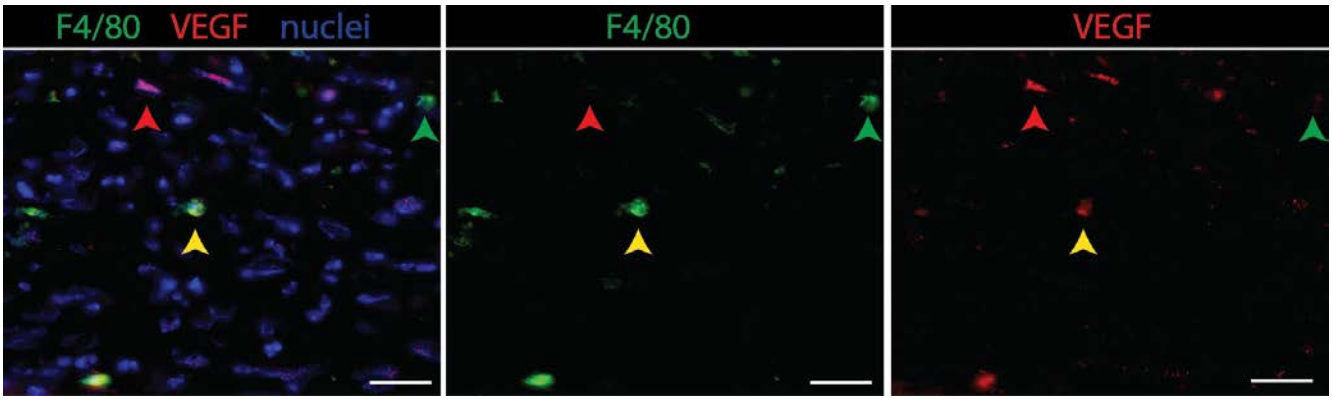
Supplemental Figure 8. Ptgs2 inhibition does not increase necroptosis in wounds four days post-injury. Immunoblot for phospho-MLKL in wounds from WT mice given vehicle or Ptgs2-inhibitor NS-398 four days post-injury. Un=Uninjured colon, W1-W4=Wound number for each mouse, L=Ladder for molecular weights, Pos=Positive control (L929 cells +Z-VAD +TNF-α +Smac mimetic), Neg=Negative control (L929 cells). *n*=11 wounds from 3 mice for vehicle treatment, *n*=8 wounds from 3 mice for NS-398 treatment. Immunoblots were probed for pMLKL first, then stripped, blocked and re-probed for actin.



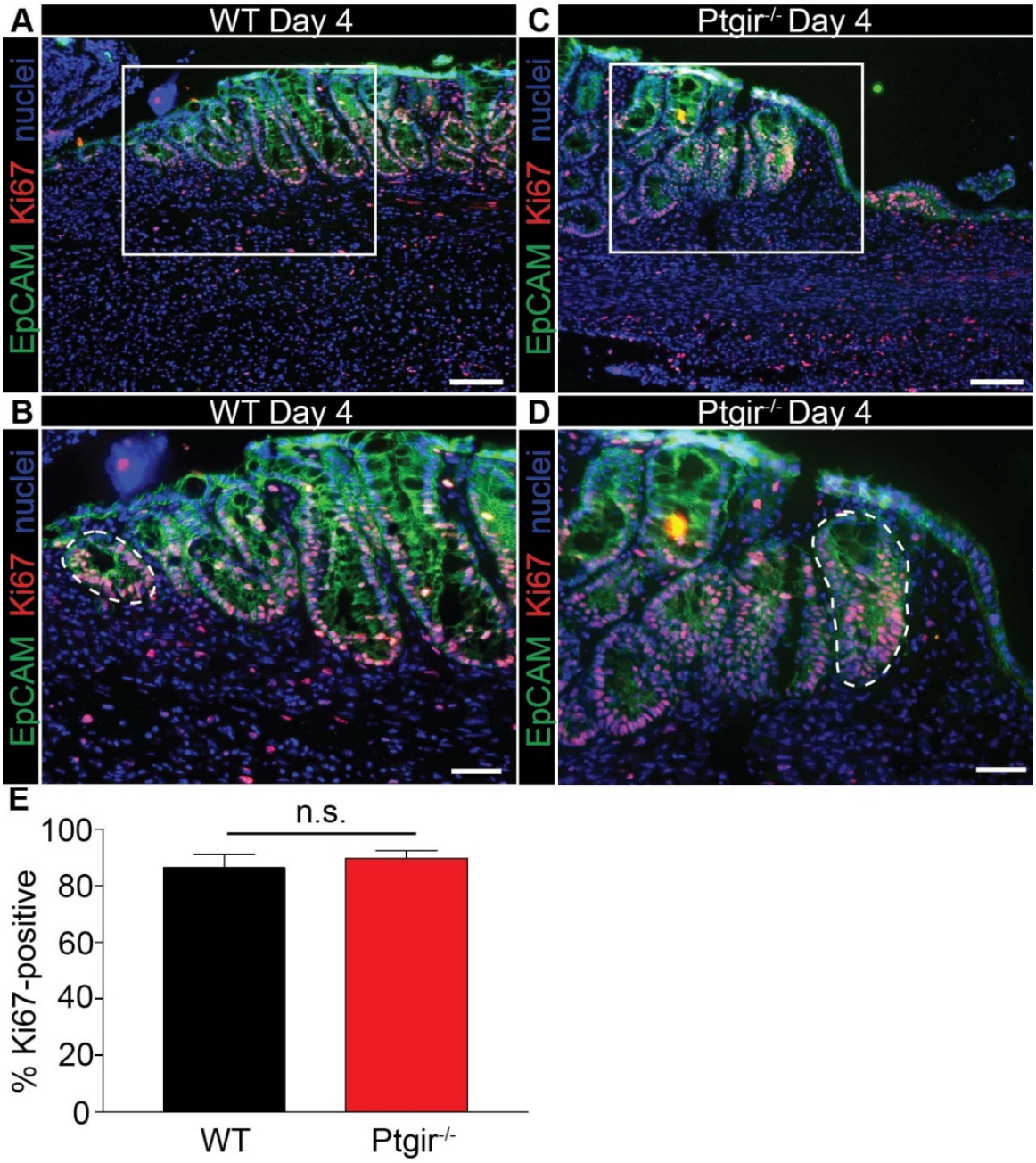
Supplemental Figure 9. ***Ptgs2*$^{-/-}$ mice have hypoxic cells in wound beds as detected by EF5 retention four days after biopsy injury.** (**A**-**B**) Representative sections of colon (*n*=5-8 wounds per group from 3-4 mice per group in 3 experiments) from WT (**A**) and *Ptgs2*$^{-/-}$ mice (**B**) four days after colonic biopsy injury were stained with anti-EF5 antisera (green) and bis-benzimide (blue). White arrowheads indicate numerous EF5-positive cells in *Ptgs2*$^{-/-}$ mice. White solid box indicates area for magnification in (**A'**) and (**B'**). Bars=100µm, inset bars=25µm. (**C**) Graph depicting the number of EF5 positive cells per wound bed length (mm) in WT (*n*=5 wounds from 3 mice) and *Ptgs2*$^{-/-}$ (*n*=8 wounds from 4 mice) mice four days after biopsy injury. *n*=3 experiments. Values=mean ±SEM. Student's *t*-test, **P*<0.05.



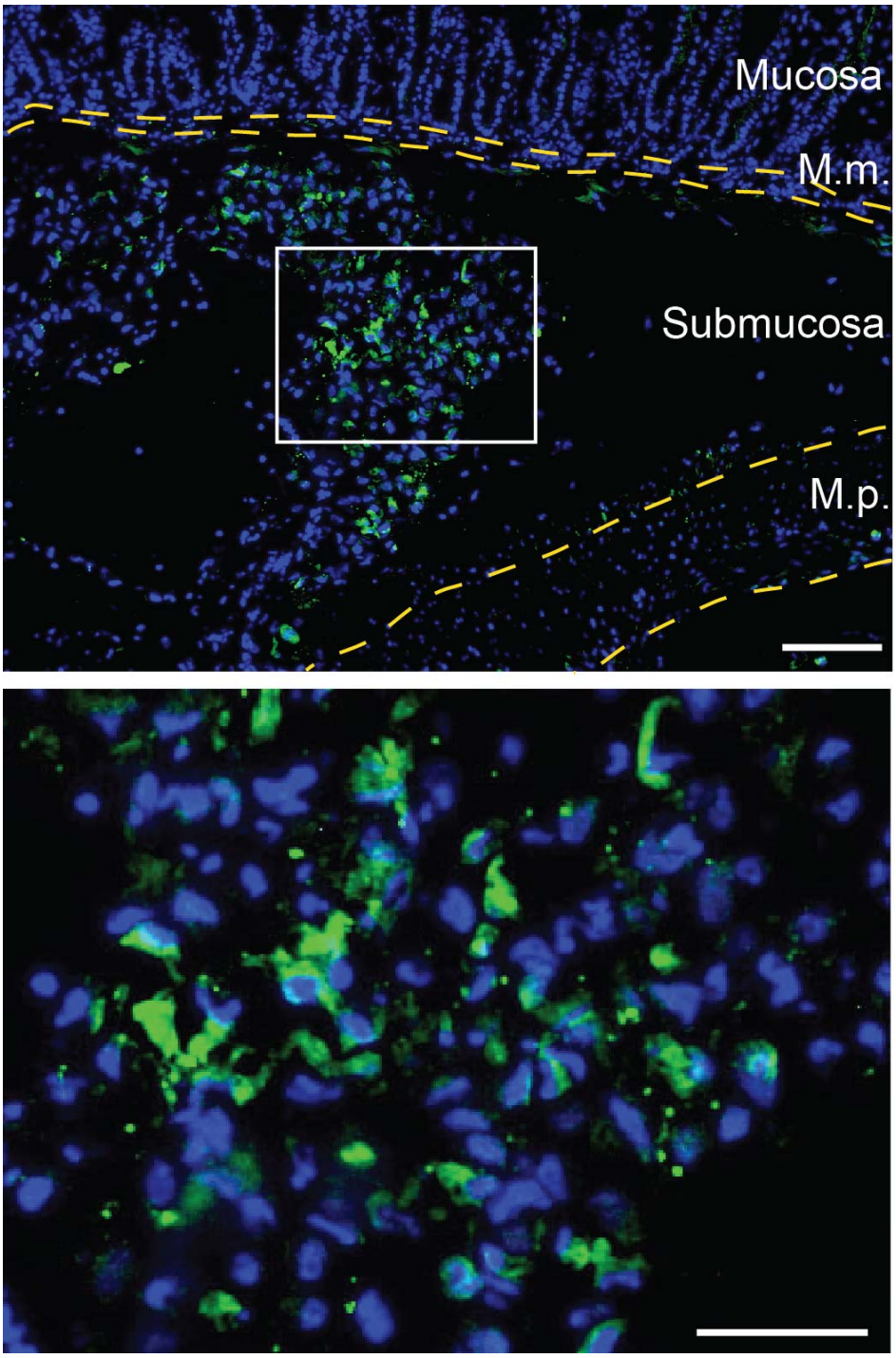
Supplemental Figure 10. *Ptgs2*$^{-/-}$ and *Ptgir*$^{-/-}$ wounds have fewer VEGF-positive cells four days post-injury compared to WT mice. (A-C) Representative images of colonic sections in 2 experiments from WT (*n*=5 wounds from 2 mice) (**A**), *Ptgs2*$^{-/-}$ (*n*=5 wounds from 2 mice) (**B**), and *Ptgir*$^{-/-}$ (*n*=7 wounds from 3 mice) (**C**) mice four days post-injury. Sections were stained with anti-VEGF antisera (red) and bis-benzimide (blue, nuclei). Solid white box indicates area for magnification to the right. Bars=50µm (insets) and 100µm.



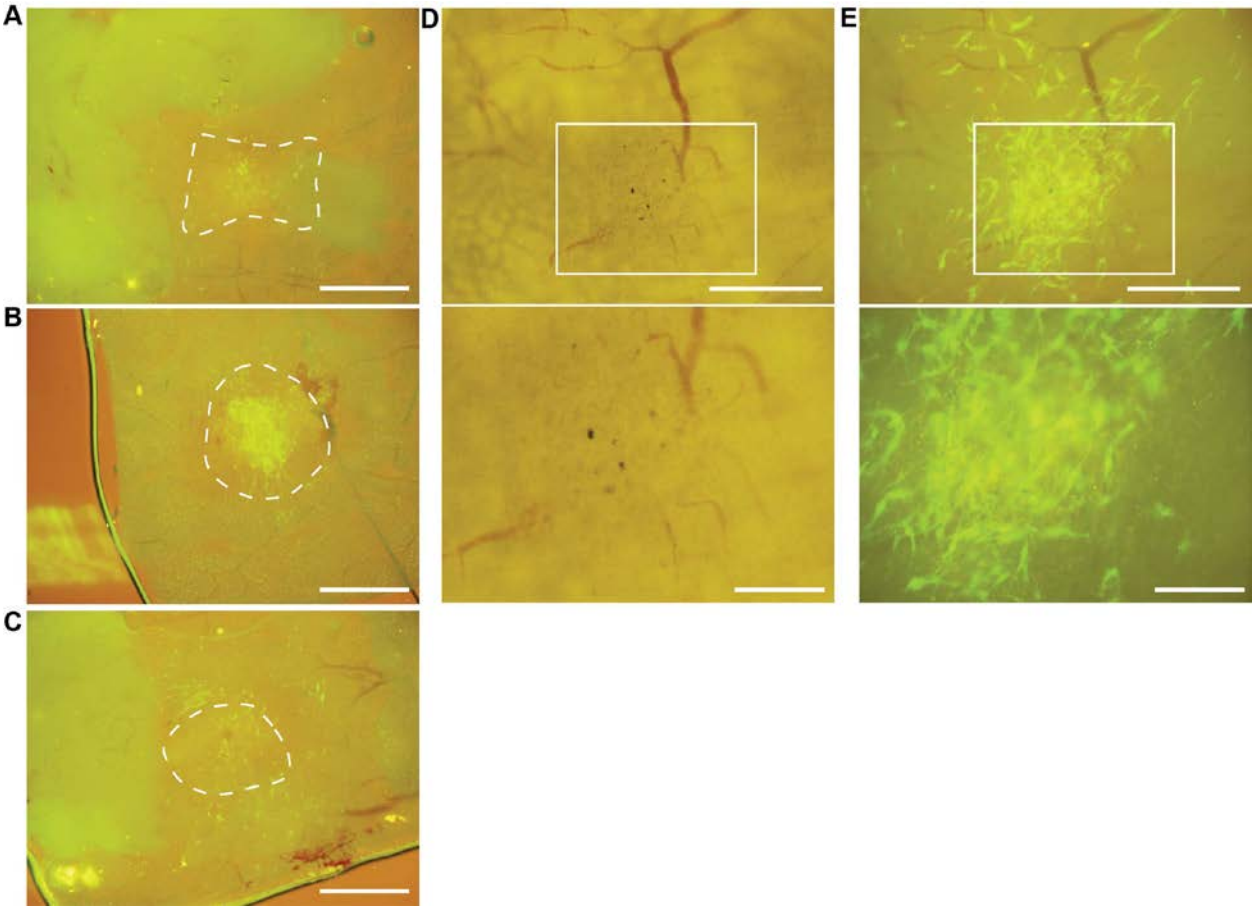
Supplemental Figure 11. **VEGF-positive cells in the wound bed in day four wounds are multiple cell types that include macrophages.** Representative section of colon (*n*= 4 wounds from 2 mice in 2 experiments) from WT mice four days post-injury was stained with anti-F4/80 antisera (green, macrophages), anti-VEGF antisera (red), and bis-benzimide (blue, nuclei) and split into separate channels in middle and right image. The yellow arrowhead indicates an F4/80 and VEGF double-positive cell, the green arrowhead indicates an F4/80 single-positive cell, and the red arrowhead indicates a VEGF single-positive cell. Bars=20μm.



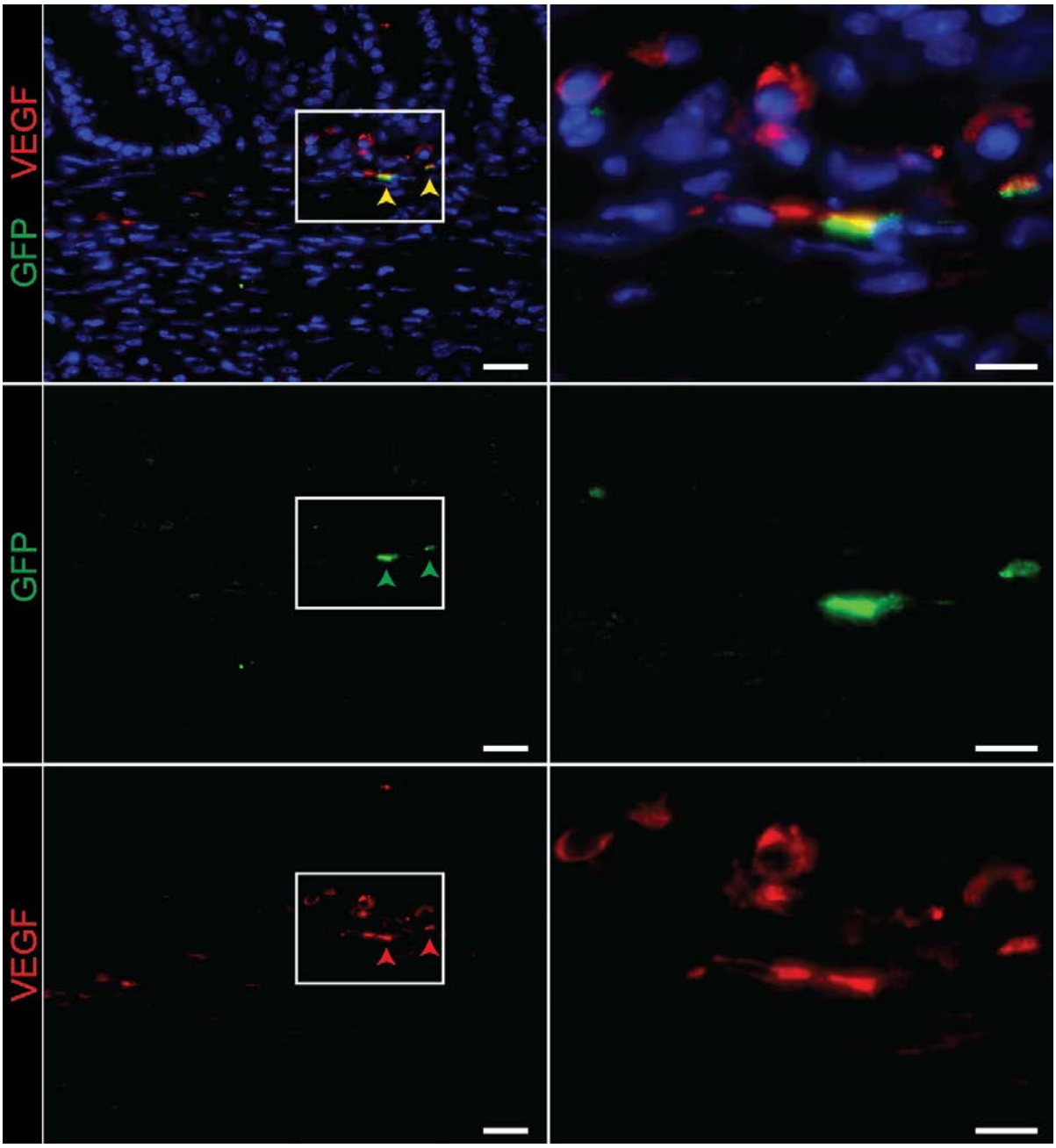
Supplemental Figure 12. WT and *Ptgir*$^{-/-}$ mice have highly proliferative epithelial cells immediately adjacent to wounds four days post-injury. (A-D) Representative images of colonic sections (*n*=8 wounds per group from 3 mice per group in 3 experiments) from WT and *Ptgir*$^{-/-}$ mice four days post-injury stained with anti-EpCAM antisera (green, epithelium), anti-Ki67 antisera (red, proliferation), and bis-benzimide (blue, nuclei). Solid white box indicates the area for magnification below, dotted white line denotes the crypt adjacent to the wound used for quantification. Bars=100μm and 50μm (insets). (E) Graph showing the percentage of nuclei that are Ki67-positive in the crypts immediately adjacent to wound beds in WT and *Ptgir*$^{-/-}$ mice four days post-injury. *n*=8 wounds per group from 3 mice per group in 3 experiments. *P*=.5301 by Student's *t*-test, n.s. indicated not significant.



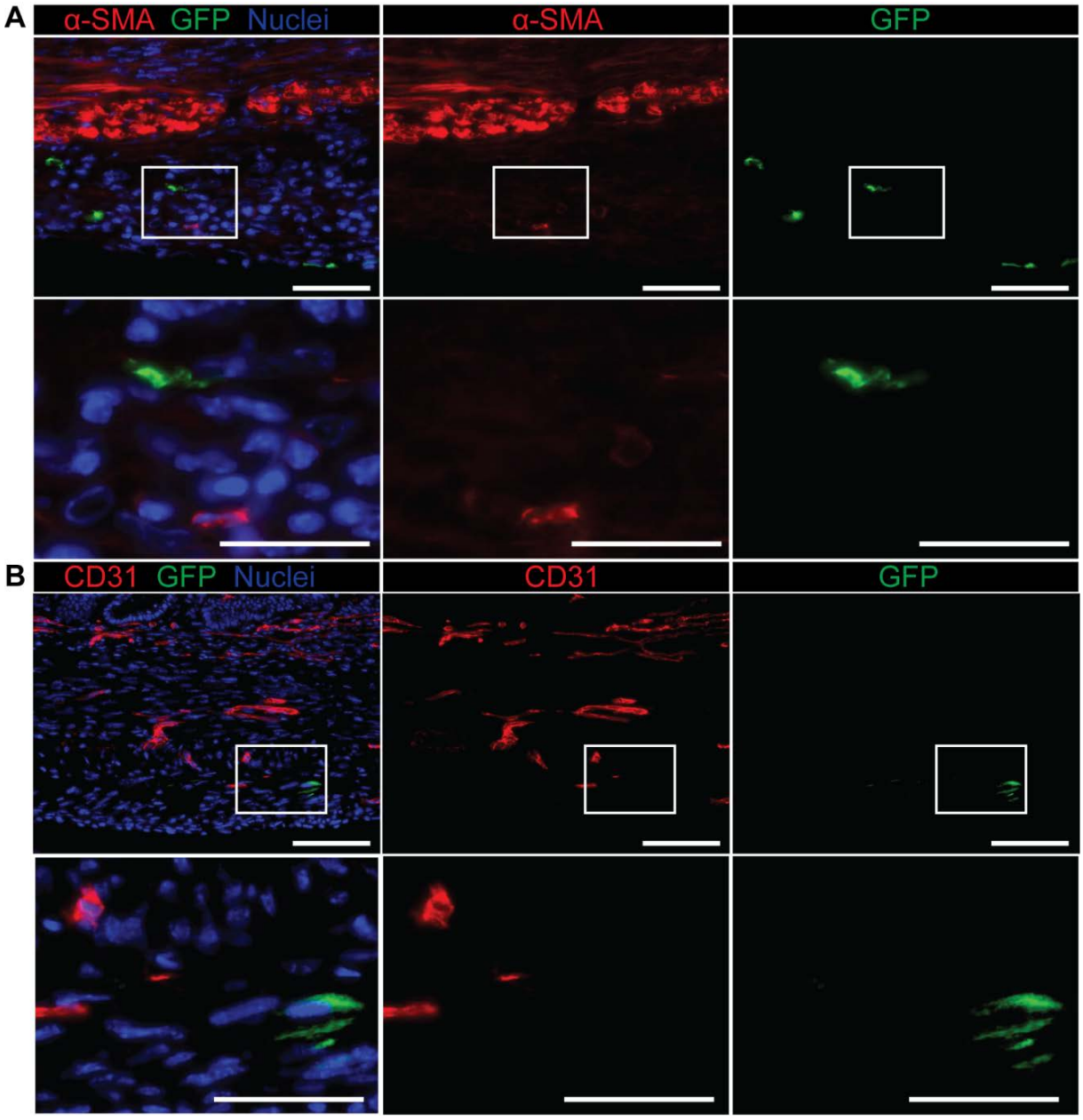
Supplemental Figure 13. **Mucosal injection of GFP-expressing cMSCs into the submucosa.** Representative image of colonic section (*n*=3 mice from 3 experiments) one hour after mucosal injection of GFP-expressing cMSCs. The section was stained with anti-GFP antisera (green) and bis-benzimide (blue, nuclei). Dashed yellow lines indicate the muscularis mucosae (M.m.) and muscularis propria (M.p.), solid white box indicates area for magnification below. Bars=100µm and 50µm (inset).



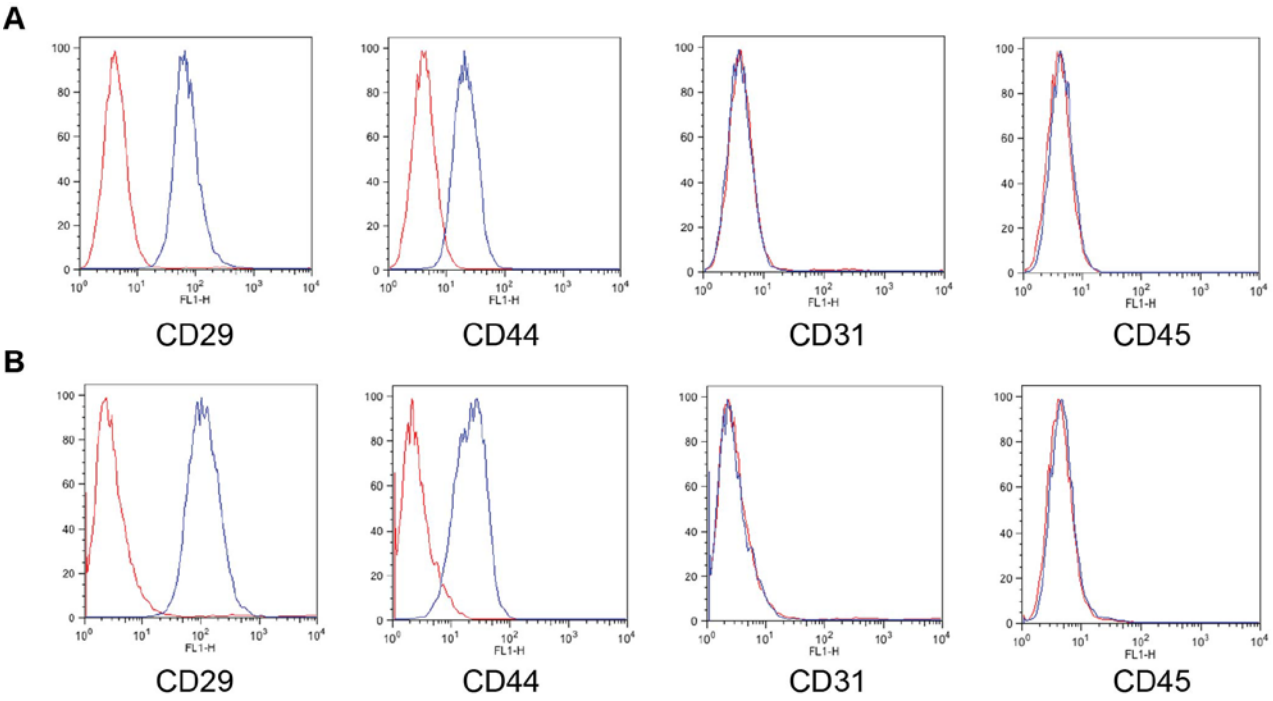
Supplemental Figure 14. GFP-expressing cMSCs migrate to all wounds throughout the distal colon. (**A-C**) Light with fluorescence whole mount images of wounds from the same *Ptgir*$^{-/-}$ mouse five days post-mucosal injection of GFP-expressing cMSCs. The wound in (**A**) is farthest from the injection site, (**B**) is in the middle, and (**C**) is closest to the injection site. All three wounds contain GFP-expressing cMSCs, and the number of cMSCs within wounds did not correlate with distance from the ano-rectal junction injection site in all mice examined (*n*=4 mice in 4 experiments). Bars=1mm. (**D-E**) Representative light (**D**) and fluorescence (**E**) whole mount images of a *Ptgir*$^{-/-}$ wound five days post-mucosal injection of GFP-expressing cMSCs. Solid white boxes indicate area for magnification below. Bars=500μm and 200μm (insets). *n*=8 wounds from 4 mice in 4 experiments.



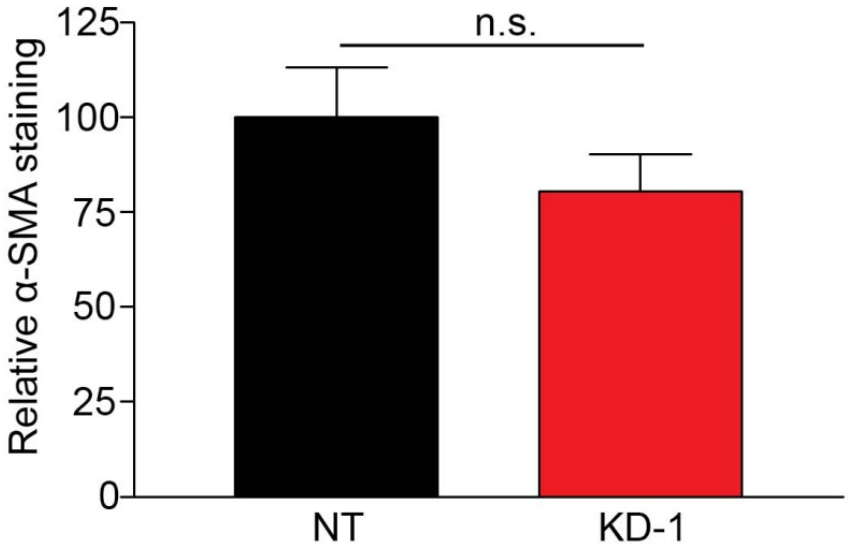
Supplemental Figure 15. GFP-expressing cMSCs increase the number of VEGF-expressing cells within *Ptgir*$^{-/-}$ wounds four days post-injury. Representative image of a colonic section (*n*=7 wounds from 4 mice in 4 experiments) from a *Ptgir*$^{-/-}$ mouse four days post-injury and three days after intramucosal injection of GFP-expressing cMSCs. The section was stained with anti-GFP antisera (cMSC, green), anti-VEGF antisera (red), and bis-benzimide (blue, nuclei). Solid white box indicates area for magnification on the right. Bars=20μm and 10μm (insets).



Supplemental Figure 16. cMSCs do not differentiate into smooth muscle cells or blood vessels by six days post-injury. (A-B) Representative images of colonic wound sections (*n*=11 wounds from 4 mice in 4 experiments) from *Ptgir*$^{-/-}$ mice five days after injection with GFP-expressing cMSCs. Sections were stained with anti-α-SMA antisera (red) in **A** or anti-CD31 antisera (red) in **B**, anti-GFP antisera (green), and bis-benzimide (blue, nuclei). There were no GFP-expressing cells that were positive for α-SMA or CD31 staining. Solid white box indicates area for magnification below. Bars=200μm and 100μm (insets).



Supplemental Figure 17. Non-targeted and VEGF shRNA knockdown cMSCs have similar cMSC surface markers. (A-B) Representative histograms of flow cytometry of non-targeted (**A**) and VEGF shRNA knockdown KD-1 (**B**) cMSCs stained for positive (CD29 and CD44) and negative (CD31 and CD45) cMSC markers (blue lines). Controls were antibodies of the same isotype (red lines). Representative of *n*=2 sequential passages of each cMSC line.



Supplemental Figure 18. Injection of 1 million VEGF shRNA knockdown cMSCs is able to partially rescue smooth muscle loss in *Ptgir*$^{-/-}$ mice. Graph showing the relative α-SMA staining six days post-injury in *Ptgir*$^{-/-}$ mice injected with 1 million non-targeting (NT) and knockdown (KD-1) cMSCs. Values=mean ±SEM. *P* =0.4031 by Student's *t*-test, n.s. indicated not significant. *n*=7 wounds per group from 3 mice per group in 3 experiments.