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Igf2bp1 is Required for Full Induction of *Ptgs2* mRNA in Colonic Mesenchymal Stem Cells in Mice

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Abstract

Background & Aims—Prostaglandin-endoperoxide synthase (Ptgs)2 is an enzyme involved in prostaglandin production during the response to mucosal damage. Its expression is regulated, in part, by mRNA-binding proteins that control the stability of *Ptgs2* mRNA. We used a precise system of colonic injury and repair to identify *Ptgs2* mRNA-binding proteins.

Methods—We used endoscopy-guided mucosal excision to create focal injury sites in colons of mice. Wound beds from wild-type, *Ptgs2*$^{-/-}$, *Ptgs2*$^{+/-}$, and Myd88$^{-/-}$ mice were analyzed at 2-day intervals following injury for aspects of repair and Ptgs2 expression. We used cultured colonic mesenchymal stem cells (cMSCs) that express Ptgs2 to identify and analyze molecules that regulate Ptgs2 expression.

Results—*Ptgs2*$^{-/-}$ mice had defects in wound repair, validating the biopsy technique as a system to study the regulation of Ptgs2. *Ptgs2*$^{+/-}$ mice had similar defects in wound healing, so full induction of Ptgs2 is required for wound repair. In wild-type mice, levels of *Ptgs2* mRNA increased significantly in the wound bed 2 and 4 days after injury; the highest levels of Ptgs2 were observed in cMSCs. In a functional small hairpin RNA knockdown screen, we identified Igf2bp1, a VICKZ mRNA-binding protein, as a regulator of Ptgs2 expression in cMSCs. Igf2bp1 also interacted physically with Ptgs2 mRNA. Igf2bp1 expression was induced exclusively in wound-bed cMSCs, and full induction of Ptgs2 and Igf2bp1 during repair required Myd88.

Conclusions—We identified Igf2bp1 as a regulator of *Ptgs2* mRNA in mice. Igf2bp1 is required for full induction of *Ptgs2* mRNA in cMSCs.

Keywords

Cox-2; Imp-1; ZBP1; colorectal cancer; inflammatory bowel disease

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Introduction

Ptgs2 (Prostaglandin-endoperoxide synthase 2) is an important enzyme for localized prostaglandin production during the response to mucosal damage and the pathogenesis of colorectal cancer.[1] Ptgs2 and Ptgs1 are isoforms that are the two rate-limiting enzymes in the synthesis of all prostaglandins, which affect downstream responses such as angiogenesis, proliferation, and apoptosis.[1] Ptgs1 is widely expressed and constitutive, whereas Ptgs2 is focally inducible with limited constitutive expression (primarily colon and testes).[2] During inflammation, damage signals and cytokines signal through Myd88 to activate NF-κB and induce Ptgs2 transcription.[3] Specialized mRNA binding/stabilizing proteins bind the AU-rich elements (ARE) in the 3'-UTR of Ptgs2 mRNA and prevent its degradation.[4] Additional, unknown mRNA-binding proteins likely regulate Ptgs2 expression, since mutations in the 3'-UTR outside of the ARE regions also affect Ptgs2 stability.[5]

To study the regulation and effects of Ptgs2 *in vivo*, we and others have used various mouse models of intestinal injury including dextran sodium sulfate (DSS),[6–9] 2,4,6-trinitrobenzene sulfonic acid,[10] and irradiation.[11] Here, Ptgs2 affects epithelial proliferation and resolution of inflammation. Despite the requirement for Ptgs2 during repair, understanding the mechanisms that control Ptgs2 expression has been challenging due to the variable timing and location of injuries in these models. To discover novel Ptgs2 regulatory proteins expressed after mucosal damage, we created small, focal injury sites in the mouse colon using endoscopy-guided forceps.[12–14] The precise timing and location of injuries in this system allowed us to investigate the cellular source, target, and timing of specific genes that regulate Ptgs2 expression.

We previously showed that repair following biopsy injury proceeds reproducibly in defined stages.[12] In the first phase, neutrophils are recruited to the wound and an immature protective epithelial barrier (wound associated epithelium; WAE) forms over the nascent wound bed. This WAE layer consists of a single layer of post-mitotic cells that emerge from crypts that are adjacent to the wound and migrate towards that center of the wound bed.[12] The second phase of healing involves increased epithelial proliferation in the adjacent crypts as well as expansion of the wound bed mesenchyme by the influx and/or proliferation of macrophages and additional stromal cells.[12] In the final stage, the wound bed is replaced by new crypts.

In this study, we were able to utilize this precise injury system to identify a novel Ptgs2 mRNA-binding protein, Insulin-like Growth Factor 2 Binding Protein 1 (Igf2bp1). We found that maximal Ptgs2 expression was required for proper mucosal healing, and that the highest levels of Ptgs2 expression were localized to colonic mesenchymal stem cells (cMSCs). Using an shRNA knockdown screen in cultured cMSCs, we demonstrated that Igf2bp1, a VICKZ mRNA binding protein, was necessary for maximal expression of Ptgs2 mRNA. Igf2bp1 interacted with Ptgs2 mRNA and expression of both genes was induced in wound bed cMSCs in a Myd88-dependent manner. These data demonstrate that Igf2bp1 was necessary for the maximal Ptgs2 expression in cMSCs that was required for proper colonic injury repair.

Materials and Methods

Mice

Animal experiments were performed in accordance with approved protocols from the Washington University School of Medicine Animal Studies Committee. *Ptgs2*$^{+/-}$ mice[15] were bred to generate littermate WT, *Ptgs2*$^{+/-}$ and *Ptgs2*$^{-/-}$ progeny for experiments.

Myd88$^{+/-}$ mice[16] from Jackson Lab were bred to generate Myd88$^{-/-}$ and Myd88$^{+/-}$ littermate controls. All mice were on a C57Bl/6 background.

Colonic biopsy

We used a high-resolution miniaturized colonoscope system to visualize the lumen of the colon and discretely injure the mucosal layer in 10–16 week old anesthetized mice. After inflating the colon with PBS, we inserted 3 French flexible biopsy forceps into the sheath adjacent to the camera. We removed 3–5 full-thickness areas of the entire mucosa and submucosa that were distributed along the dorsal side of the colon. All genetically modified mice were injured with the same technique. For this study, we evaluated wounds that averaged ~1 mm^2, which is equivalent to removal of ~250–300 crypts.

Colonic tissue preparation

Wounded mice were sacrificed 2–6 days post-injury and each wound was individually frozen in OCT to make frozen sections. See Supplementary Materials and Methods for detailed descriptions.

In situ hybridization

A cDNA clone for Ptgs2 (clone ID: 30059181) was purchased from Thermo Fisher Scientific to create digoxigenin-labeled antisense RNA probes. See Supplementary Materials and Methods for detailed descriptions.

Immunofluorescence

Staining was performed as previously published[17]. See Supplementary Materials and Methods for detailed descriptions.

RNA isolation and quantitative RT-PCR

For wound RNA isolation, the wound bed mucosa or adjacent uninjured mucosa was dissected with forceps under the guidance of a whole mount microscope, leaving the underlying muscularis propria intact. RNA was isolated using a NucleoSpin RNA II kit (Machery-Nagel, Duren, Germany). cDNAs were synthesized using Superscript III reverse transcriptase (Invitrogen). Quantitative RT-PCR reactions using SYBR-green master mix (Clontech) were analyzed on an Eppendorf realplex Mastercycler. RNA from wounds and uninjured mucosa were normalized to Gapdh. See Supplementary Materials and Methods for primer sequences.

Immunoprecipitation and RNA isolation in NIH3T3 cells

The open reading frame of Igf2bp1 was cloned into p3XFLAG-CMV-14 (Sigma-Aldrich). See Supplementary Materials and Methods for detailed descriptions.

shRNA Transfection and immunoblotting

HEK293T cells were transfected with Mission shRNA constructs specific for Igf2bp1 or non-targeting controls. Lentivirus obtained from transfected cells was used to infect cMSCs for knockdown of specific genes. See Supplementary Materials and Methods for detailed descriptions.

Cell isolation and culture conditions

Colonic mesenchymal stem cell isolation and culture was performed as previously reported.[17] See Supplementary Materials and Methods.

Statistics

All statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software). Significant differences were evaluated by Student's *t* test, Analysis of Variance (ANOVA) followed by Tukey's Multiple Comparison test, or Fisher's exact test, as noted.

Results

Two functional Ptgs2 alleles are required for colonic mucosal wound repair

To determine if Ptgs2 expression was required for colonic mucosal repair of biopsy injuries, we assessed the gross and microscopic features of wound beds from *Ptgs2*$^{-/-}$ and control WT littermate mice at two-day intervals post-injury. Starting at day 6 post-injury (second stage of repair), *Ptgs2*$^{-/-}$ mice displayed severe defects that included loss of muscularis propria and an incomplete WAE barrier (Figure 1*A-E*). Specifically, the muscularis propria showed complete loss of α-smooth muscle actin (α-SMA) underlying wound beds of *Ptgs2*$^{-/-}$ but not WT mice (Figure 1*A-D*). The damaged muscularis propria was replaced by granulation tissue comprised of endothelial cells and myeloid cells that extended through the serosa (Supplemental Figure 1 *A–F*). This phenotype resembled the histology of perforated human colonic ulcers that can occur after prolonged NSAID use.[18] Importantly, the α-SMA staining pattern was intact in the muscularis propria at day 2 post-injury in *Ptgs2*$^{-/-}$ mice, indicating that this phenotype was a delayed biological response to injury and was not due to a more fragile mucosa in the *Ptgs2*$^{-/-}$ mice (Supplemental Figure 1*G-I*).

The epithelial barrier at day 6 post-injury was also defective in *Ptgs2*$^{-/-}$ mice as WAE cells incompletely covered these wounds. WAE cells are claudin-4-positive, ZO-1-negative post-mitotic epithelial cells that emerge from adjacent crypts and completely migrate across the wound bed during the first stage of repair.[12] In contrast to WT mice, most *Ptgs2*$^{-/-}$ wounds were only partially covered by an epithelial barrier at day 6 post-injury as shown by gross morphologic examination (Supplemental Figure 2) and by immunofluorescence microscopy (Figure 1*A,C,E*). In addition, the epithelial cells that partially covered *Ptgs2*$^{-/-}$ wounds were still immature WAE cells, whereas WT wounds were covered by a complete layer of mature surface epithelial cells (Supplemental Figure 3*A-D*). There were also defects in the final stage of healing in *Ptgs2*$^{-/-}$ wounds as they contained fewer regenerated crypts at 14 day post-injury (Supplemental Figure 4*A-D*). In summary, the complex repair defects in *Ptgs2*$^{-/-}$ mice were observed in the latter phases of repair.

Interestingly, by these parameters, littermate *Ptgs2*$^{+/-}$ mice also demonstrated defective wound repair (Figure 1*B*). At day 6 post-injury, *Ptgs2*$^{+/-}$ mice showed loss of the muscularis propria underlying the wound bed (Figure 1*D*) and an incomplete epithelial layer (Figure 1*E*). These data demonstrated that loss of just one functional *Ptgs2* allele had severe effects on colonic mesenchymal and epithelial regeneration. Because this injury system was sensitive to a two-fold decrease of gene dosage, we proposed that it would be useful to discover novel proteins that regulate maximal Ptgs2 expression.

Ptgs2 expression is transiently and focally elevated during colonic wound repair

Given the profound wound repair defects in *Ptgs2*$^{+/-}$ mice, we next evaluated the timing and location of Ptgs2 expression in WT colonic wounds during two-day intervals post-injury. We first quantified Ptgs2 mRNA levels by qRT-PCR using mRNAs isolated from the mucosa of biopsy wounds. Compared to uninjured colonic mucosa, Ptgs2 levels peaked during the first stage of repair (an average of 1,640-fold higher at day 2 and 2,170-fold at day 4) and were subsequently down-regulated during the second stage of repair (only 93-fold elevation at day 6) (Figure 2*A*). In contrast, Ptgs1 mRNA levels did not differ between wounds and adjacent mucosa (Supplemental Figure 5). Interestingly, Ptgs2 mRNA

expression was also significantly lower in the wound beds of *Ptgs2*$^{+/-}$ mice compared to WT mice at day 4 post-injury (Supplemental Figure 6). This intermediate expression of Ptgs2 in *Ptgs2*$^{+/-}$ mice was not sufficient for proper wound repair (Figure 1*D,E*), supporting the conclusion that maximal induction of Ptgs2 is a critical component of mucosal repair.

We then performed *in situ* hybridization for Ptgs2 in WT mice at two-day intervals after injury. Because we wanted to identify cells that expressed the highest levels of Ptgs2, we limited the exposure time of the detection reagent. Throughout injury, Ptgs2 high-expressing cells were only detected within the mesenchyme of the wound bed and were not observed in either the underlying muscularis propria or crypts adjacent to the wound bed (Figure 2*B*). The Ptgs2 high-expressing cells were scattered in the wound bed at day 2 post-injury and were clustered beneath WAE cells at days 4 and 6 post-injury (Figure 2*B*). At day 6 post-injury, the number of Ptgs2 high-expressing cells was greatly diminished (Figure 2*B*). Thus, the cells with the highest levels of Ptgs2 mRNA in the upper wound during the first phase of repair were not detected during the second stage of repair.

We corroborated the *in situ* findings by immunofluorescence localization of Ptgs2 protein in wound bed sections. We used dilutions of primary antibody (1:5,000) that did not detect constitutive Ptgs2 expression in colonic mesenchymal stem cells (cMSCs) located outside of the wound bed or other cell types known to express lower levels of Ptgs2.[7] In WT wound beds, we found a similar spatial and temporal pattern of Ptgs2 protein induction (Figure 2*A*). The number of Ptgs2 high-expressing cells in the wound bed was significantly higher at days 2 and 4 post-injury as compared to day 6 post-injury (Figure 2*C*). As with *in situ* hybridization, Ptgs2-high expressing cells detected by immunofluorescence were present only in the wound bed mesenchyme (Figure 2*D*). As additional controls, we performed in parallel *in situ* hybridization and immunofluorescence studies for localization of Ptgs2 mRNA and protein within wounds of *Ptgs2*$^{-/-}$ mice. We found no detectable signal in *Ptgs2*$^{-/-}$ mice for either assay (Supplemental Figure 7). Thus, Ptgs2 was maximally induced in upper wound bed mesenchymal cells during the first stage of repair (Figure 2*E*).

Ptgs2 high-expressing cells in the wound bed are colonic mesenchymal stem cells (cMSCs)

In order to identify novel Ptgs2 regulators during colonic repair, we first defined the mesenchymal cell lineages that produced the highest levels of Ptgs2. Candidates included, myeloid cells,[19] endothelial cells,[20] colonic mesenchymal stem cells,[7] and myofibroblasts.[21] Multi-label immunofluorescence imaging of Ptgs2 high-expressing cells and various lineage markers at day 4 post-injury (Figure 3) demonstrated that these cells were neither endothelial nor hematopoietic lineages as they did not co-label with either CD31 or CD45, respectively (Figure 3*A*). Instead, Ptgs2 high-expressing cells showed dual labeling for multiple cMSC markers [17] including CD44, CD29, and CD54 (Figure 3*B*). Additional studies at days 2 and 6 post-injury showed that the Ptgs2 high-expressing cells showed dual labeling with these same markers (data not shown). Since myofibroblasts and cMSCs share many of these markers, we evaluated definitive myofibroblast markers. We did not detect α-SMA cells in cells that expressed high levels of Ptgs2 (Figure 3*A*). Additionally, IFN-γ is expressed in wounds[12] which can down-regulate α-SMA[22] and increase MHC class II on myofibroblasts[23]. Therefore, we showed that Ptgs2 high-expressing cells were also MHC class II-negative in the wound bed to exclude resting or activated myofibroblasts as Ptgs2 high-expressing cells (Figure 3*A*).

We hypothesized that cMSCs appeared in wound beds in part by migration from adjacent uninjured tissue. We injected GFP-expressing cultured cMSCs into the mucosa adjacent to wounds. We found that cMSCs migrated into wounds indicating these cells have the potential to migrate from the adjacent mucosa (Supplemental Figure 8). As a control for our

isolation technique, we showed that Fstl1, a marker that distinguishes myofibroblasts from intestinal MSCs,[24] was expressed in cultured cMSCs but not MIC 216 cells (Supplemental Figure 9).

To demonstrate that the defects seen in *Ptgs2*$^{-/-}$ mice were due to loss of prostaglandin synthesis, we injected stable analogues of PGE2 and PGI2 into *Ptgs2*$^{-/-}$ mice twice daily. This procedure rescued the muscle degradation phenotype at day 6 post-injury (Supplemental Figure 10). We chose these two prostaglandins as cMSCs express detectable levels of prostaglandin synthases for PGE2 and PGI2 but not for PGD2 or PGF2α.[17] Since the Ptgs2 high-expressing cells in wounds were consistent with cMCSs, we further investigated cultured cMSCs for novel regulators of Ptgs2.

Igf2bp1 interacts with Ptgs2 mRNA

Our previous studies in cMSCs showed that mRNA-binding proteins including CUGbp2 play a major role in the post-transcriptional regulation of Ptgs2 expression through mRNA stabilization.[17, 25] However, the ARE-binding protein CUGbp2 does not account for complete stabilization of Ptgs2 mRNA in cMSCs.[17] Furthermore, previous reviews have proposed that additional, unknown mRNA-binding proteins will regulate Ptgs2 mRNA in a cooperative fashion.[4]

We hypothesized that additional transacting protein(s) outside of the ARE-binding family will interact with and regulate Ptgs2 mRNA in cMSCs. Because cMSCs express 10-fold higher levels of Ptgs2 than bone marrow MSCs, [17] we analyzed microarray data for these two cell types[17] in order to identify candidate mRNA-binding proteins that were preferentially expressed in cMSCs. This screen identified known ARE-binding proteins (HuR, CUGbp1and CUGbp2) as well as candidate Ptgs2 mRNA-binding proteins (Rmb3, Rbms3, Rbpms and Igf2bp1). We functionally screened the non-ARE-binding protein candidates individually by shRNA-mediated knockdown in cMSCs. We generated multiple shRNAs for each gene, and the two shRNAs that had the greatest knockdown of the target gene (at least 80% knockdown efficiency) were used in further experiments (Figure 4*A*). Of all these candidates, we found that Igf2bp1-deficient cMSCs expressed the lowest levels of Ptgs2 mRNA (Figure 4*B*). Igf2bp1 was of further interest because it is an mRNA-binding protein[26] that had not been previously shown to interact with Ptgs2 mRNA.

We next tested if Igf2bp1 interacted with Ptgs2 mRNA. We immunoprecipitated FLAG-tagged Igf2bp1 and eluted Igf2bp1 from the pellet fraction with 3X FLAG peptide. We then immunoblotted total protein, eluate, and pellet fractions to confirm immunoprecipitation of correct size proteins (Figure 5*A*). Protein was degraded in each fraction and the associated mRNAs were quantified by qRT-PCR (Figure 5*B*). Ptgs2 mRNA was enriched in the eluate as compared to the total fraction indicating immunoprecipitation of Igf2bp1 with Ptgs2 mRNA. (Figure 5*B*). As a control, 18S RNA was not enriched in the eluate. These data demonstrate the interaction of Igf2bp1 protein and Ptgs2 mRNA.

We then designed two FLAG-tagged truncation mutants of Igf2bp1, one containing two RRM RNA-binding motifs in the amino terminus (RRM-FLAG) and a second containing the four KH RNA-binding motifs in the carboxy terminus (KH-FLAG) (Figure 5*C*). We observed that KH-FLAG similarly enriched for Ptgs2 mRNA while RRM-FLAG did not (Figure 5*D,E*). These data suggested that the KH domains of Igf2bp1 were required for interaction with Ptgs2 mRNA.

Igf2bp1 is induced in Ptgs2 high-expressing cMSCs during the early phase of repair

We next wanted to determine the spatial and temporal expression of Igf2bp1 in wound-associated cMSCs as well as the mechanism that controls its expression. We found that

Igf2bp1 mRNA was enriched in the wound mucosa at days 2 and 4 post-injury (but not day 6 post-injury) as compared to uninjured mucosa (Figure 6*A*). This temporal pattern of Igf2bp1 induction/reduction was similar to Ptgs2 expression during repair. We also assessed whether Igf2bp1 co-localized with Ptgs2 high-expressing cMSCs by multi-label immunofluorescence and found that 100% of the Igf2bp1 signal co-labeled with Ptgs2 high-expressing cMSCs (Figure 6*B*). Thus, Igf2bp1 was expressed during wound repair at the appropriate time (first phase of healing) and location (cMSCs) to regulate maximal Ptgs2 expression.

Myd88 signaling is required for induction of Igf2bp1 in cMSCs

The temporal and cellular co-induction of Igf2bp1 and Ptgs2 suggested that these genes may be regulated by the same pathway. One candidate that regulates Ptgs2 expression in various cell types is Myd88 signaling[3]. Therefore, we hypothesized that Myd88 was required for induction of Igf2bp1 and maximal Ptgs2 expression in cMSCs. We first biopsy-injured *Myd88*$^{-/-}$ mice and found a significant repair defect as indicated by loss of α-SMA expression in the muscularis propria at day 6 post-injury (Figure 7*A*). This phenotype was similar to wounded *Ptgs2*$^{+/-}$ mice (Figure 1*D*), suggesting that Myd88 signaling was required for maximal Ptgs2 expression. Therefore, we next evaluated Ptgs2 expression in wounds 2 days post-injury, and found that the magnitude of Ptgs2 induction was 30-fold lower in *Myd88*$^{-/-}$ compared to WT mice (Figure 7*B*). We hypothesized that the dramatic difference in Ptgs2 expression in wounded *Myd88*$^{-/-}$ mice was due to defective Ptgs2 regulation in Ptgs2 high-expressing cMSCs. In support of this hypothesis, we found that in contrast to WT littermate controls, Igf2bp1 was not induced in *Myd88*$^{-/-}$ mice at day 2 post-injury (Figure 7*C*). To test whether cell-intrinsic Myd88 signaling was required in cMSCs, we isolated cMSCs and found significantly lower expression levels of Igf2bp1 in *Myd88*$^{-/-}$ compared to WT cMSCs (Figure 7*D*). From these data, we conclude that Myd88 signaling was required for induction of Igf2bp1 expression in cMSCs.

Since Ptgs2 can also be induced in macrophages by a Myd88-dependent, cell-intrinsic mechanism[3], we tested whether Myd88 signaling was required for the expression of Igf2bp1 in this cell type. We treated WT bone marrow-derived macrophages with LPS, which dramatically induced Ptgs2 mRNA expression as expected (Figure 7*E*). We found that Igf2bp1 expression was not detectable in either LPS-treated or untreated macrophages. Thus, not all cell types capable of expressing Ptgs2 also express Igf2bp1 (Figure 7*F*). Therefore, the Myd88-dependent upregulation of Igf2bp1 may be a specific mechanism in MSCs.

Discussion

Igf2bp1 is a novel regulator of Ptgs2 in cMSCs

Igf2bp1 is a VICKZ mRNA-binding protein and its homologs found in other organisms have multiple names (Vg1RBP, Imp1, CRD-BP, KOC, and ZBP-1).[26] ZBP-1 was identified as a zip-code binding factor that localizes β-actin mRNA to the leading edge of chicken embryo fibroblasts.[27] Igf2bp1 regulates target mRNAs by several, complex mechanisms[26] including: i) direct binding to target mRNAs that affect their stability or translation;[4] ii) scaffolding with other mRNA-binding proteins;[28] and iii) controlling mRNA intracellular localization through the formation of Igf2bp1-containing ribonucleoprotein granules (complex structures of mRNAs and proteins). Since Igf2bp1 has both shared and unique mechanisms of regulation compared to the known ARE-binding proteins, Igf2bp1 will be a key tool to elucidate principles that mediate the overall regulation of Ptgs2 in cMSCs.

The expression pattern of Igf2bp1 and the phenotype of *Igf2bp1*-deficient mice demonstrate that Igf2bp1 is important in development.[29, 30] Igf2bp1 is an oncofetal protein that is expressed in embryos and various cancers but not adult tissues. *Igf2bp1*-deficient mice had dwarfism and impaired gut formation,[30] thus, colonic biopsy of *Igf2bp1*-deficient mice was not feasible. An inducible Igf2bp1 deletion is required to test function in the adult.

Igf2bp1 is overexpressed in certain cancers including 81% of colorectal carcinomas.[31] Since Ptgs2 is important for the progression of colon cancer and Igf2bp1 is frequently expressed in these cancers, the mechanism of the Ptgs2/Igf2bp1 interaction is highly relevant. Additionally, as Igf2bp1 is virtually undetectable in healthy tissue, its interaction with and stabilization of Ptgs2 makes it an intriguing candidate target for drug development. Drugs inhibiting the interaction of Igf2bp1 with Ptgs2 mRNA may potentially have fewer side effects than traditional NSAIDs or Ptgs2-inhibitors.

MSCs as mobilizable sources of Ptgs2 during repair

Although many cell types can express Ptgs2 in the colon, we showed that cMSCs expressed the highest detectable levels of Ptgs2 mRNA and protein after mucosal biopsy. Because *Ptgs2*$^{+/-}$ mice had defective healing similar to *Ptgs2*$^{-/-}$ mice, we concluded that maximal expression of Ptgs2 was necessary for proper mucosal repair. Previous studies identified numerous cell types capable of expressing Ptgs2,[19–21] and lineage-specific Ptgs2 knockouts demonstrated that myeloid and endothelial expression of Ptgs2 was necessary for proper intestinal healing after DSS.[8] We acknowledge that cMSCs are not the only cell type that expresses Ptgs2 during intestinal repair, and these previous studies highlight the importance of multiple Ptgs2-expressing cells, including myofibroblasts[32], during healing. The relative contributions of different cell types are unknown. However, we show that cMSCs express the highest levels of Ptgs2 after severe mucosal injury; therefore the regulation of Ptgs2 in these cells is highly relevant to mucosal healing.

MSCs are an emerging cellular therapy for inflammatory diseases, such as inflammatory bowel disease.[33] MSCs have at least two possible functions following intestinal injury; differentiation into stromal lineages to replace damaged tissue and expression of immunomodulatory molecules, including prostaglandins.[33] The location of Ptgs2 high-expressing cMSCs directly beneath the epithelium may be functionally relevant since prostaglandins have a short half-life and act within a small area. Also, Ptgs2 is expressed during the first phase of repair when WAE cells begin migrating, and *Ptgs2*$^{-/-}$ mice have a defective epithelial barrier. We were able to rescue the muscle degradation phenotype in *Ptgs2*$^{-/-}$ mice, demonstrating that the main function of Ptgs2 is the production of prostaglandins. We were also able to inject cMSCs adjacent to wounds and show that they were capable of migrating towards sites of inflammation from adjacent uninjured tissue. We believe this may be one major method of homing for cMSCs, though we cannot rule out the migration of cMSCs from the bone marrow.

The accepted markers for MSCs are a constellation of positive markers including CD29, CD44 and CD54, as well as negative lineage markers.[33, 34] A challenge in the field is that there is no single marker that only marks cMSCs. Because Igf2bp1 protein was only found in cMSCs in wound beds, we will further investigate whether Igf2bp1 may be such a marker.

In conclusion, we demonstrate the importance of maximal expression of Ptgs2 during mucosal repair, and identify Igf2bp1 as a novel mRNA-binding protein necessary for maximal Ptgs2 expression in cMSCs. Our study has implications for understanding complex mechanisms of Ptgs2 regulation by mRNA-binding proteins. Additional work will be

needed to determine how Igf2bp1 regulates Ptgs2 expression and whether it interacts with ARE-binding proteins in cMSCs or affects Ptgs2 localization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Igf2bp1	Insulin-like growth factor 2 binding protein 1
Ptgs2	Prostaglandin-endoperoxide synthase 2
ARE	AU-rich elements
cMSC	colonic mesenchymal stem cell
WAE	wound associated epithelial cell

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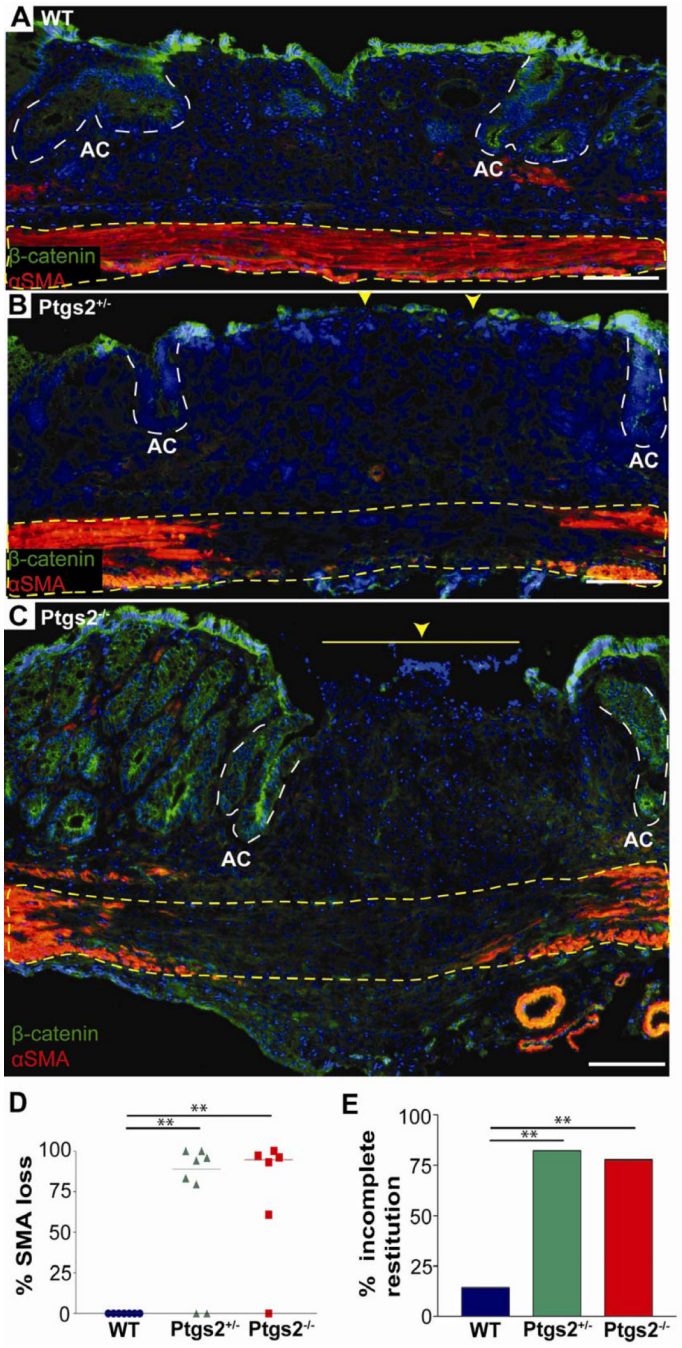

Figure 1. Two functional alleles of *Ptgs2* are required for proper mucosal healing

(A) Colonic sections from WT, (B) *Ptgs2*$^{+/-}$, and (C) *Ptgs2*$^{-/-}$ mice 6 days post-injury were stained with antibodies against α-smooth muscle actin (α-SMA) (red), β-catenin (green), and bis-benzamide (nuclei, blue). Dotted yellow lines=muscularis propria, white dashed lines=adjacent crypts (AC), yellow arrowheads=epithelial barrier interruptions. Bars=100μm. (D) Graph of the percent loss of α-SMA underlying day 6 wound beds (gap length/wound bed length) that included data points (n≥6/genotype), median bar and significance by one-way ANOVA and post hoc Tukey's test: $F_{2,18}$=10.33, $^{**}P$<0.01. (E) Graph of the percentage of wound bed sections with incomplete epithelial coverage at day 6 post-injury. Wound bed sections were scored as either fully or incompletely covered by

epithelial cells (β-catenin-positive). Fisher's exact test for the resulting data contingency table compared incomplete restitution in WT (2/14) to *Ptgs2*$^{+/-}$ (14/17) (P<0.001) and WT to *Ptgs2*$^{-/-}$ (7/9) (P<0.001).

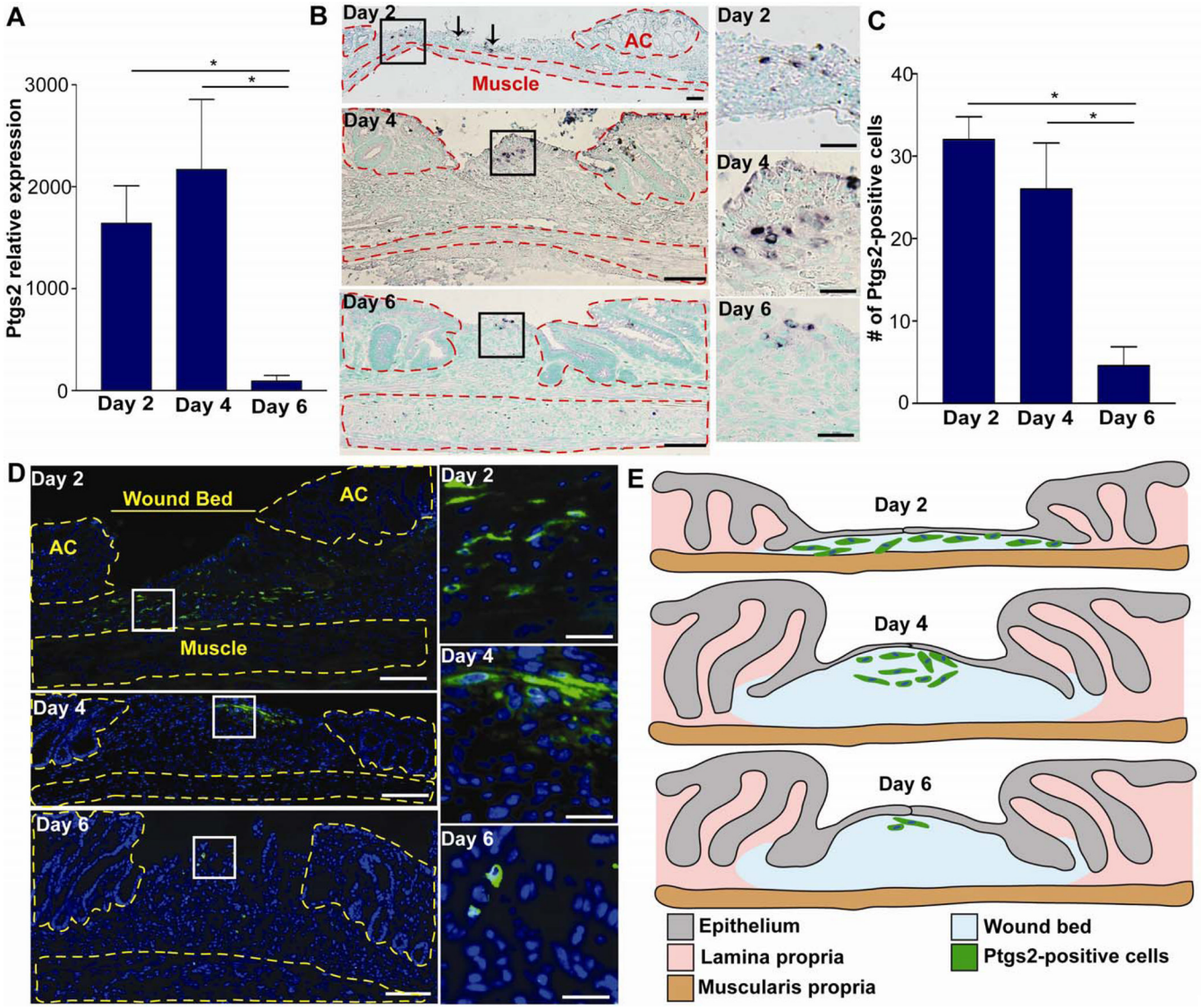

Figure 2. Ptgs2 is induced in colonic wounds in a spatial and temporal manner

(A) Graph of Ptgs2 mRNA expression (qRT-PCR) wounds at days 2, 4 and 6 post-injury (baseline=uninjured mucosa; n≥5 wounds per day). Data displayed as means ±SEM and significance by one-way ANOVA and post-hoc Tukey's test: $F_{2,18}$=5.33, **P*<0.05. (B) Colonic sections from WT mice at days 2, 4 and 6 post-injury stained by *in situ* hybridization for Ptgs2. Solid black boxes=insets (right panels). Arrows indicated additional scattered Ptgs2 high-expressing cells outside of boxed area at day 2. The adjacent crypts (AC) and muscularis propria were outlined in red dashed lines. Bars=200μm; (50μm insets). (C) Graph of the number of Ptgs2 high-expressing cells per wound section. Data displayed as means ±SEM and significance by one-way ANOVA and post-hoc Tukey's test: $F_{2,9}$=11.6, **P*<0.05. (D) Colonic sections from WT mice at days 2, 4 and 6 post-injury stained with anti-Ptgs2 antisera (green) and bis-benzamide (blue). White boxes=insets (right panels). Yellow dashed lines marked adjacent crypts (AC) and muscularis propria. Bars=100 μm (25μm inset). (E) Cartoon depicting the typical positional and temporal relationship of Ptgs2 high-expressing cells in the wound bed post-injury.

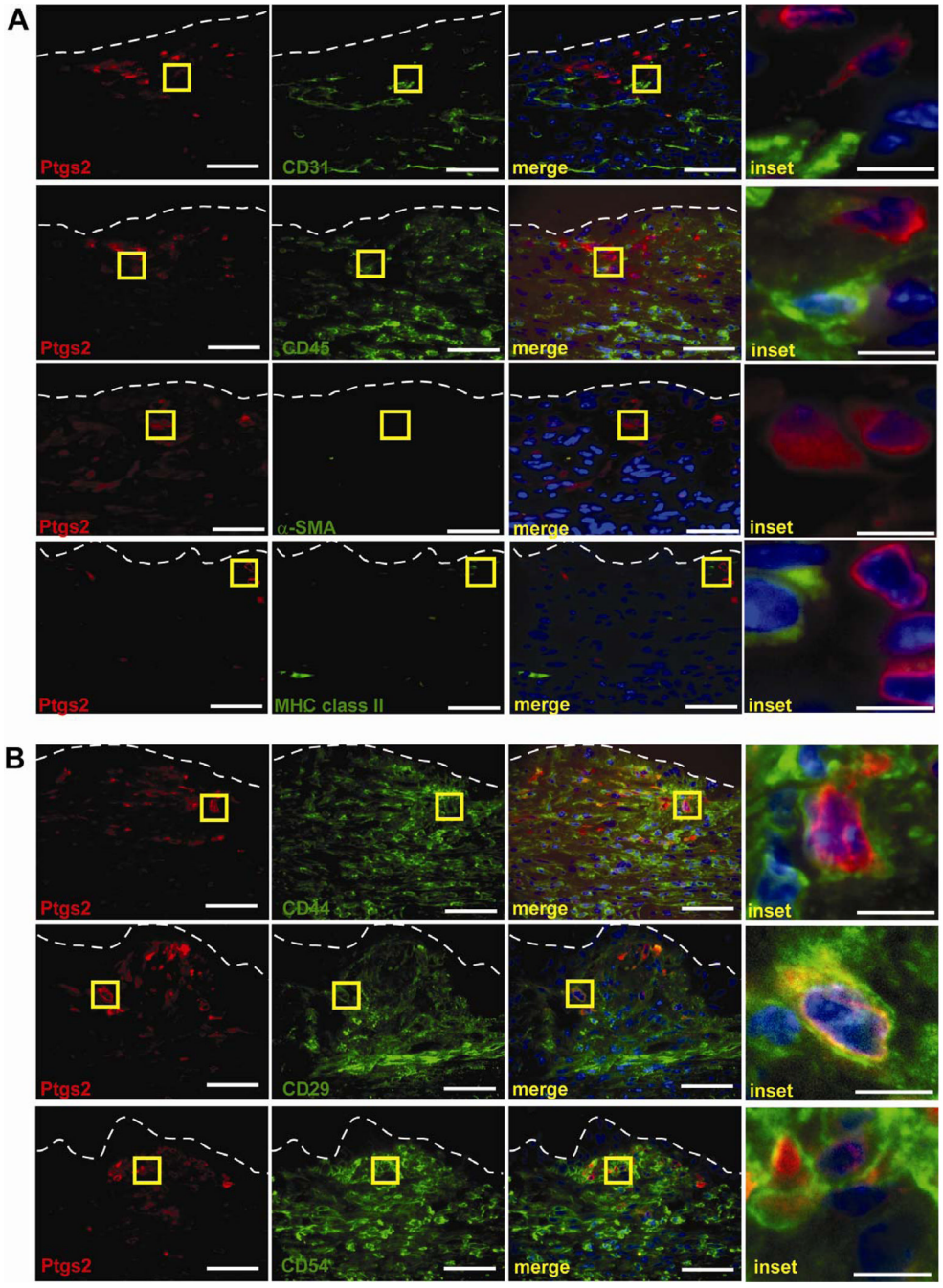

Figure 3. Ptgs2 high-expressing cells in colonic wound beds are cMSCs

(A) Sections of WT wounds at day 4 post-injury labeled with anti-Ptgs2 (red) lineage markers (green) for endothelial cells (anti-CD31), hematopoietic cells (anti-CD45), and mesenchymal cells (α-SMA and MHC class II). (B) WT day 4 wounds labeled with anti-Ptgs2 (red) and cMSC markers anti-CD44, anti-CD29, and anti-CD54. Yellow boxes=insets (right). White dashed lines=WAE basal surface. Bars=50 μm (10μm inset).

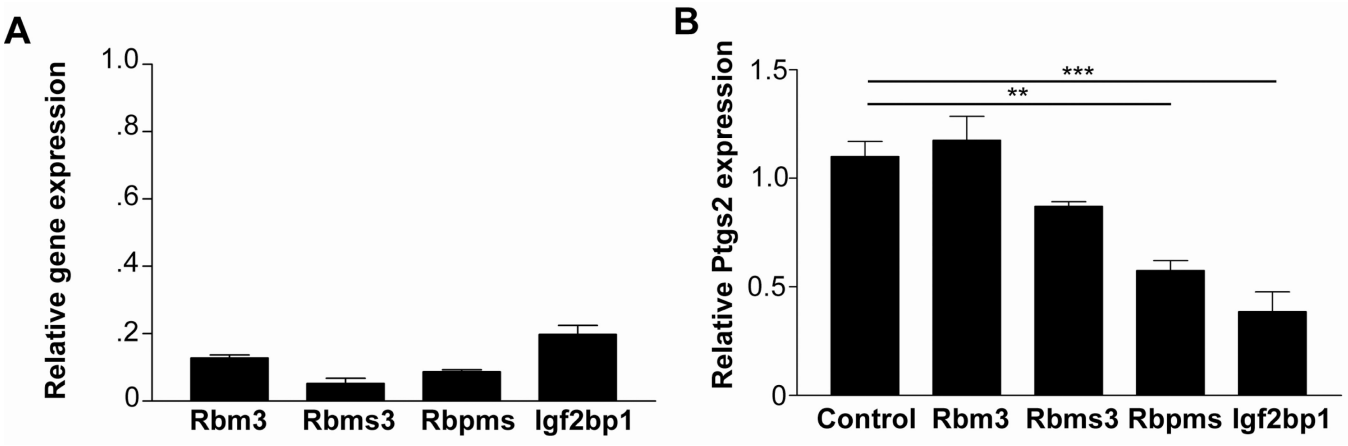

Figure 4. Igf2bp1 is required for maximal Ptgs2 expression in cMSCs

(A) Graph depicting the relative mRNA expression (means ±SEM) of candidate Ptgs2 regulators after shRNA knockdown (two most efficient for each gene) versus control shRNA cells. (B) Graph depicting the relative expression of Ptgs2 mRNA in cMSCs that contain knockdown of candidate mRNA regulators (n=4). Data displayed as means ±SEM and significance by one-way ANOVA and post-hoc Tukey's test: $F_{4,15}=20.25$, $^{**}P<0.01$, $^{***}P<0.001$.

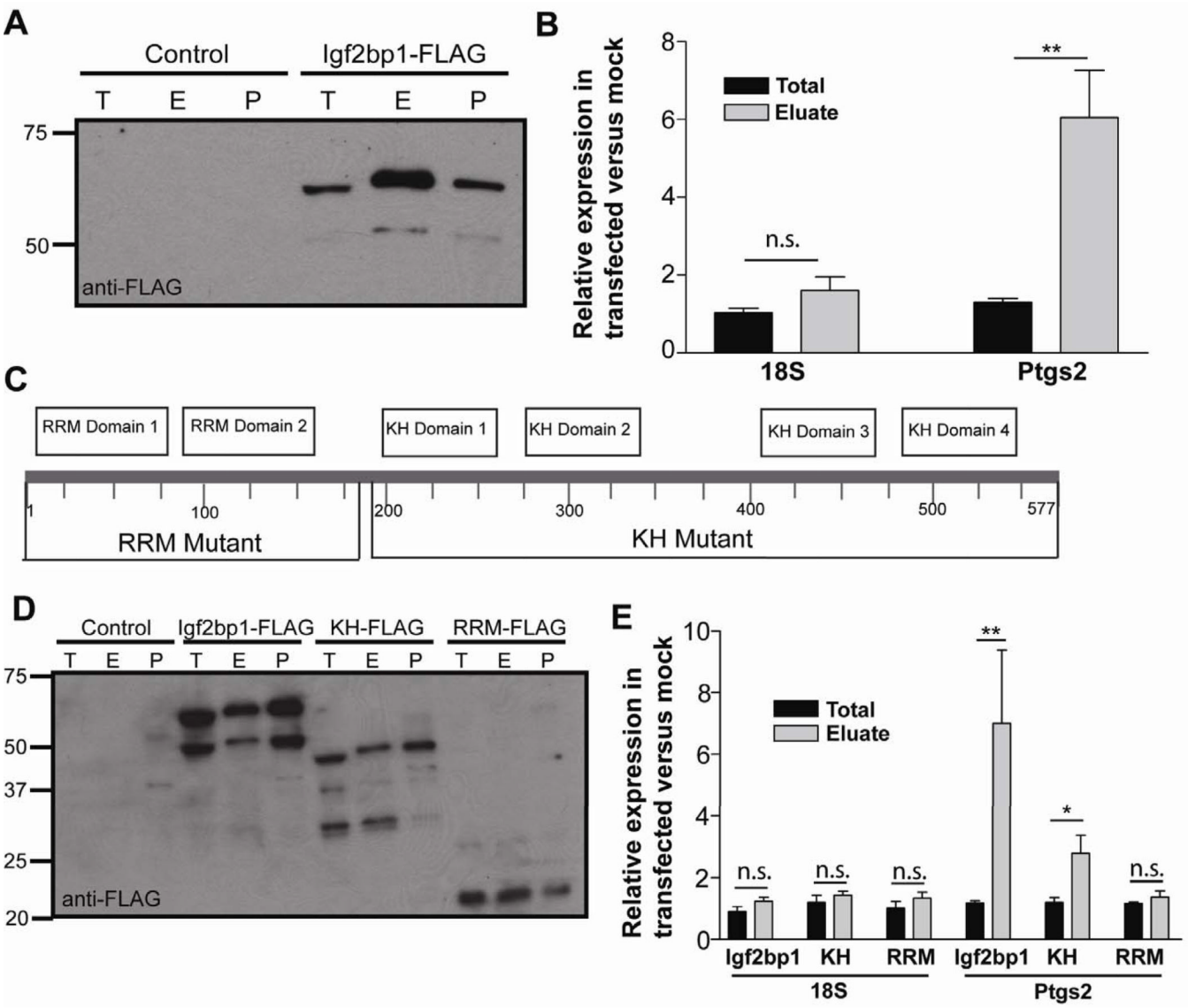

Figure 5. Igf2bp1 interacts with Ptgs2 mRNA

(A) Immunoblot of mock-transfected (control) and Igf2bp1-FLAG transfected cells for anti-FLAG-HRP. T=total fraction, before immunoprecipitation, E=elution fraction, post-3X-FLAG incubation, and P=pellet fraction post-elution. (B) Graph of the relative RNA fractions from transfected versus mock-transfected cells. Data displayed as means ±SEM with significance by one-way ANOVA and post-hoc Tukey's test: $F_{3,16}=14.04$, $^{**}P<0.01$. (C) Graphical representation of the Igf2bp1 gene displaying domains and constructs. Amino acids were numbered at intervals. A C-terminal FLAG tag was included for RRM-FLAG (aa185) KH-FLAG (aa577) and Igf2bp1-FLAG. (D) Immunoblot with anti-FLAG-HRP for control, and cells transfected with Igf2bp1-FLAG and deletion mutants. (E) Graph of the relative expression of RNAs in transfected versus mock-transfected cells. Data displayed as means ±SEM with significance by one-way ANOVA and post-hoc Tukey's test: $F_{11,28}=6.14$, $^{*}P<0.05$, $^{**}P<0.01$. Immunoblots represent>3 experiments. Graphs (N=3–4 experiments).

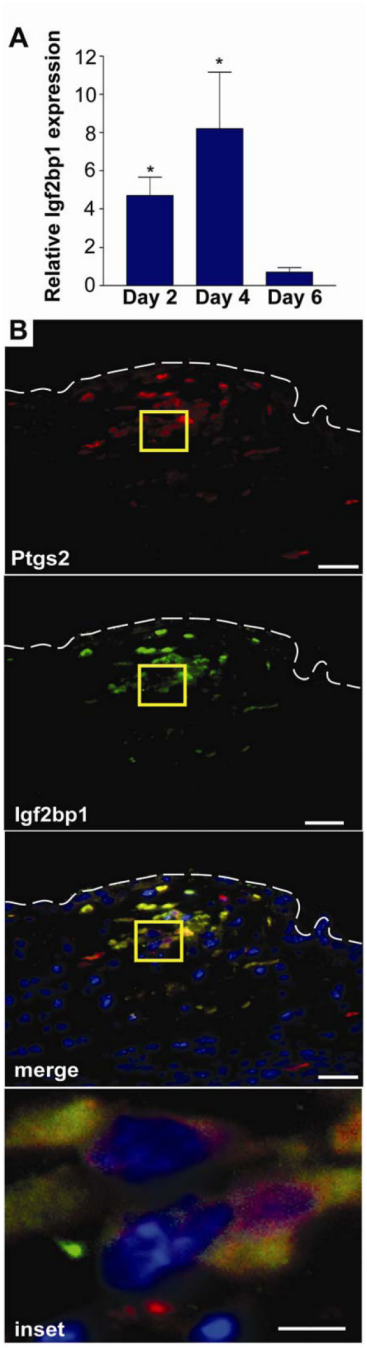

Figure 6. Igf2bp1 is expressed in Ptgs2 high-expressing cMSCs during the first phase of wound repair

(A) Graph of relative Igf2bp1mRNA expression (biopsy-injured compared to uninjured mucosa) at days 2, 4 and 6 post-injury (n≥5 wounds per day). Data displayed as means ±SEM with Student's t-test comparing injured to uninjured mucosa at each day; **P*<0.05. (B) A section of WT colon 2 days post-biopsy was stained with anti-Ptgs2 (red) and goat anti-Igf2bp1 (green) antibodies. Individual and merged channels were shown. White dashed lines=WAE cells basal surface. Yellow box=inset. Bars=50 μm (10μm inset).

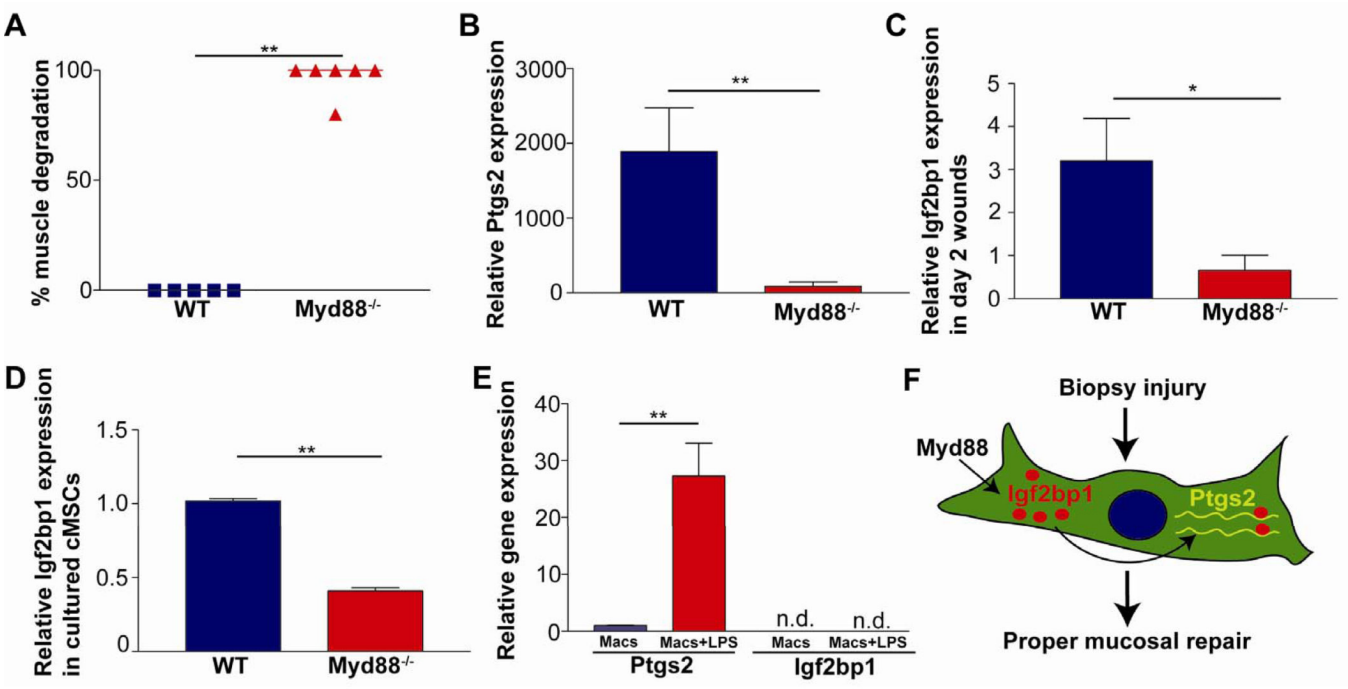

Figure 7. Myd88 is required for Igf2bp1 expression in cMSCs

(A) Graph of loss of α-SMA in WT and *Myd88*$^{-/-}$ sections 6 days post-injury (n≥5 per genotype). (B–C) Graphs showing relative Ptgs2 (B) or Igf2bp1 (C) mRNA expression comparing day 2 wounds to uninjured mucosa (n≥ 4 per genotype) for WT and *Myd88*$^{-/-}$ mice. (D) Graph of Igf2bp1 mRNA relative expression comparing cultured *Myd88*$^{-/-}$ and WT cMSCs. (n=3 experiments with 3–4 replicates/sample). (E) Graph of relative Ptgs2 and Igf2bp1 mRNA expression comparing unstimulated bone marrow macrophages (Macs) to stimulated macrophages (Macs+LPS) (n=4/treatment). Data displayed as means ±SEM and Student's t-test; *P<0.05; ***P*<0.01. (F) Model: In WT mice, Myd88 signaling induced Igf2bp1 expression in cMSCs during the early phase of mucosal repair. Igf2bp1 interacted with Ptgs2 mRNA and was required for maximal Ptgs2 expression. Maximal expression of Ptgs2 was required for proper mucosal healing observed during the later phase of repair.