A phenotype of IGFBP-3 knockout mice revealed by dextran sulfate-induced colitis

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Abstract

Background and Aim: Insulin-like growth factor-1 (IGF-1) bioactivity has been shown to be attenuated by insulin-like growth factor binding protein-3 (IGFBP-3), one of six IGF-binding proteins. While prior work revealed no major phenotype associated with IGFBP-3 knockout mice, we explored the possibility that a phenotype could be revealed under specific conditions of gastrointestinal stress.

Methods: The dextran sodium sulfate (DSS) murine model of ulcerative colitis was used for this study.

Results: Insulin-like growth factor binding protein-3 knockout mice had significantly reduced colitis on exposure to DSS as measured by lower levels of pro-inflammatory cytokines IL-6 (P < 0.0001), TNF-α (P = 0.0035), and IL-1β (P = 0.0112), reduced weight loss (P < 0.0001), reduced myeloperoxidase activity (P = 0.0025), and maintenance of colorectal length (P < 0.05), all relative to wild-type mice exposed to DSS. IGFBP-3 knockout mice also exhibited increased colon epithelial cell proliferation (P < 0.0001) following DSS exposure. Semi-quantitative immunohistochemistry showed greater IGF-1 receptor activation in colon epithelial cells of IGFBP-3 knockout mice compared with control mice following DSS exposure.

Conclusion: Our data demonstrate that IGFBP-3 influences severity of DSS-induced colitis. The observations suggest that in the absence of IGFBP-3, enhanced IGF bioactivity leads to increased epithelial proliferation and mucosal barrier repair, thereby lessening inflammation.

Introduction

Insulin-like growth factor binding protein-3 (IGFBP-3) is the most abundant binding protein that transports and controls the bioavailability of insulin-like growth factors (IGFs). It accounts for 80% of serum IGF-binding capacity. The liver generates IGF-1 in response to growth hormone (GH) stimulation, IGF-1 is also produced locally by many extra-hepatic tissues and can act as an autocrine or paracrine factor. The primary action of IGF-1 is mediated by binding to its specific receptor, which is widely expressed. Activation of the IGF-1 receptor, a tyrosine kinase, initiates an intracellular signaling cascade leading to activation of the phosphatidylinositol 3-kinase (PI3Kinase)/mechanistic target of rapamycin (mTOR) pathway, which stimulates cell growth and proliferation, and inhibits apoptosis.

In view of prior evidence linking IGF signaling to inflammation, we hypothesized that IGFBP-3 expression may influence inflammatory bowel disease. Prior work with IGFBP-3 knockout mice demonstrated no major phenotype under unstressed conditions. We used these mice in the dextran sodium sulfate (DSS) colitis model to determine if the absence of IGFBP-3 influences experimental colitis.

Metformin, an anti-diabetic drug, has been demonstrated in experimental models to display anti-inflammatory and anti-cancer activity. Metformin has been recently shown to attenuate the severity of colitis induced by DSS administration by inhibiting the activation of the NF-kB pathway, and we therefore also studied the combined effects of IGFBP-3 knockout and metformin administration in the DSS model.

Methods

Animals. Insulin-like growth factor binding protein-3 knockout mice were generated at Lexicon Pharmaceuticals Inc. (The Woodlands, TX, USA), as previously described, and were kindly provided by Dr David R. Powell. The maintenance of the IGFBP-3 knockout colony was as previously described. To obtain IGFBP-3 knockout mice on a pure C57BL/6 background, heterozygous IGFBP-3 female mice were first bred with a C57BL/6 male mouse for more than 10 generations of backcrossing, and then homozygous IGFBP-3 knockout and wild-type animals were generated and used in this study.
Genotyping was performed by PCR as previously described\(^9\) using the following primers: UTT022-7: 5′-TGCAAGGCCCTTAAGCACCTACTTCT; UTT022-8: 5′-CCCAGGTTCACATTTTCCAACCTT; UTT022-15: 5′-TAAGTTTCTCCAGACCTCAAGTG; UTT022-15: 5′-CCCTAGAATGCTCGTCAAGA.

All mice were maintained in a climate-controlled environment with conditions of 14 h light:10 h darkness, a temperature of 22 ± 2 °C, food \textit{ad libitum}, and a relative humidity of 30–60%. Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the Institute and McGill University’s Animal Ethics Committee. The protocol was approved by the McGill University and its Affiliated Hospitals’ Research Institutes (Animal Use Protocol #2011-5951). All surgery were performed under isoflurane anesthesia, and all efforts were made to minimize suffering. All procedures were performed in the specific pathogen free (SPF) animal quarters.

Response of insulin-like growth factor binding protein-3 knockout mice to dextran sodium sulfate colitis. Six- to eight-week-old male IGFBP-3 wild-type (WT: IGFBP-3 +/+ ) and knockout (KO: IGFBP-3 −/− ) mice were included for study. Both groups were exposed to 3% DSS (MP Biomedicals, Solon, OH, USA) in their drinking water for 5 days. On day 5, mice were given regular water. Mice were weighed every morning for the purpose of primary experimental outcomes and were euthanized on the morning of day 8 by cervical dislocation following inhalation of isoflurane, at which point, colon and blood samples were collected for histological and inflammatory assessment for secondary experimental outcomes. There were five to six mice for each of the two experimental groups and two control groups, and mice of the same group were kept in the same cage. Mice of the same genotype were randomly separated between experimental and control groups. All of the mice from each of the two experimental groups and two control groups were included in the analysis of the results.

Response of insulin-like growth factor binding protein-3 knockout mice to dextran sodium sulfate colitis with metformin treatment. In a separate experiment, 6- to 8-week-old male IGFBP-3 wild-type and knockout mice received 3% DSS in their drinking water for 5 days, as mentioned earlier. Half of the mice from the wild-type and IGFBP-3 knockout groups received metformin (Toronto Research Chemicals, Ontario, Canada; 600 mg/kg) in their drinking water 1 week prior to being exposed to DSS. Starting on day 1 of the DSS protocol, mice randomized to receive metformin received daily gavage treatment of metformin (600 mg/kg) in 0.5% methylcellulose. Control groups for wild-type and IGFBP-3 knockout mice had no DSS exposure, either with or without metformin exposure. Mice were weighed every morning for the purpose of primary experimental outcomes and were euthanized on the morning of day 8 by cervical dislocation following inhalation of isoflurane, at which point, colon and blood samples were collected for histological and inflammatory assessment for secondary experimental outcomes. There were four to five mice for each of the four experimental groups and four control groups, and mice of the same group were kept in the same cage. Mice of the same genotype were randomly separated between experimental and control groups. All of the mice from each of the four experimental groups and four control groups were included in the analysis of the results.

Colon tissues. At the time of sacrifice, the colon was removed and a small portion taken in the distal region (from rectum) (approximately 1–2 cm or 50–100 mg) was cut open longitudinally to have its content removed. The segment was then rolled up longitudinally (mucosa inwards) using a wooden stick as previously described.\(^11\) The most distal region was in the center of the roll. The resulting “Swiss roll” is then carefully transferred into a tissue cassette and placed in formalin for paraffin wax embedding. Colon tissue samples were stained with hematoxylin and eosin (HE), and immunohistochemical staining was performed for detection of Ki-67 and phosphorylated IGF-IR (Tyr1346).

TNF-α, IL-6, IL-1β, and IGF-1 ELISAs. Tissue isolated from the distal colon was homogenized in phosphate-buffered saline supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Laval, QC, Canada). The tissue samples, as well as ethylenediaminetetraacetic acid plasma samples, were assayed for mouse TNF-α, IL-6, IL-1β, and IGF-1 using the R&D Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Immunohistochemistry. Immunohistochemistry was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital, Montreal, QC, Canada). Briefly, tissue samples were cut at 4 μm, placed on SuperFrost/Plus slides (Fisher), and dried overnight at 37 °C. Immunostaining was performed using a Discovery XT Autostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Slide-mounted tissues were deparaffinized and rehydrated before heat-induced epitope retrieval was performed at pH 6.0 in RiboCC buffer (Ventana). Tissues were then blocked using inhibitor CM solution (Ventana) and thereafter incubated for 30 min with primary rabbit IGF-1R (Tyr1346) antibody (phospho-specific) (Origene, Rockville, MD, USA) and rabbit polyclonal antibody Ki-67 (Novus, Oakville, ON, Canada). Horseradish peroxidase-conjugated Omnimap anti-rabbit multimer (Ventana) was subsequently applied. 3,3′-Diaminobenzidine-tetrachloride was used as chromogen and hematoxylin was used for counterstaining before the slides were mounted with cover glasses. Negative controls included omission of the primary antibody. All slides were later scanned at 20x using a ScanScope AT Turbo (Leica Biosystems, Concord, ON, Canada) with default settings. Aperio ePathology Cytoplasmic v2 algorithm (Leica Biosystems) with default input parameters was utilized for quantification of Ki-67 stained proliferative cells in the distal colonic crypts. We evaluated the activity of the IGF-1R with the use of a grading scheme to help qualify the intensity of the
staining. A focus was placed on the distal regions of the colon (same defined portion of the distal colons analyzed for proliferative activity), and the staining intensity observed was given a value based on a tiered scale: 0, 1, 2, and 3 (for none, light, medium, or dark staining, respectively).

**Histological grading of colon tissue.** Hematoxylin and eosin-stained colon samples were graded on a scale of 0 to 6 to determine the extent of damage induced by DSS administration, according to the grading system shown in Table 1. Crypt architecture/ulcer presence and neutrophil localization were the two elements retained for our grading according to previous publications.12–14 The grade was obtained by adding the scores for crypt architecture changes to the scores for neutrophil presence (each scaled on a grade from 0 to 3).

**Statistical analysis.** The data presented are means ± SEM for IGF-1, TNF-α, IL-6, IL-1β, histological grading of colon, and Ki-67 levels. The distribution of variables was tested for normality. The significance of differences among all treatment groups was determined using generalized linear model or mixed procedures. One or two-way analysis of variance (ANOVA) was used to determine the effects different variables. The multivariate and univariate procedures of the repeated measures ANOVA were performed on weekly growth curves (weight) with week as a repetition factor. Additionally, least-squares means post hoc for multiple comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.3 (SAS Institute, Cary, NC, USA), with *P* values <0.05 considered significant.

**Results**

Response of insulin-like growth factor binding protein-3 knockout mice to dextran sodium sulfate colitis. Insulin-like growth factor binding protein-3 KO mice showed markedly less severe symptoms after DSS exposure relative to WT mice. They exhibited less severe diarrhea (through observation over the 7-day period) and a smaller percentage of weight loss on day 8 compared with the WT group under the DSS protocol (*P* <0.0001) (Fig. 1).

There was no significant difference in the colon length of the wild-type mice versus the knockout mice under control conditions. However, the colon lengths of IGFBP-3 knockout mice given DSS were longer than the wild-type colitic mice (*P* = 0.0476) indicating a decrease in the severity of the colitis (Fig. 2a).

Table 1  Histological grading of colon tissue

<table>
<thead>
<tr>
<th>Grading</th>
<th>Crypt architecture</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Acute regenerative changes</td>
<td>In lamina propria</td>
</tr>
<tr>
<td>2</td>
<td>Focal ulcer(s) all ≤ 1 mm</td>
<td>In submucosa</td>
</tr>
<tr>
<td>3</td>
<td>Confluent ulcers all &gt; 1 mm</td>
<td>In muscularis/mucosa/transmural</td>
</tr>
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To further quantify the degree of colonic inflammation, colonic MPO activity was measured. The IGFBP-3 knockout mice had significantly less neutrophil infiltration, as measured by MPO activity, following DSS exposure when compared with DSS-treated wild-type mice (*P* =0.0025) (Fig. 2b). As anticipated, there were no significant differences in MPO activity between the knockout and wild-type mice in the control state.

One of the ways by which DSS induces colonic inflammation is by increasing NF-kB expression, resulting in precursor cytokines, such as pro-IL-1β, being converted to their biologically active forms.15 The concentration of IL-1β in the colonic mucosa was significantly lower in the IGFBP-3 knockout mice receiving DSS when compared with the wild-type mice (*P* =0.0170) under the same conditions (Fig. 2c).

To further compare colitis severity between DSS-exposed IGFBP-3 knockout and wild-type mice, isolated colon tissue segments from each mouse were scored histologically. The wild-type mice under the DSS protocol had greater crypt architecture changes, as shown by increased presence of confluent ulcers ≥1 mm. Both groups receiving DSS had visible colonic neutrophil infiltration; however, higher levels were observed in the wild-type group (Fig. 3a–d), consistent with the MPO activity results. There were no visible architectural changes or differences in neutrophil presence between control and IGFBP-3 knockout mice unexposed to DSS. The histological scores revealed a trend of reduced colitis severity in the IGFBP-3 knockout mice when compared with the wild-type, but this did not reach statistical significance (Fig. 2d).

To compare proliferation in the colon epithelium between DSS-exposed IGFBP-3 knockout and wild-type mice, Ki-67 staining was used. The area of analysis studied for each mouse was 0.35 mm² (Fig. 3e–h). IGFBP-3 knockout mice exposed to DSS had significantly more proliferative cells compared with DSS-treated wild-type mice (*P* <0.0001). The percentage of proliferating cells in IGFBP-3 knockout mice on DSS was also...
significantly higher ($P=0.0001$) compared with both genotypes that did not receive DSS; however, there was a slight decrease in percentage of Ki-67 positive cells in wild-type mice on DSS compared with control, but it was not statistically significant ($P=0.0876$). There was no significant difference between the percentage of proliferative cells in the IGFBP-3 knockout and wild-type mice not exposed to DSS (Fig. 2e). As mentioned earlier, the crypt structure was lost in presence of DSS in wild-type mice, and Ki-67 staining was randomly distributed. As for the IGFBP-3 knockout mice receiving DSS, the architecture was kept but the distribution of the Ki-67 positive cells was in the whole crypt compared with only the base of the crypts in unexposed IGFBP-3 knockout mice (Fig. 3e–h).

We determined the relative levels of IGF-1 systemically and locally (in the colon) in IGFBP-3 knockout and wild-type mice under the DSS protocol to assess whether the treatment could influence the levels of IGF-1, although we recognize that our ability to detect modest changes is limited by the small number of animals. The IGFBP-3 knockout mice under the DSS protocol had lower IGF-1 levels in the serum when compared with the wild-type mice under the same conditions (1359 ± 369 vs 2884 ± 155 pg/mL), but this was not statistically significant. The same trend was observed for IGF-1 levels measured in colon tissue for the IGFBP-3 knockout versus the wild-type mice under the DSS protocol (633 ± 150 vs 1114 ± 109), but this was not a significant difference. In the absence of DSS, IGFBP-3 knockout mice had slightly lower levels of IGF-1 when compared with wild-type mice; both in serum (1182 ± 243 vs 1839 ± 488 pg/mL) and in colon tissue 423 ± 21 vs 719 ± 84 pg/mL), but this was not statistically significant. Because we did not observe any major differences in IGF-1 levels, IGF-1R activation was determined semi-quantitatively by staining colon sections for IGF-1R phospho-site Tyr1346. The majority of the IGFBP-3 knockout mice exposed to DSS was assigned a score of 3 with strong cytoplasmic staining in the distal colon. A moderate level of staining was visible for the mice not exposed to DSS; both IGFBP-3 knockout and wild-type mice were assigned a score of 2. The lowest intensity of staining was visible in the DSS-induced wild-type mice (Fig. 3i–l). The distribution of the phospho-IGF-1R (Tyr1346) positive cells was not restricted to the base of the crypts.

Figure 2  Comparing colitis severity between dextran sodium sulfate (DSS)-exposed insulin-like growth factor binding protein-3 (IGFBP-3) knockout and wild-type mice. At the end of a 7-day DSS exposure, mice were euthanized, and plasma and colon tissue were collected and processed for each assay as described in Materials and Methods section. Values are expressed as means ± SEM. n = 5–6 mice per group. (a) Colorectal lengths, *$P=0.0476$ compared with the wild-type DSS group; (b) myeloperoxidase activity (MPO), *$P=0.0025$ compared with the wild-type DSS group; (c) mouse colon tissue IL-1β concentration, *$P=0.0170$ when compared with the wild-type DSS group; (d) histological grading of colon tissue; and (e) quantification of Ki-67 immunostaining in distal regions of the colonic crypts, *$P<0.0001$ when compared with the wild-type DSS group. **$P=0.0001$ when compared with control wild-type and IGFBP-3 knockout groups.
Response of insulin-like growth factor binding protein-3 knockout mice to dextran sodium sulfate colitis with metformin treatment. Another set of experiments were carried out to define the role of metformin in our model, as this drug was recently reported to reduce inflammation in DSS colitis.8

Table 2 summarizes observations of a comprehensive experiment where either IGFBP-3 knockout or wild-type mice were treated (or not) with metformin, under control conditions or following DSS exposure. As for the previous experiment, on day 8, IGFBP-3 wild-type mice under DSS exposure lost more weight than IGFBP-3 knockout mice. This was also the case when mice received metformin (Fig. 4). Colon lengths, MPO levels, and histological scores showed trends consistent with our prior experiment. The levels of IL-6 and TNF-α were also decreased in DSS-exposed IGFBP-3 knockout mice compared with wild-type (P < 0.0001). The presence of metformin in DSS-exposed wild-type mice decreased the severity of colitis as observed with decreased levels of MPO (P < 0.0001), TNF-α (P < 0.0001), as well as a tendency of decreased IL-6 levels and the histological score compared with untreated DSS-exposed wild-type mice. This is also applied for IGFBP-3 knockout mice exposed to DSS receiving metformin: a trend in decreased levels of MPO, IL-6, TNF-α, and histological scores compared with untreated mice. Metformin and IGFBP-3 knockout each were associated with decreased inflammatory markers, where a small additive effect may be present.

Finally, Ki-67 staining of a 0.35-mm² area was used to determine joint effects of IGFBP-3 knockout and metformin in cellular proliferation (Fig. 5a–d). IGFBP-3 knockout mice under the DSS protocol had significantly increased proliferative cells compared with DSS-treated wild-type mice (P < 0.0001) and to DSS-treated wild-type mice given metformin (P < 0.0001). DSS-induced IGFBP-3 knockout mice also had significantly more proliferative cells compared with the DSS-induced knockout mice receiving metformin (P = 0.006). There was no significant change in

Figure 3  Microscopic findings in dextran sodium sulfate (DSS)-induced colitis mice. n = 5–6 mice per group. Image captured at 200 μm. Images represent a colon that has been rolled up longitudinally (mucosa outwards). (a–d) Hematoxylin and eosin staining of wild-type versus insulin-like growth factor binding protein-3 (IGFBP-3) knockout mice colons under 7-day DSS protocol; (e–h) anti-Ki-67 stained distal colonic regions of both mice; and (i–l) anti-phospho-IGF-1R (Tyr1348) stained distal colonic regions of both mice.
Discussion

We used IGFBP-3 knockout mice to determine if IGFBP-3 plays a role in DSS-induced colon inflammation. We hypothesized that the absence of IGFBP-3 would increase IGF-1 bioactivity, which would accelerate epithelial cell proliferation and repair of DSS-induced damage, lessening the inflammation seen in this model.16–19 The results of our first experiment, where we monitored the response of IGFBP-3 knockout mice to colitis induced by a 7-day DSS protocol, support our hypothesis. The DSS-exposed IGFBP-3 knockout mice exhibited significantly less severe colitis than wild-type mice, as shown by less weight loss, maintenance of colorectal lengths, and significantly lower levels of MPO activity and pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), all biomarkers of decreased colitis severity.18 Consistent with our hypothesis, DSS-exposed IGFBP-3 knockout mice had significantly increased cellular proliferation in defined portions of their distal colons in comparison to wild-type mice under the same DSS-exposed conditions, even though there was no difference between wild-type and knockout mice in epithelial proliferation in the absence of DSS. To explore whether this increase in proliferative cells was directly linked to enhanced activity of the IGF-1R, we compared receptor activity between control and knockout mice using immunostaining with an antibody against the activated IGF-1 receptor (anti-phospho-tyrosine 1346 of the IGF-1R). Association of increased IGF-1R activation with the increase of proliferative activity of the colonic epithelial cells following DSS exposure is suggested by the strong staining of the intestinal epithelium from the DSS-treated IGFBP-3 knockout mice, relative to the wild-type mice, but in view of the relatively small number of mice and semi-quantitative methods, this should be interpreted with caution.

Dextran sodium sulfate-induced damage to the intestinal epithelial barrier is followed by the entry of luminal antigens and microorganisms into the colonic mucosa, subsequently activating the pro-inflammatory NF-kB pathway.19 NF-kB stimulates precursor
cytokines, such as pro-IL-1β, to be converted to their biologically active forms. Biologically active IL-1β is able to stimulate an inflammatory cascade by increasing production of additional pro-inflammatory cytokines via a positive feedback mechanism.19 In principle, if the damage to the intestinal epithelial barrier was limited in IGFBP-3 knockout mice because of enhanced repair related to greater epithelial proliferation than controls, then these mice would also have reduced NF-kB activation. NF-kB activation is a crucial step for DSS to induce colitis because it triggers inflammatory cytokine production that subsequently damages the intestinal epithelium via neutrophil and macrophage recruitment.19 In both of our studies, DSS-exposed IGFBP-3 knockout mice had significantly decreased levels of pro-inflammatory cytokines.

In view of the recently described benefits of metformin in the DSS colitis model,8 we looked for interactions between the effects of IGFBP-3 deficiency and metformin in this model. We did not observe any statistically significant additional protection when DSS-exposed IGFBP-3 knockout mice were treated with metformin, in terms of weight loss, colorectal length, levels of pro-inflammatory cytokines, or MPO activity. It is possible that the use of a small number of animals in each group limited our power to detect small differences.

Interestingly, we observed a decrease in colon epithelial cell proliferative activity of the DSS-exposed IGFBP-3 knockout mice receiving metformin compared with the DSS-exposed IGFBP-3 knockout mice not receiving additional therapy. Not only did metformin decrease the proliferation of the epithelial cells but it also restricted the location of the proliferative cells to the base of the crypts. Metformin has been reported to decrease circulating insulin and IGF-1 levels, which decreases receptor tyrosine kinase signaling to downstream targets such as the PI3K–Akt–mTOR pathway.20 Metformin also may act to inhibit mTOR via AMP-activated protein kinase activation secondary to inhibition of oxidative phosphorylation.21 Thus, metformin may have a dual effect—any inhibition of proliferation would be expected to delay repair, worsening colitis, but on the other hand, as noted by prior studies,7,8 metformin may have separate anti-inflammatory activity, which would be expected to be beneficial. A role of metformin in modulating the gut microbiota should not be excluded to explain the effects observed here: more work needs to be carried out. Experimentally, we found that metformin does not significantly add to the protective effect of the absence of IGFBP-3. This is consistent with our hypothesis that an increase in proliferative activity of epithelial cells and enhanced repair of the

Figure 5  Anti-Ki-67 stained distal colonic regions: Immunohistochemistry images representing distal colon regions stained with the monoclonal antibody for Ki-67 of wild-type versus insulin-like growth factor binding protein-3 (IGFBP-3) knockout mice under 7-day dextran sodium sulfate (DSS) protocol ± metformin. n = 4–5 mice per group. Images captured at 200 μm.
epithelial barrier in the absence of IGFBP-3 is responsible for reduced colitis in the IGFBP-3 knockout mice.

A prior report\(^2\) demonstrated no effect of IGFBP-3 overexpression on severity of DSS-induced colitis. In contrast, our results reveal that IGFBP-3 deficiency influences DSS-induced colon inflammation, and that there is enhanced proliferation of colon epithelial cells in response to injury in IGFBP-3 knockout mice relative to control mice. While prior work\(^6\) revealed no obvious phenotype of IGFBP-3 knockout in mice under routine laboratory conditions, our observations reveal a phenotype under the stress of DSS exposure. These findings justify further research regarding roles of IGF signaling in inflammatory bowel disease. Future studies will explore the generality of this observation by determining if IGFBP-3 deletion is associated with enhanced proliferation in other contexts, for example, following wound healing.

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**References**