The effects of bevacizumab on intestinal anastomotic healing in rabbits

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Key words

bevacizumab; intestinal anastomotic healing; anastomotic leakage; angiogenesis; α-smooth muscle actin.

ABSTRACT

Purposes: The aim of this study was to investigate the effects of preoperative administration of BV on the healing process of intestinal anastomosis in a rabbit model.

Methods: Twenty male white rabbits were randomly divided into two groups. The control group received saline one week before surgery, and the BV group received intravenous BV one week before surgery. Each rabbit underwent an entero-enterostomy and a colo-colostomy. On postoperative day 7, the bursting pressures of the anastomoses, CD31 and α-smooth muscle actin (α-SMA) staining by immunohistochemistry, gene expression of α-SMA, and collagen deposition using Picro-Sirius Red at the site of anastomosis were evaluated.

Results: The bursting pressure of small bowel anastomoses was significantly lower in the BV group than in the control group (Control 184±10 mmHg vs. BV 140±9 mmHg; p=0.004). The microvessel counts in the anastomotic tissue were significantly lower in the BV group than in the control group in both the small bowel (p=0.023) and colon (p=0.008). The expression of α-SMA, and the degree of collagen deposition were decreased in the anastomotic tissue in the BV group compared with the control group.

Conclusion: Preoperative use of BV may negatively affect the rigidity of intestinal anastomosis.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death in industrialized countries [1]. In the past decade, the survival rates of unresectable and recurrent CRC have significantly improved with the development of cytotoxic drugs such as fluorouracil (5-FU), irinotecan**,** and oxaliplatin, as well as molecular-targeted drugs such as anti-vascular endothelial growth factor (VEGF) antibody and anti-epidermal growth factor receptor antibody. Several recent trials demonstrated that the median overall survival exceeds 20 months, with the effectiveness of molecular-targeted drug therapies in particular playing a prominent role in this positive trend [2, 3].

Bevacizumab (BV; Avastin®, Genentech, South San Francisco, CA, USA) is a recombinant humanized monoclonal antibody that inhibits VEGF. BV shows antitumor activity by inhibiting angiogenesis and normalizing abnormal tumor vasculature [4]. The number of oncological diseases to which BV is applied has been increasing year by year, including not only CRC but also breast cancer, glioblastoma, ovarian cancer, metastatic renal cell carcinoma, and non-small cell lung cancer. For these diseases, BV is used not only in palliative settings but also in neoadjuvant settings to increase the rate of curative resection by both ensuring an adequate surgical margin and controlling micrometastasis [5, 6].

Despite the benefit of preoperative use of BV in cases of cancer, BV is also known to cause various critical adverse events in some patients, such as gastrointestinal perforation and impaired wound healing [7]. We performed a phase II trial of neoadjuvant chemotherapy using BV in combination with 5-FU-based drugs for patients with locally advanced rectal

cancer [8]. Although this trial demonstrated the efficacy of neoadjuvant chemotherapy with BV for poor-risk rectal cancers (63.4% of partial response), it also demonstrated a high rate of anastomotic leakage (16.7%) [8]. These results indicated that BV has a negative impact on the healing process of intestinal anastomosis. However, there is no mechanistic study that elucidates whether BV administration induces adverse events in relation to the healing process of intestinal anastomosis in humans or large animals. Therefore, this study aimed to examine the effects of preoperative BV administration on the healing process of intestinal anastomosis in both the small bowel and the colon using a rabbit model.

METHODS

Animal Groups

Twenty male Japanese white rabbits, with a median weight of 2.5-3.0 kg, were used in this study. All animals were housed in the laboratory under a 12-h day:12-h night cycle at a constant temperature of 22°C. They were given standard laboratory food (CR-3; CLEA Japan Inc., Tokyo, Japan) and tap water *ad libitum*. All experiments were approved by the Institute for Laboratory Animal Research at the Nagoya University Graduate School of Medicine.

The animals were randomly divided into two groups of 10 individuals each. The control group received intravenous saline one week before surgery, the BV group received intravenous BV (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) in a single dose of 50 mg/kg of body weight one week before surgery (Fig. 1).

A single surgeon (H.N.) performed all operative procedures. All rabbits were sacrificed

seven days after the operation. Before sacrifice, the mechanical strength of the intestinal anastomoses was assessed under anesthesia by measuring their bursting pressures.

Anesthesia and Operative Technique

Anesthesia was induced by intramuscular injection of 50 mg/kg of ketamine hydrochloride and 12 mg/kg of xylazine hydrochloride, and this was maintained with an intravenous injection of 10 mg/kg/h of ketamine hydrochloride and 5 mg/kg/h of xylazine hydrochloride. After the rabbit was immobilized, the skin was shaved with electric clippers and cleansed with 10% povidone-iodine and 70% alcohol. The surgeon performed a laparotomy with a midline incision of approximately 5 cm. The Alexis® S wound retractor (Applied Medical, Rancho Santa Margarita, CA, USA) was placed in the wound upon entry into the peritoneal cavity and remained in place for the duration of the procedure. Two sites, at the small bowel and at the colon, were cut with a scalpel. Thereafter, the end-to-end anastomosis was performed for both the small bowel and the colon with one layer of 14-18 interrupted sutures with 5-0 PDS*II® (Ethicon, Somerville, NJ, USA). The abdominal muscle layers were closed by continuous sutures with 1-0 nylon, and the skin was closed with six interrupted mattress sutures with 1-0 nylon. All rabbits wore an Elizabethan collar after surgery in order to prevent them from biting or licking the wound. They were allowed access to tap water immediately after the operation, and they were allowed access to food the day after surgery.

Measurement of the Bursting Pressure of the Anastomosis

On postoperative day (POD) 7, laparotomy was again performed under the anesthesia

as described above. After identifying the colonic anastomosis, the colon was cut at 5 cm proximal to the anastomotic site. After washing out the feces, a catheter (inside diameter = 2.2 mm, outside diameter = 3.5 mm; TOP Co., Ltd, Tokyo, Japan) was inserted 1 cm toward the anastomotic segment from the cut end of the colon. The cut end of the colon was ligated around the catheter using silk sutures. The site 5-cm distal to the anastomotic site was clamped with a mosquito forceps. The catheter was connected to an infusion pump and a pressure transducer from Power Lab AD Instruments (Castle Hill, Bella Vista, Australia). Saline was injected with a flow rate of 2.5 ml/min. Bursting pressure was recorded in mmHg for each animal when there was a sudden loss of pressure or when the anastomosis started to leak. The bursting site was identified and documented. After the anastomotic tract segments were resected, one-third of this tissue was fixed in 10% formalin solution and subjected to histological examination. The rest of the resected anastomotic tissue was placed in sterile tubes, frozen in liquid nitrogen, and stored at -80°C. The bursting pressure of the small bowel anastomosis was measured in the same fashion. After all procedures were complete, the anesthetized rabbits were euthanized with an intravenous injection of 1 ml of sodium thiopental.

Histopathology

After the bursting pressure measurements, anastomotic segments of 2 cm were excised from the surrounding tissue and harvested. The anastomotic tissue samples were immediately immersed in 10% buffered formalin for 24-48 hours. The samples were dehydrated in a graded ethanol series and embedded in paraffin. Sections that were 4-µm-thick were mounted

on glass slides and stained with hematoxylin and eosin (H.E.) and Picro-Sirius Red (Polysciences, Inc., Warrington, PA, USA). Picro-Sirius Red staining was performed to detect the collagen components in the anastomotic tissue. Briefly, the sections were deparaffinized, rehydrated, and treated with Picro-Sirius Red solution for 60 min. The sections were rinsed with 0.1 N HCl and dehydrated by washing with absolute alcohol. Immunohistochemistry using antibodies for CD31 (Novus, Littleton, CO, USA), and α-smooth muscle actin (α-SMA) (Dako, Glostrup, Denmark) was also performed using a BOND-MAX autostainer (Leica Microsystems, GmbH, Wetzlar, Germany) according to the manufacturer's protocol. To obtain the microvessel count (MVC), the CD31-stained whole-mount slides were scanned using the Aperio CS Scanscope (Aperio Technologies, Inc., Vista, CA, USA), and six to eight fields (0.5 mm^2 each) in two sections were randomly analyzed with Aperio's vessel algorithm. The presence of collagen deposition and α-SMA in anastomotic tissue were assessed and scored by two independent observers blinded to the experimental groups, according to Ehrlich's modified 0 to 4 numerical scale, as follows: 0, no evidence; 1, occasional evidence; 2, light scattering; 3, abundant evidence; and 4, confluent cells or fibers [9].

Quantitative Real-time PCR

A 3 mm cube of the anastomotic tissue was harvested for the quantitative real-time PCR. To validate gene expression changes in the anastomotic tissue, quantitative real-time PCR analysis was performed with a Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated from every anastomotic tissue

section using the QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was generated from total RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each reaction was performed in a 20-μl mixture comprising TaqMan universal PCR master mix according to the manufacturer's instructions (Applied Biosystems). Expression of the gene encoding the α-SMA (assay identification no.Oc03399251_m1; Applied Biosystems) in the anastomotic tissue was determined. 18S rRNA (assay identification no. Hs99999901_s1; Applied Biosystems) was used as an endogenous control. All samples were tested in duplicate. The average of the gene expression in the small bowel of the control group was set as one-fold induction, and other data were adjusted to this baseline.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0® software (IBM Japan Ltd., Tokyo, Japan). Student's t-test was used to compare significant differences between the two groups, control vs. BV group. When criteria for parametric testing were violated, the appropriate nonparametric Mann-Whitney U-test was used. The Smirnov–Grubbs' rejection test was used to detect outliers. We considered $p < 0.05$ to be significant. Data are presented as means ± standard error.

RESULTS

Bursting Pressure

There was no evidence of intra-abdominal abscess or anastomotic leakage in either the

control or the BV group (Fig. 2A). With hematoxylin and eosin staining, macroscopic appearance of anastomoses were not much different between the two groups. In the small bowel, the bursting pressure was significantly lower in the BV group compared to the control group (Control 184±10 mmHg vs. BV 140±9 mmHg; p=0.004) (Fig. 2B). In the colon, the bursting pressure was also lower in the BV group compared to the control group although it did not reach to a statistically significant difference (Control 137±12 mmHg vs. BV 117±11 mmHg; p=0.291).

Microvessel Count in Anastomotic Tissue

Neoangiogenesis plays a central role in the anastomotic healing process during the proliferative phase, which begins on POD 2 and continues until POD 14 [10]. VEGF is one of the most important growth factors regulating this angiogenesis [10]. Therefore, we examined the microvessel counts in both the BV and control groups. The BV group exhibited significantly lower MVC compared with the control group in both the small bowel (Control 234±10 /mm^2 vs. BV 181±23 /mm^2; p=0.023) and the colon (Control 250±15 /mm^2 vs. BV 167±6 /mm^2; p=0.008) (Fig. 3A, 3B).

Histological Findings

Myofibroblasts are the most important cells for wound contraction and are also involved in the synthesis and repair of structural extracellular matrix proteins such as collagen [11]. α-SMA is commonly used as a marker of myofibroblast differentiation from fibroblast [11]. Therefore, we next examined the expression of α-SMA by immunohistochemistry and RT-PCR. The median α-SMA score in the BV group was

significantly lower than in the control group in both the small bowel (Control 3.5±0.1 vs. BV 2.6±0.2; p=0.002) and the colon (Control 3.5±0.3 vs. BV 2.8±0.2; p=0.013) (Fig. 4A, 4B).

α-SMA mRNA Expression in Anastomotic Tissue

The expression of α-SMA mRNA in the anastomotic tissue of the small bowel was significantly lower in the BV group than in the control group (p=0.013) (Fig. 4C). In the colon, the expression of α-SMA mRNA was also lower in the BV group than in the control group, although the differences were not significant.

Collagen Deposition in Anastomotic Tissue

The median collagen deposition score in the BV group was significantly lower than in the control group in both the small bowel (Control 3.5±0.1 vs. BV 2.3±0.3; p<0.001) and the colon (Control 3.4±0.2 vs. BV 2.4±0.3; p<0.001) (Fig. 5A, 5B).

DISCUSSION

Anastomosis leakage after CRC surgery is a critical morbidity that can be a cause of mortality [12]. Some clinical reports have shown that perioperative use of BV increases the rate of anastomotic complications [8, 13], while other reports have shown no correlation between the use of BV and postoperative complication rates [14, 15]. As for experimental studies, a number of reports have investigated the effects of 5-FU, a chemotherapeutic agent used extensively for CRC, on anastomotic healing [16-18], while only one experimental study has investigated the effects of BV on intestinal anastomosis [19]. The relationship between preoperative use of BV and anastomotic complication remains debatable, and basic

research to elucidate the mechanistic impact of BV on the healing process of intestinal anastomosis is required.

One study of the effects of BV on intestinal anastomosis was conducted in rats and reported that no significant differences between the BV group and the control group with regards to the bursting pressure of the colon, the degree of inflammation, the accumulation of fibroblasts, collagen deposition, or the degree of fibrosis [19]. Consequently, they concluded that administration of BV had no significant effect on anastomotic healing in the colon. However, according to the investigation by Lin et al., BV, which is a recombinant "humanized" monoclonal antibody against VEGF, does not bind to VEGF in the rat or mouse, but it does bind VEGF in the rabbit [20]. Another study also assessed the invalidity of BV in murine models using several bioassays [21]. Therefore, the results from the study that investigated the effects of BV using rats should be carefully interpreted, and the investigation using rabbits is preferable.

Assessment of anastomotic healing mainly depends on mechanical parameters, and the measurement of bursting pressure is particularly informative [22]. In our study, we found that the bursting pressure of the anastomotic segments on POD 7 was lower in the BV groups than in the control group (Fig. 2). Neoangiogenesis through the activation of VEGF-related signaling plays a central role in the anastomotic healing process. Currently, it is unclear whether an inhibition of VEGF signals by BV has any significant impact on angiogenesis in anastomotic healing, as anastomotic healing is a complex process that includes not only VEGF signals but also various other angiogenesis-related factors. This study clearly shows

that the preoperative use of BV substantially inhibits angiogenesis in anastomotic tissue on POD 7. The MVC of the anastomotic tissue was lower in the BV groups than in the control group (Fig. 3). These results are supported by a previous report by Christoforidis et al., which demonstrated that an intravitreal BV injection inhibits angiogenesis in cutaneous wound healing in rabbits [23]. Therefore, it appears that BV may inhibit angiogenesis during the healing process in the proliferative phase not only in cancerous tissue but also in normal tissue.

The expression of α-SMA in the anastomotic tissue, detected by both mRNA levels and immunohistochemistry, was lower in the BV group than in the control group (Fig. 4). α-SMA is commonly used as a marker of myofibroblast differentiation from fibroblasts [11]. Thus, our data imply that preoperative administration of BV decreases the number of α-SMA-positive differentiated myofibroblasts. These results are similar to a previous report that demonstrated that subconjunctival injections of BV inhibit myofibroblast transformation after glaucoma surgery in a rabbit model [24]. Myofibroblasts are the most important cells for wound contraction and are also involved in the synthesis and repair of structural extracellular matrix proteins such as collagen [11]. In this study, we found the lower collagen deposition at the site of anastomotic tissue in the BV group (Fig. 5). Because the integrity of the anastomosis represents equilibrium between collagen synthesis and lysis [17, 25], it is speculated that the reduction of α-SMA-positive differentiated myofibroblasts in anastomotic tissue negatively influences intestinal anastomotic healing. These results may support the lower anastomotic bursting pressure found in the BV group compared to the control group

(Fig. 2).

It should be noted that the humanized BV used in this study has a weaker affinity for rabbit VEGF, which is approximately one-fifth to one-eighth of its affinity for human VEGF [20, 26]. Therefore, the dose of BV (50 mg/kg) used in this study was higher than that normally used for humans with CRC (5 to 10 mg/kg). Additionally, the half-life of BV in the rabbit is reported to be 5.52 days, which is much shorter than that in humans (3 weeks) [27]. In this study using the rabbit model, the intervals between the BV administration and surgery were set at one week. This period correspond to approximately 3 to 4 weeks in humans. The National Comprehensive Cancer Network (NCCN) guideline of the colon recommend an interval of at least 6 weeks between the administration of BV and elective surgery, which corresponds to two half-lives of BV [28]. The results obtained in this study clearly support the cessation period of the NCCN guideline. It is necessary for a clinician to recognize that a short cessation period does not secure a normal healing process of intestinal anastomosis. Further study with different doses and cessation periods is required to precisely elucidate the impact of BV on the anastomotic healing process.

In conclusion, our study demonstrates that preoperative administration of BV inhibits angiogenesis, and decreases α-SMA accumulation and collagen deposition in the intestinal anastomotic tissue in rabbits. Preoperative use of BV may negatively affect the rigidity of intestinal anastomosis.

CONFLICT OF INTEREST

Chugai Pharmaceutical Co., Ltd, Tokyo, Japan provided the BV used in this study. All the authors declare that they have no conflicts of interest. The sponsor had no role in the study design, conduct of the study, data collection, data management and interpretation, preparation of this article, or approval of the article.

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FIGURE LEGENDS

Figure 1.

Schedules for all procedures.

Figure 2.

Bursting pressures of the small bowel and colon.

A: Representative photos of the anastomotic site in the small bowel and colon in each group on POD 7. White arrowheads indicate anastomotic sites.

B: Anastomotic bursting pressures (mmHg) of the small bowel and colon in each group. *$p<0.05$ vs. control.

Figure 3.

Immunohistochemistry for microvessels in the anastomotic tissue using CD31 antibodies.

A: Representative raw (left) and processed markup (right) images of CD31 immunohistochemistry sections of the small bowel and colon at the anastomotic site in each group (×100 objective).

B: Microvessel count (/mm^2) of the small bowel and colon in each group. *$p<0.05$ vs. control.

Figure 4.

α-SMA expression in the anastomotic tissue.

A: Representative images of H.E. (upper) and α-SMA immunohistochemistry (lower) sections of the small bowel and colon at the anastomotic site in each group (left; ×12.5 objective, right; ×200 objective).

B: The median histological score of the small bowel and colon in each group by Ehrlich's

modified 0 to 4 numerical scale. *p<0.05 vs. control.

C: α-SMA expression in the small bowel and colon in each group by real-time RT-PCR. *p<0.05 vs. control.

Figure 5.

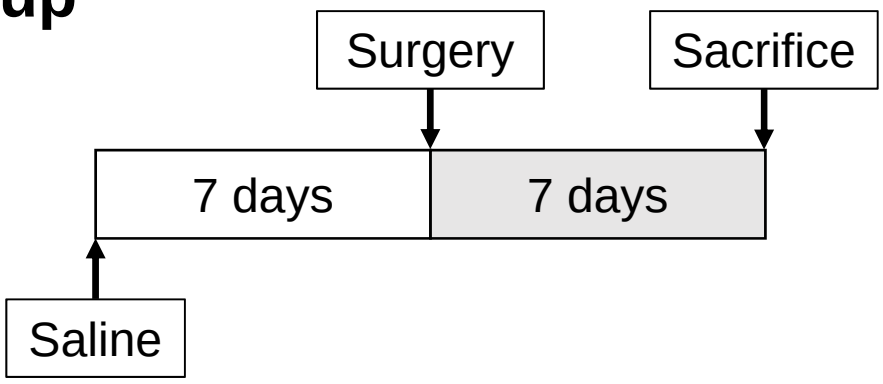
Collagen deposition in the anastomotic tissue using Picro-Sirius Red.

A: Representative images of H.E. (upper) and Picro-Sirius Red (lower) sections of the small bowel and colon at the anastomotic site in each group (left; ×12.5 objective, right; ×200 objective).

B: The median histological score of the small bowel and colon in each group by Ehrlich's modified 0 to 4 numerical scale. *p<0.001 vs. control.

Control group

n=10



BV group

n=10

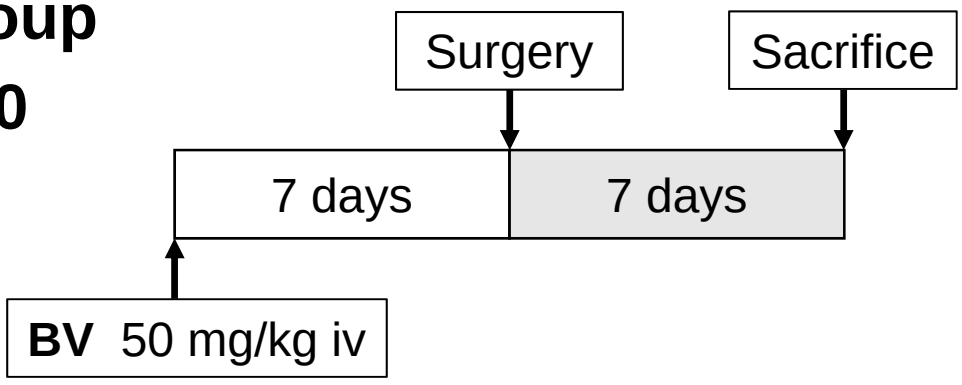


Figure 1

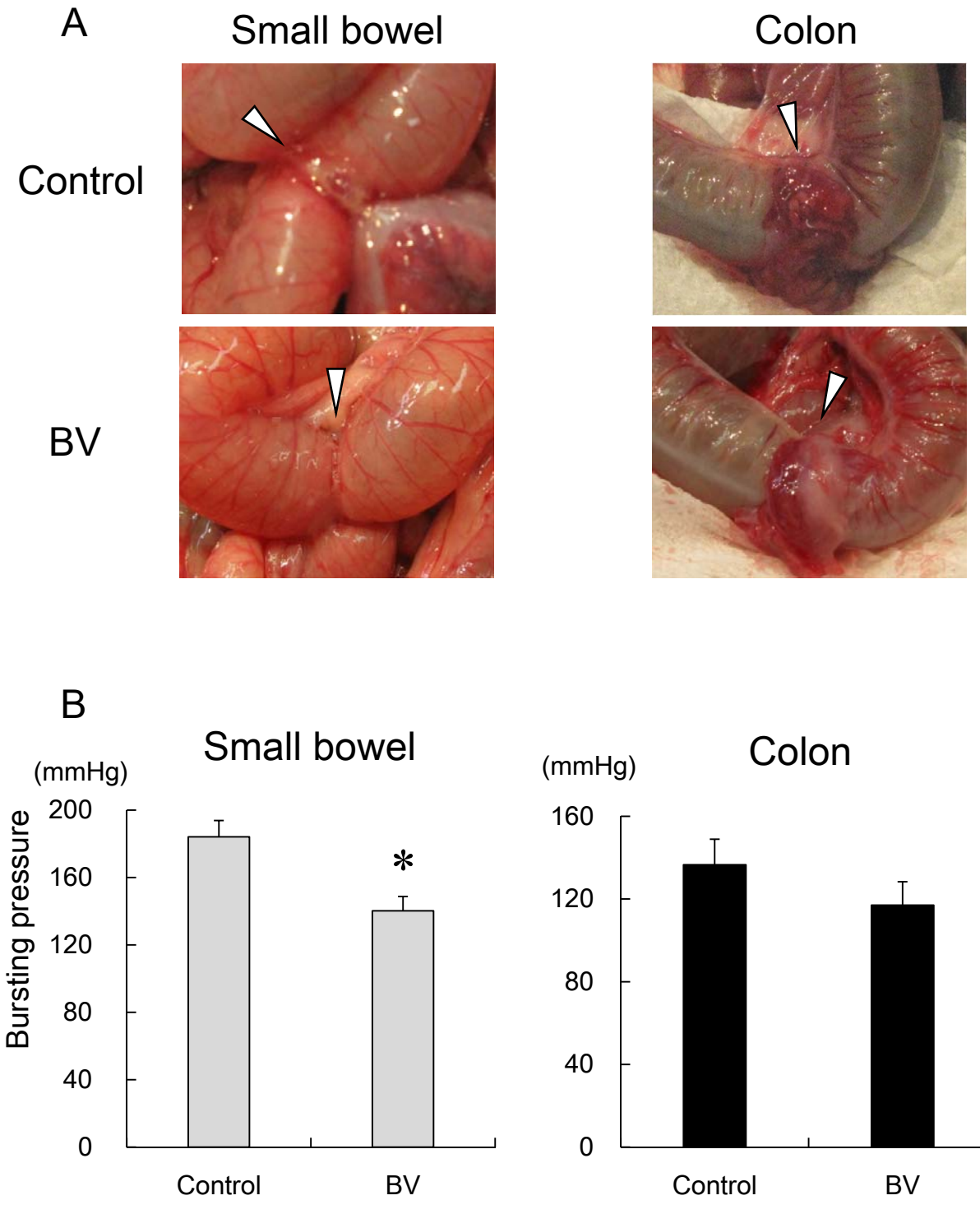
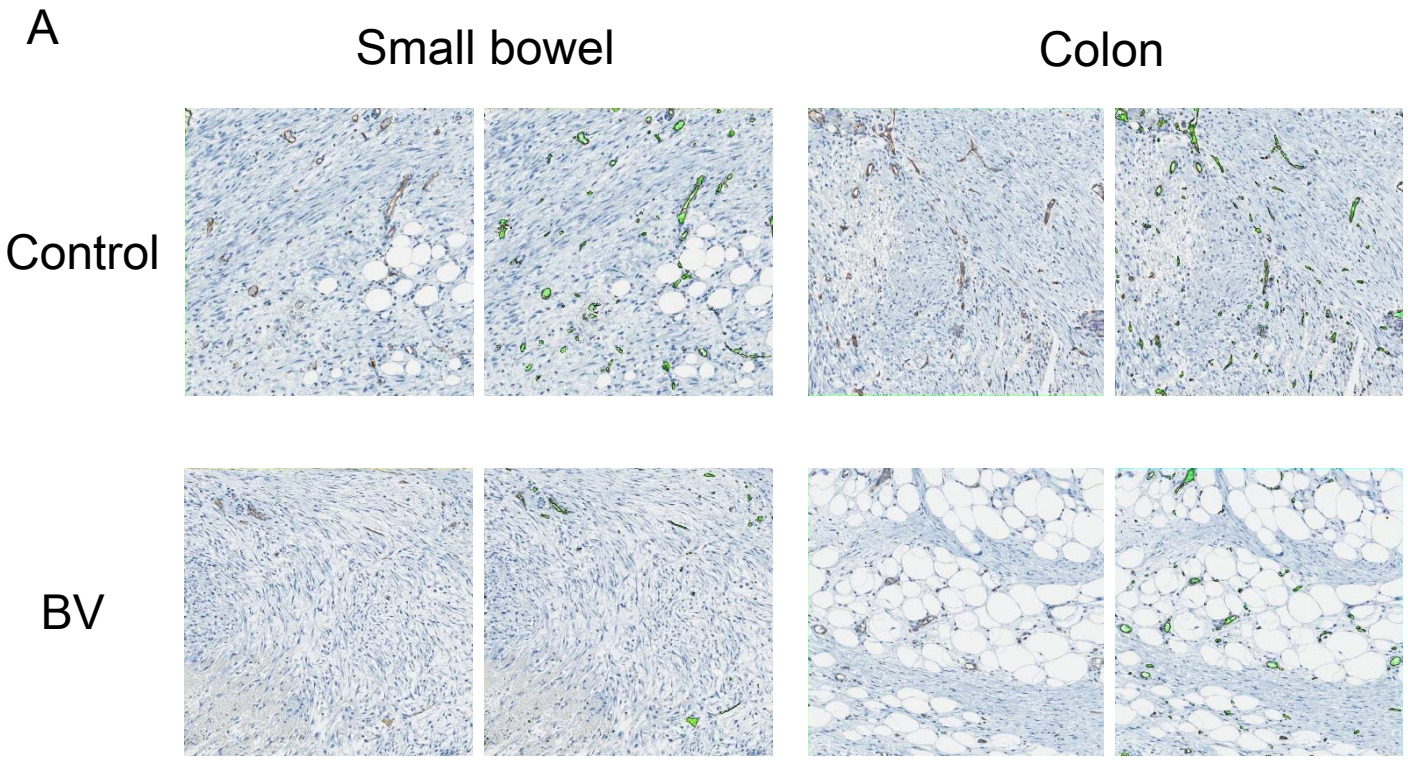

A
Small bowel
Colon
Control
BV
B
Small bowel
Colon
(mmHg)
Bursting pressure
200
160
120
80
40
0
*
Control
BV
(mmHg)
160
120
80
40
0
Control
BV

Figure 2


A
Small bowel
Colon
Control
BV

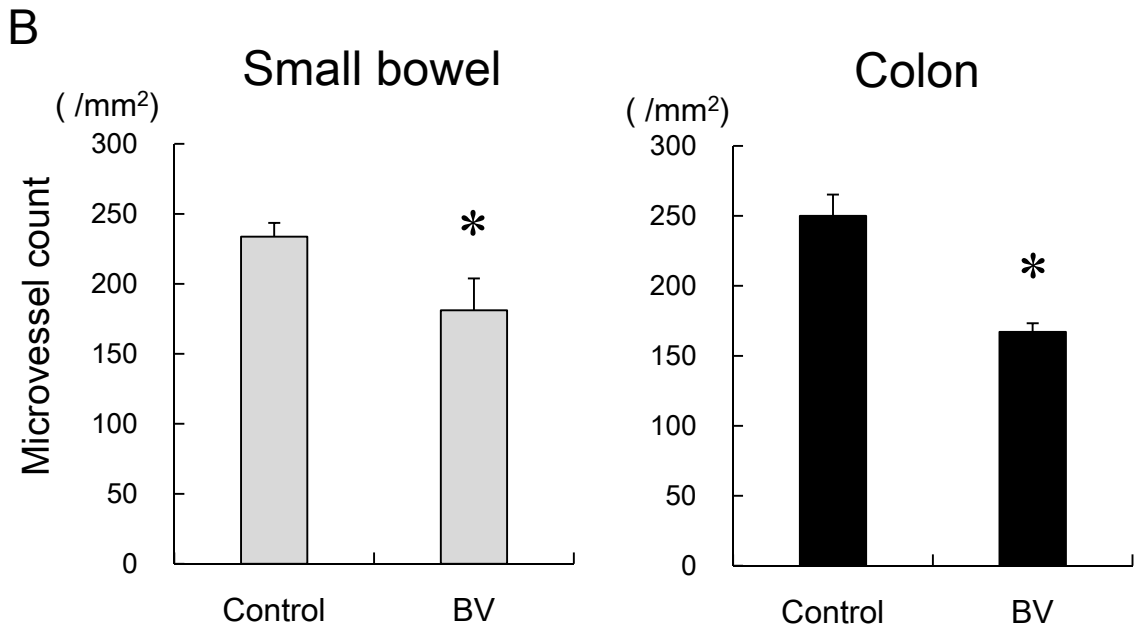
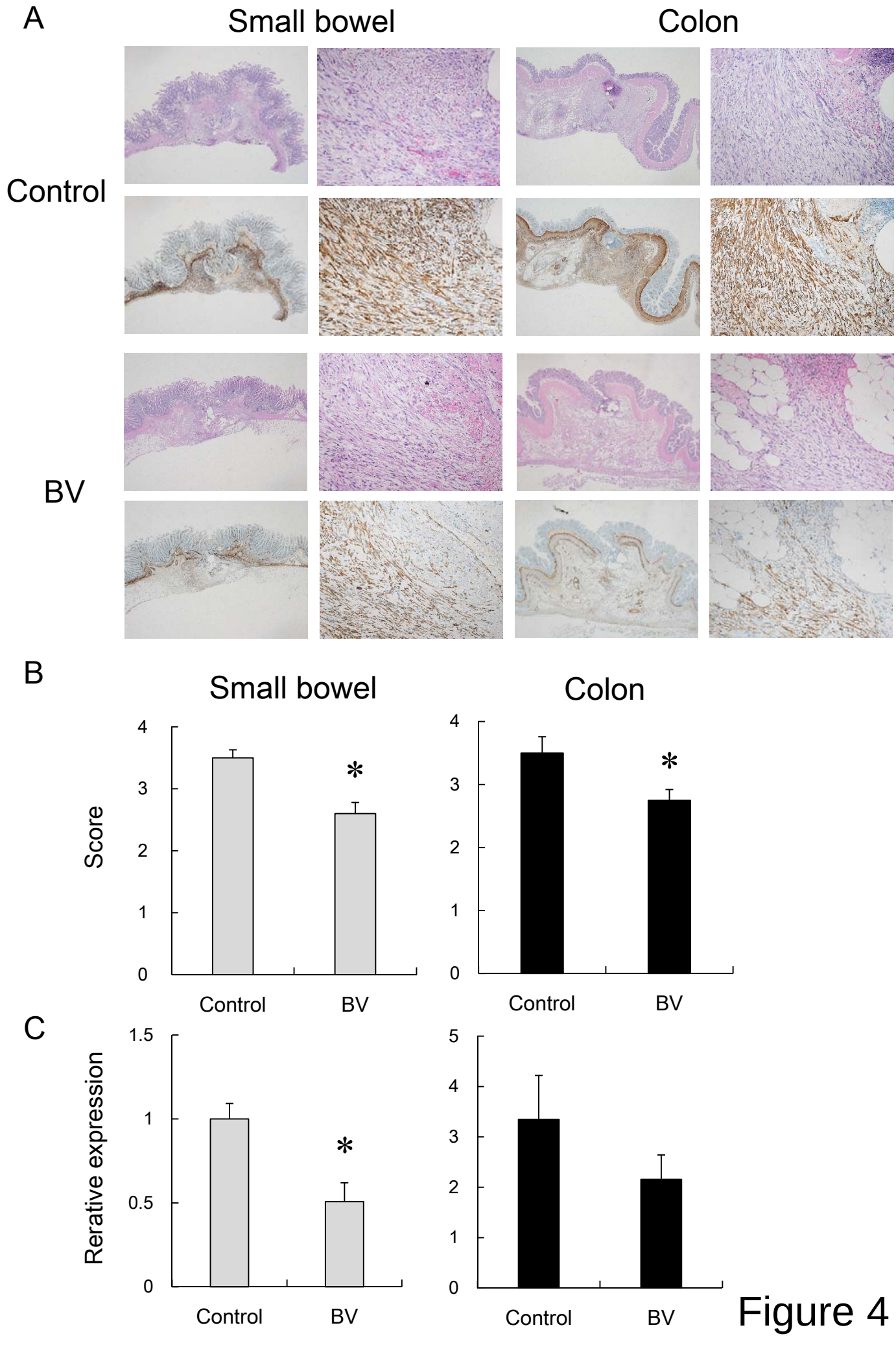

B
Small bowel
Colon
(/mm2)
(/mm2)
Microvessel count
300
250
200
150
100
50
0
*
*
Control
BV
Control
BV

Figure 3


A
Small bowel
Colon
Control
BV
B
Small bowel
Colon
Score
4
3
2
1
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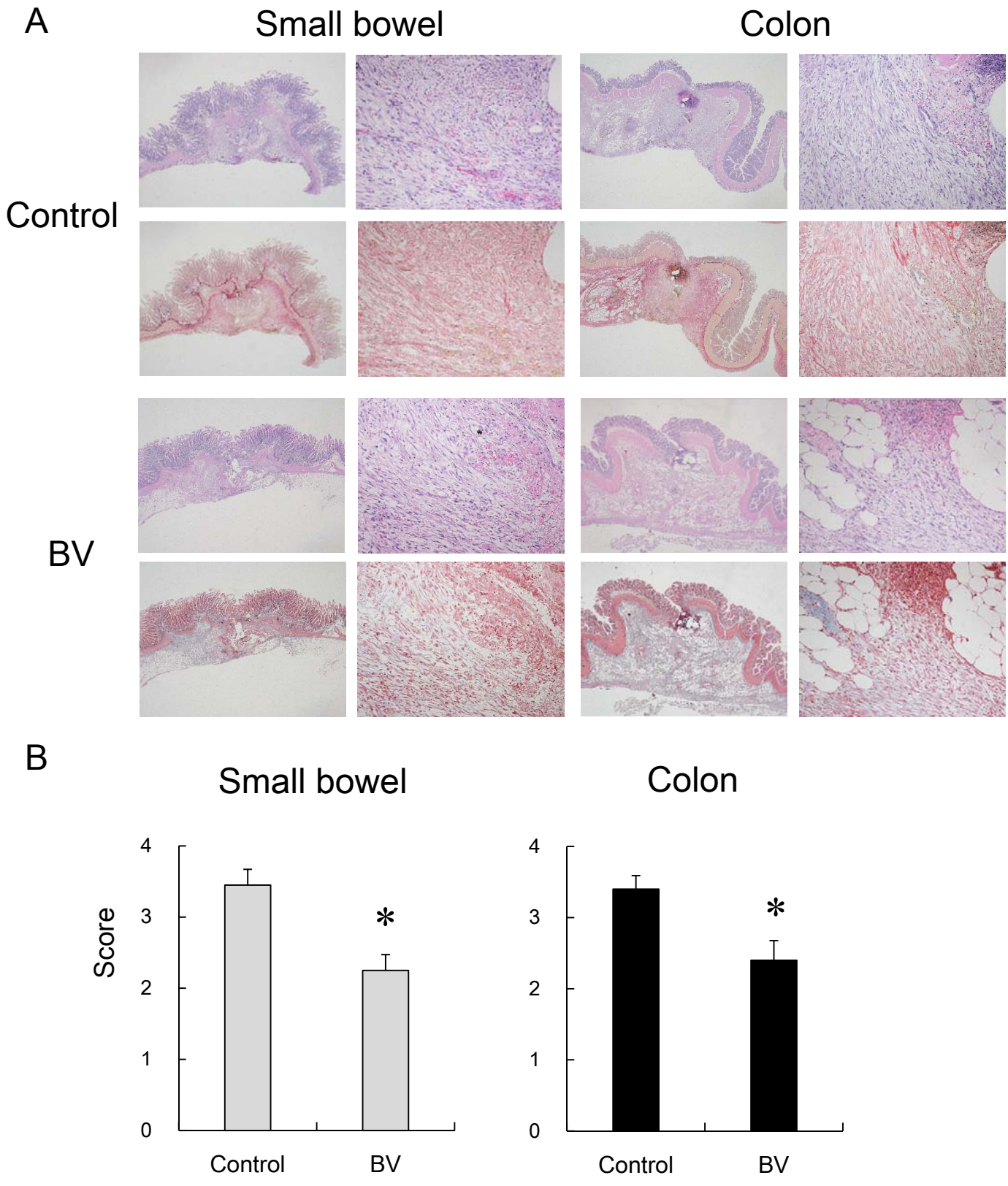
Control
BV
C
Rerative expression
1.5
1
0.5
0
5
4
3
2
1
0

Figure 4


A
Small bowel
Colon
Control
BV
B
Small bowel
Colon
Score
4
3
2
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0
*
*
Control
BV
Control
BV

Figure 5