**Saccharomyces boulardii** Improves Intestinal Cell Restitution through Activation of the α2β1 Integrin Collagen Receptor

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**Abstract**

Intestinal epithelial cell damage is frequently seen in the mucosal lesions of inflammatory bowel diseases such as ulcerative colitis or Crohn’s disease. Complete remission of these diseases requires both the cessation of inflammation and the migration of enterocytes to repair the damaged epithelium. Lyophilized *Saccharomyces boulardii* (Sb, Biocodex) is a nonpathogenic yeast widely used as a therapeutic agent for the treatment and prevention of diarrhea and other gastrointestinal disorders. In this study, we determined whether Sb could accelerate enterocyte migration. Cell migration was determined in Sb force-fed C57BL6J mice and in an *in vitro* wound model. The impact on α2β1 integrin activity was assessed using adhesion assays and the analysis of α2β1 mediated signaling pathways both *in vitro* and *in vivo*. We demonstrated that Sb secretes compounds that enhance the migration of enterocytes independently of cell proliferation. This enhanced migration was associated with the ability of Sb to favor cell-extracellular matrix interaction. Indeed, the yeast activates α2β1 integrin collagen receptors. This leads to an increase in tyrosine phosphorylation of cytoplasmic molecules, including focal adhesion kinase and paxillin, involved in the integrin signaling pathway. These changes are associated with the reorganization of focal adhesion structures. In conclusion Sb secretes motogenic factors that enhance cell restitution through the dynamic regulation of α2β1 integrin activity. This could be of major importance in the development of novel therapies targeting diseases characterized by severe mucosal injury, such as inflammatory and infectious bowel diseases.

**Introduction**

The colonic epithelium forms a continuous physical and functional barrier that protects the internal environment of the body from the fluctuating external milieu. Various substances including dietary elements, gastrointestinal secretory products and drugs are known to disrupt this epithelial barrier, leading to the shedding of epithelial cells and the development of wounds [1]. In addition inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn’s disease, are characterized by varying degrees of mucosal surface damage, chronic inflammation and mucosal ulceration, resulting in the subsequent impairment of the barrier function [2]. Moreover, infection of the colonic mucosa by bacterial pathogens including *Shigella*, *Salmonella* or *Clostridium difficile* results in the development of acute intestinal inflammatory diseases and destruction of the intestinal epithelium [3,4]. The colonic barrier has a striking ability to rapidly reseal superficial wounds, which is critical for the maintenance of barrier function and homeostasis. As with other epithelia of the gastrointestinal tract, the repair of damaged colonic mucosa initially requires cell migration to restore epithelial continuity [1]. This process, termed restitution, is followed by the proliferation and subsequent maturation and differentiation of the cells, allowing the restoration of normal architecture and absorptive/secretory function.

Intestinal restitution has been found to be influenced by a broad spectrum of factors derived from the gastrointestinal environment, including host epithelial and lamina propria cells, resident microbiota, and the dietary and non-dietary components present in the gastrointestinal lumen [2]. Moreover, dynamic and reciprocal crosstalk between receptors for soluble factors and those for the extracellular matrix (ECM) play a crucial role in the regulation of this process [5,6,7].

Integrins constitute the main cell surface adhesion receptors mediating cell-ECM adhesion. These transmembrane heterodimeric molecules are made up of non-covalently bound α and β subunits. In mammals, 18 α and 8 β subunits combine to form 24 distinct integrin receptors that bind various ECM ligands with different affinities [8]. Integrins allow a bi-directional flow of mechanochemical information across the plasma membrane and facilitate interactions between the ECM and the actin cytoskeleton.
These integrin-mediated interactions, on either side of the plasma membrane, are dynamically linked. The cytoskeleton controls the functional state of the integrins thus modulating their interaction with the ECM. Meanwhile integrin binding to the ECM changes the cell shape and the composition of the cytoskeleton beneath [8].

The nonpathogenic yeast Saccharomyces boulardii (Sb) is widely used in a lyophilized form to treat and prevent antibiotic-associated and infectious diarrhea [9]. Recent in vitro and in vivo studies indicate that Sb interacts not only with pathogenic microorganisms and resident microflora, but also with intestinal mucosa [10,11]. In addition, Sb has been shown to exert a trophic effect that restores intestinal homeostasis [11]. Furthermore, clinical trials have suggested that Sb can be effective in the treatment of IBD [12,13] through the modulation of host cell signaling pathways implicated in the proinflammatory response [14,15,16]. However, no information is currently available on the possible effects of Sb upon colonic epithelial cell restitution.

In the present study, we show that Sb secretes factors that modulate intestinal epithelial cell restitution both in vitro and in vivo. Our findings suggest that Sb increases intestinal epithelial cell migration without affecting cell proliferation. Sb exerts at least some of its motogenic effects through the activation of the α2β1 integrin collagen receptor signaling pathway, which is associated with the reorganization of focal adhesions.

Methods

Cell culture

The human colonic adenocarcinoma cell lines HCT-8/E11, CaCo2/TC7, HT29-D4 and T84, were routinely cultured as previously described [17,18,19,20]. Cells were cultured on plastic dishes until they reached confluency. These cellular monolayers consist of polarized cells joined by tight junctions, exhibiting well developed apical microvilli, which allow the study of the processes involved in intestinal epithelial cell physiology.

Yeast culture supernatants

The supernatants of Sb (Biocodex laboratories; Gentilly, France) and Saccharomyces cerevisiae (Sc, strain BY4742, a gift from C. De La Roche Saint-André; CNRS, Marseille, France) were prepared as previously described [21]. Briefly, yeast strains (100 mg/ml) were cultured overnight at 37°C in epithelial cell culture media. Conditioned media were centrifuged at 20,000 g for 15 minutes and the supernatants collected. The supernatants were passed through 0.22 μm filters (Fisher Scientific) to remove yeast cells. Serial dilutions ranging from 1/8 to 1/128 were performed in 10Sb wound, 10 measurements of wound width were recorded. To assess the role of the integrins in Sb enhanced enterocyte migration, 10 μg/ml function-blocking anti-integrin monoclonal antibodies (mAbs) (GoH3 (anti-α6), 69.6.3 (anti-αv), Lai1/2 (anti-β1), G19 (anti-α2), and SAN-1 (anti-β3); Beckman Coulter, Marseille, France), β1-activating mAb (TS2/16; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-DPP IV mAb (S. Maroux, CNRS, Marseille) were added 1 hour before wounding and again during the period of cell migration. In some samples, MAPK inhibitor (10 μM PD 98059; Tocris Bioscience, Bristol, UK) or 10 nM FAK inhibitor (PF 573228; Tocris Bioscience) were added 1 hour before wounding and again during the period of cell migration. When required, cells were treated with 2.5 μg/ml mitomycin C (Sigma) prior and during cell migration in order to block cell proliferation. In order to track the rate of cell proliferation, 10 μmol/L 5-bromo-2′-deoxyuridine (BrdU) was added 4 hours after the wound and incubation continued for an additional 1 hour period. Cells that had incorporated BrdU into their DNA were detected using the BrdU labeling and detection kit according to the manufacturer’s instructions (Roche, Mannheim, Germany). Cell proliferation in wounded monolayers was visualized by light microscopy using an inverted Olympus CKX41 microscope.

Measurement of enterocyte crypt-villus migration

Groups of 6-week old C57BL/6j female mice (n = 5) were force-fed daily with 200 μl PBS solution or without 1 mg/ml lyophilized Sb for 1 week. When required, mice were force-fed daily for one week with 200 μl Sb supernatant or Sc supernatant. Animals were injected with BrdU (50 mg/kg, BrdU; Sigma) intraperitoneally and then sacrificed 24 hours later. Segments of intestine were frozen in liquid nitrogen and cryosectioning (section thickness: 8 μm) was performed. Samples were immunostained with a rat anti-BrdU antibody mAb (Abcam) overnight at 4°C. After washing, sections were incubated with AlexaFluor 488-conjugated goat anti-rat IgG (Invitrogen) for 1 hour, and then mounted in ProLong Gold medium containing DAPI (Invitrogen) in order to counterstain nuclei. Images were captured and analyzed using a Leica DM IRBE microscope.

Cell adhesion assay

Cell adhesion assays on Laminin-111 (Sigma, Saint Quentin Fallavier, France) and type I collagen (BD-Bioscience, Bedford, MA, USA) were performed using cells incubated with or without Sb supernatant, as previously published [22]. Function blocking anti-integrin mAbs (10 μg/ml) were added to a subset of samples, to identify the receptor types involved in Sb enhanced adhesion.

Immunocytochemistry and Immunohistochemistry

Cells, incubated with or without Sb supernatant, were fixed and permeabilized as previously described [23]. Paxillin was immunostained with a mouse anti-paxillin mAb (Millipore). Tissues were fixed in acetone for 10 minutes at -20°C, and stained with rat anti-ki-67 (Abcam), rat anti-αv integrin (Beckman Coulter), rabbit anti-α2 integrin (Millipore) or rabbit anti-Y118 Paxillin (Millipore) Abs. After washing, samples were incubated with the appropriate Alexa 488- or Alexa 546-conjugated secondary Abs (Invitrogen), and then mounted in ProLong Gold medium. Images were captured and analyzed using a Leica DM IRBE microscope.

Detection of tyrosine-phosphorylated proteins

Single-cell suspensions (25 000 cells/0.1 ml), prepared in DMEM containing 0.2% BSA (adhesion buffer), were seeded onto
type I collagen-coated wells and allowed to adhere for periods ranging from 0 to 30 minutes. Adherent cells were lysed as previously published [24]. Equal amounts of cell lysates (50 μg) were resolved by SDS-PAGE and blotted onto a nitrocellulose sheet. Membranes were blocked with PBS containing 3% non-fat dry milk and probed overnight at 4°C with Abs directed against rabbit phospho ERK1/2 (Ozyme, St. Quentin en Yvelines, France), mouse anti-Y397-FAK (Invitrogen) or rabbit anti-Y118-paxillin (Millipore). Blots were then revealed by chemiluminescence after incubation with the appropriate horseradish peroxidase-conjugated secondary Ab (Amersham). Loading amounts were verified by probing the blot with rabbit-anti FAK (Ozyme), mouse anti-Erk1/ Erk2 (Santa Cruz Biotechnology), or mouse anti-paxillin (Millipore).

**Statistical analysis**

All experiments were performed a minimum of 3 times in duplicate. For statistical analysis of data, Student’s t-test was used. Values are expressed as mean ± SD. Data were considered as statistically significant at P values of <.01(**) or <.05 (*).

**Results**

**Sb supernatant improves colonic epithelial cell restitution**

To investigate any direct effect of Sb supernatant on cell restitution, we used HCT-8/E11 cells for their wound-filling capacity [25]. *In vitro*, Sb supernatant markedly enhanced wound repair (Figure 1A) in a dose-dependent manner (not shown) with a maximal effect obtained with a 1/8 dilution. We therefore used this dilution in subsequent experiments. Time lapse videomicroscopy experiments (see supplemental video S1 and S2) revealed that cells treated with Sb supernatant exhibited a higher rate of migration than cells treated with a unused culture medium for Sb (80 μm/h versus 40 μm/h). Quantification of the surface area recovered by the cells indicated that Sb supernatant exerted its effect as early as 1 hour after wounding and persisted for up to 5 hours (Figure 1B).

The motogenic effect of Sb supernatant was quite similar to that observed using type I insulin-like growth factor (IGF-I), a well known inducer of cell restitution (Figure 1C) [24].

![Figure 1. Sb increases intestinal epithelial cell restitution.](https://example.com/figure1.png)

Figure 1. Sb increases intestinal epithelial cell restitution. (A) Polarized HCT-8/E11 cell monolayers were wounded as described in Methods and incubated for 5 hours with or without Sb supernatant. Phase contrast images were acquired at the indicated times (see also Supplementary data S1 and S2). Data shown are from a representative experiment out of 5 performed. Scale bar: 300 μm. (B) HCT-8/E11 cell monolayers were wounded and incubated with or without Sb supernatant. The percentage of wound area closed was determined at hourly intervals. Data represent the mean ± SD of 3 separate experiments. ** P<.01. (C) HCT-8/E11 cell monolayers were wounded and incubated for 5 hours without (control) or with Sb or Sc supernatants, or 14 nM IGF-I (IGF-I). Results are expressed as the percentage of cell migration compared to control. Data represent the mean ± SD of 3 separate experiments. ** P<.01. (D) Polarized HT29-D4, CaCo2/TC7 and T84 cell monolayers were wounded and incubated for either 4 hours (T84) or 20 hours (HT29-D4 and CaCo2/TC7) with or without Sb supernatant. Results are expressed as the percentage of cell migration compared to control. Data represent the mean ± SD of 3 separate experiments. ** P<.01.

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S. boulardii Enhances Cell Restitution

To determine whether the enhanced wound repair observed in response to Sb supernatant was caused by an increase in cell migration and/or cell proliferation, HCT-8/E11 cells were treated with 2.5 μg/ml mitomycin C, an inhibitor of DNA replication, both before and during wound healing assays. Mitomycin C did not attenuate the Sb-dependent wound repair, indicating that Sb acts by increasing epithelial cell migration with no effect on proliferation (Figure 2A). Furthermore, as a marker of cell proliferation rate, the extent of BrdU incorporation into cells was measured. As shown in Figure 2B, few nuclei of migrating cells exhibited uptake of BrdU. Moreover, no significant difference between untreated or Sb-treated cells was observed. Taken together, these findings indicate that Sb supernatant improves intestinal epithelial restitution independently of cell proliferation.

Sb supernatant improves enterocyte crypt-villus migration

To evaluate whether Sb supernatant regulates enterocyte migration in vivo, C57BL/6j mice were force-fed with PBS solution with or without 1 mg/ml Sb, daily, for 1 week. This treatment did not modify villus length (not shown). BrdU was administrated to control and Sb force-fed mice 24 hours before death. This strategy allowed the calculation of the rate, and extent to which BrdU-labelled cells migrated from the crypts to the tips of the villi [26]. In control animals, BrdU-stained cells were detected mainly within the crypts (Figure 3A). By contrast, in Sb force-fed mice, BrdU positive cells were distributed from the crypt to the tips of the villi (Figure 3B). Statistical analysis revealed that Sb significantly enhanced cell migration along the crypt-villus axis (migration to 45% of villus height in Sb treated animals, compared to 20% of villus height in controls) (Figure 3C).

To ensure that Sb did not promote the expansion of the proliferative compartment, ki-67, a marker of proliferating cells, was immunolocalized. As depicted in Figures 3D and 3E, ki-67 positive cells were detected only within the crypts in both control and Sb force-fed mice. Moreover, Sb treatment did not modulate the number of proliferating cells in the crypts (Figure 3F). These findings indicate that Sb could modulate enterocyte migration, both in vitro and in vivo, without affecting the proliferative compartment.

To compare the effect of the whole yeast and the yeast supernatant, we daily administrated to the mice for one week, either the whole yeast either the culture supernatant of S. boulardii. A group of mice were also force-fed daily with supernatant of S. Cerevisiae. As depicted on Figures 4A and 4C, BrdU-stained cells...
were detected mainly within the crypts in both control and Sc supernatant force-fed mice. However, in Sb supernatant force-fed mice, BrdU positive cells were distributed from the crypt to the tips of the villi (Figures 4B and 4D). These data suggest that Sb supernatant, but not Sc supernatant, contains molecule(s) that induce migration of enterocytes in vivo.

α2β1 integrin collagen receptor is involved in Sb-dependent cell restitution

Enterocyte migration requires dynamic interactions between ECM molecules, such as laminin isoforms and collagens, and their cell surface receptors [27]. Therefore we determined whether Sb affected the distribution of α2 and α6 integrin subunits, receptors of collagens and laminin, respectively. In both control and Sb force-fed mice, α6 was detected at the basolateral surfaces of enterocytes on the villus and in the crypt (Figure 5A and 5B). In control mice, α2 integrin was found mainly at the lateral sites of the enterocytes whereas it was significantly redistributed to the basal sites in Sb force-fed mouse cells (Figure 5C and 5D). These results suggest that α2 integrin participates in Sb-enhanced enterocyte migration.

To confirm the role of α2β1 integrin in Sb-enhanced enterocyte migration, HCT-8/E11 cells, which express the integrins α2β1, α3β1, α5β1, αvβ5, and α6β4 (unpublished data), were incubated with function blocking anti-integrin mAbs during cell migration. As observed in Figure 6A, in control cells only the anti-αv integrin mAb inhibited the capacity of untreated HCT-8/E11 cells to migrate by approximately 50%. Interestingly, Sb-dependent cell migration was also inhibited by the anti-αv mAb (70%), and additionally with the anti-α2 (40%) mAb. Altogether these data indicate that the Sb-induced enterocyte migration depends on α2β1 integrin. It should be noted that incubation with a mixture containing anti-αv, -α6 and -β1 integrin mAbs did not totally abolish cell migration in either condition, suggesting the participation of integrin-independent cell adhesion molecules in this process.

Sb modulates α2β1 integrin-mediated adhesive properties

As adhesion is essential for cell migration, we next examined whether treatment with Sb supernatant could alter HCT-8/E11 cell attachment to ECM proteins. Cells incubated with or without Sb supernatant were allowed to attach to increasing concentrations of purified laminin-111, or type I collagen. HCT-8/E11 cells attached to both matrices (Figure 6B). Sb supernatant did not alter cell adhesion to laminin-111. However, it dramatically increased the percentage of adherent cells on the type I collagen matrix. Adhesion assays performed in the presence of anti-integrin function blocking mAbs revealed that α2β1 integrin is the sole receptor for type I collagen in these cells (Figure 7). Thus, the increase in cell adhesion to type I collagen upon Sb treatment most likely occurs through the modulation of the α2β1 integrin functional state.
We postulated that Sb supernatant exerts its effects by activating the $\alpha_2\beta_1$ integrin. To test such a possibility, we activated the $\alpha_2\beta_1$ integrin using TS2/16, a $\beta_1$ integrin activating mAb [28]. As expected, $\alpha_2\beta_1$ activation increased cell adhesion onto type I collagen (Figure 8A). This activation also led to an increase in cell migration such as that observed with Sb supernatant (Figure 8B). Pretreatment of cells with integrin $\alpha_2\beta_1$ activating factor improved neither Sb-stimulated cell adhesion nor cell restitution (Figure 8A and 8B), strongly suggesting that Sb secretes factors that activate $\alpha_2\beta_1$ integrin, either directly or indirectly.

Sb supernatant increases $\alpha_2\beta_1$ integrin activation state

After activation, integrins quickly associate within the actin cytoskeleton and cluster together to form focal adhesions, multimolecular complexes that contain signaling molecules including focal adhesion kinase (FAK), paxillin and Erk1/2. Activation of these signaling molecules leads to the modulation of cell migration. We therefore checked the phosphorylation status of these different key signaling molecules. As observed in Figure 9A, cell adhesion to type I collagen promoted phosphorylation of both FAK and paxillin on the Y397 and Y118 tyrosine residues respectively. Interestingly, Sb supernatant increased the tyrosine phosphorylation level of both proteins. Sb also upregulated the tyrosine phosphorylation status of Erk1/2, another protein associated with integrin signaling pathways (Figure 9A). We next tested the effect on cell migration of PD 98059 and PF 573228, inhibitors of MAPK and FAK respectively. As observed on Figures 9B and 9C, these inhibitors fully blocked the Sb-induced cell migration. These results confirm that Sb stimulates cell migration through the activation of multiple signaling pathways including activation of FAK and Erk1/2.

Figure 4. Sb supernatant but not Sc supernatant improves mouse enterocyte migration along the crypt-villus axis. Mice were daily force-fed for one week with unused culture medium for Sb (control), Sb supernatant (Sb sn) or Sc supernatant (Sc sn) then injected with BrdU 24 hours before sacrifice. Small intestinal tissues were processed for immunostaining. The BrdU-labeled enterocyte migration along the crypt-villus axis was traced on tissue sections by immunostaining with an anti-BrdU Ab (A, B and C) and quantitatively assessed (D) as described in Methods. (n = 5). ** P < .01. Arrow: basal site of the enterocytes. Scale bar: 150 $\mu$m.

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Figure 5. $\alpha_2\beta_1$ integrin is relocalized in the intestine of Sb force-fed mice. Intestinal tissues from control (A and C) or Sb force-fed mice (B and D) were immunostained with Abs against $\alpha_6$ (A and B) or $\alpha_2$ integrin (C and D). Each image is a representative image taken from tissue sections of five mice. Scale bar: 130 $\mu$m.

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Sb regulates the $\alpha_2\beta_1$ integrin signaling pathway

After activation, integrins quickly associate within the actin cytoskeleton and cluster together to form focal adhesions, multimolecular complexes that contain signaling molecules including focal adhesion kinase (FAK), paxillin and Erk1/2. Activation of these signaling molecules leads to the modulation of cell migration. We therefore checked the phosphorylation status of these different key signaling molecules. As observed in Figure 9A, cell adhesion to type I collagen promoted phosphorylation of both FAK and paxillin on the Y397 and Y118 tyrosine residues respectively. Interestingly, Sb supernatant increased the tyrosine phosphorylation level of both proteins. Sb also upregulated the tyrosine phosphorylation status of Erk1/2, another protein associated with integrin signaling pathways (Figure 9A). We next tested the effect on cell migration of PD 98059 and PF 573228, inhibitors of MAPK and FAK respectively. As observed on Figures 9B and 9C, these inhibitors fully blocked the Sb-induced cell migration. These results confirm that Sb stimulates cell migration through the activation of multiple signaling pathways including activation of FAK and Erk1/2.
We analyzed the impact of Sb supernatant on the organization of adherence structures (Figure 10). Paxillin staining revealed that control cells, plated on type 1 collagen, exhibited few focal adhesion structures. However, paxillin staining in Sb treated cells displayed more focal adhesion structures that appeared to be thicker than those observed in the control condition (Figure 10A and 10B). Staining of focal adhesion structures on migrating cells yielded a similar pattern (Figure 10C and 10D). This indicates that treatment with Sb supernatant is associated with functional and structural modifications of focal adhesion structures.

To explore whether Sb also regulates focal adhesion structures in vivo, we analyzed the paxillin tyrosine phosphorylation status on intestine from mice force-fed with PBS alone, or PBS containing 1 mg/ml lyophilized Sb. As depicted in Figure 10E, an anti-Y118 paxillin Ab failed to stain any structure in control C57BL6J mice. However, in Sb force-fed mice, staining was detected, confined to the basal surface of the enterocytes (Figure 10F). This strongly suggests that Sb promotes the activation of adhesion structures found in enterocytes in vivo.

Taken together these data indicate that Sb secretes factors that strengthen α2β1/collagen interactions leading to enhanced outside-in signaling and focal adhesion structure reorganization. This was associated to an increase in cell migration.

**Discussion**

Besides a marked infiltration of inflammatory cells, epithelial cell damage is also frequently observed in the mucosal lesions of IBD such as ulcerative colitis, Crohn’s disease or in infectious gastroenteritis [2]. Therefore, complete remission of such diseases requires both the cessation of inflammation and the repair of damaged epithelium. The development of novel therapies that accelerate the repair of intestinal epithelium has recently begun and various molecules are now being considered for clinical use including epidermal growth factor in combination with mesalamine [29], keratinocyte growth factor [30], and hepatocyte growth factor [31]. However, a better understanding of the biological effects of these molecules must be ascertained, not least to identify any undesired secondary effects, such as tumorigenesis.

In the present study, we analyzed the effect of Sb on the capacity of intestinal epithelial cells to reseal a wound. According to our findings, the impact of Sb on epithelial cells can be summarized as

![Figure 6. Sb-stimulated cell restitution requires modulation of the interaction between α2β1 integrin and collagen. (A) HCT-8/E11 cell monolayers were wounded and incubated with or without Sb supernatant. The monolayers were further incubated with or without (−) function-blocking anti-integrin mAbs (10 μg/mL) 1 hour before wounding and during cell migration. Wound closure was determined as described in Methods. Results are expressed as the percentage of cell migration compared to control. Data represent the mean ± SD of 5 separate experiments. * P<.01; ** P<.001. A mouse anti-DPP IV (DPPIV) mAb was used as a control for anti-integrin mAb specificity. (B) Isolated HCT-8/E11 cells were treated with or without Sb supernatant and plated on either laminin-111 or type I collagen at the indicated concentrations. Cell-ECM adhesion was evaluated as described in Methods. Results are expressed as the percentage of cell adhesion. Data represent the mean ± SD of 3 separate experiments. doi:10.1371/journal.pone.0018427.g006]
follows: (1) Sb supernatant enhances the restitution of various differentiated human intestinal epithelial cell lines in vitro; (2) in the mouse intestine, Sb improves migration of enterocytes along the crypt-villus axis; (3) Sb does not influence enterocyte proliferation either in vitro or in vivo; (4) Sb stimulates α2β1 integrin, thus activating molecules such as FAK, paxillin and ERK1/2, which are associated with integrin signaling pathways and which modulate cell-type I collagen I interaction; (5) concomitant with this activation, Sb modulates both the structure and the activity of focal adhesions. Taken together, these effects could contribute to the enhancement of intestinal cell restitution.

Intestinal wound healing is initiated by the migration of epithelial cells adjacent to the injured surface into the wound to cover the denuded area. This process, termed epithelial restitution, occurs within a period of minutes to hours and does not require cell proliferation [2]. Through the use of a relevant in vitro model [32], we have shown that Sb supernatant increased the capacity of differentiated enterocytes to recolonize a wound. The effect of Sb on wound healing was associated with an increase in cell motility rather than a stimulation of cell proliferation. Indeed, wound repair was unaffected by treatment with DNA synthesis inhibitors such as mitomycin C and BrdU incorporation experiments indicated that only a few proliferating cells were found in wounded areas. Also Sb did not alter cell growth (not shown). Furthermore, videomicroscopy experiments showed that Sb supernatant doubled the migration rate of HCT-8/E11 cells and Sb increased the rate of migration of enterocytes along the crypt-villus axis in mice without affecting the proliferative compartment. According to these data, we postulated that Sb supernatant contains motogenic factor(s) that improve intestinal cell restitution.

Although the physiological pathways involved in intestinal restitution remain partially unexplained, it has been proposed that changes in cell-ECM interactions occur spatially and temporally during this process. Studies carried out on gut tissue revealed that ECM proteins including fibronectin, type I collagen and laminins play a crucial role in regulating cell restitution [6,7,33]. They have also demonstrated the requirement for the binding and interaction of ECM-integrins such as α3β1, α6β1, α6β4 (laminins receptors), α2β1 (collagens receptor), or αvβ3 or αvβ6 (fibronectin receptors) for intestinal restitution [6,34]. In line with these results, we have provided evidence for the requirement of α2β1 integrin facilitates cell-ECM interaction for Sb-induced restitution. Sb was shown to promote the redistribution of the collagen receptor from the lateral to the basal membrane domain of enterocytes. Inhibition assays using anti-integrin mAbs demonstrated that α2β1 integrins participate in the Sb-induced cell migration, suggesting that collagens support this process. Also, adhesion assays suggested that Sb specifically modulates the α2β1-dependent cell interaction with type I collagen.

The mechanisms by which Sb modulates integrin-ECM interactions and subsequent intestinal restitution need to be explored in more depth. However, at least two possibilities exist. Firstly, cell restitution could be modulated through the regulation of the integrin functional state. In accordance with this, some data have highlighted dynamic changes in integrin affinities for ligands. These changes are thought to occur in response to extracellular cues, and can modify cell migration [35]. In line with these data, we demonstrated that factors secreted by Sb can activate α2β1 integrin. Indeed, cell treatment with the β1 integrin activating factor TS2/16 mAb [28,36] promoted the same increase in cell adhesion as Sb. Moreover, cell pretreatment with TS2/16 mAb did not enhance the impact of Sb on cell adhesion or cell restitution. In addition, Sb did increase α2β1 integrin signaling pathways.
A

\[
\begin{array}{cccc}
\text{Tyr-397 FAK} & \text{Control} & \text{Sb} \\
100 & 121 & 122 & 275 & 280 & 285 \\
\text{FAK} & \\
\text{Tyr-118 Paxillin} & \text{Control} & \text{Sb} \\
100 & 317 & 291 & 230 & 378 & 439 \\
\text{Paxillin} & \\
\text{Thr-202 Erk1} & \text{Control} & \text{Sb} \\
100 & 183 & 333 & 70 & 921 & 736 \\
\text{Tyr-204 Erk2} & \\
\text{Erk 1} & \text{Control} & \text{Sb} \\
100 & 150 & 187 & 50 & 347 & 307 \\
\text{Erk2} & \\
\text{Time (min)} & 0 & 15 & 30 & 0 & 15 & 30 \\
\end{array}
\]

B

![Graph A]

C

![Graph B]
Sb has been shown to generate transduction pathways, including activation of Erk1 and Erk2 in intestinal mucosal cells [11]. In the present study, we demonstrated that Sb can modulate pathways leading to cell migration. A large number of molecules, including FAK, have been suggested as candidates for recruitment in adhesion structures upon integrin activation. Furthermore these molecules may serve as conduits for the transmission of the force that is necessary for cell migration and for bidirectional signaling between the cell interior and its environment [37]. FAK activation leads to the stimulation of other signaling proteins such as paxillin, thereby activating various signaling pathways crucial in the regulation of cell adhesion and migration [37]. In the present study, we demonstrated that Sb secretes factor(s) that alter the FAK/paxillin signaling pathways. In mice, relocalization of α2β1 integrin to the basal domain of the enterocyte following Sb treatment is associated to paxillin phosphorylation of the Y118 residue of the same domain. Also Sb increased the tyrosine phosphorylation levels of FAK, paxillin and Erk1/2. Furthermore, Sb-treated cells exhibited focal structures that appeared thicker when compared to control cells. These observations clearly indicate that cell treatment with Sb resulted in a strengthening of α2β1/collagen interactions as shown by enhanced outside-in signaling and focal adhesion reorganization.

Sb has been proposed to act as a shuttle able to liberate at least 1500 as yet not fully characterized molecules during intestinal transit [38].

Figure 9. Sb activates FAK, paxillin and MAPK. (A) HCT-8/E11 cells were allowed to adhere on type I collagen after Sb pretreatment. The phosphorylation of tyrosine residues in FAK (Tyr-397 FAK), paxillin (Tyr-118 paxillin) and Thr-202/Tyr-204 in MAPK (Erk1 and Erk2) was determined after cell lysis at the indicated times of cell adhesion. Samples were analyzed by western blot analysis. Equal amounts of protein were analyzed and loading amounts were verified by probing the blot with anti-FAK, anti-paxillin or anti-Erk1/Erk2 Abs. The intensity of the bands was measured after scanning of the autoradiograms. Values presented on the blots correspond to percentage of band intensity compared to control. (B and C) HCT-8/E11 cell monolayers were wounded and incubated with or without Sb supernatant. The monolayers were further incubated with or without PD 98059, a MAPK inhibitor (B) or with or without PF 573228, a FAK inhibitor (C), 1 hour before wounding and during cell migration. Wound closure was determined as described in Methods. Results are expressed as the percentage of wound repair. Data represent the mean±SD of 3 separate experiments. ** P<.01.

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Figure 10. Sb modulates integrin signaling pathway both in vitro and in vivo. HCT-8/E11 cells were pretreated (B) or not (A) for 2 hours with Sb supernatant, and then allowed to adhere to type I collagen-coated surfaces (3 μg/ml) for 30 minutes. After fixation and permeabilization, cells were stained with an anti-paxillin Ab, the binding of which was revealed with an AlexaFluor 488-conjugated anti-mouse secondary Ab. Scale bar: 20 μm. HCT-8/E11 cell monolayers were wounded and incubated without (C) or with (D) Sb supernatant for 5 hours. Paxillin was revealed using a specific anti-paxillin Ab. Scale bar: 20 μm. Intestinal tissues from control (E) or Sb force-fed mice (F) were immunostained with an Ab against Tyr-118 paxillin. Stainings were analyzed using a Leica confocal microscope. Scale bar: 100 μm.

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peptic factors may at least partially explain why *Sb* has pleiotropic effects on intestinal mucosa and therapeutic effects on such a wide variety of gastrointestinal disorders [9,30,31]. Some of the yet unidentified molecules may interfere with host cell signaling pathways and thereby modulate host cell behavior [30]. Indeed, *Sb* has been shown to secrete peptides that are implicated in intestinal cell maturation, enzyme expression and membrane transport mechanisms [38]. In addition, *Sb* produces other factors that reduce inflammation by blocking NF-xB activation [21,40] and enhancing PPAR-γ expression [41]. The motogenic molecule(s) secreted by *Sb* remain to be elucidated. However, our preliminary data suggest that the motogenic factor(s) secreted by *Sb* is (are) an heat instable molecule(s) (result not shown). Further studies are now needed to explore the molecular mechanisms by which supernatant of *Sb* alters cell migration.

In conclusion, this report demonstrates for the first time that *Sb* secretes motogenic factors that can improve intestinal restitution. These factors exerted their effects through dynamic fine regulation of integrin-mediated adhesion to the ECM. This could be of major importance in the future treatment of diseases characterized by severe mucosal injury, such as IBD or infectious diarrhea and colitis.

**Supporting Information**

**Video S1** HCT-8/E11 cell monolayers were wounded as described in Methods, then placed in a temperature and CO$_2$-controlled chamber mounted on a Nikon TE2000 inverted microscope. Images were captured every 5 minutes for a total observation duration of 5 hours using a Cool Snap HQ camera (Princeton Instrument) through a 10x objective lens.

**Video S2** HCT-8/E11 cell monolayers were wounded as described in Methods and incubated with *Sb* supernatant. Plates were placed in a temperature and CO$_2$-controlled chamber mounted on a Nikon TE2000 inverted microscope. Images were captured every 5 minutes for a total observation time of 5 hours using a Cool Snap HQ camera (Princeton Instrument) through a 10x objective lens.

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**Author Contributions**

Conceived and designed the experiments: FA VR DC AC. Performed the experiments: AG CS EP RPB LP MPM CC CZ VR FA. Analyzed the data: FA VR. Contributed reagents/materials/analysis tools: FA VR. Wrote the manuscript: FA.

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