Intestinal breast cancer resistance protein (BCRP) requires Janus kinase 3 activity for drug efflux and barrier functions in obesity

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Breast cancer resistance protein (BCRP) is a member of ATP-binding cassette (ABC) transporter proteins whose primary function is to efflux substrates bound to the plasma membrane. Impaired intestinal barrier functions play a major role in chronic low-grade inflammation (CLGI)–associated obesity, but the regulation of BCRP during obesity and its role in maintaining the intestinal barrier function during CLGI-associated obesity are unknown. In the present study, using several approaches, including efflux assays, immunoprecipitation, immunoblotting, immunohistochemistry, paracellular permeability assay, FACS, cytokine assay, and immunofluorescence microscopy, we report that obese individuals have compromised intestinal BCRP functions and that diet-induced obese mice recapitulate these outcomes. We demonstrate that the compromised BCRP functions during obesity are because of loss of Janus kinase 3 (JAK3)–mediated tyrosine phosphorylation of BCRP. Our results indicate that JAK3-mediated phosphorylation of BCRP promotes its interactions with membrane-localized β-catenin essential not only for BCRP expression and surface localization, but also for the maintenance of BCRP-mediated intestinal drug efflux and barrier functions. We observed that reduced intestinal JAK3 expression during human obesity or JAK3 knockout in mouse or siRNA-mediated β-catenin knockdown in human intestinal epithelial cells all result in significant loss of intestinal BCRP expression and compromised colonic drug efflux and barrier functions. Our results uncover a mechanism of BCRP-mediated intestinal drug efflux and barrier functions and establish a role for BCRP in preventing CLGI-associated obesity both in humans and in mice.

Obesity is a worldwide epidemic and a major risk factor for several health complications including diabetes, hypertension, and colorectal cancer (1). Accumulating evidence suggests that chronic low-grade inflammation (CLGI) plays a central role in the pathogenesis of obesity (2) and associated metabolic disorders (3–7). High fat diet–mediated imbalance in gut homeostasis is indicated to play a role in CLGI during obesity (8–10). Studies from our group and others show that controlling or reversing CLGI could be beneficial during obesity-associated metabolic dysregulation (11–14). However, the mechanism for the onset and perpetuation of CLGI is unknown.

Breast cancer resistance protein (BCRP) is a member of ATP-binding cassette (ABC) transporter family with a primary function to use the energy generated by ATP hydrolysis to efflux the substrate bound to the plasma membrane (15). ABC drug transporters are expressed particularly in cells and tissues that interface with body fluids and outer environment and help coordinating the efflux of xenobiotics, drugs, and small molecules involved in signaling, thereby regulating cellular redox, nutritional states, and rate-limiting steps of key metabolisms (16, 17). In the intestine, ABC transporters are important components for barrier against dietary toxins such as carcinogens from foods, drugs, and toxins from luminal bacteria (18). Altered expression of these transporters contributes to the accumulation of carcinogens and other harmful substances including bacterial toxins. Although intestinal CLGI are reported to have impaired BCRP expression (15) and drug efflux activities (19, 20), the mechanisms of reduced BCRP expression and the compromised intestinal drug efflux functions during obesity are not known.

In the present study, we demonstrate the molecular basis of compromised intestinal drug efflux and barrier functions during obesity. We show that posttranslation regulation of intestinal BCRP by nonreceptor tyrosine kinase Janus kinase 3 played a key role in facilitating BCRP-mediated drug efflux functions through promoting BCRP expression and its surface localization. These studies have wider implications not only in our understanding of physiological and pathophysiological mechanisms of intestinal barrier functions and CLGI-associated chronic inflammatory diseases but also in protein-mediated drug efflux pharmacokinetic and pharmacodynamic characteristics of oral formulations.

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3 The abbreviations used are: CLGI, chronic low-grade inflammation; PgP, P-glycoprotein; qRT, quantitative real time; CT, cycle threshold; IP, immunoprecipitation; IB, Western blotting; AJ, adherens junction; BCRP-P, BCRP tyrosine phosphorylation.
**BCRP interacts with non-receptor tyrosine kinase**

![Diagram showing the interaction between BCRP and non-receptor tyrosine kinase]

Figure 1. Intestinal drug transport functions are compromised during obesity. A, colons from normal and obese mice were excised out, ligated in Petri dishes, injected with Hoechst 33342, and the intracellular accumulation of Hoechst 33342 measured. Contribution of BCRP and P-glycoprotein in intestinal drug efflux functions in WT mice were determined by treating with or without novobiocin or verapamil or BCRP siRNAs prior to Hoechst 33342 loading. Knockdown of BCRP expression was determined by Western blot analysis of the tissue lysates from WT mouse treated with scramble or BCRP siRNA and probed with BCRP antibody. B, paracellular permeability was determined using luminal retention of FITC-inulin probe in the ligated mice colons from WT, obese, WT treated with novobiocin or verapamil or BCRP siRNA or siBCRP-scram. C–E, colonic tissue sections (upper panels) or lysates (lower panels) from WT and obese mice were either immunostained or immunoblotted using indicated primary antibodies. Representative images are shown (n = 6) for immunostaining. Green indicates BCRP/MRP2/BCRP. Scale bar 550 μm. Colonic tissue lysates (lower panels) from the tissues of upper panels were analyzed using IB for indicated proteins using β-actin as controls. Representative blots (n = 3) are shown. Quantification of the resulting bands (lower right graphs) was done by densitometry analysis using Bio-Rad Gel Doc System equipped with ImageLab software and the results were normalized against β-actin. β-actin panels were reused in D and E. A–E, the data are representative of at least three independent experiments. Bar graphs, values are mean ± S.D. * denotes comparison with WT mice. A, obese, p = 0.0003; WT + Novo, p = 0.0005; WT + siBCRP, p = 0.0002. B, obese, p = 0.0002; WT + Novo, p = 0.0003; WT + siBCRP, p = 0.0002. E, obese, p = 0.0004.

**Results and discussion**

**Intestinal drug transport functions are compromised during obesity**

To determine the functional implications of CLGI-associated obesity in intestinal drug transport functions, we investigated the intracellular accumulation of Hoechst 33342 in colon and colonic leakiness toward Inulin in our previously reported diet-induced obesity (DIO) model of mice (13). Fig. 1A shows that normal mice had reduced intracellular accumulation of Hoechst 33342 (a substrate for both P-glycoprotein (PgP) and BCRP), indicating intact colonic drug efflux functions by drug transporter proteins. However, obesity led to a significant increase in intracellular accumulation of Hoechst, indicating a compromised drug transport function. Because Hoechst 33342 is a substrate for both PgP and BCRP, the experiments were performed using normal mice colon in presence of BCRP inhibitor novobiocin or PgP inhibitor verapamil to differentiate the compromised drug efflux functions between BCRP and PgP. Fig. 1A (third and fourth bars from left) shows that although novobiocin or verapamil had minor impact on intracellular accumulation of Hoechst 33342, inhibition by novobiocin led to a 3-fold increase in intracellular accumulation of Hoechst 33342, indicating that the compromised drug efflux functions during obesity were primarily because of compromise in BCRP functions. To confirm if the change in drug efflux functions were because of BCRP, the experiments were performed in presence of either scramble siRNA or BCRP siRNA. Fig. 1A (fifth and sixth bars from left) shows that scramble siRNA did not affect drug transport function but incubation with BCRP siRNA led to a significant increase in intracellular accumulation of Hoechst 33342 in WT mice which were comparable to the obese mice. Western blotting data on the right panel of Fig. 1A shows the knockdown of BCRP expression by siRNA. To determine whether compromised BCRP functions were associated with leaky colonic barriers, inulin assays were performed using ligated mice colons. Fig. 1B shows that although WT mice retained most of the inulin in luminal compartment, both WT and obese mice with BCRP siRNA led to a significant increase in intracellular accumulation of Hoechst 33342 in WT mice which were comparable to the obese mice. Western blotting data on the right panel of Fig. 1A shows the knockdown of BCRP expression by siRNA.
mice. To determine the underlying mechanism, as a first step, we studied the effects of obesity on the expression of major intestinal drug efflux proteins. Fig. 1, C–E (and quantification of corresponding fluorescence intensities in Fig. S1, A–C) shows that, although during obesity the expression of MRP2 (Fig. 1C) and PgP (Fig. 1D) were unaffected, the expression of BCRP (Fig. 1E) was decreased significantly. Moreover, the localization of BCRP was also affected in obese mice (Fig. 1E), where control antibody did not show any staining (Fig. S2B). To further confirm, Western blot analysis followed by densitometry quantification showed that the expressions of MRP2 and PgP decreased not significantly during obesity, but BCRP expression decreased over 7-fold (Fig. 1, C–E, lower panels).

Intestinal BCRP expression was decreased in obese humans

Next, we determined if obesity-associated changes in the intestinal expression of drug transporter proteins were also present in human obese conditions. Fig. 2A shows that BCRP was expressed toward the luminal surfaces (left panel, white arrow) and in crypts (left panel, yellow arrow) of normal human. In contrast, in obese human, the luminal expression of BCRP was significantly decreased (right panel, white arrow) and the surface localization of BCRP was almost completely absent (right panel, white and yellow arrows). Control with only secondary antibody did not show any staining (Fig. S2A). Quantifications of these images showed that similar to obese mice, obese humans also had decreased intestinal expression of BCRP (Fig. 2B). Results from Western blot analysis from obese male and female human subjects and their normal counterparts showed that irrespective of sex, obesity was associated with a significant decrease in intestinal expression of BCRP (Fig. 2C), where male subjects showed about a 5-fold decrease in BCRP expression and the female subjects showed about a 2-fold decrease compared with their normal counterparts (Fig. 2D).

Tyrosine phosphorylation of BCRP was affected during obesity

Next, we determined if transcriptional regulation was responsible for the decreased expression of BCRP. For this, we performed RT-PCR to determine the expression level of BCRP mRNA compared with the control housekeeping gene 18SRNA. Fig. 3A shows that the expression of BCRP mRNA did not show significant decrease in either obese humans (top left and middle panels) or mice (bottom left and middle panels), indicating that transcriptional regulation might not be responsible for the decreased BCRP proteins. Moreover, 18SRNA remained unaffected in both normal and obese human and mouse. Quantitative real time (qRT) PCR data further confirmed these findings where no significant differences in the cycle threshold (CT) values were found either between normal and obese human (Fig. 3A, top right) or mice (Fig. 3A, bottom right) groups. For these experiments, 18SRNA was used as an internal control in the qRT-PCR reactions. These results ruled out the transcriptional regulation as a mechanism for the decreased BCRP during obesity. Next, we determined if BCRP phosphorylation was responsible for such an effect. Fig. 3, B and C, showed that obesity was associated with a significant decrease in intestinal tyrosine phosphorylation of BCRP both in obese mouse (Fig. 3B) and in obese human (Fig. 3C). Quantitation of the Western data indicated that although there was a 3-fold decrease in phosphorylated to total BCRP protein ratios in obese mice (Fig. 3B, lower graph), there was relatively less but significant decrease in the same in obese humans (Fig. 3C, lower graph). Because our data show for the first time that tyrosine phosphorylation of BCRP is essential for its drug efflux functions, we investigated if BCRP has any potential substrate-binding sites. Using BCRP amino acid sequence, we analyzed potential phosphorylation sites by NetPhos 2.0 Server ensembles of neural networks. Our analysis revealed that BCRP has 11 tyrosine residues, 3 of which (Tyr123, Tyr173, and Tyr186) have strong NetPhos prediction score of more than 0.9 (Table S1) indicating these as potential substrate phosphorylation sites for the nonreceptor tyrosine kinase JAK3.

Compromised JAK3 expression is responsible for decreased BCRP phosphorylation during obesity

Previously we reported that loss of JAK3 led to predisposition for CLGI-associated obesity in both male and female mice (10). Because intestinal tyrosine phosphorylation of BCRP was decreased in both obese humans and mice, we determined if JAK3 expression was responsible for decreased BCRP phosphor-
**BCRP interacts with non-receptor tyrosine kinase**

Figure 3. Tyrosine phosphorylation of BCRP is affected during obesity. A. total RNA was extracted using TRizol (Invitrogen) from colons of healthy and obese subjects and WT and obese group of mice. Total RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Biochemicals). Equal amounts of cDNA as estimated using spectrophotometer were subjected to reverse transcriptase PCR using PCR machine (Bio-Rad) and analyzed using agarose gel electrophoresis. Band intensity was measured using Bio-Rad imaging system using 18SRNA as control, and relative expression of BCRP mRNA is shown. For real-time PCR, 2 μg of total RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Biochemicals). Equal amounts of cDNA as measured using NanoDrop were subjected to real-time PCR analysis using ABI PRISM 7600. A plot of CT value with respect to housekeeping gene 18S RNA are shown for absolute quantification of the BCRP gene expression as determined using ABI PRISM software (Bio-Rad). B. co-IP followed by IB studies were done using colonic tissue lysates either from normal and obese mice (B) or humans (C) probed with the indicated antibodies using β-actin as controls. Representative blots (n = 3) are shown. Densitometry analysis of the Western images were done using ImageLab software (Bio-Rad), and ratios of average densities (n = 3 experiments) between pBCRP and BCRP are shown. * indicate statistically significant differences (B, p = 0.004; C, p = 0.002).

**JAK3 expression is associated with a significant decrease in colonic expression of JAK3**

JAK3 is expressed in luminal mucosal surfaces of healthy human subjects (left panel); however, in obese subjects JAK3 expression was substantially decreased (right panel). Western blot analysis showed that obesity was associated with a significant decrease in colonic expression of JAK3 (Fig. 4B), where both male and female subjects showed about a 4-fold decrease in JAK3 expression compared with their normal counterparts (Fig. 4C). To demonstrate that the intestinal expression of BCRP depended on intestinal JAK3 expression and JAK3-mediated BCRP phosphorylation, using JAK3 KO mouse we first determined if loss of JAK3 affected the intestinal expression of BCRP. Fig. 4D shows that similar to obese human subjects, loss of JAK3 in mouse led to a significant decrease in intestinal expression of BCRP (right panel) compared with their WT littermates (left panel). Moreover, both Western blot analysis (Fig. 4E) and densitometry quantification (Fig. 4F) showed a decrease in intestinal BCRP expression thereby further confirming that JAK3 expression was required for BCRP expression. Additionally, functional analysis of mouse colon showed that loss of JAK3 expression resulted in a 2.5-fold decrease in colonic drug-transport functions (Fig. 4G) as indicated by increased intracellular accumulation of Hoechst 33342 in either JAK3 KO colon or WT colon in presence of JAK3 inhibitor tofacitinib. These indicate that JAK3 activation regulates colonic drug transport functions. To corroborate that JAK3 activation–mediated drug transport functions were because of BCRP, we performed experiments in presence of either scramble or BCRP siRNA. Fig. 4G (fourth and fifth bars from left) shows that although scrambled RNA had little effect on colonic drug efflux functions, BCRP siRNA significantly decreased drug efflux functions in WT mice. Because JAK3 activation was required for BCRP-mediated colonic drug efflux functions and IL-2 activates JAK3 (11–14), we determined colonic tissue levels of IL-2 in these mice. Indeed, Fig. 4H shows significantly high level of IL-2 in WT colon compared with JAK3 KO mice.

**JAK3 association with BCRP regulates intestinal drug transport and barrier functions**

Because BCRP-mediated drug transport functions were compromised during obesity and JAK3 activation was required for BCRP-mediated colonic drug efflux functions, we determined if BCRP associates with JAK3 in mouse colon. Indeed, Fig. 5 (white arrows) shows the localization of colonic BCRP (green), JAK3 (red), and BCRP-JAK3 complex (yellow), where BCRP not only co-localized with JAK3 in mouse colon but also in human (Fig. 5B) colon. To further confirm, immunoprecipitation studies using BCRP and JAK3 antibodies showed that BCRP antibody immunoprecipitated JAK3 from colonic tissue lysates of both mouse (Fig. 5A, lower panels) and human (Fig. 5B, lower panels). For these experiments, IP by JAK3 antibody was taken as positive control and IP by IgG as negative control. Lower blots in these panels show the immunoblotting for the equal amounts of BCRP input. To define the conditions that facilitated BCRP interactions with JAK3, we first determined if BCRP interacted with JAK3 in human colonic epithelial cells. Fig. 5C (upper panel) shows that HT-29Cl-19A cells expressed BCRP (green) and JAK3 (red) and BCRP co-localized with JAK3...
Merged, yellow). The magnified view of the images showed that most of the BCRP-JAK3 complex was localized to cellular peripheries (lower panels, white arrows) where control with secondary antibody did not show any staining (Fig. S2C). To further confirm these, IP followed by IB studies using JAK3 and BCRP antibodies showed that although JAK3 antibody immunoprecipitated JAK3 from HT-29CL19A cell lysates, BCRP antibody also immunoprecipitated JAK3 from these cell lysates. For these experiments, IP with IgG was taken as negative control (middle lane). Lower blot in the panel shows the IB for the equal amounts of JAK3 as input controls. Next, we determined the consequences of BCRP interactions with JAK3. Previously, we reported that stimulating IL-2 led to activation (tyrosine phosphorylation) of JAK3 in HT-29 cells where tofacitinib inhibited JAK3 phosphorylation in the presence of IL-2 (13, 19–27). Fig. 5D shows that although tyrosine phosphorylated BCRP was absent in serum-starved HT-29 cells, stimulation by IL-2 led to tyrosine phosphorylation of BCRP. Moreover, treatment with JAK3 inhibitor tofacitinib promoted significant decrease in IL-2–induced tyrosine phosphorylation of BCRP in these cells indicating IL-2–induced activation of JAK3 promoted tyrosine phosphorylation of BCRP. Lower blot in the panel (Fig. 5D) shows the IB for BCRP as input control. Next, we determined the functional consequences of tyrosine phosphorylation of BCRP during IL-2–induced JAK3-activation. Flow cytometric analysis of rhodamine efflux assay using HT-29 Cl-19A cells (Fig. 5E) shows that stimulation by IL-2 led to a significant decrease in the intracellular accumulation of rhodamine (middle panel) compared with serum-starved cells (left panel) indicating IL-2–induced enhanced drug efflux functions. Moreover, IL-2–induced enhanced drug efflux functions were reversed by inhibition of JAK3 by tofacitinib in IL-2–

Figure 4. Compromised JAK3 expression is responsible for decreased BCRP phosphorylation during obesity. A and D, colonic tissue sections from normal or obese human subjects (A) were immunostained using JAK3 antibody whereas colonic tissue sections from WT or JAK3 KO mice (D) were immunostained using BCRP antibody. Representative images (n = 10) are shown from each group (n = 6). B and E, Western blot analysis was done using tissue lysates from normal and obese human (upper panel, male; lower panel, female) subjects (B) or WT and JAK3 KO mice (E) using indicated antibodies with β-actin as control. Representative blots (n = 3) are shown. C and F, quantification of the resulting bands in B and E were done by densitometry and the results were normalized against controls. Values are mean ± S.D. * denotes p < 0.05 compared with healthy human subjects (C) or WT mice (F). C, obese male, p = 0.003; obese female, p = 0.005. F, JAK3 KO, p = 0.002. G, intracellular accumulation of Hoechst 33342 was measured to determine the BCRP-mediated drug efflux function in colon from WT mice, JAK3 KO mice, WT mice treated with tofacitinib, WT mice treated with scramble and WT mice treated with BCRP siRNA (n = 6 each group). H, colonic IL-2 was determined using colonic tissue lysates from WT and KO and mouse using Multi-Analyte cytokine assay kit (Qiagen), and mean values from each group (n = 6 mice per group) are shown. All the data are representative of at least three independent experiments. Values are mean ± S.D. * denotes p < 0.05 compared with WT mice. G, JAK3 KO, p = 0.0002; WT + Tof, p = 0.0005; WT + siBCRP, p = 0.0002. H, JAK3 KO, p = 0.003.
BCRP interacts with non-receptor tyrosine kinase

**Figure 5. JAK3 association with BCRP regulates intestinal drug transport and barrier functions.** A and B, upper panels, colonic tissue sections from WT mice (A) or normal human subjects (B) were immunostained using BCRP and JAK3 primary antibodies followed by FITC- and Cy3-conjugated secondary antibodies, respectively. Representative images are shown (n = 10). A and B, lower panels, co-IP followed by IB were done using tissue lysates from WT mice (A) or normal human subjects (B) and indicated antibodies with β-actin as control. C, top panels, HT-29 Cl-19a cells grown on cover slips were immunostained using indicated antibodies. C, bottom panels, and D, HT-29 Cl-19a cells grown on Petri dishes were lysed using lysis buffer and equal (input control) amounts of JAK3 (C) or BCRP (D) proteins from the lysates of HT-29 Cl-19a cells either serum starved (−IL-2/negative control) or IL-2 treated, or IL-2 treated in the presence of JAK3 inhibitor tofacitinib were subjected to IP followed by IB using indicated antibodies. E, fluorescence-activated cell sorting (FACS) analysis is presented as dot plots to measure intracellular accumulation of Hoechst using HT-29 Cl-19a cells under similar experimental conditions as in D. Relative fluorescence intensities are shown on the x axis and the cell counts for 10,000 events on the y axis. F, experiments in (E) were repeated (n = 5) and mean ± S.D values are shown. * denotes p = 0.004 compared with serum starved (−IL-2) cells. G, paracellular permeability of confluent monolayer of HT-29 Cl-19a cells under similar experimental conditions as in D was determined and apical inulin retentions are shown. Values are mean ± S.D. * denotes p = 0.0002 (IL-2), p = 0.006 (IL-2 + Tof.) compared with serum-starved (−IL-2) cells. All the Western blots shown are representative (n = 3). The data are representative of at least three independent experiments.

stimulated cells (right panel). Quantitation of flow cytometric data (Fig. 5F) shows that activation by IL-2 led to 40% decrease in intracellular accumulation of rhodamine (middle bar) compared with serum-starved cells (left bar) which were reversed by inhibition of JAK3 by tofacitinib in presence of IL-2 (right bar). To determine whether drug efflux functions also had an impact on barrier functions, using inulin flux assay (Fig. 5G), our data showed that IL-2 stimulation led to a significant increase in inulin retention to the apical compartment (middle bar) compared with serum-starved cells (left bar) which were reversed by inhibition of JAK3 by tofacitinib in presence of IL-2 (right bar). Taken together these results showed that BCRP co-localized with JAK3 in human and mouse intestine and in human HT-29 cells where these interactions facilitated BCRP tyrosine phosphorylation-mediated enhanced drug efflux and increased barrier functions.

**JAK3-mediated tyrosine phosphorylation of BCRP promotes membrane localization of BCRP**

Our data thus far suggested no significant change in BCRP mRNA between normal and obese but a significant decrease in BCRP proteins in obese where IL-2–induced tyrosine phosphorylation of BCRP enhanced the intestinal drug transport functions under normal conditions. This led us to investigate if BCRP tyrosine phosphorylation was responsible for BCRP protein expression. Fig. 6A, top left, and the corresponding magnified view of a single cell in the bottom panels, shows that although the marker for adherens junction (AJ) protein β-catenin was mostly localized to cellular surfaces of the serum-starved HT-29 CL19a cells, BCRP was mostly intracellular where β-catenin did not co-localize with BCRP (Fig. 6A, top and bottom right panels). Stimulation with IL-2 however led to surface redistribution of BCRP where most of the BCRP co-localized with AJ surface marker β-catenin (Fig. 6B, top and bottom panels). To confirm if the surface redistribution of BCRP requires activation of JAK3, IL-2–stimulated HT-29 cells were treated with tofacitinib. Fig. 6C shows that inhibition of JAK3 led to reversal of the surface redistribution of BCRP, indicating that JAK3 activation facilitated not only BCRP interactions with β-catenin but also the surface redistribution of BCRP. To confirm if JAK3–mediated tyrosine phosphorylation of BCRP was responsible for BCRP interactions with β-catenin, IP followed by IB studies using IL-2–stimulated HT-29 cells showed that although BCRP antibody immunoprecipitated BCRP (top panel right lane) that appeared tyrosine phosphorylated (middle panel right lane and Fig. 5D). For these experiments, IP using IgG antibody was taken as negative control (top panel middle lane). Bottom panel in Fig. 6D(i) shows the input control for the equal amounts of BCRP. To prove JAK3–mediated phosphorylation of BCRP is necessary for its interactions with β-catenin, we took equal amounts of commercially available BCRP proteins (Fig. 6D(ii), bottom panel) and performed an in vitro kinase assay (U. S.
patent 9739779B2) with recombinant and purified JAK3 proteins (Fig. 6D(ii), second panel from bottom) using either kinase buffer alone or with ATP in the presence of equal amounts of recombinant and purified His-tagged β-catenin proteins (Fig. 6D(ii), third panel from bottom). The reaction mixture was subjected to IP either with pY or BCRP antibody followed by IB with either BCRP or His antibody. Fig. 6D(ii) shows that autophosphorylated JAK3 (top panel) not only phosphorylates BCRP (second from top) but JAK3 autophosphorylation was required for BCRP interactions β-catenin (Fig. 6D(ii), third from top panel). Together, these results indicate that IL-2–stimulated JAK3 activation not only facilitate BCRP interactions with β-catenin but also the surface redistribution of BCRP. To demonstrate that JAK3–mediated tyrosine phosphorylation of BCRP was essential for BCRP interactions with β-catenin where these interactions regulated BCRP surface localization and BCRP expression, we determined the effects of β-catenin on BCRP expression and surface localization. Fig. 6E shows that in IL-2–stimulated cells (top panels) both BCRP (red) and β-catenin (green) were well-expressed and co-localized to the cellular surfaces. However, knockdown of β-catenin expression using β-catenin-shRNA in IL-2–stimulated cells (bottom panels) led to a decrease in β-catenin expression (green) and a significant decrease in BCRP surface localization (merged). Functionally, drug efflux function in Fig. 6F further confirms these findings which show that IL-2 stimulation led to a significant decrease in intracellular accumulation BCRP substrate Hoechst 33322 (second bar from the left) compared with control (IL-2, first bar from the left) cells. Moreover, either inhibition of JAK3 by tofacitinib (third bar from the left) or knockdown of β-catenin expression by shRNA (fourth bar from the left) led to almost complete reversal of BCRP-mediated drug efflux functions as indicated by increase in intracellular accumulation of BCRP substrate Hoechst 33322.

**BCRP interactions with B-catenin are compromised during human obesity**

Because our data showed that BCRP interactions with β-catenin regulated intestinal BCRP expression and surface localization and, in the obese subjects the expression of BCRP was compromised, we determined whether BCRP interactions with β-catenin were compromised during obesity. Fig. 7A shows that BCRP antibody immunoprecipitated BCRP proteins from colonic tissue lysates of normal human subjects but β-catenin-antibody also immunoprecipitated BCRP proteins from the aforementioned lysates indicating interactions between β-catenin and BCRP in normal human colon. For these experiments, IgG-antibody was taken as negative control (mid-
BCRP interacts with non-receptor tyrosine kinase

Figure 7. BCRP interactions with β-catenin are compromised during human obesity. A and B, co-IP followed by IB studies were done to determine BCRP interactions with β-catenin in human colons using tissue lysates from healthy (A) and obese (B) subjects and indicated antibodies with β-catenin as controls. Representative blots (n = 3 blots/subject) are shown. C, co-immunofluorescence staining of colonic mucosa of healthy and obese human subjects are shown using indicated antibodies. Representative images (n = 10) are shown from each group. Scale bar 100 μm. The data are representative of at least three independent experiments.

dle lane). To determine whether the interactions between β-catenin and BCRP was responsible for decreased BCRP expression and mislocalization, Fig. 7B shows that though BCRP antibody immunoprecipitated BCRP proteins from relatively much higher amounts of colonic tissue lysates (lower panels for total input controls) of obese human subjects, β-catenin antibody failed to immunoprecipitate BCRP proteins from the aforementioned lysates. These indicated disruption of the interactions between β-catenin and BCRP in obese human colon. For these experiments, IgG antibody was taken as negative control (middle lane). We noticed that the BCRP protein expression was much lower in obese colon where our initial trials at comparable (Fig. 7A, lower panel) proteins failed to immunoprecipitate BCRP using BCRP antibody (data not shown). For that reason, we took higher amounts of input (Fig. 7B, lower panel) to detect BCRP in positive controls (Fig. 7B, top panel first lane). Moreover, even at this higher concentration of input, BCRP failed to coimmunoprecipitate β-catenin, indicating loss of BCRP interactions with β-catenin during obesity. These results were further confirmed by immunofluorescence microscopy of colonic sections from normal and obese human subjects (Fig. 7C), which showed that β-catenin (red) and BCRP (green) co-localized (yellow) in normal human colon (upper Merge panel) but was significantly decreased in obese human colon (lower Merge panel) thereby confirming the disruption of interactions between BCRP and β-catenin during obesity. The left panels in Fig. 7C show the secondary antibody only controls.

Taken together, these results showed that IL-2–induced JAK3 activation phosphorylates tyrosine residues in BCRP and tyrosine phosphorylated BCRP interacts with β-catenin and these interactions promote surface localization of BCRP. Moreover, surface-localized BCRP not only enhances intestinal drug efflux functions but also facilitates intestinal BCRP protein expression and stabilization. During obesity, a compromise in intestinal expression of JAK3 leads to loss of BCRP tyrosine phosphorylation and disruption of BCRP interactions with β-catenin, which results in BCRP mislocalization, decreased BCRP expression, and a significant decrease in BCRP-mediated colonic drug–transport functions.

Discussion

Human obesity possesses a significant risk factor for metabolic syndrome and cancer (1, 13, 21, 22, 27–30). Although obesity associates with CLGI, the underlying mechanisms are unknown. Our previous study reported that compromised intestinal differentiation contributes to CLGI-associated obesity (13). As dysfunctional intestinal barriers are associated with various chronic inflammatory conditions, in the present study, we investigated the functional regulation of ABC family of drug transporters in general and BCRP in particular during obesity-associated compromised intestinal barrier functions. BCRP belongs to ABC family of transmembrane transporters, one of the largest and most ancient protein super families conserved between species and throughout evolution (31). These proteins transport lipids, sterols, metabolic wastes, and therapeutic drugs across intracellular and extracellular membranes. The transport processes through these proteins are ATP-driven (active transport) and directed against solute concentration gradient (32). Moreover, it also pumps a wide variety of compounds out of the cell, thereby having a xenobiotic protective function (33). Our data showed that obesity was associated with compromised drug efflux and intestinal barriers functions as indicated by increased intracellular accumulation of BCRP-substrate Hoechst 33342 and increased colonic leakiness toward inulin, respectively (Fig. 1). Additionally, in mice we showed that although colonic expression of Pgp (ABCB1) and MRP2 (ABCC2) proteins changed not significantly, the expression of BCRP (ABCG2) proteins were significantly affected dur-
ing obesity. Interestingly, our data suggested similar effects on BCRP expression in human obesity where, irrespective of sexes, both male and female obese subjects showed significant decrease in colonic BCRP expression and altered BCRP localization (Fig. 2). It was reported that the prevalence of obesity was highest among adults 40 years or above (34) and although hepatic levels of BCRP mRNA were not significantly changed at any age, BCRP protein levels were lower in higher age groups compared with children (35). Furthermore, BCRP expression in placenta was regulated by cytokines and growth factors. The expression levels and activity of BCRP and Pgp at the blood brain barriers were altered during acute inflammation, where BCRP mRNA levels at blood brain barriers were significantly reduced by IL-1β, IL-6, and TNFα. Moreover, progesterone receptor isoforms PRA and PRB differentially regulated expression of BCRP in human placental cell line, BeWo (36). Because gastrointestinal tract is a major site for human interaction with xenobiotics, our study determined the transcriptional and posttranslational mechanisms of decreased BCRP protein expression and altered localization in the gastrointestinal tract. Previously, it was reported that BCRP mRNA expression was maximal in the duodenum where colonic expression ranged from 76 to 50% of the duodenum (37). However, reports on the regulation of BCRP expression and their functional consequences during obesity were lacking. Our data showed no significant differences in colonic BCRP mRNA expression between normal and obese human subjects or mice (Fig. 3), thereby ruling out the transcriptional regulation as the reason for decreased colonic BCRP expression and function. Moreover, we showed a significant difference in tyrosine phosphorylation of BCRP in control and obese indicating a posttranslational regulation as potential underlying mechanism. Previously, it was reported that serine/threonine kinase Pim-1 phosphorylates BCRP at Thr362 that facilitates dimerization of the BCRP proteins, which in turn was required for proper efflux function (38). Moreover, mutation of Thr362 to alanine resulted in cytoplasmic compartmentalization of BCRP thereby inhibiting protein dimerization and proper localization at the plasma membrane (39). To find out the reason for decreased BCRP tyrosine phosphorylation in obese colon, we investigated potential tyrosine kinases responsible for such an effect. Previously, we reported the role of JAK3 in epithelial cell–mediated predisposition to chronic inflammatory conditions including obesity and inflammatory bowel disease (13, 21, 22, 27–30). However, the role of JAK3 in regulating drug efflux in general and intestinal drug efflux functions in particular were lacking. In the present study, our data suggested that both male and females obese human subjects had significantly decreased expression of colonic JAK3, and knockout of JAK3 in mouse led to significant decrease in colonic BCRP expression and decreased colonic drug efflux functions (Fig. 4). These indicated a role of JAK3 in regulating BCRP protein expression and functions. Furthermore, to investigate how JAK3 regulated BCRP expression and efflux functions, we showed that BCRP not only associated with JAK3 in mouse and human colons but inhibition of JAK3 also led to loss of tyrosine phosphorylation of BCRP in human colonic epithelial cells. Our data suggest that tyrosine phosphorylation of BCRP facilitated luminal membrane localization and prevented xenobiotic drugs absorption through tyrosine phosphorylation–mediated BCRP interac-

**BCRP interacts with non-receptor tyrosine kinase**

**Experimental procedures**

**Materials**

De-identified and discarded human colonic biopsy samples used in this study were obtained from Coastal Bend Pathology, HT-29 CI-19A, a permanently differentiated homogenous clone derived from human colonic epithelial cells (HT-29), was a kind gift from Dr. A. P. Naren (Cystic Fibrosis Research Center, Cincinnati, OH) (42).

**Antibodies**—Information on JAK3 and BCRP antibodies is provided in Table S2, pY20 (MP Biomedicals); IgG control (Thermo Fisher Scientific); β-catenin (Santa Cruz Biotechnology); IL-2, WH11P31, EGF, novobiocin, Inulin-FITC (all from Sigma); Pierce® BCA Protein Assay Kit (Thermo Scientific) (13, 20, 22); OneStep RT-PCR Kit (Qiagen); RNAqueous-4PCR kit (Invitrogen); Transcriptor First Strand cDNA Synthesis Kit (Roche); verapamil, β-catenin-shRNA, normal human colon lysate (Santa Cruz Biotechnology). BCRP primers, forward (5′-AGATGGGTATTTC-CAAAGCGTT CAT) and reverse (5′CCAGTTCC CAGTACGACT-GTGA) Paraffin sections of normal human colon (US Biomax, Inc.); control siRNA-A, ABCG2 (h)-PR primer (Thermo Fisher);
Methods

Cell culture, IL-2 and inhibitors treatment, and transfection—Methods for HT-29 cell maintenance, treatment, and transfection, were reported before (26, 27). Transfected HT-29 Cl-19A cells were grown to confluence followed by treatment with IL-2 (50 units/ml) as reported previously (27). For JAK3 inhibition studies, cells were treated with or without JAK3 inhibitor CP-69050 (26, 27) and expression of BCRP, PgP, MRP-2, and β-catenin were determined using Western blot analysis of the lysates.

Recombinant JAK3 and β-catenin and kinase assay—Purified proteins for His-tagged WT constructs of β-catenin and GST-tagged JAK3 were produced as reported before (24). In vitro kinase assay using recombinant JAK3 and β-catenin protein was performed as reported by our group (20, 24).

Animals—6- to 8-week-old C57BL/6 mice (WT) and C57BL/6-background JAK3-/− mice (KO) were from The Jackson Laboratory. For high-fat diet studies, male C57/B16 mice, weighing 22 ± 2 g, were fed a high-fat diet (65% cal from fat) or a normal diet (6.5% cal from fat) for 8 weeks as reported (13). The animals were housed in a temperature- and light-controlled room. The mice had food and water ad libitum. Mouse chow was purchased from Research Diets (New Brunswick, NJ). This study adhered to the institutional guidelines of Texas A & M University Institutional Animal Care and Use Committee.

Transwell inulin assay—Paracellular permeability was measured using the fluorescent dye FITC-Inulin (10 kDa). Briefly, apical compartments of Transwell plates were washed with PBS and replaced with solution containing 100 µg/ml FITC-Inulin dissolved in DMEM, and basolateral chambers replaced with fresh DMEM. After 5 h incubation at 37 °C, aliquots from the apical and basolateral compartments were collected and signal intensities were measured at 700 nm (emission) using Infinite M1000 Pro microplate reader (Molecular Devices). The C_r (µ inulin retention) was calculated as in “Inulin flux assay.”

Intracellular Hoechst 33342 accumulation studies (ex vivo)—Equal lengths of colon from WT and obese mice were excised, cleaned thoroughly to remove feces, and washed with 1× PBS. The colonic segments were tied with thread at both the ends and Krebs-Ringer buffer was added through a syringe into the colon sacs. Novobiocin (300 µM), tofacitinib at 1 µM, and verapamil (75 mg/ml) were added separately and the sacs were incubated in Krebs-Ringer buffer for 15 min at 37 °C. 10 µM Hoechst 33342 was added thereafter directly into the sacs using a syringe and incubated at 37 °C for 30 min. Then the sacs were removed, untied, and cleaned using 1× PBS, and tissue lysates were made having equal amounts of tissues per milliliter lysis buffer. The fluorescence intensities of equal amounts of the tissue lysates were measured using microplate reader at 350 nm excitation and 461 nm emission and intracellular Hoechst 33342 fluorescence values were corrected for proteins.

Efflux assay (in vitro)—HT-29CL19A cells with or without treatment were trypsinized and suspended at 0.5 to 1.0 × 10⁶ cells/ml in complete RPMI. Hoechst 33342 was added to a final concentration of 5 mg/ml and cells were incubated for 45 min in a 37 °C water bath. Cells were gently agitated every 15 min. After the staining period, the cells were washed in cold Hanks’ balanced saline solution/1 mM HEPES/2% FCS (HBSS1) and

Figure 8. Proposed models for BCRP phosphorylation mediated mucosal barrier function and predisposition to obesity.
resuspended in warm (37 °C) media and incubated for a 45-min efflux period. This poststaining efflux period served to clear cells of membrane-associated fluorescence and lower background. When efflux inhibitors were used, they were maintained at the designated concentration as mentioned above. Fluorescence intensity of the cell lysates were measured using an Infinite M1000 Pro microplate reader at 350 nm excitation and 461 nm emission.

Tissue inulin flux assay—Equal lengths of colons were dissected out and the luminal contents were flushed with 0.9% saline, ligated at both ends, and kept in a Petri dish containing 3 ml of 1× PBS. Equal volumes of FITC-inulin (50 mg/ml solution; 2 μl/g colon weight) were injected to the lumen and incubated for 1 h at 37 °C. Fluorescence of the aliquots from injected inulin (before administration) and the leaked inulin (after incubation) was collected and measured using a fluorescence plate reader. Mucosal barrier function was evaluated by measuring luminal to mucosal flux of FITC-inulin and calculated as % inulin retention using the formula CR probe = [(Ci-Ct)/Ci] × 100, where Ci is % inulin retention, Ct is initial probe concentration, and C is leaked probe concentration after incubation.

RT-PCR and quantitative real time PCR—RT-PCR was performed using a OneStep RT-PCR kit (Qiagen) as per the manufacturer’s protocol using 2 μg of total RNA and BCRP primers. 18SRNA was used as an internal control. The amplified PCR samples were separated using agarose gel, and bands’ intensities were quantified using Bio-Rad imaging system. Total RNA for the real time PCR was isolated using RNAqueous-4PCR kit (Invitrogen) and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche). Sequence specific primers for BCRP were designed using a universal probe library (Roche Applied Science) and synthesized from Integrated DNA Technologies (Coralville, IA). Primers used were as follows: BCRP, forward 5’-GGGGTTTCCTACTTGGTTCG-3’ and reverse 18SRNA control, forward 5’-CGCTACACATCCACAGGA A-3’ and 18SRNA control reverse 5’-GCTGGAATTACCGCGGCT-3’. Real-time detection of PCR products was performed using ABI PRISM 7700 Sequence Detector (Applied Biosystems). Reactions were performed in triplicate for each condition. BCRP expression (BCRP/18SRNA) was calculated using ABI PRISM 7700 Sequence Detector (Applied Biosystems). Reactions were performed in triplicate for each condition. Based on CT values from BCRP detection, normal- formed using the formula CR probe = [(Ci-Ct)/Ci] × 100, where Ci is % inulin retention, Ct is initial probe concentration, and C is leaked probe concentration after incubation.

Data analysis

All data are presented as S.E. and analyzed using Microcal Origin software version 9.2. Differences in the parametric data were evaluated by the Student’s t test. Significance in all tests was set at a 95% or greater confidence level. Statistically significant data and the corresponding p value are annotated in the figure legends.

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