Overnutrition stimulates intestinal epithelium proliferation through β-catenin signaling in obese mice

A running title: Intestinal GSK-3β/β-catenin signaling in obesity

Jiaming Mao¹, Xiaomin Hu¹, Yao Xiao¹, Chao Yang¹, Yi Ding¹, Ning Hou¹, Jue Wang¹, Heping Cheng¹,² and Xiuqin Zhang¹*

From the ¹Institute of Molecular Medicine, Peking University, Beijing, China; ²State Key Laboratory of Biomembrane and Membrane Biotechnology, Peking-Tsinghua Center for Life Sciences, Beijing, China.

*Corresponding author: Xiuqin Zhang, M.D., Ph.D. Institute of Molecular Medicine, Peking University, 5 Yiheyuan Road, Haidian District, Beijing 100871, China.

E-Mail: zhangxq@pku.edu.cn. Tel and Fax: (086)-10-6275-3420

J.M. and X.H. contributed equally to this study.

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ABSTRACT

Obesity is a major risk factor for type 2 diabetes and cardiovascular diseases. And overnutrition is a leading cause of obesity. After most nutrients are ingested, they are absorbed in the small intestine. Signals from β-catenin are essential to maintain development of the small intestine and homeostasis. In this study, we employed a hyperphagia db/db obese mouse model and a high fat diet (HFD) induced obese mouse model to investigate the effects of overnutrition on intestinal function and β-catenin signaling. The β-catenin protein was upregulated along with inactivation of glycogen synthase kinase-3β (GSK-3β) in the intestines of both db/db and HFD mice. Proliferation of intestinal epithelial stem cells, villi length, nutrient absorption and body weight also increased in both models. These changes were reversed by caloric restriction in db/db mice and by β-catenin inhibitor JW55 (a small molecule that increase β-catenin degradation) in HFD mice. Parallel, in vitro experiments showed that β-catenin accumulation and cell proliferation stimulated by glucose were blocked by the β-catenin inhibitor FH535. And the GSK-3 inhibitor CHIR98014 in an intestinal epithelial cell line increased β-catenin accumulation and Cyclin D1 expression. These results suggested that, besides contribution to intestinal development and homeostasis, GSK-3β/β-catenin signaling plays a central role in intestinal morphological and functional changes in response to overnutrition. Manipulating the GSK-3β/β-catenin signaling pathway in intestinal epithelium might become a therapeutic intervention for obesity induced by overnutrition.
Obesity, affecting about 30% of the world population, and is a major risk factor for metabolic syndrome, inflammation, type 2 diabetes (T2D) and cardiovascular diseases (CVD)(1). Epidemiological evidence suggests that body weight is regulated by complex physiological mechanisms (2; 3). However, environmental factors, especially when the energy intake from food exceeds normal physiological needs, are considered to be culprits for becoming overweight and then obese. Conversely, caloric restriction (CR) significantly reduces obesity, and incidences of T2D, and CVD in rodents, primates and humans (4-6).

Appetite and food intake are a complex physiologic process. Regulation of appetite involves numerous hormones, signals and factors defects of these appetite-related molecules and related signaling pathways cause severe obesity (7-9). These findings strongly suggested a prominent role for excess food intake and an oversupply of nutrients in obesity and related diseases. Studies show that HFD could induce intestinal epithelial proliferation, absorption and adiposity (10-12). However, the underlying mechanisms remain poorly understood.

The internal surface of the mammalian intestine is covered by a single layer of epithelial cells that protrude into the intestinal lumen to form finger-like villi that absorb nutrients from food. This single layer of cells is renewed every 3 to 5 days. Besides these villi, other specialized structures have evolved in the intestinal epithelium, termed crypts, which contain multipotent stem cells and are responsible for intestinal epithelial cell renewal. This cell renewal process is strictly controlled through a series of coordinated signaling pathways (13; 14).
In mammals, the canonical Wnt signaling pathway is essential for maintaining intestinal crypt cell proliferation during development and for intestinal epithelium homeostasis during adulthood (14-16). As a core effector of the Wnt signaling pathway, β-catenin is regulated mainly at the protein level by a proteolytic degradation complex that consists of adenomatous polyposis coli (APC), casein kinase I (CKI), glycogen synthase kinase-3β (GSK-3β) and axin. When the complex is assembled, the GSK-3β will effectively phosphorylate β-catenin, leading to β-catenin protease hydrolysis (17). However, GSK-3β is inactivated by phosphorylation at serine 9, leading to cytoplasmic β-catenin accumulation and nuclear translocation, resulting in an increase of β-catenin target gene, such as Cyclin D1 expression and cell proliferation (18).

To better understand whether and how small intestine homeostasis is involved in its morphological and functional changes induced by excess food intake and high fat diet (HFD), we used a hyperphagic db/db obese mouse model and an obese model induced by HFD to investigate the changes in absorptive surface area and related signaling in the small intestine during the occurrence of obesity. We found that intestinal epithelial cell proliferation induced by excess food intake was correlated with activation of the GSK-3β/β-catenin signaling pathway, suggesting that nutrient-induced activation of GSK-3β/β-catenin signaling in the intestinal epithelium may contributes to increased nutrient absorption and obesity development.

RESEARCH DESIGN AND METHODS

**Animal models.** Hyperphagia db/db mouse model, male and female db/+ mice obtained from Jackson Laboratories (Bar Harbor, Maine 04609, USA) were mated to generate
db/db mice. Mice were fed with standard Chow diet containing 4% fat and 50% carbohydrate. Age matched male *ad libitum* db/+ and db/db mice were used for further studies (n = 8 per group). For embryonic intestine sample collection, the pregnant female db/+ mice were sacrificed at the time that embryos become 18.5 days.

In the CR group, 4 week-old, male db/db mice (n = 5) were housed in individual cages and fed twice daily with a restricted amount (60% of *ad libitum*) of the standard Chow diet, these mice were sacrificed after 4 weeks of CR and intestinal samples were harvested for further studies.

For HFD induced obese model, at the age of 4 weeks, male C57BL/6 mice were fed a HFD (Cat. # D12492, Research Diets Inc. New Brunswick, NJ), that contained 35gm% (60 kcal%) fat, for 8 weeks, matching controls were fed a standard Chow diet (n = 8 per group).

To form the β-catenin inhibition group, 4 week-old, male C57BL/6 mice were randomized into 4 groups and fed for 3 weeks: standard chow diet (n=8), HFD (n=8), HFD + DMSO (n=8), or HFD + JW55 in DMSO (100 mg/kg, daily oral application) (n=4). JW55 (Tocris Bioscience, US) is a small molecule that increases β-catenin degradation (19).

All mice were maintained under specific pathogen-free conditions in the AAALAC-accredited animal facility at Peking University (PKU), mice had free access to water and body weight was measured weekly or twice a week. The present study was approved by the Institutional Animal Care and Use Committee of PKU and was in accordance with the principles of laboratory animal care of the National Academy of Sciences/National Research Council.
Intestinal sample collection. To evaluate epithelial cell proliferation and migration in the small intestinal villi, 1 h or 24 h after an intraperitoneal injection of bromodeoxyuridine (BrdU, Sigma, MO, USA) at 100 mg/kg body weight, mice were anesthetized with sodium pentobarbital (100 mg/kg body weight) and sacrificed by cervical dislocation. The entire intestine was then removed, weighed, and rinsed rapidly with iced Ringer buffer (in mM: NaCl 115, NaHCO₃ 25, MgCl₂ 1.2, CaCl₂ 1.2, K₂HPO₄ 2.4, and KH₂PO₄ 0.4, pH 7.3) supplemented with PMSF. Small intestinal tissue was dissected and the jejunum was snap frozen in liquid nitrogen for isolation of total RNA and protein, or was fixed in ice-cold 4% paraformaldehyde (PFA) overnight for histological and immunohistochemical analysis. Embryonic intestinal sample collection was performed as previously described (20). In brief, embryos were removed from time-mated females at E18.5 1 h after an intraperitoneal injection of BrdU. For histological and immunohistochemical analysis, the intestines were fixed in 4% PFA for paraffin sections preparation. For isolation of total RNA and protein, the small intestine was frozen in liquid nitrogen immediately after dissection.

Histology and immunohistochemistry. The PFA-fixed intestines were embedded in paraffin and 5-μm sections were prepared. The sections were stained with hematoxylin and eosin (H&E) for histological analysis. For immunohistochemistry, sections were incubated with anti-BrdU (BD, CA, USA) or anti–Cyclin D1 (Santa Cruz, CA, USA) antibodies overnight at 4°C, followed by anti-rabbit or mouse horseradish peroxidase–labeled secondary antibodies (Dako Diagnostics) for 1 h at room temperature. Slides were developed using 3,3’-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.
To measure length of villi, the H&E-stained sections were imaged with a light microscope BX51 (Olympus, Japan). One hundred well-orientated villi were randomly chosen from ten 100× images and measured with Image-Pro MC 6.0 software (Olympus). For cell proliferation assays, the BrdU- or Cyclin D1-positive cells were imaged within 100 well-oriented crypts from ten 100× images and counted per crypt using Image-Pro.

**Western blot.** Total intestinal protein was extracted using an RIPA lysis buffer supplemented with a protease inhibitor cocktail (Sigma), phosphorylation protease inhibitor (Roche) and PMSF. After rapid homogenization, the homogenate was incubated in ice for 30 min, and centrifuged at 12,000 g for 15 min at 4°C. Protein samples (30-100 µg) were separated by 10% SDS-PAGE. Blots were incubated at 4°C overnight with primary antibodies: rabbit anti-mouse GLUT2 (1:1000, MILLIPORE, USA), rabbit total GSK3β and anti-phosphorylated Ser9-GSK-3β (1:1000, Cell Signaling Transduction), mouse anti-β-catenin (1:1000, BD), rabbit anti-Cyclin D1 (1:1000, Santa Cruz Biotech) and mouse anti-mouse β-actin (1:1000, Santa Cruz Biotech), followed by a 1-h incubation with anti-rabbit or mouse horseradish peroxidase–labeled secondary antibodies (Santa Cruz Biotech) at room temperature. An ECL detection system (Bio-Rad) was used to reveal the peroxidase label. Relative abundance was quantified by densitometry using Quantity One 4.6.7 software (Bio-Rad).

**Intestinal glucose absorption.** Glucose uptake was performed as described previously (21). Briefly, intestinal rings were incubated in an oxygenated Ringer solution containing 50 mM D-[U-3H] glucose (0.1 µCi/ml, PE) for 10 min. Then the intestine was lysed overnight in 10% HNO3 at 4°C. The radioactivity of the lysate was measured by liquid scintillation. Counts were expressed as nM/cm ± SEM.
**Cell culture and cell proliferation assay.** The mouse colon epithelial cell line (CT26) was from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C under 5% CO₂ and 95% O₂ in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, CA, USA). To determine the effect of high glucose exposure on cell proliferation, after 24-h culturing, cells were serum-starved for 24 h and then incubated for another 48 h in fresh medium supplemented with 0 mM or 20 mM D-glucose. To test the function of GSK-3β/β-catenin signaling in cell proliferation, 5 µM FH535 (an inhibitor of β-catenin/Tcf–mediated transcription) (Sigma) (22) or CHIR98014 (GSK-3 inhibitor) (Selleckchem, Houston, TX) (23) were added to culture medium containing 20 mM D-glucose. For proliferation assays, MTT was performed as described previously (24; 25). Absorbance was measured at 490 nm and converted into cell number. Three independent experiments were performed.

To determine the signaling pathways involved in CT26 cell proliferation in response to high glucose or FFA (Palmitic acid; Sigma), cells were seeded in 6-well plates in RPMI 1640 medium to reach 70-80% confluence. After 24 h of serum starvation, the cells were incubated with serum–free RPMI 1640 medium containing 20 mM D-glucose, or serum–free RPMI 1640 medium containing 2% BSA and 0.5 mM FFA for 15, 30, 60, 120, or 240 min. The cells then were lysed in RIPA buffer to extract protein.

**Statistic analysis.** All data are expressed as mean ± standard error (SEM). Student’s t test was used to compare the differences between groups. A p value <0.05 was considered statistically significant.
RESULTS

Intestinal growth and glucose absorption increased in db/db mice, and was reversed by CR. Nutrient absorption in the intestine after food intake is the gateway for the body’s energy supply. Energy overload in excess of expenditure is the basis of obesity, while CR significantly reduces the incidence of obesity and its related diseases (4; 5; 26). To investigate changes in intestinal absorption related to excess food intake and developing obesity, we first looked at hyperphagic db/db mice. We found that, from the age of 4 weeks, food intake in db/db mice was increased significantly compared to age-matched lean db/+ mice (Fig. 1A) in concordance with body weight (Fig. 1B). Restricting food intake caused a distinct reduction in body weight in db/db mice compared with their ad libitum db/db littermates (Fig. 1B). Intestinal length and weight also dramatically increased in db/db mice. CR did not alter db/db mice intestinal length in db/db mice, but robustly decreased intestine weight (Fig. 1C and D).

To test if the absorption capacity per unit length of intestine also increased in db/db mice, we carried out glucose uptake assays in jejunal rings of 8 week-old db/db, db/+, and db/CR mice using a $^3$H-labelled glucose tracer (21). The data showed that the intestinal glucose absorption of the jejunal rings increased in db/db mice compared with the same length of db/+ mouse intestine (Fig. 1E). Consistently, the glucose transporter protein type 2 (GLUT2) protein, which mediates glucose transport from the intestinal lumen to the blood stream (21; 27), was increased dramatically in db/db intestines (Fig. 1F and G). CR significantly reduced glucose uptake and GLUT2 expression in db/db mice (Fig. 1E, F and G). These results suggested that excess food intake, along with the
increased intestinal absorption capacity, may contributed to obesity progression, and that this can be reversed by CR.

**Intestinal absorption area increased in db/db mice, CR reduced absorption area in db/db mice.** The intestinal mucosa is the main site of nutrient absorption. Villi in the mucosa increase the surface area over which absorption takes place. We studied morphological changes to determine if the overall absorption surface area of the small intestine in db/db mice was increased, and the influence of CR on the absorption surface area. H&E staining of the jejunum showed that the villi lengthened by approximately one third (36.65%, p<0.001) in db/db mice, and CR significantly reduced the length of villi (Fig. 2A and B), indicating that the increased absorption area may have contributed to the increased absorption capacity in db/db mice, and CR reduced absorption by reducing growth of villi.

In adult mice, epithelial cells in the villi are renewed every 3-5 days through cell proliferation, differentiation, migration and apoptosis from multipotent stem cells located in the intestinal crypts. During the cell renewal process, these stem cells proliferate, differentiate and convert into several types of functional mature epithelial cells that migrate along the villi (15). To evaluate the epithelial cell proliferation and migration in the small intestinal villi, we injected BrdU (i.p.) into 8-week-old mice to label S-phase cells. One hour after BrdU was injected, the BrdU-positive rate in the jejunal crypts was significantly higher in db/db mice. The increase in BrdU-positive cells was reduced by CR in db/db mice (Fig. 2C and D). In addition, we performed immunohistochemistry 24 h after BrdU was injected to trace the proliferation and migration of BrdU-labeled cells. After 24 h, the number of BrdU-positive cells increased significantly in db/db mice (Fig.
and $F)$ and the distance of BrdU-positive cells from the crypt/villi axis was significantly longer in the db/db mice. Nearly 100% of BrdU-positive cells in the control mice were distributed within 25 cells from the base of the crypt, while more than half of the BrdU-positive cells were located between cell positions 25 and 40 in db/db mice (Fig. 2G). These data suggest that increased proliferation and migration of stem cells from the crypts, was likely responsible for the observed villi elongation and increased absorption in db/db mice.

**Activation of GSK-3β/β-catenin axis in the mucosa of small-intestinal of db/db mice.**

Cellular proliferation regulated by β-catenin plays a pivotal role in crypt stem cells and epithelial progenitor cells during gut development and in colon cancer (14; 15; 28). To assess if β-catenin signaling also is involved in villi elongation in db/db mice, we examined β-catenin expression and signaling in the jejunum of 8-week-old db/db and db/+ mice. β-catenin mRNA expression within the intestine did not differ between these mice when assayed by qRT-PCR (Fig. 3A). However, the protein level was significantly increased in db/db mice compared with db/+ mice (Fig. 3B and C). This suggested that β-catenin proteins accumulation is increased in the db/db mouse intestine, and that this accumulation was reduced with CR (Fig. 3B and C). Analysis of GSK-3β phosphorylation showed that the ratio of Ser9 phosphorylation (p-GSK-3β) to total GSK-3β levels was upregulated in intestines of db/db. While the total GSK-3β (t-GSK-3β) protein expression did not change, the ratio of p-GSK-3β to t-GSK-3β was reduced by CR (Fig. 3B and D). As expected, when using immunohistochemistry to labeling the β-catenin target gene Cyclin D1, positive cells also increased in the intestine of db/db mice, and decreased after CR (Fig. 3E and F). These results mean that excess food intake
stimulated GSK-3β/β-catenin signaling activation might be involved in proliferation of intestinal epithelium.

**Leptin signaling defects did not affect proliferation of intestinal epithelium in the embryonic stage.** Because db/db mice are leptin receptor mutant mice, we investigated whether increased intestinal epithelial proliferation was derived from abnormal gut development caused by the loss of leptin signaling. E18.5 db/db and db/+ littermates showed no difference in body weight or intestinal length (Fig. 4A and B). H&E staining also did not show gross differences in intestinal histology and villi length (Fig. 4C and D). In addition, there was no difference in cryptal BrdU labeling between the two groups (Fig. 4E and F). β-catenin protein level, GSK-3β phosphorylation (Fig. 4G, H and I) and Cyclin D1-positive cells (data not shown) were similar in embryonic db/db and db/+ mouse intestines. These findings indicated that the genetic defect in the leptin receptor did not affect β-catenin signaling and cell proliferation in the embryonic intestinal epithelium. Combined with the earlier results, these results support a model in which increased intestinal epithelial cell proliferation was due to the excess food intake stimulation in adult db/db mice, and suggested that compensatory intestinal epithelial cell proliferation/migration was important in increasing intestinal absorption, and might contribute to obesity.

**A high fat diet increased intestinal epithelial cell proliferation through activation of GSK-3β/β-catenin signaling pathway.** As described above, we observed that proliferation of epithelial cells in the intestine of db/db mice was increased significantly and could be reversed by CR. Because the db/db mice were leptin receptor mutant and
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hyperphagia, the intestinal changes in adult mice also could have been caused either by genetic background or physical stimulation due to the increased volume of food consumed. To confirm of this, we subjected 4-week-old, wild type C57BL/6 mice to HFD. After 8 weeks of HFD, the mice had significantly greater body weight than mice fed a standard Chow diet (Fig. 5A, B). Concurrently, increased villi length and epithelial cell proliferation were found in the small intestines of HFD mice (Fig. 5C, D, E and F). Moreover, GSK-3β phosphorylation, β-catenin protein and Cyclin D1 level, all were increased (Fig. 5G, H, I and J). These results confirmed that it was the energy density in the food behind the pronounced signaling of GSK-3β/β-catenin, and stimulated intestinal epithelial proliferation and absorption, rather than genetic background and volume of food intake.

To investigate further if β-catenin was responsible for proliferation and morphological changes of intestinal cell from over nutrition, we treated mice fed the HFD with JW55. JW55 is a small molecule inhibitor of the β-catenin signaling pathway, it stabilizes AXIN2, a member of the β-catenin destruction complex, and increases degradation of β-catenin (19). After 3 weeks treatment with JW55, β-catenin and Cyclin D1 were significantly lower compared with HFD DMSO control treatment group, but no differences were found with CD group (Fig. 6A, B, C and D). Also, villi length and BrdU positive cells were lower in JW55 treated group (Fig. 6E, F, G and H). The absolute increased body weight in mice treated with JW55 was lower, but not significantly, compared mice treated with DMSO (8.25 ± 0.37 g and 9.23 ± 0.46 g, for JW55 and DMSO treatments, respectively). In our prior studies, significant differences in body weight between chow diet and HFD fed C57BL/6 mice usually appeared after 4-weeks.
The present result might be due, in part, to the shorter duration of HFD feeding and treatment. These data indicated that an activated β-catenin signaling pathway was essential for proliferation intestinal epithelium in response to HFD feeding.

**High glucose and FFA induces GSK-3β phosphorylation and β-catenin accumulation in epithelial cells.** Carbohydrate is the main component of regular mouse chow. Most ingested carbohydrate is digested and broken down into glucose in the small intestine and transported into the blood stream. Previous studies have reported that exposure to high glucose levels induces proliferation in some cell types (29; 30). In addition, high sucrose diets promote intestinal epithelial cell proliferation and tumorigenesis in APC\textsuperscript{min} mice (31). Therefore, we performed an in vitro study to determine whether glucose directly stimulates intestine epithelial cell proliferation. When CT26 epithelial cells were exposed to high glucose (HG, 20 mM), phosphorylation of GSK-3β increased, and the expression of β-catenin and its target gene, Cyclin D1, were upregulated after 1 h of HG stimulation (Fig. 7 A and B). After mice were fed a HFD, the primary nutrient absorbed in the intestine was free fatty acid (FFA). To confirm whether FFA directly activates GSK-3β/β-catenin signaling, we added FFA to CT26 cell culture medium. GSK-3β phosphorylation and β-catenin accumulation were increased after 1 h of FFA stimulation. The expression of the β-catenin target gene, Cyclin D1, also was upregulated after FFA stimulation (Fig. 7 C and D). However, treating CT26 cells with FH535, an inhibitor of β-catenin/TCF transcription, reduced β-catenin and significantly blocked expression of Cyclin D1 (Fig. 8 A, B and C) and cell growth induced by HG (Fig. 8D). Similarly, when the GSK-3 inhibitor CHIR98014 was add to CT26 cells, the phosphorylation of GSK-3β, accumulation of β-catenin and expression of Cyclin D1 all
were upregulated significantly (Fig. 8 E, F, G and H). These data support the supposition that high nutrient levels directly activate GSK-3β/β-catenin signaling and stimulate intestinal epithelial cell proliferation.

**DISCUSSION**

The increasing incidence of obesity and its association with disease have become a great challenge to global health. Obesity increases the risk of cardiovascular disease, premature death, insulin resistance, T2D and many types of cancer (32; 33). However, few drugs for anti-obesity are approved by the FDA in the United States, such as phentermine and orlistat (34). Unfortunately, because of insufficient weight loss and significant gastrointestinal side effects, these anti-obesity drugs are less than ideal. So there is an urgent need to find new therapeutic targets for obesity.

The etiology of obesity is very complex and pathological mechanisms have been studied widely in adipose tissue, liver and muscle (35; 36). Several genes have been identified that regulate adipose mass and are associated with the development of obesity. Recent findings regarding the role of intestine-secreted hormones in metabolic diseases and the effects of gastric bypass bariatric surgery makes the intestine a primary site in the pathophysiology of obesity and T2D (37-39). However, aside from the impact of gut-derived endocrine hormones on T2D, there has been little study of the contribution of the intestine to excess food intake-related obesity.

Some studies describing gene regulation in the intestine during the development of obesity induced by a HFD found that many dietary fat-induced molecular changes are associated with lipid metabolism, the cell cycle, inflammation and the immune response.
But, details of mechanisms that affect the functionality of intestine during the progression of obesity have not been studied. In a regular diet, carbohydrates are the most prevalent nutrient. Monosaccharides in the intestinal lumen interact with the intestinal epithelium after carbohydrate digestion. Whether this interaction affects intestinal function is unknown. β-catenin dependent intestinal stem cell proliferation is a prerequisite for the maintenance of epithelial homeostasis and absorption (13).

Free β-catenin proteins accumulating in cells is an important feature of activating β-catenin downstream signaling (16). Chen, et al., found that retinal tissue sections from diabetics displayed increased β-catenin expression and nuclear translocation (43). Anagnostou and Shepherd also verified that high glucose induces upregulation of β-catenin in two macrophage cell lines (44). In this study, nutrient over load in animals and high glucose or FFA in cultured intestinal epithelial cells induced β-catenin accumulation and intestinal epithelial cell proliferation. In confirmation of this, high glucose did not stimulate intestinal cell proliferation and Cyclin D1 expression after treatment with the inhibitor of β-catenin transcription, FH535. Giving a β-catenin inhibitor JW55 to mice fed a HFD also prevented intestinal epithelial cell proliferation. Together, these results indicated that nutrients may prevent β-catenin degradation and promote downstream transcriptional activation that increases intestinal epithelial cell proliferation. Functionally, GLUT2, a main transporter of dietary sugar in the intestine (27), increased in hyperphagic obese db/db mice. On the contrary, food restriction in db/db mice reduced intestinal cell proliferation and absorption. The indication being that intestinal absorptive capacity was increasing along with the increased food intake and intestinal epithelial proliferation.
GSK-3β-based protein degradation is a key event in regulating intracellular β-catenin protein accumulation in canonical Wnt signaling (14; 16). The ninth serine in the N terminus is very important for GSK-3β activity. Once this serine is phosphorylated, GSK-3β fails to bind and phosphorylate β-catenin for degradation. We found that excess food intake promoted phosphorylation of the ninth serine of GSK-3β in intestine. By adding CHIR98014 (a GSK-3 inhibitor) alone, or with high glucose, to epithelial cells could increases GSK-3β phosphorylation, and then induced β-catenin accumulation and Cyclin D1 expression. This suggests that the increased β-catenin protein in intestinal epithelial cells may depend on phosphorylation of the ninth serine of GSK-3β.

Recently, in addition to Wnts, a variety of signaling molecules involved in the regulation of intracellular transcriptional activity of β-catenin/TCF in different contexts have been found, including insulin, insulin-like growth factor-1 (IGF-1), PDGF, glucagon-like peptide-2 (GLP-2) and FOXO (45-47). Studies have shown that, in breast cancer cells, IGF-1 stabilizes intracellular β-catenin and promotes the transcription activity of β-catenin/TCF. IGF-1 and insulin are high in the plasma of obese animals, and are closely related to nutrient status. GLP-2 is one of the best studied peptide secreted from enteroendocrine L-cells of the intestine that closely related to intestinal function (48; 49). Studies show that GLP-2 could stimulate cell proliferation of intestine epithelium thought IGF-1 signaling and β-catenin alterations (46; 47; 50). And GLP-2 also involved in intestinal epithelial proliferation after HFD stimulation (49). After 3 weeks of HFD feeding, blood glucose and insulin were increased significantly. Though GLP-2 had no clear increase in the HFD group. Phosphorylation of Akt increased significantly in adipose tissue, but not in the intestine (data not shown). Nutrients seemed to have a direct
and important effect on the proliferation of intestinal epithelial cells, at least in the early period of nutrient overload. However, we cannot exclude that GLP-2, inflammation, hyperglycemia or hyperinsulinemia might also have contributed to intestinal epithelial cell proliferation in long term nutrient overload or obesity status.

Taken together, our findings confirm the importance of nutrition on initiating proliferation of intestinal epithelium, and provided a possible link between intestinal metabolism and obesity development. Targeting epithelial GSK-3β/β-catenin signaling in the small intestine may provide a novel strategy to prevent obesity related to diet. Further investigation into the mechanisms responsible for interactions between diet and the intestine will advance our knowledge of the pathogenesis of obesity.

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J.M. contributed to the experiments, data analysis and wrote the manuscript. X.H. contributed to the experiments and contributed to all revised experiment. Y.X., C.Y., Y.D., and N.H. contributed to the experiments. H.C. and J.W. contributed to the discussion and reviewed/edited the manuscript. X.Z. contributed to the study design, data collection, and interpretation and wrote the manuscript. X.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

FIG. 1. Intestinal growth and glucose absorption are increased in adult db/db mice, and it can be reversed by caloric restriction (CR). A: Food intake in db/db and db/+ mice. B: Body weight in db/db, db/+ and db/CR mice. C: Whole length of small intestine in db/db, db/+ and db/CR mice. D: Small intestine weight per cm length in db/db, db/+ and db/CR mice. E: Glucose uptake in jejunal rings of 8-week old db/db, db/+ and db/CR mice. Counts are expressed as nM/cm. F and G: Intestinal GLUT2 protein expression in 8-week old db/db, db/+ and db/CR mice. Western blot data from at least three independent experiments and are expressed as mean ± SEM; mice n = 5 - 8 per group. *p <0.05, **p <0.01.

FIG. 2. Enhanced epithelial proliferation in the small intestine of adult db/db mice. A: H&E staining of paraffin-embedded jejunum section show longer villi length in db/db
mice, and decreased length in db/CR mice. B: Quantification of the villi length. C: Cell proliferation identified by BrdU immunohistochemical staining of paraffin section. Brown color nucleus indicates BrdU positive cells. D: Quantification of the BrdU positive cell. E: Epithelial cell proliferation and migration detected by BrdU immunohistochemical staining. A brown nucleus indicates BrdU positive cells. F: Quantification of BrdU positive cell. G: Quantification of BrdU(+) cell migration. Data are expressed as mean ± SEM; mice n = 5 - 8 per group. *p <0.05, **p <0.01. Scale bar, 50 µm.

FIG. 3. GSK-3β/β-catenin signaling is activated in db/db mouse intestinal epithelium. A: β-catenin transcription level was detected by real-time RT-PCR. mRNA expression was calculated relative to β-actin. B and C: β-catenin accumulation was increased in db/db mice compared with db/+ mice, and the β-catenin accumulation in db/db mice was reduced by CR. B and D: GSK-3β Ser9-phosphorylation was increased in db/db mice compared with db/+ mice, and the increased GSK-3β Ser9-phosphorylation in db/db mice was reversed by CR. E: β-catenin target gene Cyclin D1 was detected by immunohistochemical staining. F: β-catenin target gene Cyclin D1 was increased in db/db mice intestine, and was decreased by CR. PCR or Western blot data are from at least three independent experiments and are expressed as mean ± SEM; *p <0.05, **p <0.01. Scale bar, 50 µm.

FIG. 4. Embryonic intestinal epithelium proliferation did not differ between db/db and db/+ mice. A: Body weight of E18.5 db/db and db/+ embryos. B: Intestinal length of E18.5 db/db and db/+ embryos. C: H&E staining of E18.5 embryonic intestines. D: Length of embryonic intestine villi E: E18.5 embryonic intestinal epithelial cell
proliferation identified by BrdU staining. 

F: Quantification of BrdU positive cells in E18.5 embryonic intestine. 

G, H and I: β-catenin, GSK-3β Ser9-phosphorylation and total GSK-3β expression detected by western blot in E18.5 db/db and db/+ embryonic jejunum, β-actin was used as a loading control. Western blot data are expressed as mean ± SEM from at least three independent experiments; embryos n = 10 per group. Scale bar, 20 µm.

FIG. 5. Enhanced jejunal epithelial proliferation and GSK-3β/β-catenin signaling activation in high-fat diet (HFD) mice. 

A: Body weight of chow diet (CD) and HFD mice. 

B: Food intake in CD and HFD mice. 

C: H&E staining CD and HFD mice intestine. 

D: Quantification of the villi length. 

E: BrdU immunohistochemical staining of CD and HFD mice intestine. 

F: Quantification of BrdU positive cell. 

G: β-catenin, GSK-3β Ser9-phosphorylation, total GSK-3β and Cyclin D1 expression was detected by western blot in CD and HFD mice jejunum, β-actin was used as a loading control. 

H, I and J: Quantification of β-catenin, GSK-3β Ser9-phosphorylation and Cyclin D1 expression. Western blot data from at least three independent experiments and expressed as mean ± SEM; mice n = 6~8 per group. *p <0.05, **p <0.01. Scale bar, 50 µm.

FIG. 6. Increased degradation of β-catenin attenuated proliferation of intestinal epithelium in high-fat diet (HFD) fed mice. 

A: β-catenin, GSK-3β Ser9-phosphorylation, total GSK-3β and Cyclin D1 expression detected by western blot, β-actin was used as a loading control. 

B, C and D: Quantification of β-catenin, GSK-3β Ser9-phosphorylation and Cyclin D1 expression. 

E: H&E staining show longer villi length in HFD and HFD + DMSO mice, and shorter villi length in HFD + JW55 mice. 

F: Quantification of villi length. 

G: Cell proliferation identified by immunohistochemical staining with BrdU. Brown nucleus indicates BrdU positive cells. 

H: Quantification of
the BrdU-positive cells. Western blot data from at least three independent experiments and are expressed as mean ± SEM; mice n = 4–8 per group. *p <0.05, **p <0.01. Scale bar, 20 µm.

**FIG. 7.** High-concentration glucose (HG) and FFA activate GSK-3β/β-catenin signaling pathway in epithelial cell. A: Expression of β-catenin, GSK-3β Ser9-phosphorylation and Cyclin D1 was detected by western blot in CT26 cells after stimulation with glucose. B: Expression of β-catenin, GSK-3β Ser9-phosphorylation and Cyclin D1. C: Expression of β-catenin, GSK-3β Ser9-phosphorylation, total GSK-3β and Cyclin D1 expression was detected by western blot in CT26 cell after FFA stimulation. D: expression of β-catenin, GSK-3β Ser9-phosphorylation and Cyclin D1. Data are expressed as mean ± SEM; n = 3 independent experiments. *p <0.05, **p <0.01, vs. time 0.

**FIG. 8.** β-catenin inhibitor FH535 reduces expression of Cyclin D1, GSK-3 inhibitor increases Cyclin D1 expression. A, B and C: FH535 blocked β-catenin accumulation and expression of Cyclin D1 induced by HG. D: FH535 blocked intestinal epithelial cell proliferation induced by HG. E, F, G and H: CHIR98014 alone or combined with HG increases GSK-3β Ser9-phosphorylation, β-catenin accumulation and Cyclin D1 expression in CT26 cells. Data are expressed as mean ± SEM; n = 3 independent experiments. *p <0.05, **p <0.01.
FIG. 1.
FIG. 2.
FIG. 3.

A

![Graph](graph.png)

B

![Images](images.png)

C

![Graph](graph.png)

D

![Graph](graph.png)

E

![Images](images.png)

F

![Graph](graph.png)
FIG. 4.
FIG. 5.

**Bar Graphs and Images:***

**Panel A:**
- Graph showing body weight (g) over age (week) for CD and HFD groups.
- The graph indicates a significant difference (**) between the two groups.

**Panel B:**
- Graph showing food intake (g/day) over age (week) for CD and HFD groups.
- The graph shows a significant difference (**) between the two groups.

**Panel C:**
- Images of intestinal tissue stained with hematoxylin and eosin, comparing CD and HFD groups.

**Panel D:**
- Graph showing villus length (um) over age (week) for CD and HFD groups.
- The graph indicates a significant difference (**) between the two groups.

**Panel E:**
- Images of intestinal tissue showing BrdU (+) cell/crypt, comparing CD and HFD groups.

**Panel F:**
- Graph showing BrdU (+) cell/crypt count for CD and HFD groups.
- The graph shows a significant difference (**) between the two groups.

**Panel G:**
- Western blot images of β-catenin, p-GSK-3β, t-GSK-3β, Cyclin D1, and β-actin for CD and HFD groups.

**Panel H:**
- Band intensity of β-catenin in Western blot analysis for CD and HFD groups.
- The graph shows a significant difference (*).”

**Panel I:**
- Band intensity of p-GSK-3β in Western blot analysis for CD and HFD groups.
- The graph shows a significant difference (*).”

**Panel J:**
- Band intensity of Cyclin D1 in Western blot analysis for CD and HFD groups.
- The graph shows a significant difference (*).”

**Diabetes:**
- Description or analysis related to diabetes research.
FIG. 6.
FIG. 7.
FIG. 8.

A Glu (20mM) - + - +
FH535 (5 μM) - - + +
β-catenin
Cyclin D1
β-actin

B

\[ \text{β-catenin} \]

\[ \text{Cyclin D1} \]

\[ \text{β-actin} \]

C

D

E Glu (20mM) - + - +
CHIR (5 μM) - - + +
β-catenin
p-GSK-3β
t-GSK-3β
Cyclin D1
β-actin

F

G

H

Diabetes

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