

# Enhanced intestinal epithelial cell proliferation in diabetic rats correlates with $\beta$ -catenin accumulation

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## Abstract

The Wnt/ $\beta$ -catenin signaling cascade is implicated in the control of stem cell activity, cell proliferation, and cell survival of the gastrointestinal epithelium. Recent evidence indicates that the Wnt/ $\beta$ -catenin pathway is activated under diabetic conditions. The purpose of this study was to evaluate the role of Wnt/ $\beta$ -catenin signaling during diabetes-induced enteropathy in a rat model. Male rats were divided into three groups: control rats received injections of vehicle; diabetic rats received injections of one dose of streptozotocin (STZ); and diabetic–insulin rats received injections of STZ and were treated with insulin given subcutaneously at a dose of 1 U/kg twice daily. Rats were killed on day 7. Wnt/ $\beta$ -catenin-related genes and expression of proteins was determined using real-time PCR, western blotting, and immunohistochemistry. Among 13 genes identified by real-time PCR, seven genes were upregulated in diabetic rats compared with control animals including the target genes *c-Myc* and *Tcf4*. Diabetic rats also showed a significant increase in  $\beta$ -catenin protein compared with control animals. Treatment of diabetic rats attenuated the stimulating effect of diabetes on intestinal cell proliferation and Wnt/ $\beta$ -catenin signaling. In conclusion, enhanced intestinal epithelial cell proliferation in diabetic rats correlates with  $\beta$ -catenin accumulation.

## Key Words

- ▶ Wnt/ $\beta$ -catenin
- ▶ diabetes
- ▶ intestine
- ▶ mucosa
- ▶ cell proliferation
- ▶ stem cell

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## Introduction

Growing evidence indicates that Wnt signaling is implicated in regulation of adipogenesis, insulin resistance, pancreatic function, and inflammation (Almario & Karakas 2015). Wnt/ $\beta$ -catenin signaling includes  $\beta$ -catenin-dependent (canonical) and  $\beta$ -catenin-independent (non-canonical) pathways (Lu *et al.* 2004, Turashvili *et al.* 2006). The canonical Wnt signaling pathway regulates cell fate

and proliferation, and this signaling is initiated by the binding of Wnt ligands to Frizzled (FZD) family receptors and the LDL receptor-related proteins 5 and 6 (LRP5/LRP6) co-receptors. Under inactive condition,  $\beta$ -catenin is bound to Axin in adenomatous polyposis coli (APC) and interacts with glycogen synthase kinase 3 beta (GSK3 $\beta$ ) for phosphorylation at N-terminal residues, which then

leads to ubiquitin-mediated proteasomal degradation of  $\beta$ -catenin (Hwang *et al.* 2009). In contrast, when Wnt signaling is activated by various intercellular stimulators, Wnt ligands can activate FZD and LRP targeting APC and Axin, leading to dephosphorylation of GSK3 $\beta$  and can recruit the cytosolic protein Dishevelled (Dvl), which inhibits phosphorylation of  $\beta$ -catenin, thus causing  $\beta$ -catenin to accumulate. Subsequently,  $\beta$ -catenin translocates to the nucleus and activates T-cell factor (TCF) and lymphoid enhancer factor to regulate the expression of Wnt target genes (Schinner *et al.* 2009). Formation of a  $\beta$ -catenin/Tcf4 complex results in the direct upregulation of *c-Myc*, which in turn represses p21CIP1/WAF1 by direct promoter binding, thereby allowing cells to proliferate (van de Wetering *et al.* 2002). There are several secreted protein antagonists of Wnt signaling that have been previously identified, including secreted Frizzled-related proteins and Dickkopf (DKK) protein, which are thought to function as negative-feedback regulators of the Wnt/ $\beta$ -catenin pathway (Glinka *et al.* 1998).

Recently, emerging evidence has indicated that the Wnt/ $\beta$ -catenin pathway is activated under diabetic conditions. Zhou *et al.* (2012) have shown that the levels of  $\beta$ -catenin and Wnt proteins are upregulated in the kidney tissues of both types 1 and 2 diabetic animal models. Treatment with insulin in this study attenuated the activation of WNT signaling via lowering blood glucose levels. In recent clinical trials, enhanced proliferation, accompanied by increased aerobic glycolysis, was detected in colorectal epithelium of patients with diabetes and was correlated with  $\beta$ -catenin accumulation (Li *et al.* 2014).

The understanding of the mechanisms by which diabetes stimulates intestinal cell proliferation may have important clinical implications. The results of many trials have indicated that diabetic patients have an increased risk of malignant disease, including gastrointestinal malignancies. Hyperinsulinemia, alone or in combination with other features of the insulin resistance syndrome, represents a biologically plausible mechanism for increased colorectal cancer risk among persons with diabetes (Ahmadi *et al.* 2014). Hyperinsulinemia is present during the early stages of diabetes. The results of preclinical studies have indicated that insulin stimulates proliferation, decreases apoptosis and promotes tumorigenesis within the gastrointestinal tract (Limburg *et al.* 2006). Evaluation of the main mechanisms of intestinal stem cell (ISC) activity in diabetes emphasizes the need for further understanding of the biological mechanisms involved in diabetes-associated colorectal cancer risk.

We have shown recently that experimental diabetes results in a significant increase in epithelial cell proliferation and a concomitant increase in cells, indicating accelerated cell turnover within the gastrointestinal tract (Sukhotnik *et al.* 2011). The current study is an extension of our previous work and was designed to investigate the role of Wnt/ $\beta$ -catenin signaling in the development of diabetes-induced intestinal mucosal hyperplasia in a rat model.

## Materials and methods

### Animals

This experiment and animal care were conducted in compliance with the guidelines established by the 'Guide for the Care and Use of Laboratory Animals', Rappaport Faculty of Medicine, Technion (Haifa, Israel). Male Sprague–Dawley rats (250–300 g) were used in this study. Animals were housed in wire-bottomed cages and were acclimatized at 21 °C on a 12 h light:12 h darkness cycle for a minimum of 3 days before the experiment. The rats were allowed access to water and food *ad libitum*.

### Experimental design

The animals were divided randomly into three experimental groups of ten rats each. Group A, control rats (CONTR), received injections of vehicle; group B, diabetic rats (DIAB), received injections of one dose of streptozotocin (STZ); and group C, DIAB–INS rats, received injections of STZ and were treated with insulin given subcutaneously at a dose of 1 U/kg, twice daily as we described previously (Houghton *et al.* 1999, Sukhotnik *et al.* 2005).

On the morning of day 7, rats were anesthetized with *s.c.* pentobarbital (70 mg/kg) and were killed by open pneumothorax. Blood samples (1 ml) were obtained from the left ventricle. Plasma was prepared by centrifugation (750 g for 10 min at 4 °C) of blood samples. Plasma glucose was measured using the glucose oxidase method.

### Intestinal mucosal samples

The small intestine was removed, stripped of adherent fat and mesentery, washed with normal saline and dried. The removed bowel was divided into two segments: proximal jejunum (10 cm from the ligament of Treitz) and distal ileum (10 cm proximal to the ileocecal junction). Each segment was weighed, and mucosa were scraped off using a glass slide and weighed. The mucosal samples were stored at –80 °C for further gene and protein investigation.

### Crypt cell proliferation and apoptosis

Standard 5-bromodeoxyuridine (5-BrdU) labeling reagent (Zymed Laboratories, Inc., San Francisco, CA, USA) was injected intraperitoneally at a concentration of 1 ml/100 g body weight 2 h before killing. Crypt cell proliferation was assessed using 5-BrdU labeling with a kit (Zymed Laboratories, Inc.).

Immunohistochemistry for caspase 3 (caspase 3 cleaved concentrated polyclonal antibody; dilution 1:100; Biocare Medical, Walnut Creek, CA, USA) was performed for identification of apoptotic cells using a combination of streptavidin–biotin–peroxidase according to the manufacturer's instructions. All measurements were performed by a qualified pathologist blinded as to the source of the intestinal samples.

### Real-time PCR

RNA was isolated using TRIzol (Invitrogen) reagent according to the manufacturer's instructions, and quantification of RNA was performed using 260/280 nm spectrophotometry. The method was extended using reverse transcriptase (PrimeScript RT reagent Kit, TaKaRa, Shiga, Japan) to convert 500 ng of total RNA into cDNA, which was then amplified by PCR-Thermal Cycler (2720 Thermal Cycler, Applied biosystems, Israel). Gene Expression of *Wnt3*, *Wnt3a*, *Wnt5a*, *Wnt5b*, *Wnt7b*, *Wnt10a*, *Wnt11*, *Fzd2*, *Dkk1*, *Dvl2*, *c-Myc*, *Tcf4*, and *Mmp7* mRNA was determined by quantitative real-time PCR ABI-PRISM 7000 (Applied Biosystems) on cDNA samples using Cyber Green Master Mix (Takara) with the exception of template and primers. The results were normalized to the expression of the *GAPDH* reference gene.

### Western blotting

Tissue was homogenized in RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 2 mM EDTA, supplemented with a cocktail of protease (Roche Diagnostics), and phosphatase cocktail inhibitors (Sigma). Protein concentrations were determined using Bradford reagent according to the manufacturer's instructions. Samples containing equal amounts of total protein (30  $\mu$ g) were resolved by SDS–PAGE under reducing conditions. After electrophoresis, proteins were transferred to PVDF membrane and probed with anti- $\beta$ -catenin antibody (1:1000 dilution, sc-59737), anti-GLUT1 (1:500 dilution, Millipore (Darmstadt, Germany), 07-1401), anti-caspase 3 (1:1000 dilution, Oncogene Science (Cambridge,

MA, USA), AM-20), anti-c-Myc (dilution 1:1000, sc-40), anti-p-ERK (1:500 dilution, sc-7383), anti GSK3 $\beta$  (1:500 dilution, sc-8257), and anti- $\gamma$ -tubulin antibody (1:10 000 dilution, Sigma, T6557). HRP-conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) and then developed by ECL (Lumina Classico from Millipore). The optical density of the specific protein bands was quantified using a densitometer (Vilber Lourmat, Lyon, France).

### Immunohistochemistry

Immunohistochemistry for  $\beta$ -catenin ( $\beta$ -catenin cleaved polyclonal antibody, dilution 1:100, ab16667), Musashi 1 (dilution 1:40, Epitomics (Buringame, CA, USA), #1877-1), cyclin D1 (dilution 1:50, Epitomics, #1677-1), and c-Myc (dilution 1:50, BioVision (Milpitas, CA, USA), #3501-100) was performed for identification of positive cells using a combination of the streptavidin–biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues according to the manufacturer's protocols. The distribution of  $\beta$ -catenin was scored as follows: M (membranous) when the positivity was localized on the intercellular borders of enterocytes; H (heterogeneous) when the immunolabeling was localized on the intercellular borders of some cells mixed with negative cells; and C (cytoplasmic) when the immunolabeling was evenly distributed throughout the cytoplasm. The intensity of immunolabeling was scored (+) when the positivity was strong, ( $\pm$ ) when the positivity was weak, and (–) when it was absent. A qualified pathologist blinded as to the source of intestinal tissue performed all measurements.

### Statistical analysis

The data are expressed as the mean  $\pm$  S.E.M. A Kruskal–Wallis test followed by a *post hoc* test for multiple comparisons was used for statistical analysis. Prism Software was used (GraphPad Software, Inc., San Diego, CA, USA), and statistical significance was defined as  $P < 0.05$ .

## Results

### Diabetic model

Diabetes was induced by i.p. injection of STZ (75 mg/kg body weight; Sigma Chemical) dissolved in a citrate buffer (pH 4.5). Blood glucose levels vary between 400–550 in diabetic rats and 300–350 in diabetic rats treated with insulin. The animals had no Clinical signs of hypoglycemia.

**Table 1** Effect of diabetes and treatment with insulin on intestinal epithelial cell proliferation and apoptosis

Cell turnover	CONTR	DIAB	DIAB-INS
Cell proliferation (BrdU positive cells per ten crypts)			
Jejunum	135 $\pm$ 3	151 $\pm$ 5*	139 $\pm$ 2 <sup>†</sup>
Ileum	148 $\pm$ 3	165 $\pm$ 4*	139 $\pm$ 4 <sup>†</sup>
Cell apoptosis (apoptotic cells per ten villi)			
Jejunum	3.56 $\pm$ 0.37	2.23 $\pm$ 0.23*	2.16 $\pm$ 0.19
Ileum	3.03 $\pm$ 0.65	2.5 $\pm$ 0.24*	2.09 $\pm$ 0.5*

CONTR, control rats; DIAB, diabetic rats; INS, insulin. \* $P < 0.05$  DIAB and DIAB-INS versus CONTR and <sup>†</sup> $P < 0.05$  DIAB-INS versus DIAB.

### Cell proliferation and apoptosis

Diabetic rats (group B) demonstrated a significant increase in enterocyte proliferation rates in jejunum (12%,  $P = 0.004$ ) and ileum (10%,  $P = 0.001$ ) compared with control animals (group A) (Table 1). Treatment of diabetic rats with insulin (group C) induced a significant decrease in the proliferation index in jejunum (8%,  $P = 0.01$ ) and ileum (16%,  $P = 0.03$ ) compared with diabetic animals (group B).

Diabetic rats (group B) demonstrated a significant decrease in cell apoptosis in jejunum (37%,  $P = 0.004$ ) and ileum (17%, NS) compared with control animals (group A) (Table 1). Treatment of diabetic rats with insulin (group C) resulted in a trend toward an additional decrease in the apoptotic index in jejunum and ileum; however, this trend was not statistically significant.

### Wnt/ $\beta$ -catenin-signaling-related genes

The effect of diabetes and insulin on Wnt/ $\beta$ -catenin-signaling-related genes throughout the gastrointestinal

tract was determined using real-time PCR (Table 2). Thirteen Wnt/ $\beta$ -catenin-pathway-related genes were investigated. Diabetes (group B) resulted in a significant upregulation of *Wnt3a* (31%,  $P < 0.05$ ), *Wnt7b* (54%,  $P < 0.05$ ), *Wnt10a* (threefold increase,  $P < 0.05$ ),  $\beta$ -catenin (29%,  $P < 0.05$ ), and *Mmp7* (twofold increase,  $P < 0.05$ ) mRNA levels compared with control animals (group A). Wnt11 was detectable at low levels in diabetic (versus control) intestinal specimens. Wnt/ $\beta$ -catenin target genes were also upregulated in diabetic rats compared with control animals (*Tcf4* (38% increase,  $P < 0.05$ ) and *c-Myc* (38% increase,  $P < 0.05$ )). Diabetic rats demonstrated a trend toward an increase in *Wnt3* and *Dvl2* gene expression as well as a trend towards a decrease in *Wnt5a* and *Fzd2* gene expression; however, this trend was not statistically significant. Treatment with insulin resulted in a significant decrease in *Wnt3a* (twofold decrease,  $P < 0.05$ ), *Wnt7b* (32% decrease,  $P < 0.05$ ), *Wnt10a* (27% decrease,  $P < 0.05$ ), *Mmp7* (32% decrease,  $P < 0.05$ ), and *Tcf4* (22% decrease,  $P < 0.05$ ) as well as a trend toward a decrease in *Wnt3*, *Wnt5b*, *Dvl2*, and *c-Myc* mRNA levels; however, this trend did not achieve statistical significance.

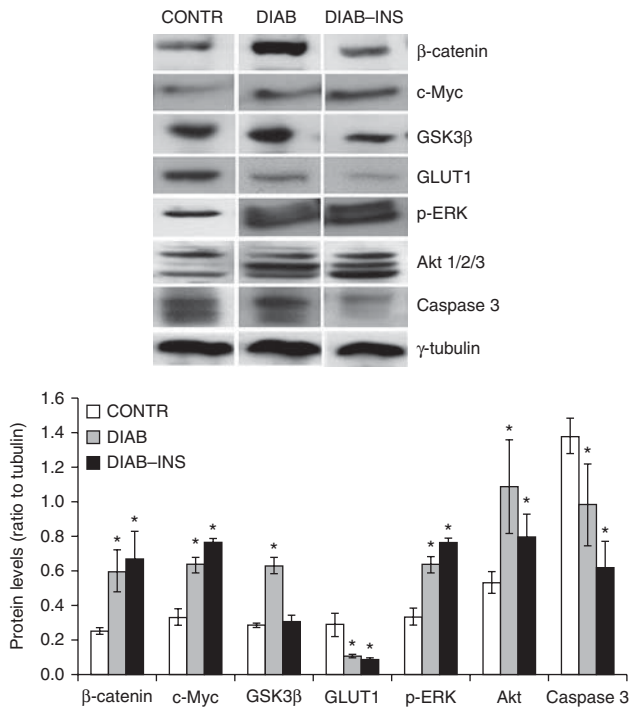
### Western blot

A significant upregulation of Wnt/ $\beta$ -catenin-signaling-related genes in diabetic animals (group B) was accompanied by a significant increase in  $\beta$ -catenin protein levels in jejunum and ileum compared with those of control animals (Fig. 1). Diabetic rats demonstrated also a significant increase in c-Myc protein levels (target, twofold increase), GSK3 $\beta$  (twofold increase), Akt (twofold increase), and p-ERK (twofold increase) compared with control

**Table 2** Effect of diabetes and insulin on Wnt/ $\beta$ -catenin related genes expression (real-time PCR)

Wnt/ $\beta$ -catenin signaling related genes	CONTR	DIAB	DIAB-INS
<i>Wnt3</i>	1.38 $\pm$ 0.17	1.50 $\pm$ 0.17	1.44 $\pm$ 0.22
<i>Wnt3a</i>	1.12 $\pm$ 0.24	1.47 $\pm$ 0.26* ( $\uparrow$ 31%)	0.63 $\pm$ 0.10* <sup>†</sup> (twofold $\downarrow$ )
<i>Wnt5a</i>	1.72 $\pm$ 0.17	1.58 $\pm$ 0.20	1.66 $\pm$ 0.11
<i>Wnt5b</i>	1.11 $\pm$ 0.17	1.12 $\pm$ 0.17	0.997 $\pm$ 0.12
<i>Wnt7b</i>	1.14 $\pm$ 0.24	1.76 $\pm$ 0.26* ( $\uparrow$ 54%)	1.20 $\pm$ 0.23 <sup>†</sup> ( $\downarrow$ 32%)
<i>Wnt10a</i>	0.28 $\pm$ 0.06	0.73 $\pm$ 0.20* (threefold $\uparrow$ )	0.53 $\pm$ 0.13* ( $\downarrow$ 27%)
<i>Wnt11</i>	1.45 $\pm$ 0.13	1.10 $\pm$ 0.15* ( $\downarrow$ 24%)	1.02 $\pm$ 0.09
<i>Fzd2</i>	1.45 $\pm$ 0.28	1.27 $\pm$ 0.20	1.28 $\pm$ 0.21
<i>Dvl2</i>	1.04 $\pm$ 0.12	1.17 $\pm$ 0.13	1.02 $\pm$ 0.13
$\beta$ -catenin	0.84 $\pm$ 0.08	1.08 $\pm$ 0.10* ( $\uparrow$ 29%)	0.95 $\pm$ 0.11
<i>Tcf4</i>	1.16 $\pm$ 0.25	1.60 $\pm$ 0.40* ( $\uparrow$ 38%)	1.24 $\pm$ 0.32 ( $\downarrow$ 22%)
<i>c-Myc</i>	1.35 $\pm$ 0.14	1.80 $\pm$ 0.29* ( $\uparrow$ 33%)	1.69 $\pm$ 0.15*
<i>Mmp7</i>	0.68 $\pm$ 0.11	1.25 $\pm$ 0.20* (twofold $\uparrow$ )	0.85 $\pm$ 0.16 <sup>†</sup> ( $\downarrow$ 32%)

CONTR, control rats; DIAB, diabetic rats; INS, insulin. \* $P < 0.05$  DIAB and DIAB-INS versus CONTR and <sup>†</sup> $P < 0.05$  DIAB-INS versus DIAB.

**Figure 1**

Relative expression of  $\beta$ -catenin, caspase 3, GSK3 $\beta$ , c-Myc, ERK, Akt, and GLUT1 protein, as determined by western blot analysis. A significant upregulation of Wnt/ $\beta$ -catenin-signaling-related genes in diabetic animals was accompanied by a significant increase in  $\beta$ -, c-Myc (target), GSK3 $\beta$ , Akt, and p-ERK protein levels compared with those for control animals. Decreased rates of cell apoptosis in diabetic rats correlated with a significant decrease in caspase 3 protein levels compared with those of control animals. Insulin-treated rats demonstrated a trend towards a decrease in GSK3 $\beta$  and Akt levels; however, this trend was not statistically significant. CONTR, control rats; DIAB, diabetic rats; INS, insulin. Values are mean  $\pm$  s.e.m. \* $P$  < 0.05 DIAB and DIAB-INS versus CONTR.

animals. Decreased rates of cell apoptosis in diabetic rats correlated with a significant decrease in caspase 3 protein levels compared with control animals. Diabetic rats also showed a significant decrease in GLUT1 protein levels in intestinal mucosa compared with those of control animals. Treatment of diabetic rats with insulin (group C) did not change  $\beta$ -catenin, caspase 3, and GLUT1 protein levels significantly compared with those of DIAB non-treated animals (group B). Insulin-treated rats demonstrated a trend towards a decrease in GSK3 $\beta$  and Akt levels; however, this trend was not statistically significant.

### Immunohistochemistry

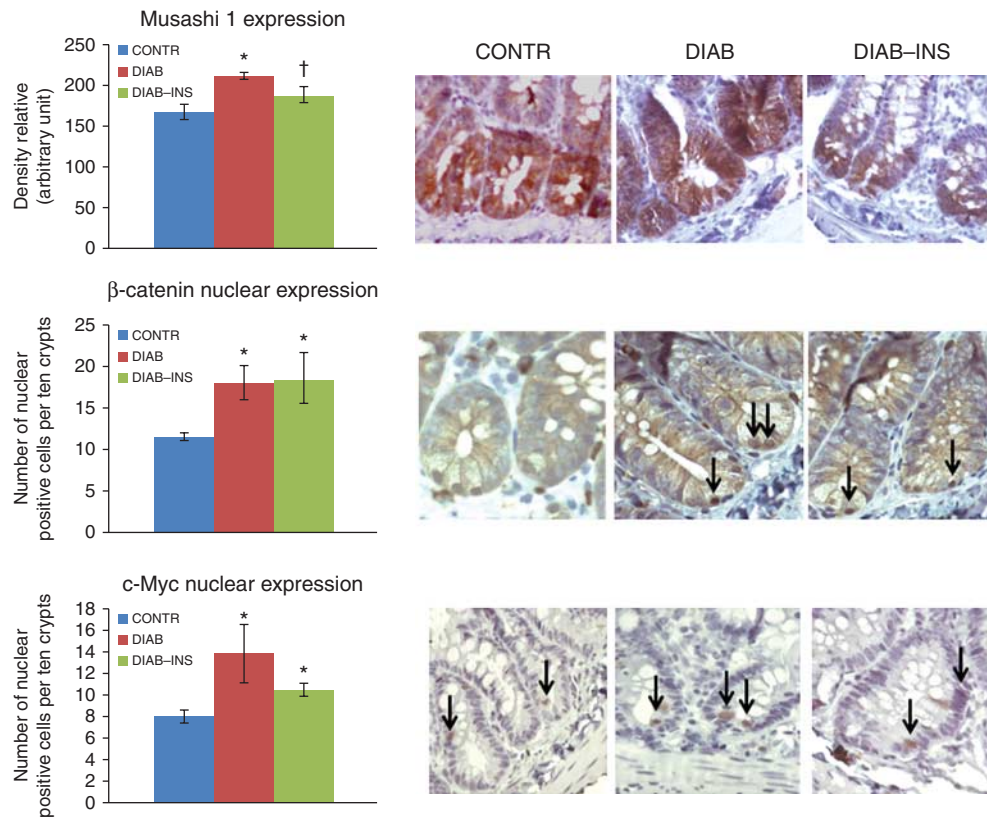
Diabetic rats demonstrated a significant increase in the intensity of Musashi 1 immunostaining compared with that for control animals, indicating elevated stem cell

activity (Fig. 2). Given the significant changes in crypt architecture that were observed, we next undertook immunohistochemical localization for total (phosphorylated and dephosphorylated forms)  $\beta$ -catenin to determine whether  $\beta$ -catenin was present and localized to proliferating epithelial intestinal cells. In control animals, the crypt cells were stained weakly for  $\beta$ -catenin in the cytoplasm and membranes. In diabetic animals, the signal in villus enterocytes was largely restricted to the plasma membrane, whereas in crypt cells  $\beta$ -catenin was detected strongly in the cytoplasm and nucleus, indicating higher activity compared with that in control animals. The total number of nuclear positive cells increased in diabetic rats compared with control animals. Diabetic rats also demonstrated an increased number of c-Myc-positive cells (nuclear staining) compared with controls. Treatment with insulin resulted in a significant decrease in the intensity of Musashi 1 immunostaining compared with sham-treated animals, indicating decreased stem cell activity, which was accompanied by a trend towards a decrease in the number of  $\beta$ -catenin- and c-Myc-positive cells (nuclear staining); however, this trend was not statistically significant.

### Discussion

The integrity of the gastrointestinal epithelia is regulated by the activity of ISCs. ISCs are fundamental cornerstones of intestinal biology, ensuring homeostatic self-renewal of intestinal mucosa and providing a reserve pool of cells that can be activated after tissue injury (ischemia-reperfusion or chemotherapy) or after bowel resection. ISCs are characterized by their ability to self-renew as well as to differentiate into specialized cells – properties critical for tissue maintenance and regeneration. Proliferation and differentiation of ISCs are regulated by multiple signaling pathways. Growing evidence indicates that Wnt/ $\beta$ -catenin signaling plays a pivotal role in homeostatic self-renewal of stem cells, including gastrointestinal mucosa (Lu *et al.* 2004, Turashvili *et al.* 2006). Two Wnt pathways have been identified: the canonical or Wnt/ $\beta$ -catenin pathway and a noncanonical pathway or  $\beta$ -catenin-independent pathway (Hwang *et al.* 2009). In the canonical pathway, Wnt binding leads to the stabilization of  $\beta$ -catenin, which enters the nucleus to regulate Wnt pathway-target genes.

The results of several experiments have revealed an activated Wnt/ $\beta$ -catenin pathway during diabetic conditions. Abiola and colleagues have shown that the activation of Wnt/ $\beta$ -catenin signaling in skeletal muscle cells improved insulin sensitivity by i) decreasing

**Figure 2**

Effect of diabetes and insulin on intestinal expression of  $\beta$ -catenin, Musashi 1, and c-Myc (immunohistochemistry). As expected, diabetic rats demonstrated a significant increase in the intensity of Musashi 1 immunostaining compared with that for control animals, indicating elevated stem cell activity. In addition, diabetic rats demonstrated higher  $\beta$ -catenin staining in cytoplasm and nucleus as well as an increased number of c-Myc-positive cells (nuclear staining (arrows)) compared with the results for control rats. Treatment with insulin resulted in a significant decrease in the intensity of

Musashi 1 immunostaining compared with that for sham-treated animals, indicating decreased stem cell activity, which was accompanied by a trend towards a decrease in the number of  $\beta$ -catenin- and c-Myc-positive cells (nuclear staining (arrows)); however, this trend was not statistically significant. CONTR, control rats; DIAB, diabetic rats; INS, insulin. Values are mean  $\pm$  S.E.M. \* $P < 0.05$  DIAB and DIAB-INS versus CONTR and † $P < 0.05$  DIAB-INS versus DIAB.

intramyocellular lipid deposition through downregulation of SREBP1c; ii) increasing insulin effects through a differential activation of the Akt/protein kinase B (PKB) and AMPK pathways; and iii) inhibiting the MAPK pathway. The authors suggest that a crosstalk between these pathways and Wnt/ $\beta$ -catenin signaling in skeletal muscle opens the exciting possibility that organ-selective modulation of Wnt signaling might become an attractive therapeutic target in regenerative medicine and for treating obese and diabetic populations (Abiola *et al.* 2014). Accumulating evidence has indicated that Wnt/ $\beta$ -catenin exhibits a pivotal function in the progression of diabetic nephropathy (Xiao *et al.* 2013). In a recent report, García-Jiménez *et al.* (2013) have suggested that the diabetes-cancer link relies on enhanced WNT/ $\beta$ -catenin signaling due to high glucose. Aberrant WNT signaling is

present in 40–90% of gastrointestinal cancers (White *et al.* 2012), which are the specific cancer sites more tightly associated with diabetes. There is now new and solid evidence that demonstrates that WNT stimulation alone is not enough to promote nuclear accumulation of  $\beta$ -catenin, but that this process requires and depends on high glucose to increase its nuclear retention in a variety of human tumor-derived cell lines related to cancers associated with hyperglycemia and diabetes (Chocarro-Calvo *et al.* 2013).

Since Wnt/ $\beta$ -catenin signaling has established roles in regulating intestinal cell metabolism and survival, the present study was designed to investigate whether Wnt/ $\beta$ -catenin signaling is required for an effective response to diabetes-induced intestinal damage. Herein, we have demonstrated that diabetes results in a significant upregulation of cell proliferation rates in jejunum and ileum

and a concomitant decrease in cell apoptosis compared with control animals. Both mechanisms may be responsible for the increase in the number of enterocytes; however, the role of elevated cell proliferation seems more significant. The elevated rates of cell proliferation in diabetic rats correlated with a significant increase in the intensity of Musashi 1 immunostaining, indicating elevated stem cell activity. This stimulating effect of diabetes on cell proliferation coincided with stimulated Wnt/ $\beta$ -catenin signaling. Among 13 investigated genes, seven genes were upregulated in diabetic rats compared with control animals (*Wnt3a* (31%), *Wnt7b* (31%), *Wnt10a* (threefold increase),  $\beta$ -catenin (29%), and *Mmp7* (twofold increase)). This observation is consistent with data reported by other investigators that *Wnt1*, *Wnt2*, *Wnt2B*, *Wnt3*, *Wnt3a*, *Wnt7b*, and *Wnt8a* have a preferential ability to signal through  $\beta$ -catenin (Horvay & Abud 2013). Conversely, we have shown that expression of *Wnt11* and *Wnt5a* is lower in diabetic rats compared to control animals. Similarly *Wnt4*, *Wnt5a*, and *Wnt11* have been reported by various researchers to signal by alternative intracellular signaling mechanisms, including  $\text{Ca}^{2+}$ -mediated pathways or the c-Jun N-terminal kinase cascade (Farin *et al.* 2012). In most systems tested, the various Wnts that signal through  $\beta$ -catenin appear to promote cell proliferation, whereas Wnt proteins that signal through alternative or noncanonical pathways seem to promote differentiation and oppose proliferation and cellular multipotency. Specifically, *Wnt5A* has been shown to promote differentiation in a variety of tissue types while opposing the proliferative effects of canonical Wnts, including *Wnt1* and *Wnt3A* (Liang *et al.* 2003). We further chose to examine the mRNA expression of genes, which are known transcriptional targets of  $\beta$ -catenin. In particular, we focused on *c-Myc* (He *et al.* 1998) and *Tcf4* (van de Wetering *et al.* 2002), both involved in induction of cell proliferation.  $\beta$ -catenin translocates to the nucleus and activates *Tcf4*. Formation of a  $\beta$ -catenin/*Tcf4* complex results in the direct upregulation of *c-Myc*, which in turn represses p21CIP1/WAF1 by direct promoter binding, thereby allowing cells to proliferate. We have demonstrated that both *c-Myc* and *Tcf4* mRNA are upregulated in diabetic rats compared with control animals. Increased  $\beta$ -catenin protein levels in our study as well as increased  $\beta$ -catenin nuclear staining (by immunohistochemistry) were consistent with increased cell proliferation and upregulated Wnt/ $\beta$ -catenin gene expression. The levels of *c-Myc* protein were also upregulated in diabetic rats compared with control animals, which was correlated with elevated *c-Myc* mRNA levels. Elevated  $\beta$ -catenin

protein levels in diabetic rats were accompanied by a significant increase in GSK3 $\beta$  protein levels. GSK3 is a serine (S)/threonine (T) kinase that is responsible for ubiquitin-mediated proteasomal degradation of  $\beta$ -catenin (Hwang *et al.* 2009). GSK3 $\beta$  is a critical central target in many cell-signaling pathways and also an important regulator of several transcription factors, which can affect the expression of numerous genes and cell viability; GSK3 $\beta$  is a component of the Wnt signaling pathway, which regulates the stability of  $\beta$ -catenin; and p-GSK3 $\beta$  is known to stabilize  $\beta$ -catenin. In the current study, we documented the upregulation of the MAPK and Akt signal complexes in diabetic intestine. MAPKs are S/T-specific protein kinases that regulate various cellular activities, such as gene expression, mitosis, cell proliferation, differentiation, and apoptosis (Pearson *et al.* 2001). One of the MAPK signaling pathways triggered by cytokines or growth factors is the ERK pathway. The other important pathway may be the recently discovered phosphatidylinositol 3 kinase (PI3K)/S/T kinase (Akt) signaling pathway. The S/T protein kinase Akt, also known as PKB or RAC-PK, was initially identified as one of the downstream targets of PI3K. Activated Akt plays a key role in mediating signals for cell growth, cell survival (anti-apoptotic), cell-cycle progression, differentiation, transcription, translation, and glucose metabolism (Kandel & Hay 1999). Activation of PI3K leads to phosphorylation of AKT and its downstream target, GSK3 $\alpha$  and GSK3 $\beta$  (Torres-Arzyus *et al.* 2004).

Diabetic mice also showed a significant decrease in GLUT1 protein levels in intestinal mucosa compared with the levels for control animals. These data are consistent with the results of previous studies indicating an interaction between the GLUT1 and Wnt/ $\beta$ -catenin pathways. The upregulation of glucose transporters in the cell membrane by insulin in order to increase glucose uptake from the bloodstream is partially mediated by activation of Wnt/ $\beta$ -catenin signaling, which can increase a cell's sensitivity to insulin. In particular, *Wnt10b* is a Wnt protein shown to increase this sensitivity in skeletal muscle cells (Abiola *et al.* 2014). Treatment of diabetic rats with insulin attenuated partially the stimulating effect of diabetes on cell proliferation. This inhibitory effect was accompanied by a significant downregulation of five Wnt/ $\beta$ -catenin related genes: *Wnt3a* (twofold decrease), *Wnt7b* (32%), *Wnt10a* (27%), *Mmp7* (32%), and *Tcf4* (22%) as well as by a trend towards a decrease in *Wnt3*, *Wnt5b*, *Dvl2*, and *c-Myc* mRNA levels; however, this trend did not achieve statistical significance. Decreased  $\beta$ -catenin protein levels in DIAB-INS rats as well as decreased

$\beta$ -catenin nuclear staining (by immunohistochemistry) are in agreement with decreased cell proliferation and downregulated Wnt/ $\beta$ -catenin gene expression. Insulin-treated rats also demonstrated a trend toward a decrease in GSK3 $\beta$  and Akt levels. Under most conditions involving different injuries, Akt-dependent phosphorylation of GSK3 $\beta$  is known to inhibit its pro-death functions (Cohen & Downey 2011).

Together, these data provide the first *in vivo* evidence that  $\beta$ -catenin is a key component of an effective intestinal tissue-specific response to diabetes.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

T D, Y P, and R S designed the study, performed the experiment, collected data and assisted in generating the figures and drafting the manuscript. J B provided the microscope images and revised the pathological content of the manuscript. A G C designed the study and revised the manuscript for intellectual content. I S designed the study, performed the experiment, collected data, and drafted the manuscript.

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