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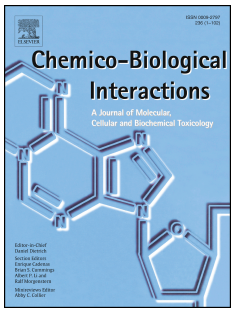

Diethylnitrosamine enhances hepatic tumorigenic pathways in mice fed with high fat diet (Hfd)

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DIETHYLNITROSAMINE ENHANCES HEPATIC TUMORIGENIC PATHWAYS IN MICE FED WITH HIGH FAT DIET (HFD).

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Running Title: HFD increases the sensitivity to DEN-induced HCC in mice

Key Words: High fat diet (HFD); Type 2 Diabetes Mellitus (T2DM); Hepatocarcinogenesis (HCC); Diethylnitrosamine (DEN); Wnt/β-catenin; TGF-β1/Smads

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ABSTRACT

Obesity has been implicated in the genesis of metabolic syndromes including insulin resistance and Type 2 Diabetes Mellitus (T2DM). Given the association between T2DM and the risk of hepatocellular carcinoma (HCC), our specific goal was to determine whether the liver of HFD-induced T2DM mice is more sensitive to the carcinogen diethylnitrosamine (DEN), due to a modification of the molecular pathways implicated in the early stages of HCC pathogenesis. C57BL/6 male mice (five-week-old) were divided into 4 groups: C, C+DEN, HFD and HFD+DEN. Mice were euthanized twenty-five weeks after DEN-injection. Livers of HDF-fed mice showed a higher proliferative index than Control groups. In line with this, HFD groups showed an increase of nuclear β-catenin, and interestingly, DEN treatment led to a slight increase in the expression of this protein in HFD group. Based on these results, and to confirm this effect, we analyzed β-catenin target genes, finding that DEN treatment in HFD group led to a significant increase of *Vegf, c-myc, c-jun* and *cyclin D1* expression levels. According to our results, the expression of TCF4 showed to be significantly increased in HFD+DEN vs. HFD. In this regard, the β-catenin/TCF4 complex enhanced its association with pSmads 2/3, as we observed an increase of nuclear Smads expression in HFD+DEN, suggesting a possible role of TGF-β1/Smads signaling pathway in this phenomenon. Our results show that the liver of HFD fed model that resembles early T2DM pathology in mice, is more sensitive to DEN, by inducing both Wnt/β-catenin and TGF-β1/Smads tumorigenic pathways.

1. INTRODUCTION

Obesity is a major cause of insulin resistance and could be aggravated by metabolic dysregulation, including hypertension and dyslipidemia (it is collectively known as metabolic syndrome) which is a precursor of Type 2 Diabetes *mellitus* (T2DM). T2DM represents a significant global health problem. The incidence of disease increases with age, obesity, physical inactivity, unhealthy diet, and ethnicity (Hispanics, Africans, and Aboriginals) and the rates are increasing among children [1, 2]. In the last years, several studies have demonstrated that chronic and low-grade inflammation is closely involved in the development of T2DM. Experimental models of high-fat diet (HFD) feeding in mice elevates circulatory fatty acids and influences glucose and fat metabolism, inducing the genesis of metabolic syndromes including insulin resistance and T2DM [3].

Extensive epidemiological studies have revealed that the diabetic population is at higher risk of developing cancers of the liver, pancreas, endometrium, colon and others. Such increased risk may be attributed to diabetes treatment, hormonal disorders, chronic inflammation and metabolic alterations underlying the diseases. Given the increase in the prevalence of overweight, obesity and T2DM, the incidence of metabolic disease-related hepatocellular carcinoma (HCC) is expected to rise, further increasing the burden of liver diseases in years to come. In this connection, HCC is the most common type of liver cancer and among the leading causes of cancer-related death in humans [4, 5]. Rodent models of HCC have been proven useful in revealing aspects of its multistep pathogenesis and preclinical testing of anti-HCC treatments. Mice have been shown to be particularly useful in this case and a wide variety of genetically engineered, xenograft and chemically induced models are available for HCC research. Among them, the experimental model that utilizes diethylnitrosamine (DEN) for HCC initiation is widely used and is well-characterized. This model recapitulates aspects of liver injury, fibrosis and hepatitis, the basis of human HCC [6, 7]. For this reason, and because it is comparable to its human

counterpart in terms of cancer-associated gene expression patterns and carcinogenic pathways, it is considered among the best-fit experimental models of HCC [8].

Common molecular mechanisms of HCC pathogenesis involve alterations in Wnt/β-catenin pathways. Wnt genes encode a large family of secreted glycoproteins that act as extracellular cell signaling molecules. Their binding to the transmembrane Frizzled (FZD) receptors activates the Wnt/β-catenin pathway that eventually results in cytoplasmic accumulation and nuclear translocation of the β-catenin protein (a well-known tumor marker) [9]. Even more, intranuclear β-catenin binding to T-cell factor 4 (TCF4) consequently upregulates the expression of many different cancer-related genes, including *c-myc*, *c-jun* and *cyclin D1* [10], and also, genes involved in the regulation of angiogenic factors, such as vascular endothelial growth factor (*Vegf*) [11]. When nuclear β-catenin and TCF4 form an active complex, the activated receptor of Smad 2 synergistically enhances the transcriptional activity of Wnt/β-catenin targets genes [12–14]. Smad 2 is phosphorylated when TGF-β1 activates its type 1 receptor, which in turns phosphorylates Smad 2 and Smad 3 proteins. These activated proteins, then associated with Smad 4, translocate to the nucleus where they regulate transcription, by associating to nuclear transcription factors and/or by binding directly to DNA [15]. Edlund *et al.* (2005), suggested that the specific effects of β-catenin on different promoters are modulated by alterations in the nuclear amounts of the β-catenin/TCF4 complex, as well as of Smad 2/3 and Smad 4 [16].

Wang Y. *et al.* (2009) demonstrated that the hepatic carcinogenesis induced by a non-necrogenic dose of DEN was enhanced in a dietary model of nonalcoholic steatohepatitis in Sprague-Dawley rats [17]; however, reports about the molecular pathways involved in this phenomena are not available. In this regard, we aimed at deepening the study of the involvement of Wnt/-catenin and TGF-1/Smads molecular pathways in these processes. In line with this, and to our knowledge, there are very few studies that analyze the early alterations in the initiation of hepatocarcinogenesis induced by DEN associated to chronic inflammation and to the metabolic alterations

underlying diabetes in mice [18, 19]. Thus, our specific goal was to determine whether the liver of HFD-induced T2DM mice is more sensitive to DEN-induced alterations in Wnt/β-catenin and/or TGF-β1/Smads molecular pathways that are implicated in the early stages of HCC pathogenesis.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

Five-week-old C57BL/6 male mice were provided by the School of Medicine, National University of Rosario, and were maintained at the animal facilities of the School of Biochemistry of National University of Rosario. Animals received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (National Research Council, Washington D.C.: National Academy Press, 1996). All the experimental protocols were performed according to the Regulation for the Care and Use of Laboratory Animals (Expedient 6109/012 E.C. Resolution 267/02) and approved by the Institutional Committee for Animal Use of the National University of Rosario, Argentina.

Mice were randomly divided into 4 experimental groups (n=5 per group), housed in plastic cages, kept on hardwood bedding, in animal facilities with a 12-hour light/dark cycle, controlled temperature (23 ± 2 °C) and ventilation. Water and diets were provided *ad libitum* throughout the experiment. Mice of two groups were fed with regular chow diet (C) (GEPSA, http://www.gepsa.com) or with a 40% HFD, *ad libitum* for 41 weeks [20]. The C and HFD (HFD-induced T2DM) groups were injected i.p. with saline solution DEN-vehicle at week 21, or injected i.p. with a single dose of DEN (75 mg/kg body weight) leading to C+DEN and HFD+DEN groups [19]. During the whole treatment, body weight and food intake were recorded every ten days. Mice were euthanized twenty-five weeks after saline solution or DEN injection with a mixture of ketamine (100 mg/kg bw) and xylazine (10 mg/ kg bw). After O/N fasting, blood was

obtained by cardiac puncture and glucose levels were spectrophotometrically determined (Wiener Lab, Rosario, Argentina) in plasma samples. Livers were promptly removed, frozen in liquid nitrogen and stored at -70 °C until analytical assays were performed. Samples of liver tissue were fixed in 4% buffered formalin for immunohistochemistry studies [20]. A scheme of the experimental protocol is shown in Figure 1.

2.2 Determination of proliferative index (PI)

To assess alterations in the proliferation activity among the experimental groups, liver slides were examined by immunohistochemical staining with anti-Proliferating Cell Nuclear Antigen (PCNA) antibody [21]. The PCNA proliferative index is defined as the number of proliferative cells (in G1, S, G2, and M phases) per 100 hepatocytes counted in ten high-power fields [19].

PCNA, as a naturally occurring cell marker of proliferating cells, offers an alternative method for investigating cell proliferation. Molecular studies indicate that the synthesis of PCNA is initiated in the nucleus in late G1 phase and continues during the S phase. The different staining patterns recognized in this study are believed to reflect individual phases of the cell cycle. Cells expressing no staining in the nucleus or cytoplasm are expected to be quiescent Go phase cells. Minimal nuclear staining would be consistent with G1 phase cells. Deep red nuclear staining is characteristic of S phase. G2 cells present speckled nuclear and cytoplasmic staining. In mitosis (M) the nucleoplasm and cytoplasm coalesce with the loss of nuclear boundaries. This could account for the diffuse speckled cytoplasmic staining specifically observed in all actively mitotic cells [22].

2.3 Immunoblot assay

Tissue samples were homogenized in 300 mM sucrose with protease inhibitors. Cytosolic and nuclear extracts were prepared as described previously [19]. Protein

concentration was determined by the Lowry method [23]. Equal amounts of protein were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted (Bio-Rad, Hercules, CA, USA) onto polyvinyl difluoride (PVDF) membranes (Perkin Elmer Life Sciences, Inc., Boston, MA). Membranes were blocked with PBS-Tween-10% non-fat milk, washed, and incubated overnight at 4°C with primary antibodies (Cyclin D1 (H-295): sc-753, Cyclin E1 (E-4): sc-25303, TCF-4 (H-125): sc-13027, PCNA (PC10): sc-56, pSmad2/3 (Ser423/425): sc-11769, p-b-catenin (BC-22): sc-57535, Santa Cruz Biotechnology (Santa Cruz, CA, USA), Smad4 #9515, GAPDH (D4C6R) #97166 Cell Signaling Technology (Boston, MA, USA), β-catenin BD: 610154, GSK-3β pY216 BD: 612312, GSK-3β BD: 610201 BD Biosciences (San Jose, CA, USA). Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Rockford, IL). The immunoreactive bands were quantified by densitometry using the Image J software (imagej.nih.gov). Equal loading and protein transference were checked: cytosolic fraction by GAPDH, and nuclear fraction by *Ponceau S* staining of the membranes. According to the literature, several nuclear loading controls are modified in diabetes or HCC; then, *Ponceau S* staining is a reliable loading control in nucleus of HFD or DEN-treated liver tissue [24, 25].

2.4 RNA isolation, cDNA synthesis and real time quantitative polymerase chain reaction

Total liver RNA was isolated by the TriZOL method (Life Technologies, Inc., Gaithersburg, MD, USA) according to manufacturer's instructions. One microgram of total liver RNA was treated with DNase I (Thermo Fisher Scientific) and cDNA was synthesized using an oligo-dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) assay was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, U.S.A.) with SYBR Green I

(Invitrogen). The PCR conditions were: 10 min at 95 °C, followed by 40 cycles of a two-step PCR denaturation at 95 C for 15 s and annealing/extension at 60 °C for 60 s. For each sample we analyzed *β-actin* expression to normalize target gene expression. Primers were designed using Primer3 software [26], and were then manufactured by Invitrogen. The list of genes with their primer sequences is given in Table 1. Relative changes in gene expression were determined by the $2^{-\Delta\Delta Ct}$ method [27].

Table 1.

Gene	***Primer sequence***
Vegf	F: 5'- AACGATGAAGCCCTGGAGTG -3' R: 5'- GCTGGCTTTGGTGAGGTTTG -3'
c-myc	F: 5'-CCTAGTGCTGCATGAGGAGA-3' R: 5'-TCCACAGACACCACATCAATTT-3'
c-jun	F: 5'-CCAGAAGATGGTGTGGTGTTT-3' R: 5'-CTGACCCTCTCCCCTTGC-3'
Ccnd1	F: 5'-CCGTCCATGCGGAAGATC-3' R: 5'- GAAGACCTCCTCCTCGCACT-3'
β-actin	F: 5'-CTTCCTCCCTGGAGAAGAGC-3' R: 5'-AAGGAAGGCTGGAAAAGAGC-3'

Gene Identity and sequence of primers used in Real-Time Quantitative Polymerase Chain Reaction. F, forward primer; R, reverse primer

2.5 In vitro studies. Isolation and culture of mouse hepatocytes

In another set of experiments, hepatocytes were isolated from non-fasting male mice from the 4 experimental groups by collagenase perfusion [20]. Cells were cultivated in 6-wells plates and cultured in DMEM/F12 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS for 4 h. Cells were serum-starved for 2 h and further stimulated with TGF-β1 (5ng/ml) for 16 h. Tissue culture plates were from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Tissue culture media were from Gibco (InvitrogenTM, Grand Island, NY, USA).

2.6 Data analysis

Data are expressed as means ± SEM. Statistical significance was determined by Student's t-test or the one-way analysis of variance followed by Tukey's test. To assess a significant relationship between two variables, Pearson's correlation was calculated. Analysis was performed by using the statistical software GraphPad Prism 5. A $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Determination of DEN effect on weight gain curves and blood glucose levels

Heydemann *et al.* (2016) reviewed evidence that support that high fat diet (HFD)-induced diabetes with obesity has proved to be the most popular experimental model in rodents, being closely correlated to the known pathology of T2DM [28]. In line with this, we have worked with a HFD-feeding model that resembles early pathogenesis of T2DM in mice. In this regard, our group developed and validated a model of HFD-induced obesity and insulin resistance in mice that resembles hallmarks of the disease assessed by glucose tolerance test (GTT) and insulin tolerance test (ITT) [20]. In addition, biochemical assays as glycaemia, plasma insulin, cholesterol and triglycerides levels were measured in order to characterize the our model of insulin resistance and have been recently published by our group [20]. Aiming at corroborating that DEN treatment does not modify the establishment of HFD-induced obesity and insulin resistance in our model, we analyzed weight gain curves and blood glucose levels. Weight gain curves were performed by periodically determining animals weight for 46 weeks (Figure 2A). As we previously described, HFD groups showed an increase in body mass when compared to C [20]. Likewise, DEN treatment did not modify the effect of HFD on weight gain. Besides, plasma levels of glucose in O/N-

fasted HFD mice at 46 weeks showed to be higher than those of C group, and DEN treatment did not modify the effect of HFD (Figure 2B).

3.2 Analyses of proliferative status

Figure 3A shows representative images for immunohistochemical detection of PCNA- positive cells in liver slices of the 4 experimental groups. As it is depicted in Figure 3B, livers showed the quiescent status of normal adult liver [19] while DEN treatment led to a significant increase in cell proliferation. When we analyzed the differences between HFD+DEN and HFD, we observed an increase in the PI associated to DEN treatment (+99 % vs. HFD), whilst between C and C+DEN such increase was of 21%. This suggests that there is a higher sensitivity to DEN treatment in HFD-fed mice livers. Additionally, we determined the percentages of hepatocytes in each phase of the cell cycle (Figure 3C). Significantly higher percentages of cells in G1 phase were observed in HFD+DEN group when compared to HFD (+54%). In line with this, a similar pattern of increase in the number of cells in S phase was found in HFD+DEN (+23% vs. HFD), suggesting a higher rate of entrance into the cell cycle. Accordingly, HFD+DEN group showed an increase in the percentage of cells in M phase. Even there is a significant increase in the percentage of cells in all phases of the cell cycle in HFD livers when compared to C that correlates with an increased PI, we underline the fact that there is a major sensitivity of HFD-fed mice livers to DEN.

3.3 Expression of cell cycle regulatory proteins

Cyclin D1 and cyclin E1 nuclear expression are depicted in Figure 4A and B, respectively. These proteins are hallmarks of the G1 to S phase progression in the cell cycle. We found an increase of cyclin D1 nuclear localization in HFD group when compared to C groups. Also, the nuclear localization of cyclin E1 in HFD showed a similar pattern. Anyway, we did not find differences between HFD and HFD DEN-treated group. These results suggest that HFD-fed condition is sufficient to promote

nuclear accumulation of two important cyclins that regulate the cell cycle.

3.4 Evaluation Wnt/β-catenin pathway

Nuclear localization of β-catenin is a key feature of Wnt/β-catenin signaling dysregulation, and it is a well-known tumor marker [29]; for this reason we analyzed the expression of β-catenin in nuclear extracts. As shown in Figure 5A, levels of nuclear β-catenin are significantly increased in HFD groups, highlighting the role of HFD in promoting β-catenin nuclear accumulation. On the other hand, we found a slight increase in the nuclear localization of this protein induced by DEN-treatment in HFD group.

Additionally, we determined total expression of β-catenin in liver extracts, finding a significant increase only in HFD+DEN group (Figure 5B). These results allowed us to hypothesize that in the HFD group there could be an increase in β-catenin phosphorylation, since its phosphorylation in Ser^{37}, Thr^{41} and Ser^{33} turns this protein recognizable for ubiquitination and subsequent microsomal degradation [30]. As shown in Figure 5C, we observed that phosphorylated β-catenin levels are significantly higher in HFD+DEN when compared to HFD.

Based on these results, we wanted to determine if there could be an alteration in the expression of the enzyme involved in the phosphorylation of β-catenin in HFD, so we evaluated the cytosolic expression of total glycogen synthase kinase 3β (total GSK-3β) and the inactive form GSK-3β pY216, in the 4 experimental groups. Figure 5D shows a significantly increase of total GSK-3β levels in both HFD groups. According to these results, we observed a diminution of GSK-3β pY2016 in HFD and HFD+DEN (Figure 5E). Given all the results obtained so far, we suggest that phosphorylated β-catenin is degraded in HFD groups, thus explaining the lack of increase observed in cytosol. Besides, in HFD+DEN the accumulation of phosphorylated β-catenin in cytosol, could be due, at least in part, to an alteration in the degradation process [31,

32].

Based on the slight increase of nuclear β-catenin localization observed in HFD+DEN when compared to HFD, we set out to analyze several β-catenin target genes in these groups in order to confirm a likely functional effect. We analyzed the main target genes of β-catenin: *Vegf, c-myc, c-jun* and *cyclin D1* by quantitative PCR [10, 11]. As shown in Figure 6A we found a significant increase in mRNA of each of the genes analyzed in the HFD+DEN group compared to HFD, suggesting that the slight increase observed in β-catenin nuclear accumulation is enough to induce an enhanced response of the target genes in DEN-treated HFD mice. Taking into account these data, and knowing that transcription of target genes is activated when non-phosphorylated active β-catenin is translocate into the nucleus to form a complex with T-cell factor 4 (TCF4) [33], we evaluated the expression of TCF4 in nuclear fraction. Indeed, we found that after HFD feeding, there exists a positive correlation between β-catenin and TCF4 nuclear expression (Pearson r=0.9764, p=0.0236). Moreover, and it is depicted in Figure 6B, there is a significant increase in the nuclear levels of TCF4 in HFD+DEN vs. HFD groups, in agreement with the increase observed in the levels of the evaluated target genes. These results demonstrate that HFD induced a greater sensitivity to the treatment with the carcinogen DEN, as can be seen by the higher increase in TCF4 expression found in HFD+DEN group.

3.5 Evaluation TGF-β1/Smads pathway

Several studies have demonstrated a functional interaction between the canonical Wnt/β-catenin and TGF-β1 signaling pathways [34]. In order to assess whether the increase observed in nuclear β-catenin protein level affects the contents of Smads proteins, we evaluated TGF-β1/Smads signaling in our model. The hallmark of TGF-β1 signaling activation is the increment of phosphorylated Smad 2/3 and their association with Smad 4 [15]. The amounts of phosphorylated Smad 2/3, were

significantly higher in nuclear extracts of HFD groups, and showed a positive correlation with β-catenin nuclear expression (Pearson r=0.9808, p=0.0192). It should be noted that the increase is significantly greater in HFD+DEN, evidencing an additive effect of DEN to the HFD condition (Figure 7A). As can be seen in Figure 7B, levels of Smad 4 only showed a tendency to increase in the HFD group, whereas it increased significantly in HFD+DEN when compared to control groups. These data indicate that Smad 2/3 and Smad 4 levels are increased in HFD and HFD+DEN groups, in accordance with the observed rise of both β-catenin and TCF-4 in nuclear fraction, suggesting a potential synergism between these two signaling pathways in our model.

3.6 In vitro studies: incubation with TGF-β1 cytokine

Taking into account the precedent data, we performed a new set of experiments in which isolated and cultured hepatocytes from non-fasting mice of the 4 experimental groups were stimulated with TGF-β1.

As shown in Figure 8A, TGF-β1 induced a significant increase of pSmad2/3 levels in hepatocytes from both HFD (+29%) and HFD+DEN (+39%) when compared to its corresponding controls. In addition, we analyzed the expression of nuclear β-catenin, and we observed that treatment with TGF-β1 led to higher levels of β-catenin in hepatocytes isolated from HFD groups, and that this increase was greater in HFD+DEN group (Figure 8B). These results could explain our observations in the *in vivo* studies, suggesting that pSmad 2/3 and β-catenin pathways modification could be driven by TGF-β1. In accordance with other studies [14, 35], we observed an interaction between the β-catenin and TGF-β1 pathways.

4. DISCUSSION

Obesity, insulin resistance and its associated complications such as metabolic syndrome, dyslipidemia, hypertension and T2DM, are increasing dramatically and have become an important global health issue. T2DM results in a status of relative insulin deficiency leading to hyperglycemia, and presents a skeletal muscle defect in insulin action and accounts for the overhelming majority of cases of insulin resistance reported for the human condition [36]. Diabetes-related factors including steatosis, nonalcoholic fatty liver disease (NAFLD), and cirrhosis, may also enhance susceptibility to liver cancer (HCC) [37]. Moreover, there is now clear evidence that T2DM and HCC are closely linked, owing to their association with obesity, impaired insulin sensitivity and NAFLD [38]. Very few studies address the early alterations in the initiation of hepatocarcinogenesis with the background of diseases such as metabolic syndrome and T2DM. In the present study we used mice with a C57BL/6 genetic background that are reported to be more susceptible to obesity and diabetes when subjected to a HFD [39, 40]. This model has been exhaustively analyzed, and the authors suggested that a straightforward HFD may be the optimal diet to model early T2DM hallmarks in mice. Likewise, among the variety of basic approaches used to uncover the pathogenesis of complications in T2DM, high fat diet (HFD)-induced diabetes with obesity has proved to be the most popular experimental model in rodents, being closely correlated to the known pathology of T2DM [28, 41–43]. In this sense we have work with a HFD fed mice that it is a model early T2DM.

HFD-treated mice showed an increase in body mass and higher levels of plasma glucose, when compared to Control. On the other hand, we used the genotoxic agent DEN to induce HCC. As we were interested in analyzing the very early events of the initiation of HCC, we performed a single injection of DEN and carried out our studies 25 weeks later [18, 19]. Treatment with DEN did not modify the effect of HFD on the studied markers of the experimental model.

The relationship between factors involved in cell cycle regulation and cancer has been extensively reported. In this regard, Masaki *et al.* (2000), described the roles of

cell cycle-related proteins in spontaneous HCC in Long-Evans Cinnamon rats, and suggested that cyclin D1 and cyclin E1 are involved in the transition from normal liver to HCC [44]. The cyclin D1 proto-oncogene is an important regulator of G1 to S-phase transition in numerous cell types from diverse tissues. During cell cycle progression, protein levels of cyclin D1 begin to rise early in G1, prior to its rapid nuclear export and degradation within the cytoplasm. Interestingly, the nuclear export and/or degradation of cyclin D1 is required for S-phase progression as failure to remove the cyclin results in G1 arrest [45]. Therefore, this protein is considered the “rate-limiting” step in hepatocyte proliferation, suggesting that administration of DEN leads to up-regulation of the cell cycle and the expression level of cyclin D1, the inhibition of apoptosis and consequently, it may lead to HCC [18]. In this study, we observed that HFD represents a risk factor that increase the sensitivity to DEN-induced hepatic cell cycle alteration indicated by an increased PI, which is essential, in the early stages, for malignant transformation. On the other hand, our results demonstrate that in livers of HFD mice there are higher nuclear levels of proteins involved in cell cycle regulation as cyclin D1 and cyclin E1. The strong effect of HFD-induced T2DM in the promotion of both cyclins activation could explain the lack of an increase after DEN treatment.

Recent evidence suggests that deregulation of the Wnt/β-catenin signaling pathway contributes to HCC development and growth. Moreover, β-catenin is a central player in Wnt signaling pathway and plays a key role in the genesis and development of tumors. When β-catenin is phosphorylated by GSK-3β, which is in turn activated by dephosphorylation of GSK-3β pY216, and ubiquitin-dependent degradation is inhibited, β-catenin concentrates in the cytoplasm and it forms a complex with the transcription factor TCF, which is subsequently transported into cell nuclei. This transcription complex activates the expression of downstream target genes, resulting in abnormal cell proliferation and cell carcinogenesis [46, 47]. We analyzed the expression of both GSK-3β and GSK-3β pY216, and we observed a similar over-expression of GSK-3β in

HFD and HFD+DEN groups. Besides, we observed a decrease of GSK-3β pY216 in both HFD groups. Then, we assessed the expression of phosphorylated β-catenin in cytosol fraction, and we found higher levels in HFD+DEN group. As phosphorylated β-catenin undergoes ubiquitination and degradation, its increase in cytoplasm in HFD+DEN group could be due to a functional inactivation of the destruction complex as it was described in other experimental models [32]. Further analyses would be necessary to confirm this presumption.

It is known that Wnt/β-catenin signal cascade is an important player in liver development and growth when β-catenin accumulates and translocate to the nucleus to activate target genes. In this connection, we assessed whether β-catenin pathway is involved in the sensitization of the liver of HFD-treated mice to DEN in the early stages of HCC development. In this regard, we observed a significant increase of nuclear β-catenin in HFD groups and, interestingly, a slight increase in HFD+DEN, when compared to HFD. Aberrant activation of Wnt/β-catenin pathway gives rise to the accumulation of β-catenin in the nucleus and promotes the transcription of many oncogenes [30] . In this sense, an increase of nuclear β-catenin expression in HFD reveals a sensitization of the liver derived from the dietary model that would predispose it to the development of HCC. Interestingly, we found that nuclear β-catenin expression was slightly higher in HDF-DEN group. In the nucleus, β-catenin forms a complex with a member of the TCF/LEF family of DNA binding proteins, TCF4, an effector of the Wnt pathway that promote the transcription of target genes such as *jun*, *c-myc* and *cyclinD-1* most of which encode oncoproteins [29]. Herein, the expression of nuclear TCF4 was found increased after HFD feeding and showed a positive correlation with β-catenin nuclear expression. Moreover, the increment found was greater in HFD+DEN group. Based on these results, we analyzed β-catenin target genes *Vegf, c-myc, c-jun* and *cyclinD1* in the HFD and HFD+DEN groups, observing a significant increase of the expression levels of all these genes in HFD+DEN, when compared to HFD. Ours

results demonstrate that HFD+DEN increased TCF4/β-catenin complex capability and transcriptional activity, associated with an upregulation of the endogenous TCF4/β-catenin target genes, suggesting an enhanced sensitivity of HFD fed mice to DEN action.

It is known that both Wnt/β-catenin and TGF-β1/Smads pathways coordinately regulate pattern formation during tumor initiation and progression. Moreover, in mesenchymal cells, activation of TGF-β1 signaling synergistically induces the transcriptional activity of canonical Wnt/β-catenin signaling to control cell growth. Global gene expression analysis of genetically manipulated mice revealed that TGF-β1/Smad and Wnt/β-catenin signaling pathways are firmly intertwined [48].

After recognition of HFD and HFD+DEN impact on Wnt/β-catenin pathway, we decided to analyze TGF-β1 pathway. We found a nuclear increase of pSmad2/3 and Smad4 that could implicate an enhanced sensitization of the liver of HFD mice, which might lead to the development of HCC.

In addition, it has been observed that plasma TGF-β1 is augmented in hypertension and other cardiovascular diseases, indicative of its role in metabolic syndrome. Even more, elevated TGF-β1 has been associated with a higher risk for T2DM in a prospective case-cohort study [49]. Importantly, circulating TGF-β1 levels are significantly elevated in obese humans, *ob/ob* mice, and HFD-induced obese mice [44, 45]. By regulating expression of its target genes, such as PGC-1α and PPAR-γ, elevated TGF-β1/Smad 3 signaling is associated with systemic insulin resistance and hepatic steatosis [51, 52]. Taking this into account, and based in the data obtained in ours *in vivo* studies, we explored if TGF-β1 was involved in DEN-induced liver sensitization in HFD-induced T2DM mice. To achieve this, we performed *in vitro* studies using isolated hepatocyte cultures from the 4 experimental groups that were used exclusively for this experimental design, which were incubated in the presence or absence of TGF-β1. A potentially relevant observation of our study was that TGF-β1

increased not only the level for nuclear pSmad 2/3, but there also was observed a significant increase in nuclear β-catenin, thus suggesting a potential synergism between these two signaling pathways in our model. Our results of the synergism between both tumouric pathways are in agreement with what has been demonstrated by other authors in different systems [53, 54].

In conclusion, in a HFD fed model that resembles early T2DM pathology in mice, our results suggest that in HFD-fed group, liver cells are not quiescent and exhibit alterations in some proteins involved in cell cycle progression. Besides, the exposure to a carcinogen such as DEN could lead to an enhanced progression of the hepatocarcinogenic process after HFD-treatment. In this sense, it becomes highly relevant that both tumorigenic pathways Wnt/β-catenin and TGF-β1/Smads are basally deregulated in liver of HFD group, prior to the stimulation with DEN. Our results support the notion that TGF-β1 would be involved in this liver sensitization by activating both tumorigenic pathways. In this regard, our work was focused in the very early stages of the initiation of HCC, when no manifestation of neoplasia is observable and there are only alterations in some signaling pathways. This makes our work totally original, thus enabling new information to shed light to the subject of study.

Identifying the molecules involved and understanding the processes that underlie T2DM-associated hepatocarcinoma will empower the development of new therapies for the prevention of the development of this specific type of cancer in diabetic patients. Understanding the cellular and molecular mechanisms leading to HCC in HFD-induced T2DM, and most importantly those connected to systemic/metabolic influences, has therefore become an urgent and imperative issue. Without this knowledge, developing efficient preventive, diagnostic and therapeutic counter-measures is bound to fail.

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FIGURES LEGENDS

Figure 1*. Experimental protocol.* Five-week-old male mice C57BL/6 were randomly divided into 4 experimental groups (n = 5 per group) in two similar rounds. Mice of two groups were fed with regular chow diet (C) or with a 40% high-fat diet (HFD), *ad libitum* for 41 weeks. The C (Chow diet) and HFD (HFD-induced T2DM) groups were injected i.p. with saline solution DEN-vehicle at week 21, or injected i.p. with a single dose of DEN (75 mg/kg body weight) leading to C+DEN and HFD+DEN groups. During the whole treatment, body weight and food intake were recorded every ten days. Mice were euthanized 25 weeks after saline solution or DEN injection (at 46 weeks of age).

Figure 2. *Determination of DEN effect on weight gain curves and blood glucose levels.* **A)** Body weight was registered periodically in all groups. **B)** Glucose plasma levels were measured. Data are expressed as percentage of C group and mean ± S.E.M., a representative experiment from two similar rounds (n =5). *$p < 0.05$ vs C, $^{\#}p < 0.05$ vs C+DEN group.

Figure 3*. Proliferative status of liver.* **A)** Representative images of proliferating cell nuclear antigen (PCNA)-positive cells obtained by optical microscopy (objective: 20x and ocular: 10x). **B)** Proliferative Index (Number of Proliferative Cells in each phase/100 hepatocytes). **C)** Percentage of hepatocytes in each phase of the cell cycle. Data are expressed as mean ± S.E.M., a representative experiment from two similar rounds. *$p < 0.05$ vs C; $^{\#}p < 0.05$ vs C+DEN and $^{\dagger}p < 0.05$ vs HFD group.

Figure 4*. Expression of cell cycle regulatory proteins.* Markers of cell cycle progression were determined by Western blot in nuclear fraction: **A)** cyclin D1 and **B)** cyclin E1 expression. *Ponceau S* was probed as loading control in nuclear fraction. Data are expressed as percentage of C group and mean ± S.E.M., a representative experiment from two similar rounds. *$p < 0.05$ vs C; $^{\#}p < 0.05$ vs C+DEN group.

Figure 5. *Expression levels of Wnt/β-catenin pathway*. **A)** Nuclear β-catenin, **B)** Total β-catenin, **C)** p-β-catenin, **D)** GSK-3β and **E)** GSK-3β pY216 were analyzed by Western blot. *Ponceau S* was probed as loading control in nuclear fraction and GAPDH was probed as loading control in cytosolic fraction. Data are expressed as percentage of C group and mean ± S.E.M., a representative experiment from two similar rounds. *$p < 0.05$ vs C; $^{\#}p < 0.05$ vs C+DEN group and $^{\dagger}p < 0.05$ vs HFD group.

Figure 6. *Expression levels of β-catenin target genes mRNA and TCF-4.* **A)** *Vegf, c-myc, c-jun* and *cyclin D1* mRNA expression level was measure in liver by RT-qPCR. β-actin was probed as loading control and **B)** TCF-4 protein were evaluated by Western blot in nuclear extracts. *Ponceau S* was probed as loading control in nuclear fraction and. Data are expressed as percentage of C group and mean ± S.E.M., a representative experiment from two similar rounds. $^{\dagger}p < 0.05$ vs HFD group.

Figure 7. *Expression levels of Smads pathway*. **A)** p-Smad2/3 and **B)** Smad4 were analyzed by Western blot. *Ponceau S* was probed as loading control in nuclear fraction. Data are expressed as percentage of C group and mean ± S.E.M., a representative experiment from two similar rounds. *$p < 0.05$ vs C; $^{\#}p < 0.05$ vs C+DEN group and $^{\dagger}p < 0.05$ vs HFD group.

Figure 8. *In vitro studies: expression levels of proteins triggered by TGF-β1*. **A)** p-Smad2/3 and **B)** β-catenin were analyzed by Western blot. *Ponceau S* was probed as loading control in nuclear fraction. Data are expressed as fold change of 5 ng/ml respect to 0 ng/ml mean ± S.E.M., a representative experiment from two similar rounds. *$p < 0.05$ vs C; $^{\#}p < 0.05$ vs C+DEN and $^{\dagger}p < 0.05$ vs HFD group.

Figure 1

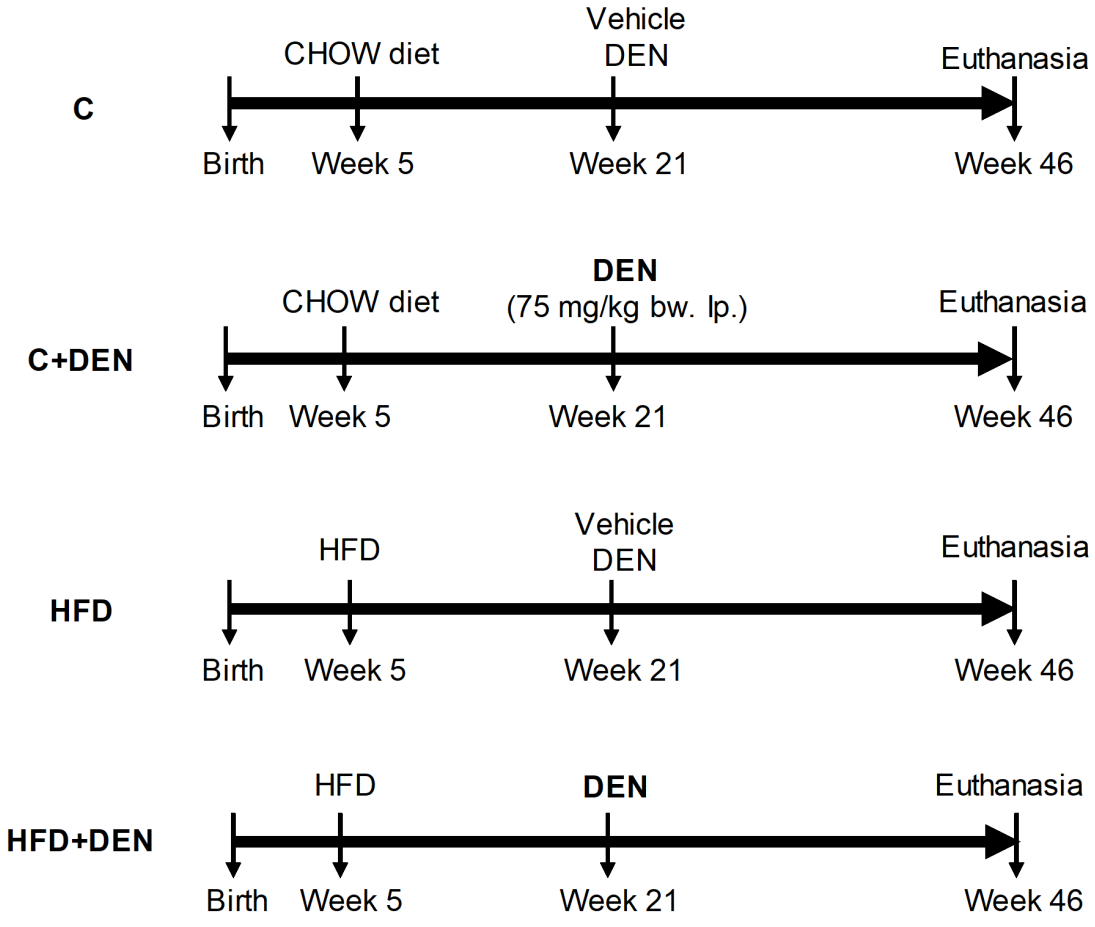

C
CHOW diet
Vehicle
DEN
Euthanasia
Birth
Week 5
Week 21
Week 46
C+DEN
CHOW diet
DEN
(75 mg/kg bw. Ip.)
Euthanasia
Birth
Week 5
Week 21
Week 46
HFD
HFD
Vehicle
DEN
Euthanasia
Birth
Week 5
Week 21
Week 46
HFD+DEN
HFD
DEN
Euthanasia
Birth
Week 5
Week 21
Week 46

Figure 2

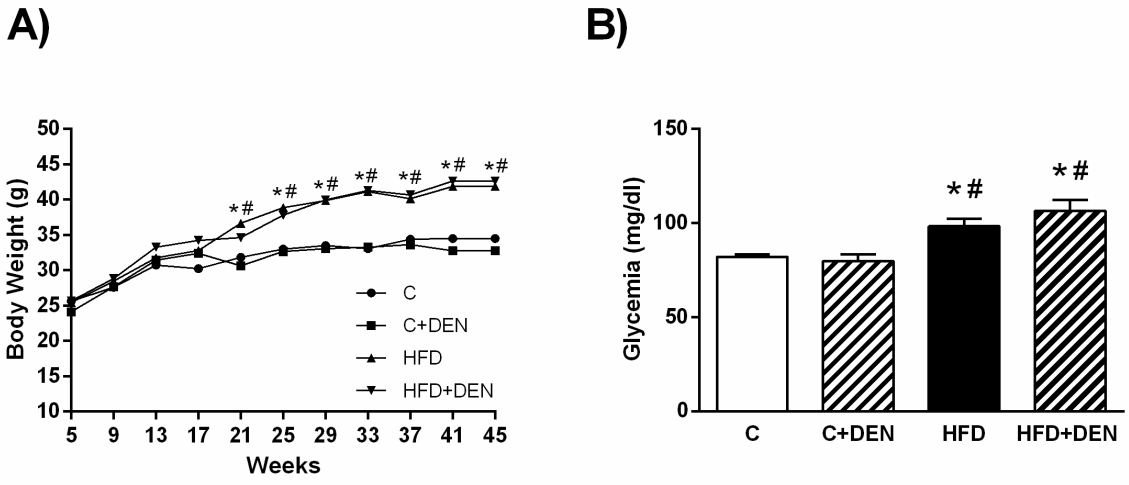
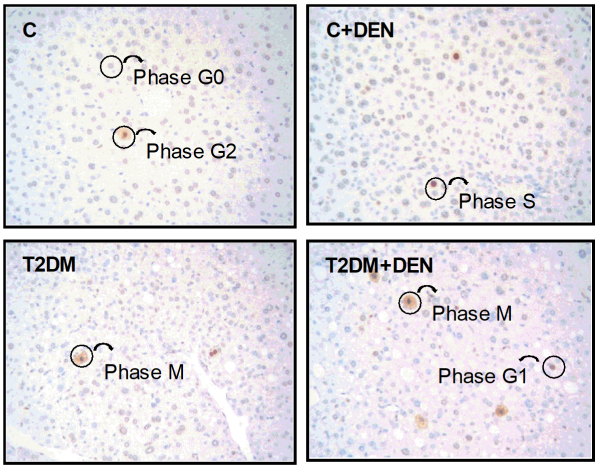


Figure 3

A)



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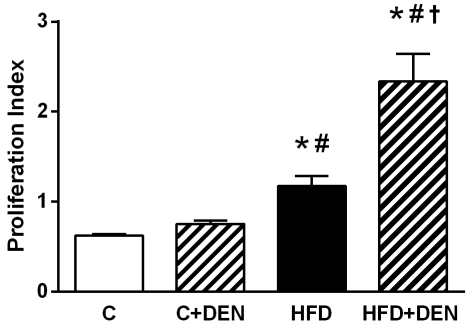

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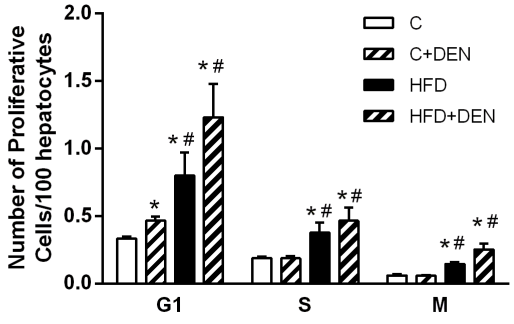


Figure 4

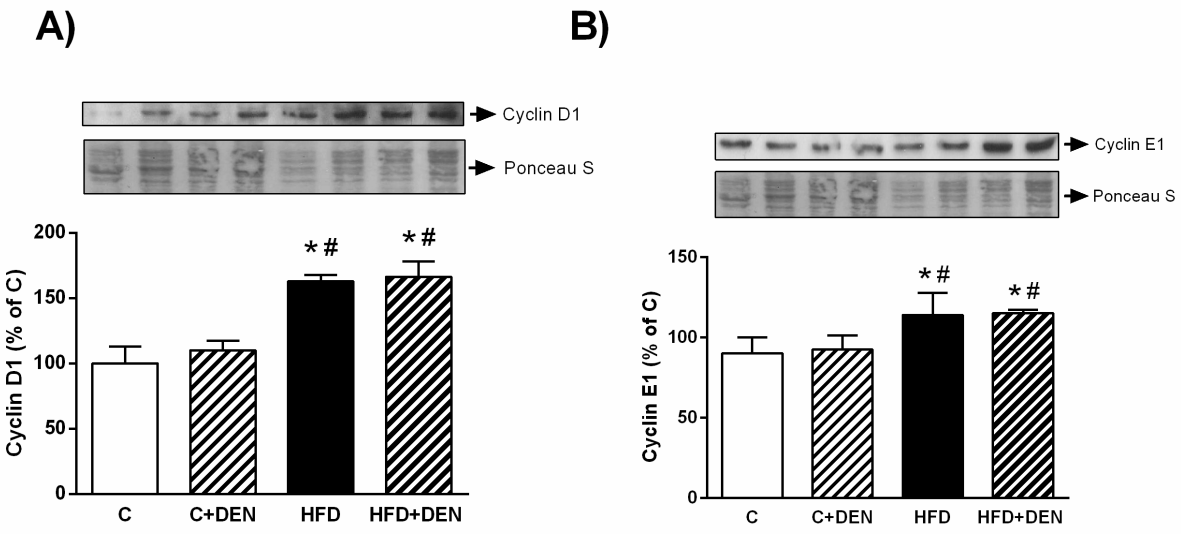


Figure 5

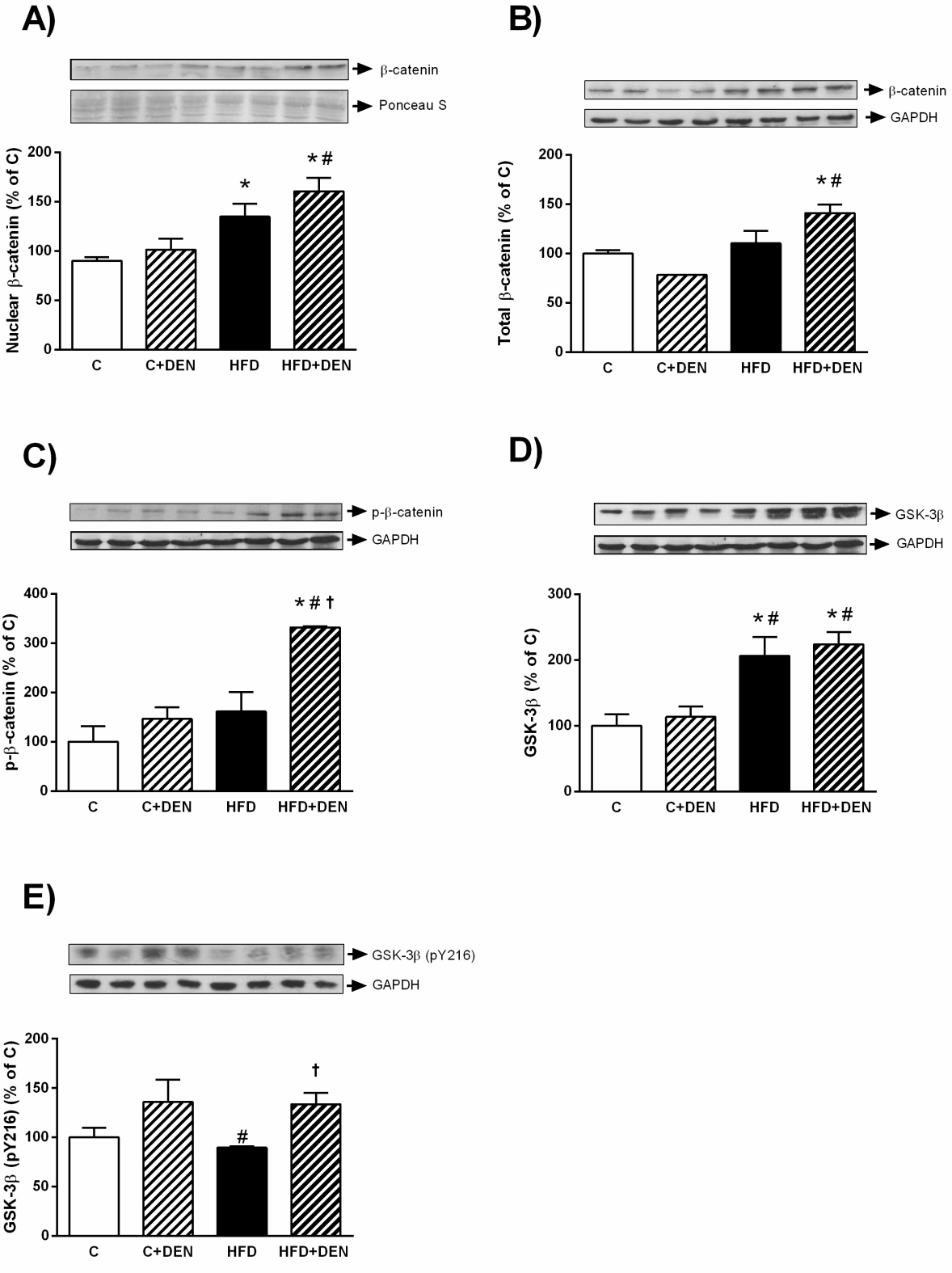

A)
β-catenin
Ponceau S
Nuclear β-catenin (% of C)
200
150
100
50
0
*
*#
C
C+DEN
HFD
HFD+DEN
B)
β-catenin
GAPDH
Total β-catenin (% of C)
200
150
100
50
0
*#
C
C+DEN
HFD
HFD+DEN
C)
p-β-catenin
GAPDH
p-β-catenin (% of C)
400
300
200
100
0
*# †
C
C+DEN
HFD
HFD+DEN
D)
GSK-3β
GAPDH
GSK-3β (% of C)
300
200
100
0
*#
*#
C
C+DEN
HFD
HFD+DEN
E)
GSK-3β (pY216)
GAPDH
GSK-3β (pY216) (% of C)
200
150
100
50
0
#
†
C
C+DEN
HFD
HFD+DEN

Figure 6

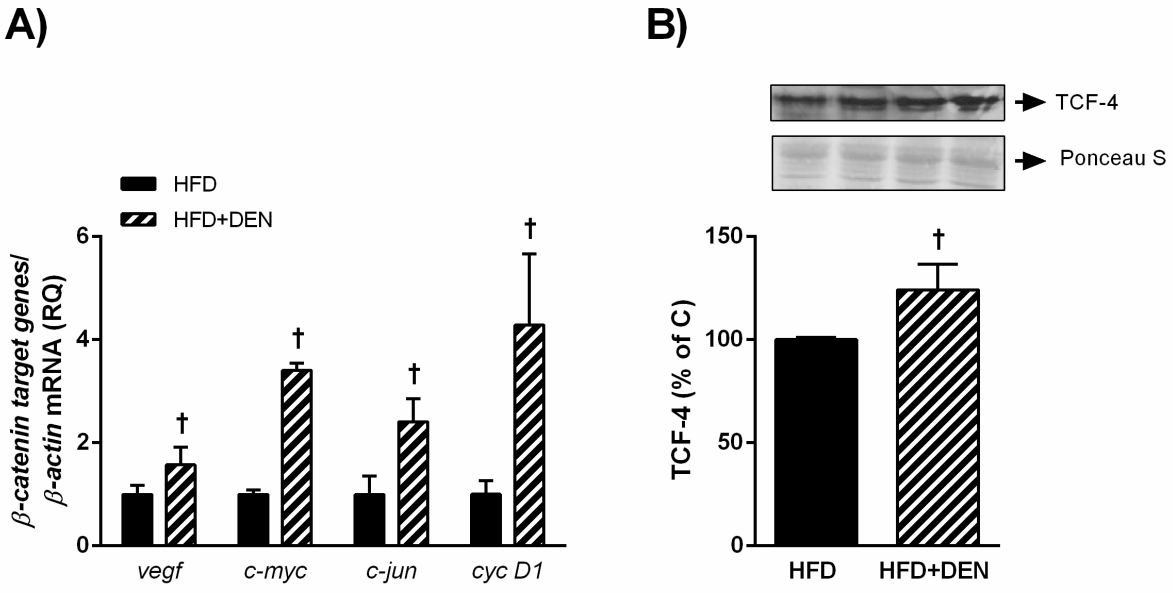


Figure 7

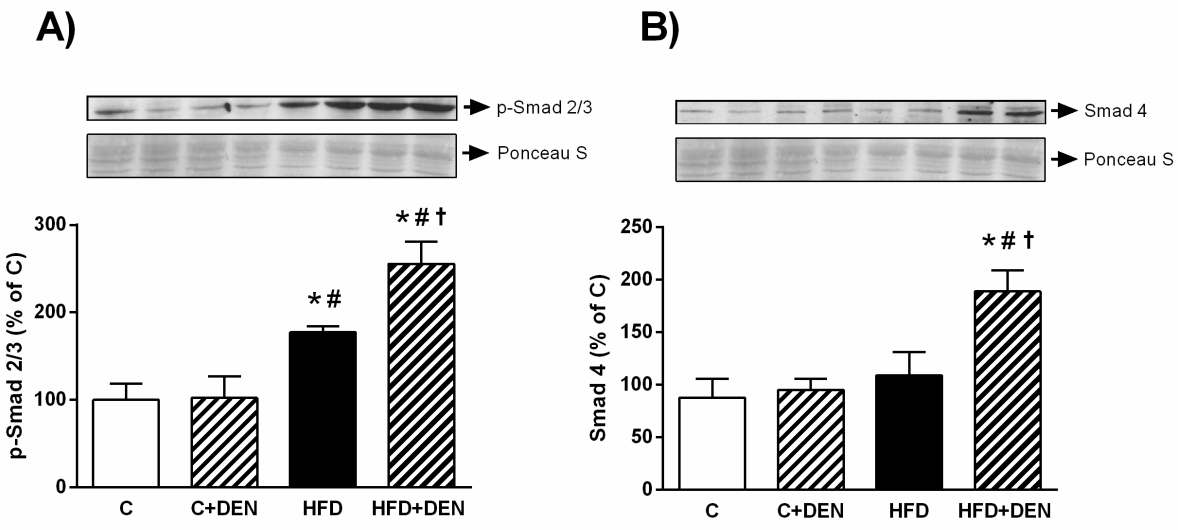
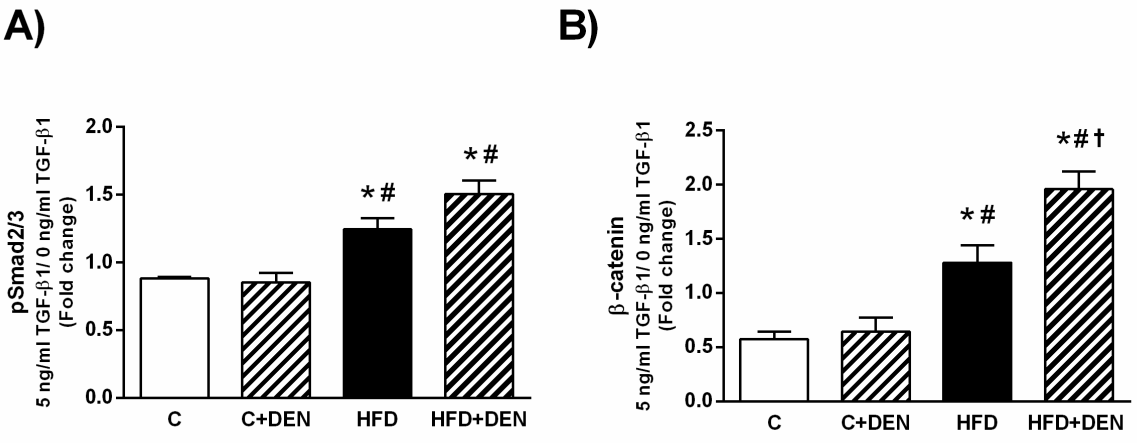


Figure 8



HIGHLIGHTS

- HFD-induced type 2 diabetes sensitizes mice to DEN-induced HCC.
- Wnt/βcatenin and TGF-β1/Smads molecular pathways are implicated in early HCC.
- New insights into molecular mechanisms leading to HCC in T2DM are describe.