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Attenuation of the Wnt/β-catenin/TCF pathway by *in vivo* interferon-α2b (IFN-α2b) treatment in preneoplastic rat livers

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Abstract

Wnt/β-catenin/T cell factor (TCF) pathway is activated in several types of human cancers, promoting cell growth and proliferation. Forkhead box containing protein class O (FOXO) transcription factors compete with TCF for β-catenin binding, particularly under cellular oxidative stress conditions. Contrary to β-catenin/TCF, β-catenin/FOXO promotes the transcription of genes involved in cell cycle arrest and apoptosis. We have previously demonstrated that *in vivo* interferon-α2b (IFN-α2b) administration induces apoptosis in preneoplastic livers, a mechanism mediated by reactive oxygen species (ROS) and transforming growth factor-β_1 (TGF-β_1). This study was aimed to assess the status of the Wnt/β-catenin/TCF pathway in a very early stage of rat hepatocarcinogenesis and to further evaluate the effects of *in vivo* IFN-α2b treatment on it. We demonstrated that the Wnt/β-catenin/TCF pathway is activated in preneoplastic rat livers. More important, *in vivo* IFN-α2b treatment inhibits Wnt/β-catenin/TCF pathway and promotes programed cell death possibly providing a link with FOXO pathway.

Keywords: *Hepatocarcinogenesis, β-catenin, TCF, FOXO, oxidative stress*

Introduction

The canonical Wnt/β-catenin/T cell factor (TCF) pathway is a highly conserved signal transduction cascade present during normal embryogenesis. Through the transcription of specific target genes, it determines the cell fate and regulates proliferation, migration, motility, and polarity (Cadigan and Nusse 1997; Zeng et al. 1997). In the absence of Wnt signaling, free cytoplasmic β-catenin is recognized and phosphorylated by a multiprotein complex consisting of adenomatous polyposis coli, axin, glycogen synthase kinase 3β (GSK-3β), and casein kinase I (Behrens et al. 1998; Hart et al. 1998; Liu et al. 2002; Yanagawa et al. 2002). Phosphorylated β-catenin is recognized for ubiquitination by the E3 ubiquitin ligase β-TrCP and subsequent proteasomal degradation (Aberle et al. 1997). When Wnt ligands bind to the Frizzled transmembrane receptor family and to the co-receptor low-density lipoprotein-related protein (LRP5/6), a signaling cascade is initiated, β-catenin degradation is blocked, and the result is an accumulation of cytoplasmic unphosphorylated β-catenin and subsequent translocation to the nucleus (Yanagawa et al. 1995; Tamai et al. 2000; Mao et al. 2001). Nuclear β-catenin interacts with transcription factors of the TCF/lymphoid enhancing factor (LEF) family and activates the transcription of a specific subset of genes involved in cell growth and proliferation (Brabletz et al. 1999; Shtutman et al. 1999; Yan et al. 2001; Willert et al. 2002; Fujimura et al. 2007). A defect on the regulation of

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the Wnt/β-catenin/TCF pathway leading to an aberrant activation is associated with several disorders, especially cancer. In fact, Wnt/β-catenin/TCF activation was detected in a wide variety of human cancers such as colorectal cancer (Morin et al. 1997), melanoma (Rubinfeld et al. 1997), pancreatic cancer (Tanaka et al. 2001), and hepatocellular carcinoma (HCC) (Thorgeirsson and Grisham 2002; Bruix et al. 2004). HCC is one of the most common tumors worldwide and is the major liver malignancy (Parkin et al. 2005). Although the etiological agents of the majority of the cases are well known (i.e. chronic hepatitis B and C virus infection, chronic alcohol consumption, or dietary exposure to aflatoxin B1), there is no much knowledge about the molecular mechanism underlying HCC development (Thorgeirsson and Grisham 2002; Bruix et al. 2004). Recent studies have reported the presence of genetic alterations in the Wnt/β-catenin pathway in human HCC (de La Coste et al. 1998; Miyoshi et al. 1998) and in transgenic (Calvisi et al. 2001; Merle et al. 2005) and chemically induced hepatocarcinogenic mouse models (Devereux et al. 1999). These alterations led to an anomalous activation of the pathway manifested by a post-transcriptional stabilization of β-catenin. Most alterations are activating mutations (missense mutations or deletions) in the GSK-3β phosphorylation region of the β-catenin gene (de La Coste et al. 1998; Miyoshi et al. 1998; Devereux et al. 1999; Calvisi et al. 2001). However, there could be changes in other components of the pathway that leads to wild-type β-catenin stabilization. For instance, overexpression of Frizzled-7 was reported both in human HCC (Merle et al. 2004), and in transgenic mouse models (Merle et al. 2005) and it was suggested that contributes to β-catenin stabilization and accumulation.

In addition to the role of β-catenin in Wnt signaling pathway, it has been recently reported that β-catenin is also a cofactor for the FOXO subfamily of transcription factors (Essers et al. 2005). In contrast to TCF activation, FOXO family members are involved in the induction of cell cycle arrest and expression of genes involved in apoptosis (Dijkers et al. 2000; Burgering and Kops 2002; Furukawa-Hibi et al. 2005). It was demonstrated that β-catenin binding to FOXO inhibits TCF transcriptional activation (Hoogeboom et al. 2008). In addition, this binding is enhanced under cellular oxidative stress conditions (Essers et al. 2005; Hoogeboom et al. 2008). Hence, β-catenin would play a decisive role in cell fate, favoring proliferation or apoptosis according to the transcription factor to which it is bound.

On the other hand, interferon-alpha (IFN-α) has been described as an essential cytokine for antiviral immunity with antiproliferative and immunomodulatory effects (Pfeffer 1997). In addition, IFN-α has anti-oncogenic activity against a variety of solid tumors and hematologic malignancies (Zurita et al. 1994). Nowadays, although IFN-α is used for the treatment of hepatitis C and B as well as some forms of human cancer (Gutterman 1994; Ikeda et al. 1998; Yoshida et al. 1999), the molecular basis for the treatment is poorly understood. In this regard, it has been stated that IFN-α treatment in the very early stages of hepatocarcinogenesis could have more important clinical effects than in advanced stages (Gutterman 1994). We have previously shown that IFN-α2b administration reduces the number and volume of altered hepatic foci (AHF) in preneoplastic rat livers through an apoptotic mechanism mediated by ROS and TGF-β_1 (de Lujan et al. 2002; de Lujan et al. 2004; Quiroga et al. 2007).

In this study, we aimed to assess the status of the Wnt/β-catenin/TCF pathway in an early stage of hepatocarcinogenesis and to analyse the effects of IFN-α2b treatment on it.

Materials and methods

Chemicals

Diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-pi class of rat glutathione S-transferase (rGST-P) antibody was purchased from Stressgen Bioreagents (Ann Arbor, MI, USA). Anti-β-catenin antibody was from BD Transduction Labs (San Jose, CA, USA). Anti-TCF4 antibody was from Cell Signaling Technology (Danvers, MA, USA). Anti-*p*-β-catenin (against Ser-33 phosphorylated β-catenin), anti-Frizzled-7 and anti-FoxO3a antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488 fluorescent secondary antibody was from Invitrogen (Carlsbad, CA, USA). Cy3 fluorescent secondary antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Pierce enhanced chemiluminescence (ECL) Western Blotting was from Thermo Fisher Scientific (Rockford, IL, USA). Phosphatase inhibitor calyculin A was from BioSource International (Camarillo, CA, USA). All other chemicals were of the highest grade commercially available.

Animals and treatment

Adult male Wistar rats weighing 330–380 g were maintained in a room at constant temperature with a 12 h light–dark cycle, with food and water supplied *ad libitum*. Experimental protocols were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (Publication no. 25-28, revised 1996). Animals were divided into four groups. An overview of the experimental protocol is provided in Figure 1. Animals of the initiated–promoted (IP) group were subjected to a two-phase model of rat hepatocarcinogenesis, as previously described (de

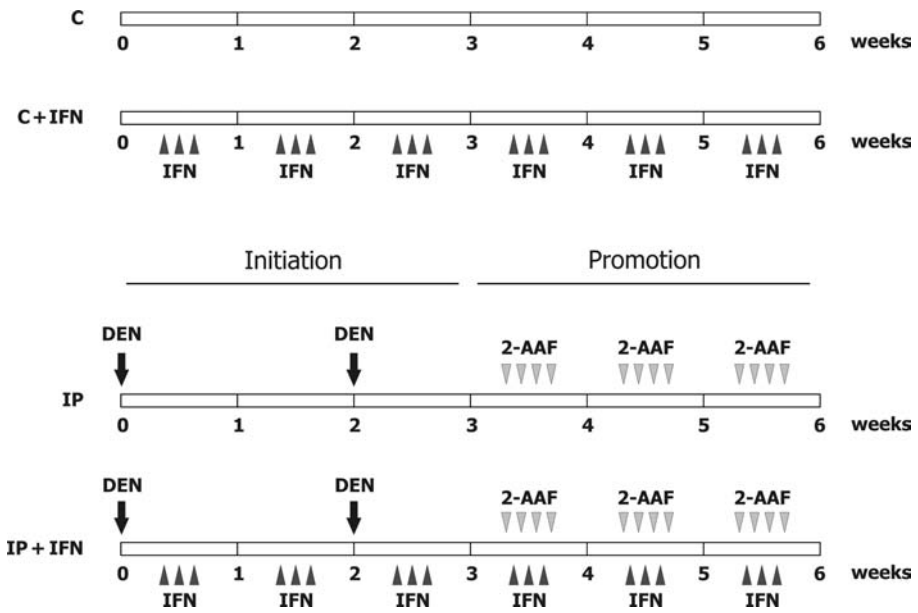

Figure 1. Scheme for the treatment of the animals. Male Wistar rats were subjected to a two-phase model of hepatocarcinogenesis. IP group received two intraperitoneal doses of DEN (150 mg/kg body weight) 2 weeks apart. One week after the last injection of DEN, the animals received 20 mg/kg body weight of 2-AAF by gavage for four consecutive days per week during 3 weeks. IP + IFN group was subjected to the same two-phase protocol and also received intraperitoneal IFN-α2b (6.5×10^5 U/kg body weight) three times per week during the entire treatment. C group received only the vehicles of the drugs. C + IFN group received only IFN-α2b according to the same schedule described for IP + IFN group. Animals were killed at the end of week 6.

Lujan et al. 2002). Briefly, animals received two intraperitoneal necrogenic doses of DEN (150 mg/kg body weight) 2 weeks apart. One week after the last injection of DEN, rats received 20 mg/kg body weight of 2-AAF by gavage for four consecutive days per week during 3 weeks. IP + IFN group was subjected to the same two-phase protocol as IP group and also received IFN-α2b (PC-Gen S.A., Buenos Aires, Argentina) 6.5×10^5 U/kg body weight administered intraperitoneally three times per week during the entire treatment. The dose used was comparable to that used for therapeutic purposes (de Lujan et al. 2002). Control rats (C group) were raised for the same period of time but receiving only the vehicles of the drugs. Finally, C + IFN group received only IFN-α2b according to the same schedule described for IP + IFN group. At the end of the six-week treatment, animals were killed and livers were removed and processed. At that time, approximately 5% of the liver is occupied by reversible preneoplastic foci (de Lujan et al. 2002).

Confocal immunofluorescence analysis

Liver tissues were fixed in 10% vol/vol formalin solution and embedded in low-melting paraffin blocks. Sections of 5 μm thickness were used for immunohistochemical staining. Sections were deparaffinized, rehydrated, and then microwaved in a 10 mM citrate buffer solution for 10 min at 96°C to perform antigen retrieval. After incubation with blocking serum (3% BSA, 0.03% Triton X-100) for 30 min, slides were incubated with primary antibodies in a humidified chamber at 4°C overnight. For determination of preneoplastic foci, rGST-P immunodetection was chosen since it is the most widely used method for identification of AHF (Pitot 1990). Slides were incubated with anti-rGST-P and β-catenin primary antibodies (dilution 1:100). For increased sensitivity, fluorescent dye-conjugated secondary antibodies (dilution 1:100) were used. Nuclei were counterstained using 4′, 6-diamidino-2-phenylindole (DAPI, dilution 1:100) for 5 min. Detection of bound antibody was accomplished by immunofluorescence in a Nikon C1 Plus microscope (Nikon, Tokyo, Japan). Slides were also stained in the absence of primary antibodies to evaluate nonspecific secondary antibodies reactions.

Preparation of tissue lysates, homogenates, and subcellular fractions

Whole liver lysates were prepared by homogenization of tissues in radioimmunoprecipitation assay buffer (RIPA) (20 mM Tris, pH 8, 200 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 1 mM NaF, 1 mM Na3VO4, 1.5 nM Calyculin A, and protease inhibitors). Whole liver homogenates and subcellular fractions enriched in plasma membranes were prepared by differential centrifugation, as previously described (Parody et al. 2007). Briefly, liver tissues were homogenizated in 0.3 M sucrose containing

phosphatase and protease inhibitors. The plasma membrane fraction was obtained by centrifugation at 200,000 × *g* for 60 min on a discontinuous 1.3 M sucrose gradient. The purity of plasma membrane fractions was confirmed measuring activity of 5′-nucleotidase using commercial kits (Wiener Lab., Rosario, Argentina) (data not shown).

Western blot analysis

Protein concentration of liver lysates, homogenates, and subcellular fractions was determined by the Lowry method (Lowry et al. 1951), using bovine serum albumin as a standard. Aliquots of 20 μg of protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (PerkinElmer Life Sciences, Boston, MA, USA). Membranes were blocked with PBS-10% nonfat milk, washed and incubated overnight at 4°C with primary antibodies (1:2000 for β-catenin and *p*-β-catenin; 1:1000 for Frizzled-7). Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by the ECL detection system and quantified by densitometry using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) software. Equal loading and transference of protein was checked by detection of β-actin and by Ponceau S staining of the membranes.

Co-immunoprecipitation assay

Whole liver homogenates were subjected to immunoprecipitation with anti-TCF4 antibody (dilution 1:25), anti-FoxO3a antibody (dilution 1:10), or anti-β-catenin antibody (dilution 1:50). Proteins bound to protein A-sepharose beads were washed with lysis buffer and resolved by 12% SDS-PAGE. Gels were blotted onto PVDF membranes and subjected to immunoblotting with specific secondary antibodies at appropriates dilution. The immunoreactive bands were detected as described above.

Chromatin immunoprecipitation assay

Chromatin was isolated from whole liver lysates previously cross-linked with 1% formaldehyde at room temperature for 15 min. Chromatin was then fragmented to a mean size <700 bp by sonication. A fraction of supernatant of sonicated samples was saved as an input material, whereas the rest of the chromatin in the supernatant was pre-cleared by incubating with protein A-sepharose beads for 2 h. Samples were then subjected to chromatin immunoprecipitation (ChIP) using specific antibodies (anti-TCF4 or anti-FoxO3a) following commercial ChIP protocol. In addition, anti-rabbit IgG was also included in the immunoprecipitation to process DNA for a negative control. DNA was purified by phenol–chloroform extraction and used as a template for PCR amplification (36 cycles; 30 s at 95°C, 60 s at 55°C, 60 s at 72°C). Specific primer pairs for the SP5 (5′-TGCCTTGG-TGCAAATCTTTA-3′ and 5′-TGGTGTGTTTGT-TGGTCTGG-3′) and p130 (5′-TCCTGCTCTGC-ACTCTTCCT-3′ and 5′-CAACCCAACAGAGAG-GATTC-3′) promoter regions were used for investigating the binding of TCF4 and FoxO3a to DNA, respectively. The PCR products were visualized on an ethidium bromide gel.

RNA isolation and semiquantitative RT-PCR

Determination of mRNA expression levels of several targets of the Wnt/β-catenin/TCF pathway (Cyclin D1, MMP-7, Axin 2, and SP5), Frizzled-7 transcript and a target of FOXO transcription factor (p130) was performed by semiquantitative RT-PCR (sqRT-PCR). Total RNA was isolated from rat liver tissues by the TriZOL method (Life Technologies Inc, Gaithersburg, MD, USA). cDNA was made from 2 μg of total RNA using an oligo-dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Two micro litres of cDNA were amplified using *Taq* polymerase (Promega) and specific set of primers for each studied mRNA. Amplification of mRNA transcript encoding β-actin, a housekeeping gene, was used as a quantitative control. See Table I for primers sequences and PCR conditions. PCR products were resolved in a 2% agarose gel stained with ethidium bromide and bands were visualized using a high-performance ultraviolet transilluminator (UVP, Upland, CA, USA). Images of the agarose gels were acquired with an imaging system EpiChemi 3 Darkroom (UVP) and quantification of the bands was performed using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) software. Each quantified band was normalized to the corresponding β-actin levels.

β-catenin mutation analysis

RT-PCR was performed using total liver RNA as a template and a set of primers which amplify the region of hotspot mutations located in β-catenin exon 2, homologous to human exon 3 (de La Coste et al. 1998) (see Table I for primers sequences and RT-PCR conditions). RT-PCR products were electrophoresed, excised, and purified from 2% agarose gels using the Wizard SV Gel and PCR Clean-Up System (Promega). Amplicons were labeled with DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and the reaction products were precipitated with ammonium acetate and ethanol to remove unincorporated dye-labeled terminators. The reaction products were resuspended in a formamide loading buffer, separated

Table I. Primers for PCR amplification and sequencing.

Primer[a]	Sequence 5′ → 3′	Cycles
β-actin F	CAACCTTCTTGCAGCTCCTC	30
β-actin R	TTCTGACCCATACCCACCAT	
Cyclin D1 F	AGGAGACCATTCCCCTGACT	30
Cyclin D1 R	GCGGATGATCTGCTTGTTCT	
MMP-7 F	GAGTGCCAGATGTTGCAGAA	36
MMP-7 R	GTCTGCAGTCCCCCAACTAA	
Axin 2 F	AAGCGAACCGGTTAATCCTT	36
Axin 2 R	GCTCAGACCCCTCCTTTTCT	
SP5 F	GTAGCGGCAAACTTCAAAGC	36
SP5 R	GTCATAGGGCACCTGAAGGA	
β-catenin F	CATGGAGCCAGACAGAAAGG	36
β-catenin R	AGCAGTTTGGTCAGCTCAGG	
p130 F	AGGCGGCTATTTGAGAGTGA	36
P130 R	CTCCAGGGGAAATTTGTTGA	
Frizzled-7 F	GGCCAACTCGCAGTACTTTC	38
Frizzled-7 R	ACAGGAAGGACGTACCGATG	

[a] Primer names refer to their target specificity and their sense (F, forward) or antisense (R, reverse) orientation. PCR conditions: reaction was started with a single step of denaturation at 95°C for 5 min. Cycling conditions consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR was finished with a 7 min, 72°C elongation step. In all cases, amplification was performed with the optimal conditions previously determined using an increasing number of PCR cycles. A linear relationship between the band intensity of the PCR product and the number of amplification cycles performed was observed. The conditions were chosen so that none of the amplification products reached a plateau at the end of the protocol.

and detected on a MegaBACE 1000 sequencing instrument (GE Healthcare Bio-Sciences) by capillary electrophoresis. Amplicons sequence determination was carried out in both directions and compared to the published β-catenin sequence. RT-PCR primers were used for the sequencing reactions.

Statistical analysis

Results were expressed as mean ± SE. Significance in differences was tested by one-way ANOVA, followed by Tukey's test. Differences were considered significant when the p value was < 0.05.

Results

In situ immunodetection of preneoplastic foci

As expected, liver slices showing rGST-P positive preneoplastic foci were observed only in preneoplastic livers (IP and IP + IFN) (Figure 2). In addition, the number and volume percentages of AHF per liver in rats treated with IFN-α2b were significantly decreased compared with the values of IP animals, as was previously reported (de Lujan et al. 2002; Quiroga et al. 2007).

Subcellular localization of β-catenin

Sections of liver tissue were immunostained with anti-β-catenin antibody. As expected, in C and C + IFN livers a strong membranous signal was observed (Figure 2). Conversely, in IP and IP + IFN livers membranous β-catenin immunostaining was markedly decreased and a strong cytoplasmic signal was present within the foci and the surrounding tissue (Figure 2). Cytoplasmic accumulation of β-catenin is a main characteristic of Wnt/β-catenin pathway activation. Consistent with confocal images, we have also confirmed decreased expression of β-catenin in plasma membrane fraction by western blotting in IP (−76.20 ± 5.97%) and IP + IFN (−81.88 ± 7.78%) animals whereas C + IFN group showed no differences compared with C (Figure 3).

β-catenin and p-*β-catenin protein levels*

Since, free cytosolic β-catenin is regulated by a phosphorylation process, expression of β-catenin and *p*-β-catenin was analysed by western blotting in whole liver lysates. While we found no differences in β-catenin level in all studied groups (Figure 4(A)), *p*-β-catenin level was significantly decreased in IP animals (−76.52 ± 3.89%), whereas it was slightly decreased in IP + IFN animals (−51.78 ± 12.12%) compared with C levels (Figure 4B).

Expression of TCF target genes

To validate the assumption that the Wnt/β-catenin/TCF pathway is activated in our liver preneoplastic model, we measured the transcription levels of TCF target genes by sqRT-PCR method. Our results show that Cyclin D1, MMP-7, Axin 2, and SP5 transcripts were significantly higher in IP animals with respect to C (Figure 5), indicating an activated Wnt/β-catenin/TCF pathway in preneoplastic livers. Furthermore, we observed that transcription levels of these genes were similar to controls in IP animals that

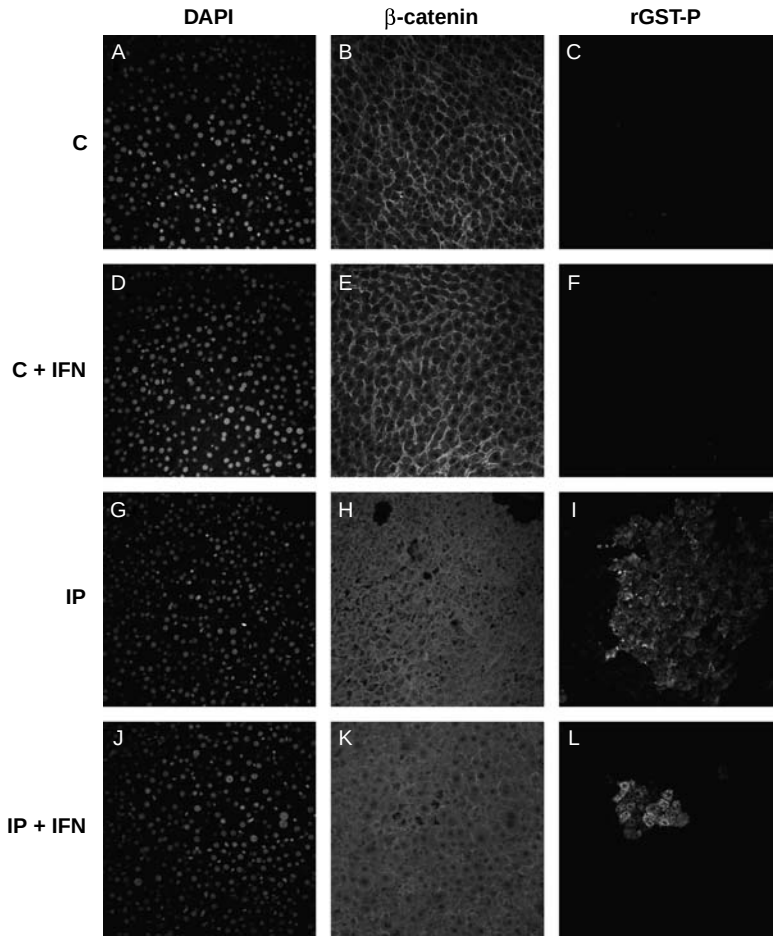

Figure 2. Immunofluorescence demonstrates cytoplasmic accumulation of β-catenin in preneoplastic rat livers. Liver slices were double immunostained with anti-β-catenin (B, E, H, and K) and anti-rGST-P (C, F, I, and L). The nuclei were counterstained with DAPI (A, D, G, and J). (B) Control liver showing homogeneous membranous β-catenin localization and (C) none rGST-P staining. (E) and (F) IFN-α2b-treated control liver showing the same pattern as control liver. (H) Preneoplastic liver displaying cytoplasmic accumulation of β-catenin uniformly distributed throughout the liver. (I) An altered hepatic focus is stained with anti-rGST-P antibody. (K) IFN-α2b-treated preneoplastic liver showing a β-catenin distribution similar to that found in IP group. (L) IFN-α2b treatment induced a significant decrease in number (IP: $5.7 \times 10^5 \pm 1.2 \times 10^5$; IP + IFN: $1.1 \times 10^5 \pm 0.4 \times 10^5$) and volume (IP: 4.2 ± 1.2%; IP + IFN: 1.4 ± 0.2%) of AHF per liver due to an increment in the apoptotic index within the foci (IP: 0.21 ± 0.01%; IP + IFN: 0.38 ± 0.06%) (Quiroga et al. 2007). Original magnification 40×. The images shown here are representative of three independent experiments.

received IFN-α2b treatment (Figure 5). This implies that IFN-α2b attenuates activation of the canonical Wnt/β-catenin/TCF pathway.

β-catenin mutation analysis

To evaluate a possible correlation of genomic mutation and activation of the Wnt/β-catenin/TCF pathway, we performed sequence analysis of a region of exon 2 of the β-catenin gene. Our results revealed that there were no mutations at the GSK-3β phosphorylation sites of β-catenin gene in the experimental groups (data not shown).

Expression analysis of Frizzled-7 receptor

Because of the cytoplasmic stabilization of wild-type β-catenin found in our preneoplastic model, it was of interest to further explore the causes for this dysregulation. Since it was reported that overexpression of Frizzled-7 leads to an aberrant activation of Wnt signaling by stabilization of wild-type β-catenin (Merle et al. 2004, 2005), we decided to measure the level of Frizzled-7 protein in plasma membrane fraction and Frizzled-7 mRNA transcript. The expression of this transmembrane receptor did significantly increased at protein (Figure 6(A)) and transcript (Figure 6(B)) levels in IP rats compared with C and this increment was blocked in animals treated with IFN-α2b.

β-catenin binding partner: switching from TCF to FOXO

To evaluate the dual role of β-catenin as a cofactor of TCF and FOXO, we have performed a co-immunoprecipitation assay. We probed that β-catenin interacts with TCF only in preneoplastic livers and IFN-α2b treatment attenuates this interaction (Figure 7(A)).

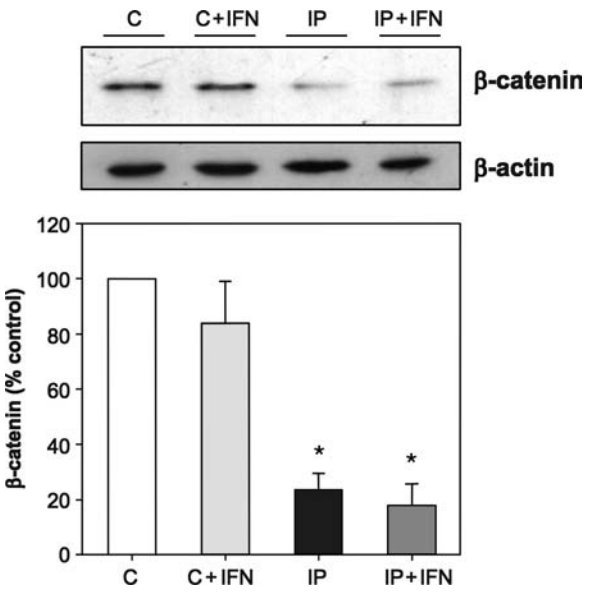

Figure 3. Expression levels of β-catenin in plasma membrane fraction by western blotting analysis. C: control rats, C + IFN: rats that received IFN-α2b 6.5×10^5 U/kg body weight, IP: rats with hepatic preneoplasia, IP + IFN: IP rats that also received IFN-α2b 6.5×10^5 U/kg body weight. Densitometric analysis was performed in at least four independent experiments. Results are expressed as percentages of control group and are the mean ± SD of four independent experiments. $*p < 0.05$ vs. C.

Moreover, β-catenin/FoxO3a interaction is enhanced in preneoplastic livers that received IFN-α2b treatment. In addition, we have also performed sqRT-PCR for p130, a target gene of FoxO3a transcription factor. We found that p130 transcripts were overexpressed in IP + IFN animals (Figure 7(B)) indicating that IFN-α2b treatment induces a switch from TCF to FOXO activation.

TCF4 and FoxO3a binding to DNA

A ChIP assay was performed to determine the binding of the TCF and FoxO3a transcription factors to their target genes promoter regions and to determine whether IFN-α2b treatment affects these interactions. Figure 8 reveals that TCF4 interacts with SP5 promoter region in all studied groups. In addition, FoxO3a is indeed more recruited to the promoter region of p130 gene in the IP + IFN group compared to all other experimental groups.

Discussion

There are several lines of evidence suggesting that Wnt/β-catenin/TCF pathway has an essential role in tumorigenesis. In the study reported in this paper, we have determined the status of the Wnt/β-catenin/TCF pathway in a preneoplastic stage of carcinogenesis and evaluated the possible effects of the IFN-α2b treatment on this pathway. The major findings of this study are related to the impairment of the canonical Wnt/β-catenin/TCF pathway in a very early stage of hepatic carcinogenesis. In addition, we demonstrated that *in vivo* IFN-α2b treatment produces an attenuation of TCF transcriptional activity

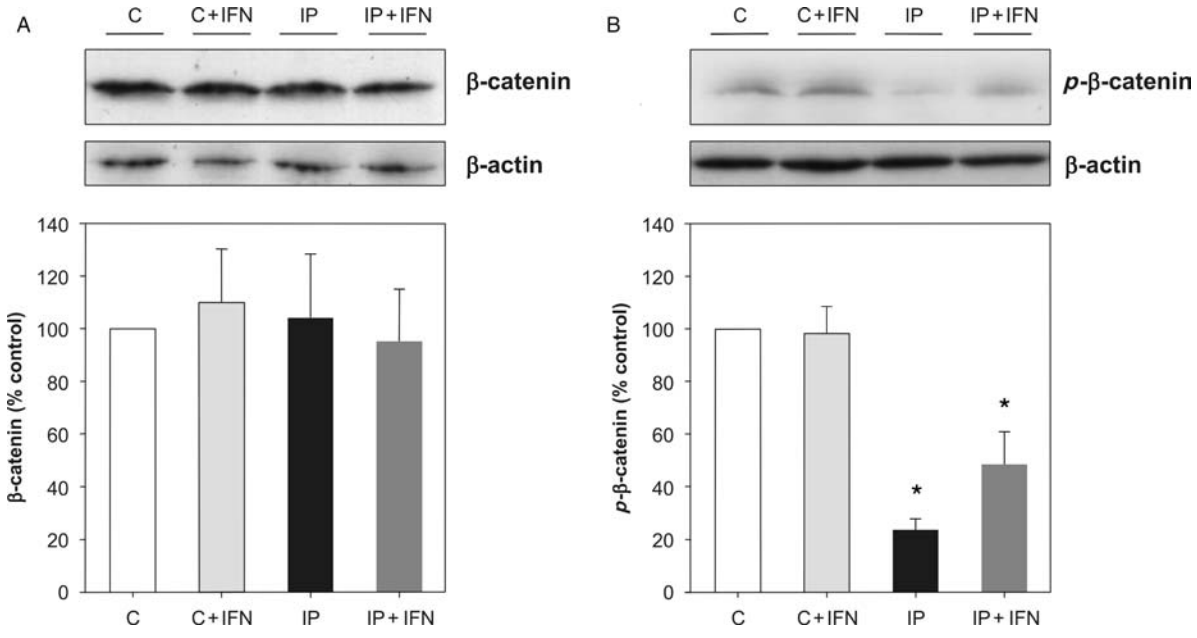

Figure 4. Expression levels of β-catenin and *p*-β-catenin in liver lysates by western blotting analysis. C: control rats, C + IFN: rats that received IFN-α2b 6.5×10^5 U/kg body weight, IP: rats with hepatic preneoplasia, IP + IFN: IP rats that also received IFN-α2b 6.5×10^5 U/kg body weight. (A) Levels of β-catenin. (B) Levels of *p*-β-catenin. Densitometric analysis was performed in at least four independent experiments. Results are expressed as percentages of control group and are the mean ± SD of four independent experiments. $*p < 0.05$ vs. C.

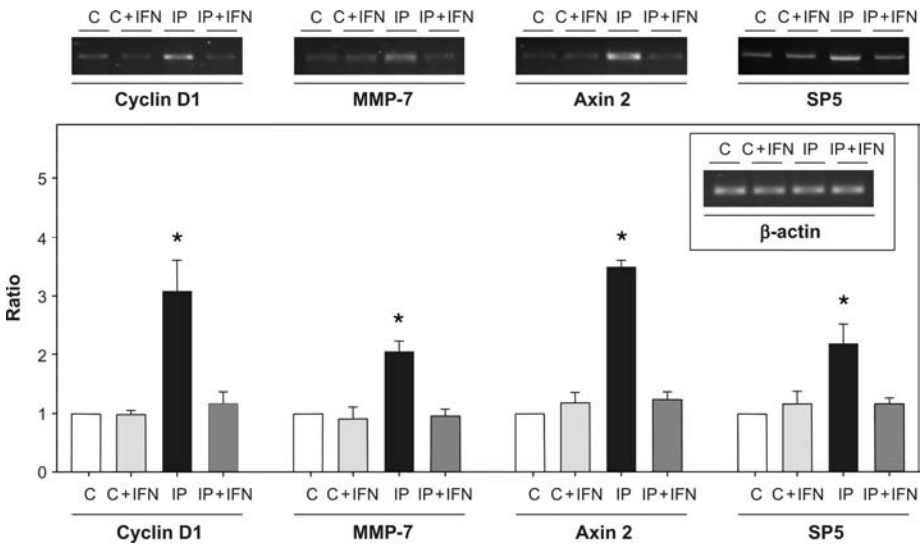

Figure 5. IFN-α2b treatment reduces the expression of β-catenin/TCF target genes in preneoplastic livers. C: control rats, C + IFN: rats that received IFN-α2b 6.5×10^5 U/kg body weight, IP: rats with hepatic preneoplasia, IP + IFN: IP rats that also received IFN-α2b 6.5×10^5 U/kg body weight. Upper panel, representative images of RT-PCR of mRNAs encoding Cyclin D1, MMP-7, Axin 2, and SP5. Lower panel, intensity of the bands were quantified and normalized to the corresponding β-actin levels. Ratios are presented in a graphical form and indicate the relative level of the target amplification product over the control sample after normalization to the housekeeping gene. Data are representative of four independent experiments. $\star p < 0.05$ vs. C.

and enhances FOXO transcriptional activity in preneoplastic livers.

The common denominator of an abnormal Wnt signaling is the stabilization and accumulation of unphosphorylated β-catenin in the cytoplasm of a cell. Eventually, this allows entry of unphosphorylated β-catenin into the nucleus where it promotes the transcription of a subset of genes implicated in cellular proliferation. This β-catenin stabilization was demonstrated in our two-phase carcinogenic model, where plasma membrane delocalization and cytoplasmic accumulation of β-catenin were observed. Moreover, significant reductions of phosphorylated β-catenin levels were found in IP animals. Since β-catenin (phosphorylated and unphosphorylated) protein levels were preserved in all studied groups, these

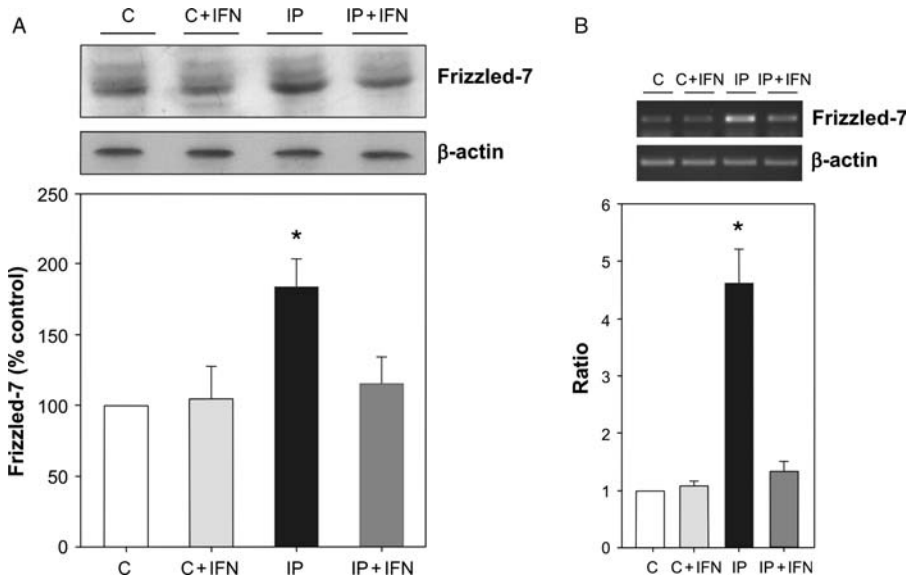

Figure 6. IFN-α2b treatment reduces expression of Frizzled-7 protein and mRNA in preneoplastic livers. C: control rats, C + IFN: rats that received IFN-α2b 6.5×10^5 U/kg body weight, IP: rats with hepatic preneoplasia, IP + IFN: IP rats that also received IFN-α2b 6.5×10^5 U/kg body weight. (A) Analysis of Frizzled-7 protein in plasma membrane fraction by western blotting. Results are expressed as percentages of control group and are the mean ± SD of four independent experiments. (B) Analysis of Frizzled-7 mRNA transcript by sqRT-PCR. Densitometric analysis was performed in at least four independent experiments. Intensity of the bands were quantified and normalized to the corresponding β-actin levels. Ratios are presented in a graphical form and indicate the relative level of the target amplification product over the control sample after normalization to the housekeeping gene. $\star p < 0.05$ vs. C.

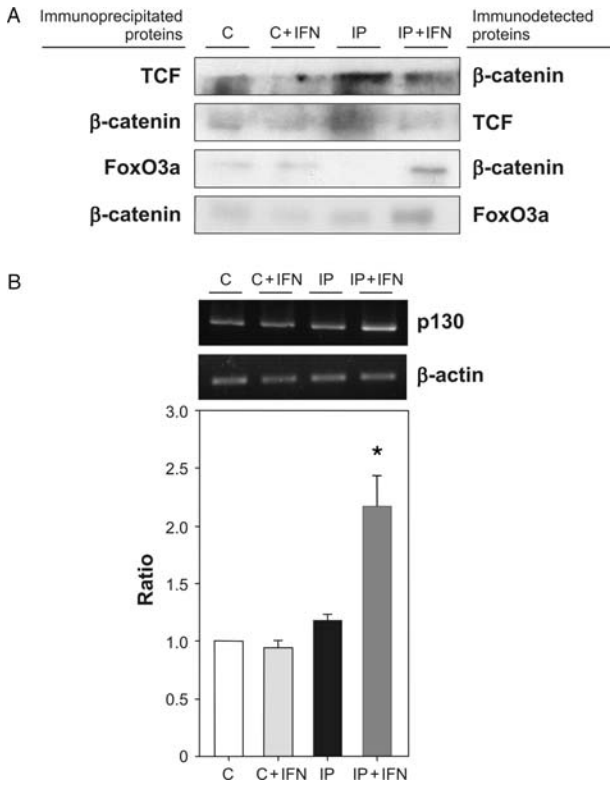

Figure 7. IFN-α2b treatment in preneoplastic livers reduces binding of β-catenin to TCF4 transcription factor and enhances binding of β-catenin to FoxO3a. C: control rats, C + IFN: rats that received IFN-α2b 6.5×10^5 U/kg body weight, IP: rats with hepatic preneoplasia, IP + IFN: IP rats that also received IFN-α2b 6.5×10^5 U/kg body weight. (A) Co-immunoprecipitation assays performed for β-catenin, TCF4, and FoxO3a. Immunoprecipitated and immunodetected proteins are indicated in the figure. (B) Expression levels of p130 mRNA transcript by sqRT-PCR. Densitometric analysis was performed in at least four independent experiments. Intensity of the bands were quantified and normalized to the corresponding β-actin levels. Ratios are presented in a graphical form and indicate the relative level of the target amplification product over the control sample after normalization to the housekeeping gene. $*p < 0.05$ vs. C.

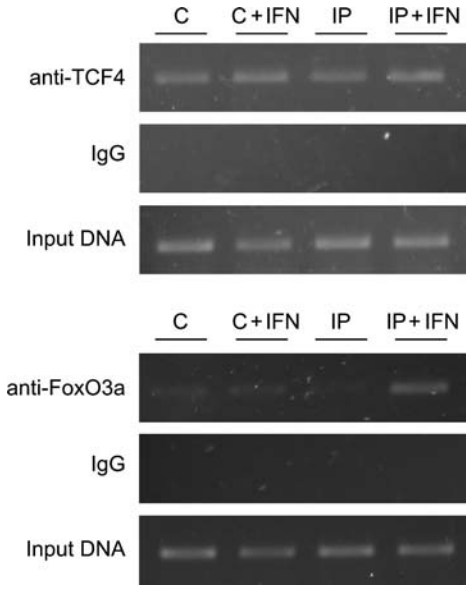

Figure 8. IFN-α2b treatment in preneoplastic livers enhances the interaction of FoxO3a transcription factor to its target gene promoter region. C: control rats, C + IFN: rats that received IFN-α2b 6.5×10^5 u/kg body weight, IP: rats with hepatic preneoplasia, IP + IFN: IP rats that also received IFN-α2b 6.5×10^5 u/kg body weight. ChIP were performed with the indicated antibodies, and PCR was performed with specific primers for the SP5 gene promoter region (upper panel) and for the p130 gene promoter region (lower panel). An anti-rabbit IgG ChIP was used as a negative control. Input DNA lanes represents PCR amplification of the total DNA samples before immunoprecipitation.

results indicate a lower phosphorylation rate of cytoplasmic β-catenin in IP rats.

As expected, we have also found upregulation of Cyclin D1, MMP-7, Axin 2, and SP5 transcripts only in livers from IP animals. Upregulation of Cyclin D1 was predicted since this protein is an important regulator of cell cycle progression, and its activity is required for G1 to S-phase transition. Overexpression of this gene has been associated with the development and progression of several cancers (Gautschi et al. 2007). In addition, it has been reported that overexpression of Cyclin D1 in tumor cells contributes with their resistance to cytotoxic drugs (Kornmann et al. 1999). In fact, inhibition of Cyclin D1 enhances the effects of several chemotherapeutic agents (Kornmann et al. 1999). In agreement with the present result, we have previously described a drug-resistance phenotype in isolated hepatocytes obtained from rat livers subjected to the initiation–promotion model (Parody et al. 2007). Thus, it is possible that the overexpression of Cyclin D1 could play a role in the drug-resistance phenotype of this model. MMP-7, a member of the matrix metalloproteinase family, acts as a specific proteolytic enzyme for degradation of certain components of the extracellular matrix. This protein was already shown to be important for the growth of early adenomas (Wilson et al. 1997) and its function is essential in more advanced stages such as tumor progression and metastasis, where an invasive growth is a highlight of these steps (Powell et al. 1993; Yamamoto et al. 1995). Hence, enhanced MMP-7 expression could be proposed as an indicator of potential tumor progression, invasiveness, and metastatic ability at a very early stage of hepatocarcinogenic development. It has been reported that the tumor suppressor Axin 2 is a target of Wnt signaling (Yan et al. 2001; Jho et al. 2002). The upregulation of Axin 2 showed in IP rats, which is known to be a negative regulator of free β-catenin (Behrens et al. 1998; Hart et al. 1998), could be an expression of a feedback preservation mechanism of the preneoplastic tissue, and might not be sufficient to prevent cytoplasmic β-catenin accumulation. SP5, a member of the SP1 transcription factor family and known target of Wnt signaling (Fujimura et al. 2007) was also over-

expressed. This protein seems to work as a transcriptional repressor, preventing the expression of genes involved in cell cycle G1 phase arrest such as p21 (Fujimura et al. 2007).

In order to determine the involvement of a mutated β-catenin protein in the activation of this pathway as was described for HCC (de La Coste et al. 1998; Miyoshi et al. 1998; Devereux et al. 1999; Calvisi et al. 2001), we performed a direct sequencing of amplicons encoding a region of exon 2 of rat liver β-catenin gene. Our results demonstrate that this sequence had no deletion or point mutations in any of the studied groups.

Even with a wild-type β-catenin, the pathway can also be triggered because of alterations in other components of the cascade signaling. The Frizzled protein family acts as a seven-span transmembrane receptor for Wnt proteins. It was recently reported an upregulation of the Frizzled-7 receptor in the presence of wild-type β-catenin in four murine transgenic models of hepatocarcinogenesis (Merle et al. 2005) and in human HCC (Merle et al. 2004) with activation of the Wnt/β-catenin/TCF pathway. Therefore, it was suggested that overexpression of Frizzled-7 could lead or contributes to activation of Wnt signaling. The obtained data in the present work showed a marked increase of this receptor in preneoplastic livers at mRNA and protein levels. Since it was reported that Frizzled-7 is also a target gene of the Wnt/β-catenin/TCF pathway (Willert et al. 2002), we presume that overexpression is rather a consequence than a cause of abnormal activation of the Wnt/β-catenin/TCF pathway. Further analysis would be necessary to confirm this presumption.

In previous works using the hepatocarcinogenic model, we have demonstrated that *in vivo* IFN-α2b treatment induces apoptosis in rat liver preneoplasia through a mechanism that involves hepatocyte production of ROS and TGF-β_1 (de Lujan et al. 2002; de Lujan et al. 2004; Quiroga et al. 2007; Alvarez et al. 2009). Moreover, we have shown that the apoptotic process is mediated by p38 MAPK via activation of NADPH oxidase (Quiroga et al. 2009). In the current study, results showed that *in vivo* IFN-α2b treatment did not prevent β-catenin dislocalization and cytoplasmic accumulation; however, it certainly inhibits activation of the pathway as measured by sqRT-PCR of four TCF target genes. In addition, results from immunoblotting and sqRT-PCR showed that Frizzled-7 levels in IP + IFN animals remained unchanged compared to control animals. These results reinforce our hypothesis that Frizzled-7 upregulation occurs as a result of the abnormal activation of the studied pathway.

In an attempt to get more insight into the regulation of Wnt/β-catenin/TCF pathway, FOXO transcription family has come into scene. Recent studies reported that FOXO interacts with β-catenin in a competitive manner with TCF, particularly under cellular oxidative stress conditions (Essers et al. 2005; Hoogeboom et al. 2008). Taking this into consideration and the fact that *in vivo* IFN-α2b treatment induces endogenous ROS formation in preneoplastic livers (Quiroga et al. 2007), we analysed interactions between β-catenin with TCF4 and FoxO3a and association of these transcription factors with their corresponding target gene promoters. Co-immunoprecipitation results showed that β-catenin/TCF4 interaction effectively occurs in preneoplastic livers and administration of IFN-α2b not only attenuates this interaction but also promotes β-catenin/FoxO3a association. Using ChIP assay, we verified that interaction of FoxO3a with the promoter region of its target gene is enhanced in preneoplastic livers treated with IFN-α2b. On the other hand, TCF4 remains associated with SP5 gene promoter region in all studied groups. It is known that TCF4 contains a conserved domain that binds DNA irrespective of its interaction with β-catenin; however, the transcriptional activity is blocked by the presence of a family of transcriptional repressors (Cavallo et al. 1998; Levanon et al. 1998). TCF4 must bind β-catenin for its transactivation and this interaction was verified by co-immunoprecipitation assays. In addition, it has been demonstrated that interaction of β-catenin with FOXO enhances its transcriptional activity (Essers et al. 2005; Hoogeboom et al. 2008), so we measured the expression of p130, a FOXO target gene whose main function is related to the maintenance of cell cycle arrest. Furthermore, it was suggested that p130 may exert a proapoptotic effect on certain tumor samples (Bellan et al. 2002). In this study, we found upregulation of p130 transcript in preneoplastic livers treated with IFN-α2b. These findings suggest that IFN-α2b treatment in preneoplastic livers decreases β-catenin/TCF interaction and consequently reduces TCF transcriptional activity probably via ROS induction. Furthermore, IFN-α2b-induced ROS production could stimulate β-catenin/FOXO interaction, thereby favoring cell cycle arrest and apoptosis. In fact, we have already reported that preneoplastic hepatocytes from animals treated with IFN-α2b showed G_1 phase arrest (de Lujan et al. 2002).

Collectively, our data demonstrate that the canonical Wnt/β-catenin/TCF signaling pathway is activated at a very early stage of the development of the hepatocarcinogenic process, even with a wild-type β-catenin. More importantly, *in vivo* IFN-α2b treatment could be an efficient therapy to attenuate Wnt/β-catenin/TCF signaling promoting diminution of preneoplastic foci by an apoptotic process.

The data presented here suggest a model in which IFN-α2b provides a link between the Wnt signaling pathway and the oxidative stress/FOXO pathway. The stress caused by IFN-α2b treatment described in previous works might strengthen the interaction

between FOXO and β-catenin and potentially inhibit the interaction with TCF. These findings may have important clinical implications, since β-catenin, TCF, or FOXO arise as molecular targets for novel therapies that can modify their interactions favoring cellular apoptosis over proliferation in patients that underwent a potential carcinogenic hepatic injury.

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