Interferon-α2b and transforming growth factor-β1 treatments on HCC cell lines: Are Wnt/β-catenin pathway and Smads signaling connected in hepatocellular carcinoma?

María Paula Ceballos, Juan Pablo Parody, María de Luján Alvarez, Paola Inés Ingaramo, Cristina Ester Carnovale, María Cristina Carrillo *

Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

ABSTRACT

Wnt/β-catenin pathway is often dysregulated in hepatocellular carcinoma (HCC). Activated β-catenin accumulates in the cytosol and nucleus and forms a nuclear complex with TCF/LEF factors like TCF4. Interferon-α (IFN-α) has recently been recognized to harbor therapeutic potential in prevention and treatment of HCC. Transforming Growth Factor-β1 (TGF-β1) is a mediator of apoptosis, exerting its effects via Smads proteins. One mode of interaction between Wnt/β-catenin and TGF-β1/Smads pathways is the association of Smads with β-catenin/TCF4. In this study we analyzed the effects of IFN-α2b and TGF-β1 treatments on Wnt/β-catenin pathway, Smads proteins levels, β-catenin/TCF4/Smads interaction and proliferation and apoptotic death in HepG2/C3A and Huh7 cell lines. IFN-α2b and TGF-β1 attenuated Wnt/β-catenin signal by decreasing β-catenin and Frizzled7 receptor proteins contents and the interaction of β-catenin with TCF4. Truncated β-catenin form present in C3A cell line also diminished after treatments. Both cytokines declined Smads proteins and their interaction with TCF4. The overall cellular response to cytokines was the decrease in proliferation and increase in apoptotic death. Treatment with Wnt3a, which elevates β-catenin protein levels, also generated the increment of Smads proteins contents when comparing with untreated cells. In conclusion, IFN-α2b and TGF-β1 proved to be effective as modulators of Wnt/β-catenin pathway in HCC cell lines holding both wild-type and truncated β-catenin. Since the inhibition of β-catenin/TCF4/Smads complexes formation may have a critical role in slowing down oncogenesis, IFN-α2b and TGF-β1 could be useful as potential treatments in patients with HCC.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Wnt/β-catenin is one of the most frequently dysregulated pathways responsible for initiating and sustaining a hepatocellular carcinoma (HCC). This pathway has a central role in embryogenesis and in adult tissue homeostasis [1]. Under normal circumstances, in the absence of Wnt receptor ligands, β-catenin is localized at the cell membrane in a complex with E-cadherin. In addition, free cytoplasmic β-catenin is recognized and phosphorylated by a destruction complex consisting of adenomatous polyposis coli, axin, glycogen synthase kinase 3β (GSK-3β), and casein kinase I (CK1). Inside this complex, CK1 phosphorylates β-catenin at Ser 45 as a priming site followed by phosphorylation of Thr 41, Ser 37 and Ser 33 residues by GSK-3β. Phosphorylated β-catenin is then recognized for ubiquitination and proteasomal degradation [2]. Wnt receptor complexes at the cell surface are composed of a seven transmembrane domain Frizzled protein and a single-pass transmembrane protein of the LDL receptor-related protein family. Once Wnt ligands engage this complex, a signal is transduced that inactivates the β-catenin destruction complex and so permits unphosphorylated β-catenin to accumulate in both cytoplasm and nucleus [3]. In the nucleus, β-catenin forms complexes with members of the TCF/LEF family of DNA binding proteins and thereby modulates the transcription of numerous target genes [4].

The genetic modifications in the Wnt/β-catenin pathway reported in human HCC led to an anomalous activation of this signaling manifested by a post-transcriptional stabilization of β-catenin. Most alterations are activating mutations in the GSK-3β

* Corresponding author at: Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Suipacha 570, 2000-Rosario, Argentina. Tel.: +54 341 4305799; fax: +54 341 4399473.

E-mail addresses: ceballos@ifise-conicet.gov.ar (M.P. Ceballos), parody@ifise-conicet.gov.ar (J.P. Parody), alvarez@ifise-conicet.gov.ar (M.d.L. Alvarez), paolaingar@gmail.com (P.I. Ingaramo), ccarnova@fbioyf.unr.edu.ar (C.E. Carnovale), carrillo@ifise-conicet.gov.ar (M.C. Carrillo).

and CK1 phosphorylation regions of the β-catenin gene [5,6]. However, there could be changes in other components of the pathway. For instance, over-expression of Frizzled7 receptor was reported in human HCC, and it was suggested that contributes to wild-type β-catenin stabilization and accumulation [7,8].

On the other hand, transforming growth factor-β1 (TGF-β1) is an important physiological mediator of apoptosis both in normal and neoplastic liver, and exerts its various effects via two transmembrane serine/threonine kinases known as type I and type II receptors [9]. The ligand-activated type II receptor associates with, phosphorylates, and activates the type I receptor, which in turn phosphorylates Smads2 and 3 proteins, members of the Smad family of signal transducers. These activated Smads then associate with Smad4 and translocate to the nucleus, where they regulate transcription by associating with nuclear transcription factors and/or by binding directly to DNA. The inhibitory Smad7 bind stably TGF-β1 receptors and interfere with ligand-induced phosphorylation of Smads2 and 3 [10]. After TGF-β1 stimulation, Smad7 translocates out of the nucleus to the plasma membrane to exert its inhibitory functions, serving as an autoregulatory negative feedback function in TGF-β1 signaling [11].

Both Wnt/β-catenin and TGF-β1/Smads pathways coordinately regulate pattern formation during embryogenesis as well as tumor progression [12–15]. Although many groups have studied the integration of Wnt/β-catenin and TGF-β1/Smads signals during embryonic development [12,13,16], the cross-talk between these pathways in carcinogenesis remains largely unexplored. One mode of interaction is by the association between Smads and the β-catenin/TCF/LEF complex in the nucleus that triggers the differential regulation of diverse target genes [14,17,18]. Smads2 and 3 and Smad4 have been shown to be directly associated with TCF/LEF while their association with β-catenin was found to be indirect [16]. On the other hand, Smad7 is not only directly associated with both β-catenin and TCF/LEF factors but also is required for the cooperation between β-catenin and the other Smads [17]. In addition, Edlund et al. [17] suggested that the specific effects of β-catenin on different promoters are moderated by alterations in the nuclear amounts of the β-catenin/TCF/LEF complex as well as of Smads2 and 3, Smad4 and Smad7.

Interferon-α (IFN-α) has been described as an essential cytokine for antiviral immunity with anti-proliferative and immunomodulatory effects [19]. Clinically, IFN-α has been used for the treatment of viral infections and others malignancies [20,21]. Furthermore, IFN-α has been shown to be useful for delaying progression of liver function impairment or prevention of HCC in patients with chronic hepatitis B or C [22,23]. Nowadays, some reports are also suggesting a potential efficacy of IFN-α therapy for the treatment of HCC [24–26].

Our group have been studied the IFN-α effects on Wnt/β-catenin signaling in a very early stage of rat hepatocarcinogenesis. We demonstrated that not only this pathway is activated in preneoplastic rat livers, but more important, that in vivo IFN-α2b treatment inhibits it [27]. Besides, we also showed an apoptotic effect of IFN-α2b on preneoplastic foci which was induced by oxidative stress and TGF-β1 release [28–30].

Since there are no published studies that integrate IFN-α, TGF-β1/Smads and Wnt/β-catenin signals, the aim of the present study was to analyze the effects of IFN-α2b and TGF-β1 treatments on Wnt/β-catenin pathway in HCC cell lines. Additionally, we explored the actions of these cytokines on Smads proteins levels and on the interaction between Smads and the nuclear complex β-catenin/TCF4. Furthermore, we investigated the consequences of IFN-α2b and TGF-β1 treatments on cell proliferation and apoptotic cell death. Finally, we evaluated the effect of an increased β-catenin protein level on the contents of Smads proteins.

2. Materials and methods

2.1. Reagents

IFN-α2b was kindly provided by PC-Gen (Buenos Aires, Argentina). Anti-Smad7 (MAB2029) antibody and recombinant TGF-β1 (240-B) and Wnt3a (1324-WN/CF) proteins were obtained from R&D Systems (Minneapolis, MN, USA). Anti-β-catenin (610154) antibody was from BD Transduction Labs (San Jose, CA, USA). Anti-active-β-catenin (#05-665) antibody was from Millipore (Temecula, CA, USA). Anti-Smad4 (#9515) antibody was from Cell Signaling Technology (Danvers, MA, USA). Anti-Frizzled7 (ab64636) antibody was obtained from Abcam (Cambridge, MA, USA). Anti-p-Smad2/3 (Ser 433/435; sc-11769), anti-TCF4 (sc-13027), anti-PCNA (proliferating cell nuclear antigen; sc-56) anti-Cytochrome c (sc-13156) and anti-Histone H1 (sc-8030) antibodies were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Anti-β-actin (A2228) and anti-α-tubulin (T5168) were from Sigma–Aldrich Corp (St. Louis, MO, USA). Phosphatase inhibitor Calyculin A (PHZ1044) was from BioSource International (Camarillo, CA, USA). All other chemicals were of the highest grade commercially available.

2.2. Cell lines and treatments

The human HCC cell lines HepG2/C3A (a clonal derivative of HepG2) and Huh7 were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. Cells were plated with the maintenance medium and the next day were starved by incubation in serum-free medium (SFM) for an hour at 37 °C. SFM is composed of Iscove's modified Dulbecco's medium (IMDM) and F-12 Nutrient Mixture (Ham) (1:1), 20 IU/mL penicillin, 20 μg/mL streptomycin and Insulin-Transferrin-Selenium supplement (Gibco, Carlsbad, CA, USA). After starvation, cells were treated with 100,000 U/mL IFN-α2b, 100,000 U/mL IFN-α2b plus 2 ng/mL recombinant TGF-β1, 2 ng/mL recombinant TGF-β1, or left untreated. Previous studies from our group have shown that IFN-α2b induces TGF-β1 production and secretion from preneoplastic hepatocytes [28–30]. However, this phenomenon does not occur in C3A and Huh7 cell lines, since IFN-α2b stimulus did not modify TGF-β1 secretion (data not shown). Taking this result into consideration, in the present work we have added recombinant TGF-β1 to the culture media.

In order to stimulate Wnt/β-catenin pathway, cells were treated with 20 ng/mL recombinant Wnt3a.

2.3. Cell proliferation assay

The sensitivity of HCC cell lines to IFN-α2b, TGF-β1 or both factors was determined by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a density of 6×10^3 cells/well for C3A and 2×10^3 cells/well for Huh7, and treated with IFN-α2b, TGF-β1 or both factors, for up to 6 days. After each treatment day, cells were incubated with the proliferation reagent and absorption at 490 nm was measured in a LD400 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

2.4. Caspase-3 activity assay

Caspase-3 activity was determined using EnzChek® Caspase-3 Assay Kit #1 (Molecular Probes Inc, Eugene, OR, USA), according to the manufacturer's suggestions. Briefly, cells were seeded in 10 cm

plates at a density of 4×10^6 cells/plate for C3A and 2×10^6 cells/plate for Huh7, treated with IFN-α2b, TGF-β1 or both factors during 48 h and then lysed and sonicated. After differential centrifugation, the cytosolic fraction from each sample was mixed with substrate solution. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm in a DTX 880 multimode detector (Beckman Coulter Inc., Fullerton, CA, USA).

2.5. Preparation of total cell lysates and subcellular fractions

Cells were seeded in 10 cm plates at a density of 4×10^6 cells/plate for C3A and 2×10^6 cells/plate for Huh7 and treated with IFN-α2b, TGF-β1 or both factors during 48 hs. In one experiment, cells were treated with Wnt3a. Total cell lysates and cytosolic and nuclear extracts were prepared as previously described [28,30]. Total cell lysates used for co-immunoprecipitation experiments were obtained in non-denaturing lysis buffer containing 20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1.5 nM Calyculin A, and protease inhibitors.

2.6. Protein concentration determination

The protein concentration was determined by Lowry method [31], using bovine serum albumin as a standard.

2.7. Western blot analysis

β-catenin, active-β-catenin, p-Smad2/3, Smad7 and Smad4 proteins were detected in cytosolic and nuclear extracts (15 μg of protein per lane). Cytochrome c and PCNA proteins were analyzed in cytosolic and nuclear extracts, respectively (15 μg of protein per lane). Frizzled7 receptor was explored in total cell lysates (25 μg of protein per lane). Samples were resolved by SDS-PAGE and electroblotted onto polyvinyl difluoride (PVDF) membranes (PerkinElmer Life Sciences, Boston, MA, USA). Immunoblots were blocked and incubated overnight at 4 °C with the primary antibody (1:2000 for anti-β-catenin and anti-active-β-catenin; 1:300 for anti-p-Smad2/3; 1:500 for anti-Smad7 and anti-PCNA; 1:1000 for anti-Smad4; 1:3000 for anti-Cytochrome c and anti-Frizzled7). Finally, they were incubated with peroxidase-conjugated secondary antibody (1:2000, Thermo Fisher Scientific, Rockford, IL, USA) and bands were detected by enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific). The immunoreactive bands were quantified using the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). Equal loading and transference of protein was checked by detection of β-actin and by Ponceau S staining (latter data not shown) of the membranes. The purity of cytosolic and nuclear extracts was confirmed by detecting α-tubulin and Histone H1, respectively (5 μg of protein per lane).

2.8. Co-immunoprecipitation assay

500 μg of total cell lysates was subjected to immunoprecipitation with 3 μg of anti-TCF4 antibody. Proteins bound to Protein A-Sepharose® beads (P3391; Sigma–Aldrich Corp., St. Louis, MO, USA) were washed and resolved by 12% SDS-PAGE. Gels were blotted onto PVDF membranes and incubated with specific primary and secondary antibodies at appropriate dilutions. The immunoreactive bands were detected as described above.

2.9. Statistical analysis

Results were expressed as mean ± SE. Significance in differences was tested by one-way ANOVA, followed by Tukey's test. In the case of
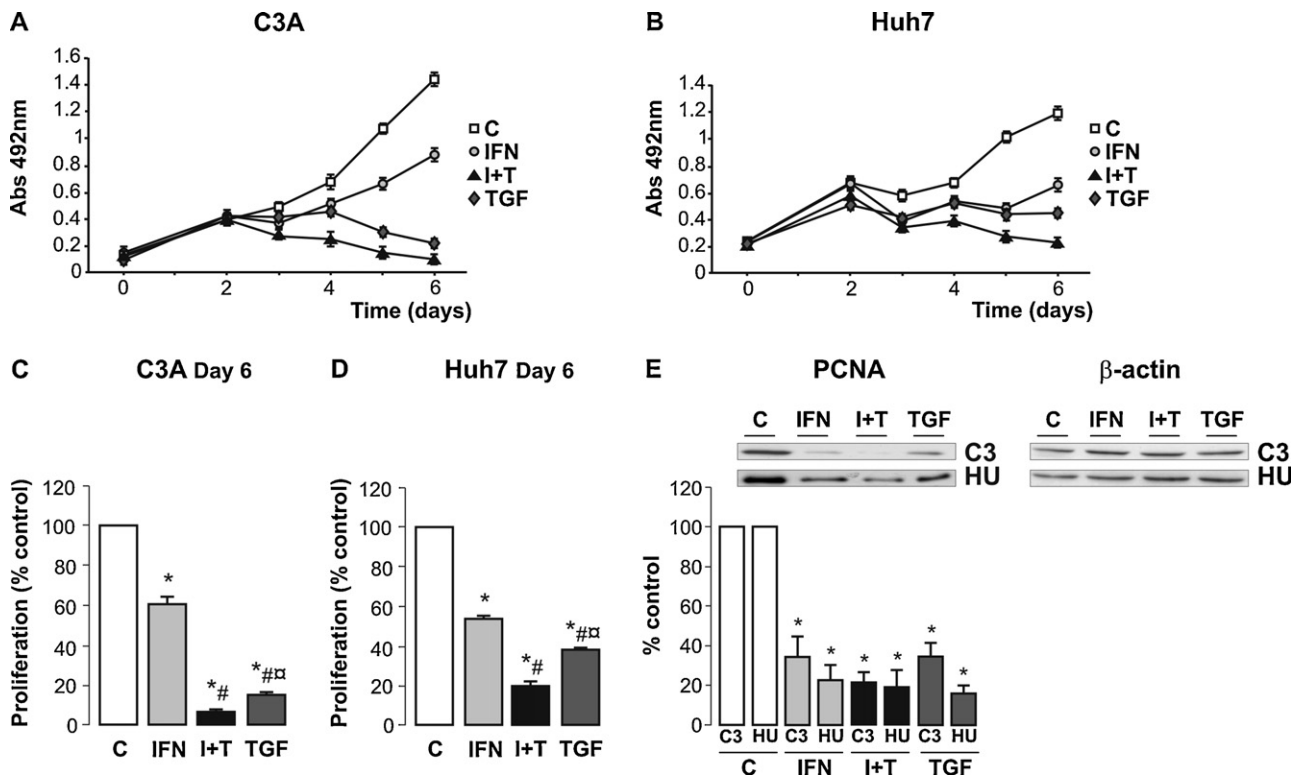
Fig. 1. Antiproliferative effects of IFN-α2b and TGF-β1 on HCC cell lines.
Proliferation assay was evaluated over 6 days (A: C3A, B: Huh7) by treating HCC cells with IFN-α2b (100,000 U/mL), TGF-β1 (2 ng/mL) or a combination of both cytokines. Results are expressed in percent values at day 6 with control cells arbitrarily considered 100% (C: C3A, D: Huh7). E) PCNA levels were assessed in nuclear extracts. β-actin was probed as loading control. Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. Statistical significance symbols were omitted in A and B figures in order to avoid confusion. C: control (untreated) cells, IFN: cells treated with IFN-α2b, I + T: cells treated with IFN-α2b plus TGF-β1, TGF: cells treated with TGF-β1, C3: C3A HCC cell line, HU: Huh7 HCC cell line. Mean ± SE; $n \geq 4$. *$p < 0.05$ vs. C, #$p < 0.05$ vs. IFN, ¤$p < 0.05$ vs. I + T.

Wnt3a experiment, significance in differences was tested by Student's *t*-test. Differences were considered significant when the p value was <0.05.

3. Results

3.1. IFN-α2b and TGF-β1 treatments cause a diminution in HCC cell lines proliferation

Both C3A and Huh7 cell lines treated with IFN-α2b, TGF-β1 and the combination of both factors, significantly reduced cellular proliferation compared with untreated cells, from the third day of treatment onwards (Fig. 1A and B). At day 6 (Fig. 1C and D) there were significant differences between all treatments in both cell lines.

On the other hand, we also quantified PCNA, by western blot. PCNA is an auxiliary protein of DNA polymerase δ that is essential for DNA replication during S-phase [32]. Treatments with IFN-α2b and/or TGF-β1 during 48 h significantly decreased PCNA protein levels in nuclear extracts of both HCC cell lines (Fig. 1E).

3.2. IFN-α2b and TGF-β1 treatments trigger the apoptotic mechanism in HCC cell lines

We evaluated activity of Caspase 3 [33] and mitochondrial release of Cytochrome c [34] as parameters of the apoptotic mechanism triggered by treatments.

As shown in Fig. 2A and B 48 h of treatment with IFN-α2b and/or TGF-β1 significantly increased Caspase 3 activity compared with untreated cells. Also, we observed significant differences between all treatments on the enzyme activity.

Furthermore, the content of Cytochrome c protein significantly increased in cytosolic extracts of C3A and Huh7 after 48 h of stimulation with IFN-α2b and/or TGF-β1 (Fig. 2C). As can be seen from the figure, the combined treatment produces the greatest increment in Cytochrome c protein levels (as well as the highest Caspase 3 activities).

3.3. Attenuation of Wnt/β-catenin pathway by IFN-α2b and TGF-β1 cytokines in HCC cell lines

HepG2/C3A cells harbor two species of β-catenin: wild-type 92-kDa form, and a more prominent truncated 75-kDa form that lacks phosphorylation sites critical for degradation. Thus, HepG2 presents abnormal activity of Wnt/β-catenin signaling since the truncated form cannot be inactivated [35]. In addition, it was found that HepG2 over-express the Frizzled7 receptor, by comparing mRNA levels with those found in normal liver [7].

On the other hand, Huh7 presents an aberrant pathway by having contents of Frizzled7 mRNA and protein much higher than that of HepG2, which activates the signaling in the presence of wild-type β-catenin [7,36].

Cytoplasmic and nuclear accumulation of β-catenin is a main characteristic of Wnt/β-catenin signaling dysregulation. Since the defective activation is accomplished by an unphosphorylated β-catenin, expression of β-catenin and active-β-catenin (unphosphorylated on Ser37 or Thr41) was analyzed by western blotting in cytosolic and nuclear extracts of C3A and Huh7 after 48 h of IFN-α2b and/or TGF-β1 stimulation.

As shown in Fig. 3, levels of β-catenin (A and B) and active-β-catenin (C and D) significantly decreased after all treatments in cytosolic and nuclear extracts of both HCC cell lines. Additionally, in the case of C3A, this behavior was found not only for the wild-type form but also for the truncated form of β-catenin (Fig. 3A).

Because Frizzled7 receptor over-expression implies an activated Frizzled7-mediated Wnt/β-catenin signaling, we also chose to
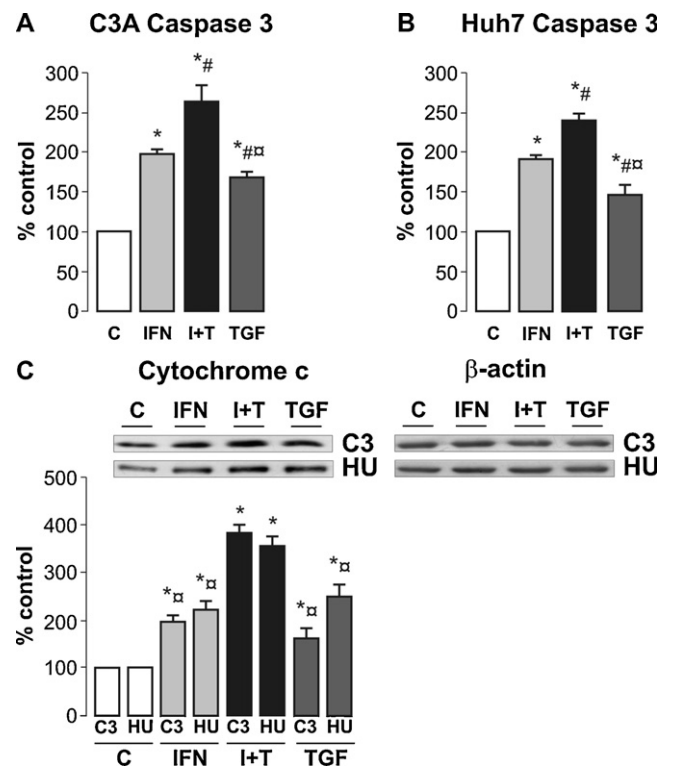
Fig. 2. Apoptosis induction in HCC cell lines after treatments with IFN-α2b and TGF-β1.

Caspase-3 activity was measured in cytosolic fractions and expressed in percent values with control cells arbitrarily considered 100%, for C3A (A) and Huh7 (B) cell lines. (C) Cytochrome c levels were detected in cytosolic fractions. β-actin was probed as loading control. Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. C: control (untreated) cells, IFN: cells treated with IFN-α2b, I + T: cells treated with IFN-α2b plus TGF-β1, TGF: cells treated with TGF-β1, C3: C3A HCC cell line, HU: Huh7 HCC cell line. Mean ± SE; $n \geq 4$. *$p < 0.05$ vs. C, #$p < 0.05$ vs. IFN, ¤$p < 0.05$ vs. I + T.

evaluate the effect of 48 h IFN-α2b and/or TGF-β1 treatment on the level of this receptor. As can be seen from Fig. 3E, Frizzled7 protein contents significantly decreased with all treatments in total cell lysates of both HCC cell lines.

3.4. IFN-α2b and TGF-β1 cytokines lead to a decrease of Smads proteins levels in HCC cell lines

The next step was to investigate the effects of IFN-α2b and TGF-β1 on the Smads intermediates. Both HCC cell lines express TGF-β1 surface receptors and Smads proteins and thus present a functional TGF-β1/Smads pathway [10,37].

Currently, the outcome of TGF-β1 stimulation on Smads levels has only been studied in the first hours of treatment. Therefore, we decided to determine the actions on Smads proteins after 48 h of treatment, when significant changes on proliferative and apoptotic mechanisms are installed (see previous results in Sections 3.1 and 3.2).

The amounts of phosphorylated Smads2 and 3, Smad4 and Smad7, significantly decreased in cytosolic and nuclear extracts of both HCC cell lines treated during 48 h with IFN-α2b and/or TGF-β1 when comparing with those found in no treated cells (Fig. 4).

### 3.5. Treatments with IFN-α2b and TGF-β1 trigger the β-catenin/TCF4/Smads nuclear complex dismantling in HCC cell lines

To analyze TCF4/β-catenin and TCF4/Smads associations under the effects of cytokine treatments, whole cell lysates were
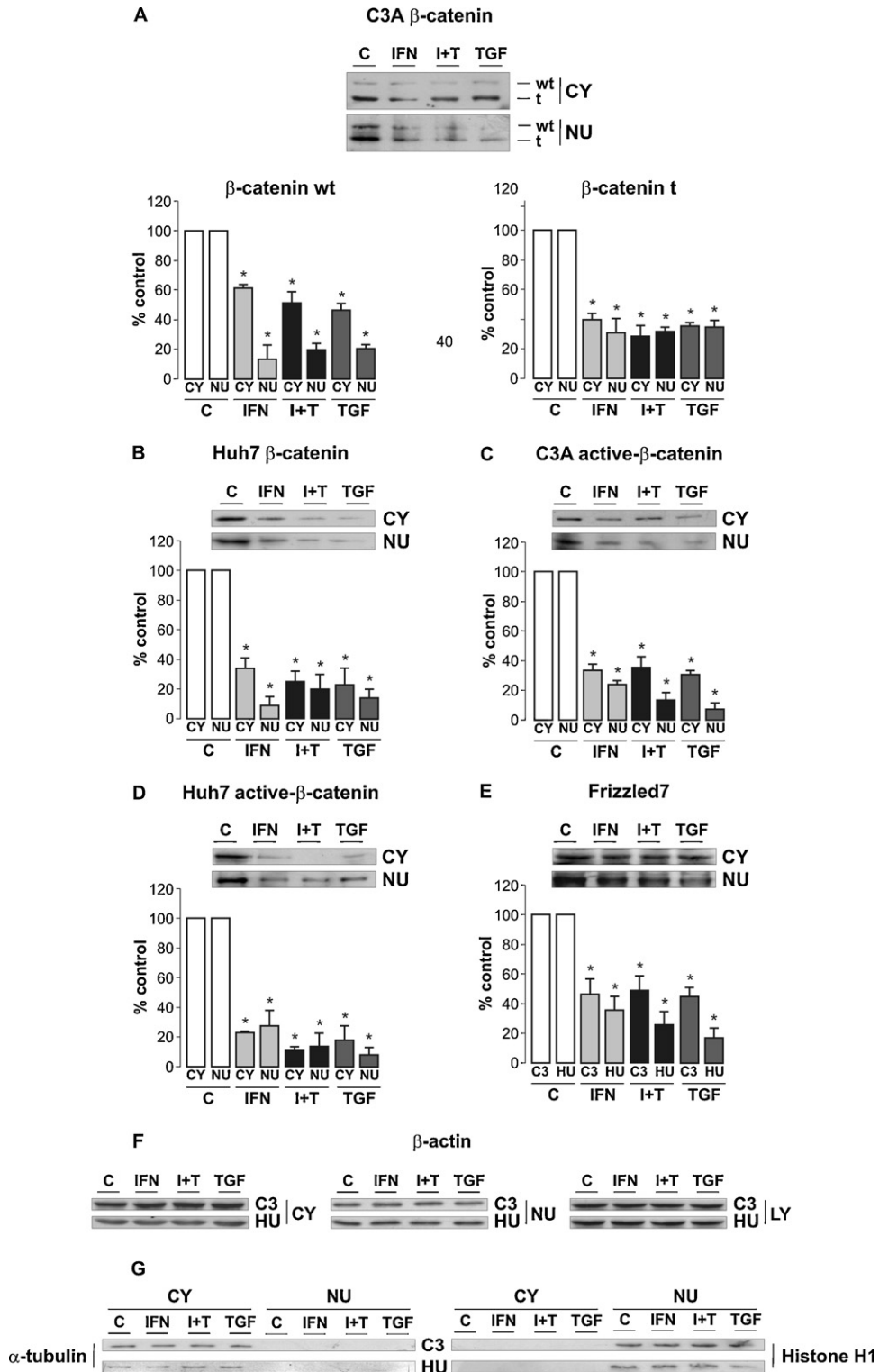
Fig. 3. IFN-α2b and TGF-β1 effectively attenuate Wnt/β-catenin signaling cascade in HCC cell lines.

Levels of β-catenin (A: C3A, B: Huh7) and active-β-catenin (C: C3A, D: Huh7) were explored in cytosolic and nuclear extracts. Frizzled7 receptor levels (E) were evaluated in total cell lysates. In all cases β-actin (F) was probed as loading control. The purity of cytosolic and nuclear extracts was confirmed by detecting α-tubulin and Histone H1, respectively (G). Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. C: control (untreated) cells, IFN: cells treated with IFN-α2b, I + T: cells treated with IFN-α2b plus TGF-β1, TGF: cells treated with TGF-β1, CY: cytosolic extract, NU: nuclear extract, LY: total cell lysate, wt: wild-type β-catenin, t: truncated β-catenin, C3: C3A HCC cell line, HU: Huh7 HCC cell line. Mean ± SE; $n \geq 4$. $^{*}p < 0.05$ vs. C.
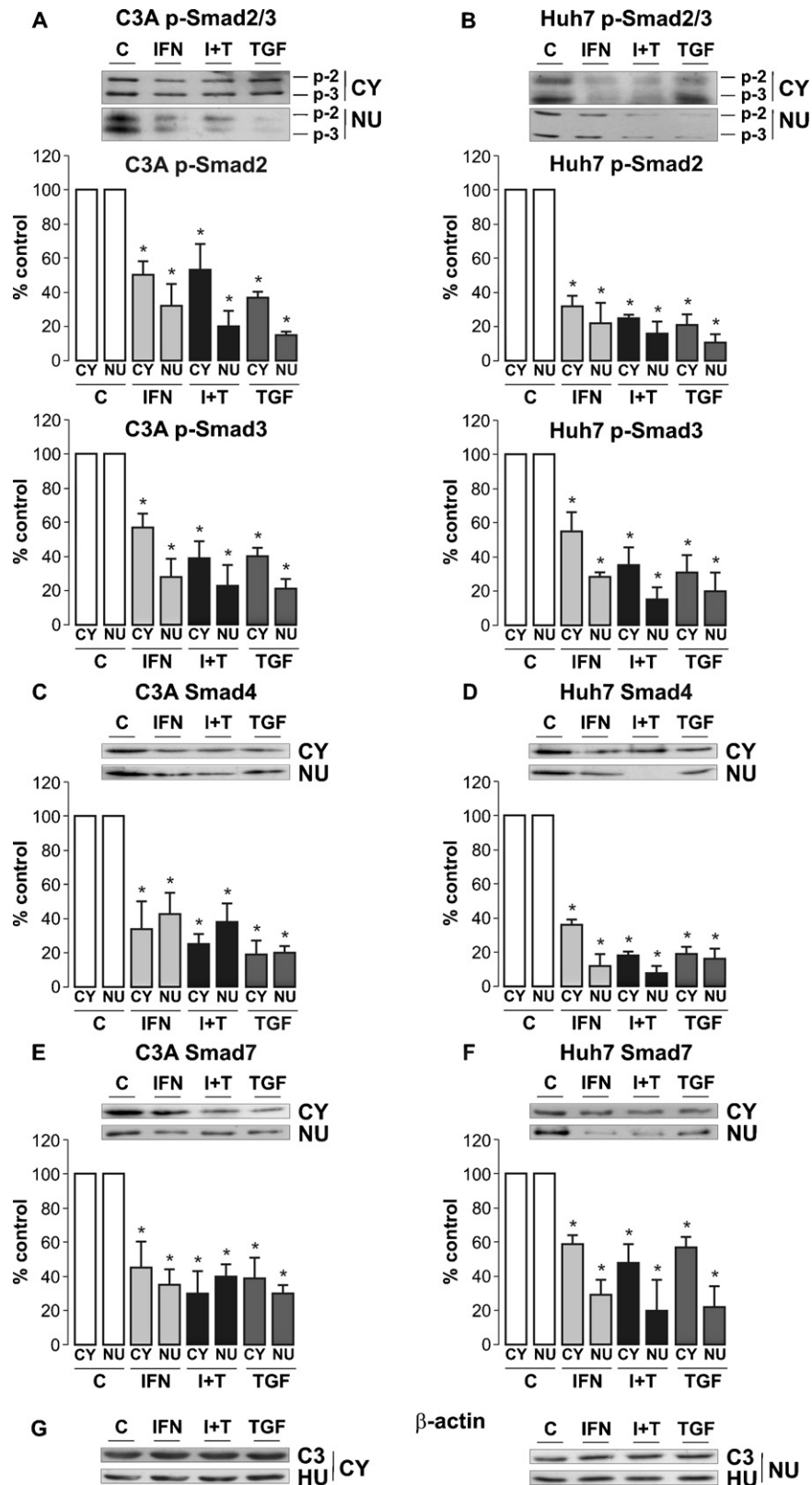
Fig. 4. Decline in Smads proteins levels by IFN-α2b and TGF-β1 treatments in HCC cell lines.Levels of p-Smad2/3 (A: C3A, B: Huh7), Smad4 (C: C3A, D: Huh7) and Smad7 (E: C3A, F: Huh7) were measured in cytosolic and nuclear extracts. In all cases β-actin (G) was probed as loading control. Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. C: control (untreated) cells, IFN: cells treated with IFN-α2b, I + T: cells treated with IFN-α2b plus TGF-β1, TGF: cells treated with TGF-β1, CY: cytosolic extract, NU: nuclear extract, p-2: phosphorylated form of Smad2 protein, p-3: phosphorylated form of Smad3 protein, C3: C3A HCC cell line, HU: Huh7 HCC cell line. Mean ± SE; $n \geq 4$. *$p < 0.05$ vs. C.
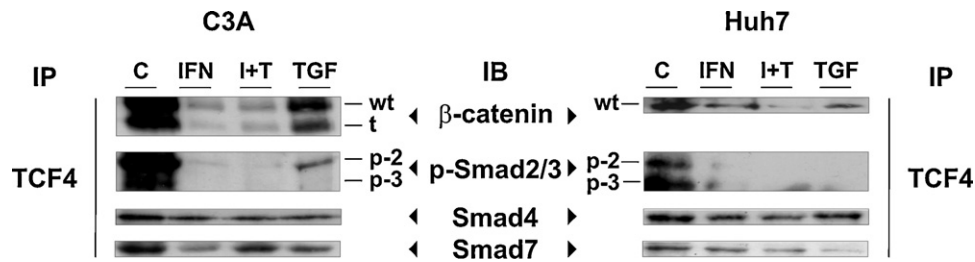
Fig. 5. Treatments with IFN-α2b and TGF-β1 prevent the assembly of β-catenin/TCF4/Smads nuclear complex in HCC cell lines.
Proteins from total cell lysates were immunoprecipitated (IP) with anti-TCF4 and were analyzed by immunoblotting (IB) with anti-β-catenin, anti-p-Smad2/3, anti-Smad4 and anti-Smad7 antibodies. C: control (untreated) cells, IFN: cells treated with IFN-α2b, I + T: cells treated with IFN-α2b plus TGF-β1, TGF: cells treated with TGF-β1, wt: wild-type β-catenin, t: truncated β-catenin, p-2: phosphorylated form of Smad2 protein, p-3: phosphorylated form of Smad3 protein.

subjected to immunoprecipitation with anti-TCF4 antibody. Treatments with IFN-α2b and/or TGF-β1 during 48 h attenuate the binding of TCF4 factor with β-catenin and with p-Smad2/3, Smad4 and Smad7 (Fig. 5).

3.6. Wnt3a treatment produces an increment in Smads proteins levels in HCC cell lines

Since 48 h cytokines stimulation of HCC cell lines led to a decrease in Smads levels, we wanted to explore the possibility that Smads proteins levels could be modulated by Wnt/β-catenin pathway status. For achieving this, we performed a treatment with recombinant Wnt3a to increase β-catenin levels. If our hypothesis was correct, we should find an increase in the contents of Smads proteins.

As speculated, 48 h of Wnt3a treatment not only significantly increased the amount of active-β-catenin protein (Fig. 6A) but also the contents of Smads proteins (Fig. 6B–D) in cytosolic and nuclear extracts of both HCC cell lines. In addition, Wnt3a treatment also caused a significant increment in PCNA protein levels in nuclear extracts of C3A and Huh7 cells (Fig. 6E).

4. Discussion

While Wnt/β-catenin signal cascade is an important player in liver development and growth, it is also known for its oncogenicity in HCC [1]. It has been increasingly important to understand the mechanisms underlying inhibition of growth and induction of apoptosis in cancer cells. In this way, both IFN-α and TGF-β present anti-proliferative and pro-apoptotic properties [9,19,20] and may serve as therapeutic strategies for HCC.

On the other hand, involvement of Wnt/β-catenin and TGF-β/Smads signaling in embryonic development, tissue homeostasis, and pathologic conditions suggests that these pathways may function within a complex interdependent and mutually regulating network.

Taking these data together, the elucidation of the signals induced by IFN-α and TGF-β in human liver tumor cells, and their possible cross-talks with other intracellular signals, would have relevance in the future design of therapeutic tools to balance the cellular responses in favor of liver tumor suppression. To gain mechanistic insights into these cooperative signals, we analyzed the effects of IFN-α2b and TGF-β1 on Wnt/β-catenin pathway and Smads intermediates in HepG2/C3A and Huh7 HCC cell lines.

To begin with this analysis, we assessed the impact of IFN-α2b and TGF-β1 on cell proliferation and apoptosis. IFN-α2b or TGF-β1 stimulations not only decreased cellular proliferation but also increased apoptotic cell death. The apoptotic and anti-proliferative effects of both cytokines separately have already been reported in HepG2 and Huh7 [37–39]. More interestingly, we demonstrated here that the combined treatment increased these effects. Until now, combined treatment with both cytokines has only been used

to analyze their impact on proliferation in human melanoma cell lines [40].

Recently, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was identified as an important mediator of TGF-β1- [41] and IFN-α- [42] induced apoptosis in hepatoma cells. It is likely that the effects described here may be mediated by TRAIL. Further studies are needed to go deeper in the involved mechanism.

Aberrant Wnt/β-catenin signaling in HCC is exemplified by β-catenin over-expression or activation. As a consequence, unphosphorylated β-catenin level is high in the cytoplasm and nuclei, activating the transcription of several target genes [1,4]. In our study, significant reductions of total and unphosphorylated β-catenin were found in cytosolic and nuclear extracts of both HCC cell lines after 48 h stimulation with IFN-α2b and/or TGF-β1. Moreover, it was found that all treatments caused a drop in the levels of β-catenin bound to TCF4 in these HCC cell lines. This alteration in the composition of the nuclear complex could implicate less transcription of Wnt/β-catenin target genes.

There is not much knowledge connecting IFN-α and Wnt/β-catenin signaling. Masuda et al. [43] reported that the expression and interaction between E-cadherin and β-catenin are potentially modulated by IFN-α. Recently, Thompson et al. [44] found that Pegylated-Interferon-α2a decreased the transcriptional activity of β-catenin/TCF4 in HepG2 and Huh7, supporting the efficacy of utilizing this interferon to inhibit Wnt/β-catenin pathway in HCC.

On the other hand, similarly to our results, treatment with TGF-β1 during 48 h interferes with β-catenin/TCF4 binding in chondrocyte cell cultures [45]. In another work, breast cancer cells treated with TGF-β1 during 24 h showed a decrease in β-catenin protein levels [15].

A potentially relevant observation in our study was the diminution in the truncated form of β-catenin in C3A after treatments. Since this form lacks the Ser/Thr residues that participate in β-catenin phosphorylation by GSK-3β and CK1, another mechanism has to be implicated in the attenuation of Wnt/β-catenin signal by IFN-α and TGF-β in this HCC cell line. Further analysis would be necessary to confirm this presumption.

Besides, given that it is proposed that Frizzled7 over-expression is frequently implicated in hepatocarcinogenesis [7,8] we decided to evaluate treatments effect on this receptor protein contents. As a result of 48 h stimulation with IFN-α2b and/or TGF-β1, the amount of Frizzled7 significantly decreased in total cell lysates of C3A and Huh7. In this way, there are no published studies that have analyzed the effect of these cytokines on Frizzled7 in HCC or others types of cancer. In this connection, we described [27] that the expression of this receptor was significantly increased at protein and transcript levels in preneoplastic rat livers compared with normal ones and that this increment was blocked in animals treated with IFN-α2b. Nevertheless, since it was reported that Frizzled7 is also a target gene of the Wnt/β-catenin signal cascade [4], we cannot discard the possibility that the reduction found in
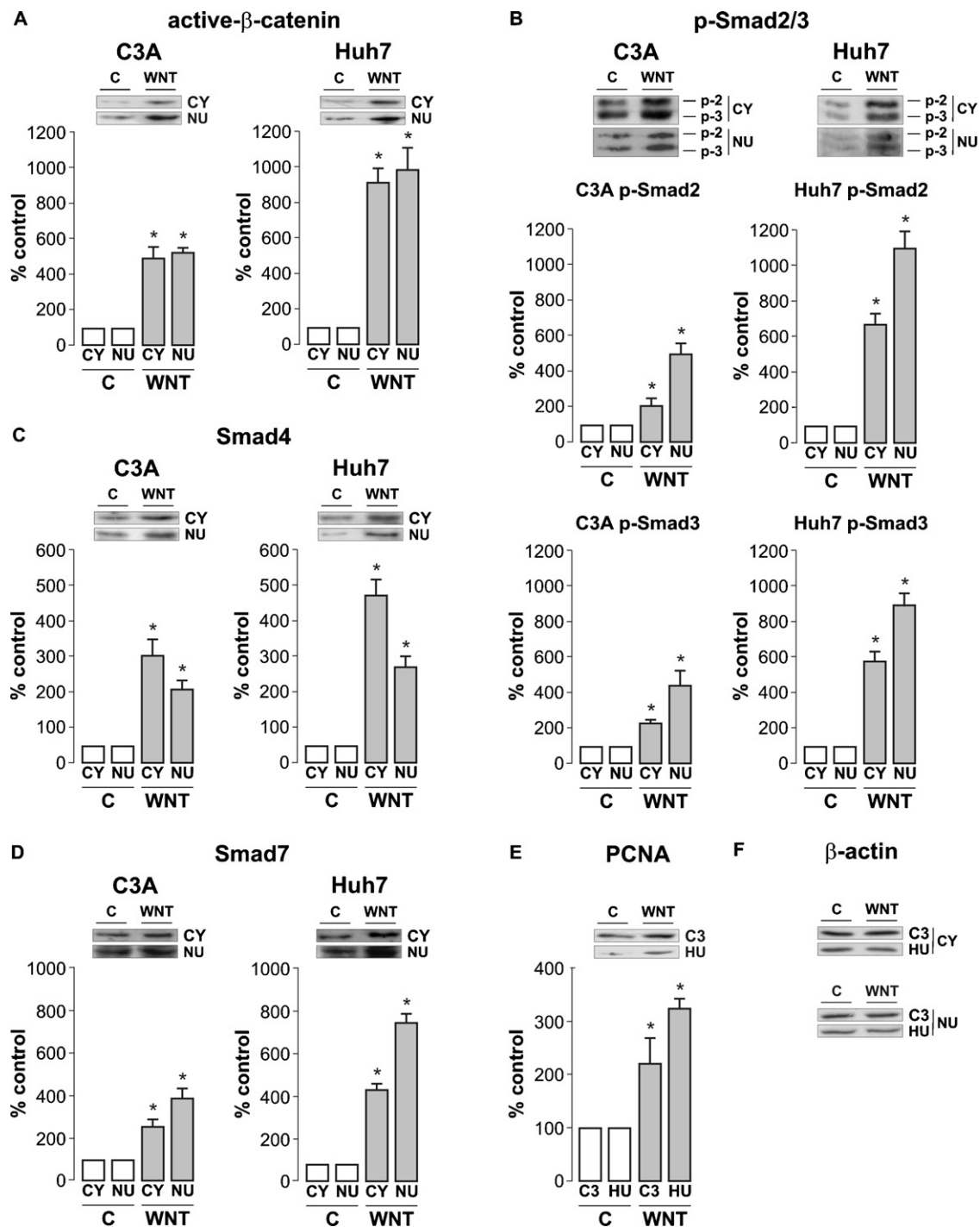
Fig. 6. Wnt3a treatment induces an increment in Smads and PCNA proteins levels in HCC cell lines.Levels of active-β-catenin (A), p-Smad2/3 (B), Smad4 (C) and Smad7 (D) were measured in cytosolic and nuclear extracts. PCNA levels (E) were detected in nuclear extracts. In all cases β-actin (F) was probed as loading control. Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. C: control (untreated) cells, IFN: cells treated with IFN-α2b, I + T: cells treated with IFN-α2b plus TGF-β1, TGF: cells treated with TGF-β1, CY: cytosolic extract, NU: nuclear extract, C3: C3A HCC cell line, HU: Huh7 HCC cell line, p-2: phosphorylated form of Smad2 protein, p-3: phosphorylated form of Smad3 protein. Mean ± SE; $n \geq 4$. *$p < 0.05$ vs. C.

Frizzled7 protein could be a consequence of treatments effect in attenuating this pathway.

After recognition of the treatments impact on Wnt/β-catenin pathway, we decided to analyze the effects of IFN-α2b and TGF-β1 on Smads proteins: 48 h stimulation with IFN-α2b and/or TGF-β1 caused a significant diminution in the levels of phosphorylated Smads 2 and 3, Smad4 and Smad7 in cytosolic and nuclear extracts of both HCC lines. Additionally, 48 h stimulation of C3A and Huh7 cells with IFN-α2b and/or TGF-β1

attenuates the binding of TCF4 factor with Smads proteins. These results were contrary to expectations, since the hallmark characteristic of TGF-β1 signaling activation is the increment of phosphorylated Smads 2 and 3 and their association with Smad4 and the rise of Smad7. But the activation effect has only been studied at the first hours of TGF-β1 treatment (generally at 30 min and 1 h) in HepG2 and Huh7 cells lines [10,46,47]. Thus, it seems that the effect of longer TGF-β1 treatments modifies the behavior of Smads proteins.

Insufficient information is available concerning TCF4/Smads association and their impact on carcinogenesis in HCC cell lines. Labbé et al. [48] and Letamendia et al. [12] reported the interaction between Smads 2, 3 and 4 and TCF/LEF in HepG2. Additionally, and similar to our findings, treatment with TGF-β1 in HepG2 reduced the amount of Smad4 protein bound to TCF/LEF and this was associated with the capacity of TGF-β1 of inhibiting cell proliferation [49]. In addition, to date, no study on IFN-α and Smads has been carried out. More insight could be gained by analyzing the amount of each Smad protein inside the β-catenin/TCF4 nuclear complex, since all Smads coexist in this complex and the balance between them could contribute to the overall cell response by differently regulating gene expression as suggested by Edlund et al. [17].

We treated HCC cell lines during 48 h with recombinant Wnt3a in order to turn-on Wnt/β-catenin signaling. This stimulation significantly increased the amounts of p-Smad2/3, Smad4 and Smad7 in cytosolic and nuclear extracts of C3A and Huh7, indicating that the activation of β-catenin signaling was triggering the increase of Smads proteins levels at 48 h. In this connection, Schohl and Fagotto [50] found an increase in p-Smad2 protein when they generated the activation of β-catenin signaling in Xenopus embryos. They concluded that the intensity and pattern of p-Smad2 was strongly dependent on β-catenin during earlier stages of Xenopus embryonic development. Knocking down β-catenin may confirm the relationship between Smads and β-catenin proteins levels.

In addition, we found an increase in PCNA protein levels after Wnt3a treatment in nuclear extracts of C3A and Huh7. Given that PCNA is essential for DNA replication during S-phase of cell cycle [32] its increment after β-catenin accumulation is probably accelerating DNA synthesis and promoting cellular proliferation.

Taken altogether, our findings clearly show a negative modulation of IFN-α2b and TGF-β1 on Wnt/β-catenin pathway. This attenuation was evidenced by a decrease in β-catenin and Frizzled7 receptor proteins levels in C3A and Huh7 and by a diminution in the amount of β-catenin bound to TCF4. Stimulation with both cytokines also caused a decrease in Smads protein contents and their association with TCF4. This treatments effect on Smads proteins seems to be linked to the decrease of β-catenin. Finally, the inhibition of β-catenin/TCF4/Smads complexes formation may have a critical role in slowing down oncogenesis, since the overall action of IFN-α2b and/or TGF-β1 treatments on both HCC cell lines was the diminution in cellular proliferation and increase in apoptotic cell death.

In conclusion, our results support the efficacy of inhibiting Wnt/β-catenin pathway in HCC cell lines through an IFN-α2b and TGF-β1 combined treatment, proving that is effective against either wild-type or truncated β-catenin. These findings open a wide therapeutic option for patients with HCC.

Disclosures

The authors have nothing to disclose.

Acknowledgements

References

[1] Takigawa Y, Brown AMC. Wnt signaling in liver cancer. Curr Drug Targets 2008;9:1013–24.

[2] Lee HC, Kim M, Wands JR. Wnt/Frizzled signaling in hepatocellular carcinoma. Front Biosci 2006;11:1901–15.

[3] Cadigan K. Wnt-β-catenin signaling. Curr Biol 2008;18:R943–7.

[4] Willert J, Epping M, Pollack JR, Brown PO, Nusse R. A transcriptional response to Wnt protein in human embryonic carcinoma cells. BMC Dev Biol 2002;2:2–8.

[5] Cui J, Zhou X, Liu Y, Tang Z, Romeih M. Wnt signaling in hepatocellular carcinoma: analysis of mutation and expression of beta-catenin, T-cell factor-4 and glycogen synthase kinase 3-beta genes. J Gastroenterol Hepatol 2003;18:280–7.

[6] Kim YD, Park CH, Kim HS, Choi SK, Rew JS, Kim DY, et al. Genetic alterations of Wnt signaling pathway associated genes in hepatocellular carcinoma. J Gastroenterol Hepatol 2008;23:110–8.

[7] Merle P, de la Monte S, Kim M, Herrmann M, Tanaka S, Von Dem Bussche A, et al. Functional consequences of frizzled-7 receptor overexpression in human hepatocellular carcinoma. Gastroenterology 2004;127:1110–22.

[8] Kim M, Lee HC, Tsedensodnom O, Hartley R, Lim YS, Yu E, et al. Functional interaction between Wnt3 and Frizzled-7 leads to activation of the Wnt/beta-catenin signaling pathway in hepatocellular carcinoma cells. J Hepatol 2008;48:780–91.

[9] Thorgeirsson SS, Teramoto T, Factor VM. Dysregulation of apoptosis in hepatocellular carcinoma. Semin Liver Dis 1998;18:115–22.

[10] Matsuzaki K, Date M, Furukawa F, Tahashi Y, Matsushita M, Sugano Y, et al. Regulatory mechanisms for transforming growth factor beta as an autocrine inhibitor in human hepatocellular carcinoma: implications for roles of Smads in its growth. Hepatology 2000;32:218–27.

[11] Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, et al. Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J Biol Chem 2001;276:12477–80.

[12] Letamendia A, Labbé E, Attisano L. Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. J Bone Joint Surg Am 2001;8:3-A. S31-9.

[13] Warner DR, Greene RM, Pisano MM. Cross-talk between the TGFbeta and Wnt signaling pathways in murine embryonic maxillary mesenchymal cells. FEBS Lett 2005;579:3539–46.

[14] Tian X, Du H, Fu X, Li K, Li A, Zhang Y. Smad4 restoration leads to a suppression of Wnt/beta-catenin signaling activity and migration capacity in human colon carcinoma cells. Biochem Biophys Res Commun 2009;380:478–83.

[15] Tang Y, Liu Z, Zhao L, Clemens TL, Cao X. Smad7 stabilizes beta-catenin binding to E-cadherin complex and promotes cell-cell adhesion. J Biol Chem 2008;283:23956–63.

[16] Nishita M, Hashimoto MK, Ogata S, Laurent MN, Ueno N, Shibuya H, et al. Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. Nature 2000;403:781–5.

[17] Edlund S, Lee SY, Grimsby S, Zhang S, Aspenström P, Heldin CH, et al. Interaction between Smad7 and beta-catenin: importance for transforming growth factor beta-induced apoptosis. Mol Cell Biol 2005;25:1475–88.

[18] Hirota M, Watanabe K, Hamada S, Sun Y, Strizzi L, Mancino M, et al. Smad2 functions as a co-activator of canonical Wnt/beta-catenin signaling pathway independent of Smad4 through histone acetyltransferase activity of p300. Cell Signal 2008;20:1632–41.

[19] Pfeffer LM. Biologic activities of natural and synthetic type I interferons. Semin Oncol 1997;24. S9-63-9.

[20] Jonasch E, Haluska FG. Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities. Oncologist 2001;6:34–55.

[21] Friedman RM. Clinical uses of interferons. Br J Clin Pharmacol 2008;65:158–62.

[22] Masuzaki R, Yoshida H, Omata M. Interferon reduces the risk of hepatocellular carcinoma in hepatitis C virus-related chronic hepatitis/liver cirrhosis. Oncology 2010;78:17–23.

[23] Wong GL, Yiu KK, Wong VW, Tsoi KK, Chan HL. Meta-analysis: reduction in hepatic events following interferon-alfa therapy of chronic hepatitis B. Aliment Pharmacol Ther 2010;32:1059–68.

[24] Sakaguchi Y, Kudo M, Fukunaga T, Minami Y, Chung H, Kawasaki T. Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. Intervirology 2005;48:64–70.

[25] Shen YC, Hsu C, Chen LT, Cheng CC, Hu FC, Cheng AL. Adjuvant interferon therapy after curative therapy for hepatocellular carcinoma (HCC): a meta-regression approach. J Hepatol 2010;52:889–94.

[26] Nagano H. Treatment of advanced hepatocellular carcinoma: intraarterial infusion chemotherapy combined with interferon. Oncology 2010;78:142–7.

[27] Parody JP, Alvarez ML, Quiroga AD, Ceballos MP, Frances DE, Pisani GB, et al. Attenuation of the Wnt/beta-catenin/TCF pathway by in vivo interferon-alpha2b (IFN-alpha2b) treatment in preneoplastic rat livers. Growth Factors 2010;28:166–77.

[28] de Luján Alvarez M, Ronco MT, Ochoa JE, Monti JA, Carnovale CE, Pisani GB, et al. Interferon alpha-induced apoptosis on rat preneoplastic liver is mediated by hepatocytic transforming growth factor beta (1). Hepatology 2004;40:394–402.

[29] Quiroga AD, Alvarez MdeL, Parody JP, Ronco MT, Francés DE, Pisani GB, et al. Involvement of reactive oxygen species on the apoptotic mechanism induced by IFN-alpha2b in rat preneoplastic liver. Biochem Pharmacol 2007;73:1776–85.

[30] Alvarez MdeL, Quiroga AD, Parody JP, Ronco MT, Francés DE, Carnovale CE, et al. Cross-talk between IFN-alpha and TGF-beta1 signaling pathways in preneoplastic rat liver. Growth Factors 2009;27:1–11.

[31] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.

[32] Naryzhny SN. Proliferating cell nuclear antigen: a proteomics view. Cell Mol Life Sci 2008;65:3789–808.

[33] Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nat Cell Biol 2000;2:156–62.

[34] Zimmermann KC, Green DR. How cells die: apoptosis pathways. J Allergy Clin Immunol 2001;108:S99–103.

[35] Carruba G, Cervello M, Miceli MD, Farruggio R, Notarbartolo M, Virruso L, et al. Truncated form of beta-catenin and reduced expression of wild-type catenins feature HepG2 human liver cancer cells. Ann N Y Acad Sci 1999;886:212–6.

[36] Wei W, Chua MS, Grepper S, So SK. Soluble Frizzled-7 receptor inhibits Wnt signaling and sensitizes hepatocellular carcinoma cells towards doxorubicin. Mol Cancer 2011;11:10–6.

[37] Damdinsuren B, Nagano H, Kondo M, Natsag J, Hanada H, Nakamura M, et al. TGF-beta1-induced cell growth arrest and partial differentiation is related to the suppression of Id1 in human hepatoma cells. Oncol Rep 2006;15:401–8.

[38] Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA, Lebrun JJ. The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. EMBO J 2005;24:3247–58.

[39] Murata M, Nabeshima S, Kikuchi K, Yamaji K, Furusyo N, Hayashi J. A comparison of the antitumor effects of interferon-alpha and beta on human hepatocellular carcinoma cell lines. Cytokine 2006;33:121–8.

[40] Foser S, Redwanz I, Ebeling M, Heizmann CW, Certa U. Interferon-alpha and transforming growth factor-beta co-induce growth inhibition of human tumor cells. Cell Mol Life Sci 2006;63:2387–96.

[41] Herzer K, Grosse-Wilde A, Krammer PH, Galle PR, Kanzler S. Transforming growth factor-beta-mediated tumor necrosis factor-related apoptosis-inducing ligand expression and apoptosis in hepatoma cells requires functional cooperation between Smad proteins and activator protein-1. Mol Cancer Res 2008;6:1169–77.

[42] Herzer K, Hofmann TG, Teufel A, Schimanski CC, Moehler M, Kanzler S, et al. IFN-alpha-induced apoptosis in hepatocellular carcinoma involves promyelocytic leukemia protein and TRAIL independently of p53. Cancer Res 2009;69:855–62.

[43] Masuda T, Saito H, Kaneko F, Atsukawa K, Morita M, Inagaki H, et al. Up-regulation of E-cadherin and β-catenin in human hepatocellular carcinoma cell lines by sodium butyrate and interferon-alpha. In Vitro Cell Dev Biol Anim 2000;36:387–94.

[44] Thompson MD, Dar MJ, Monga SP. Pegylated interferon alpha targets Wnt signaling by inducing nuclear export of β-catenin. J Hepatol 2011;54:506–12.

[45] Dong Y, Drissi H, Chen M, Chen D, Zuscik MJ, Schwarz EM, et al. Wnt-mediated regulation of chondrocyte maturation: modulation by TGF-beta. J Cell Biochem 2005;95:1057–68.

[46] Sugano Y, Matsuzaki K, Tahashi Y, Furukawa F, Mori S, Yamagata H, et al. Distortion of autocrine transforming growth factor beta signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. Oncogene 2003;22:2309–21.

[47] Liu X, Yang Y, Zhang X, Xu S, He S, Huang W, et al. Compound Astragalus and Salvia miltiorrhiza extract inhibits cell invasion by modulating transforming growth factor-beta/Smad in HepG2 cell. J Gastroenterol Hepatol 2010;25:420–6.

[48] Labbé E, Letamendia A, Attisano L. Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. Proc Natl Acad Sci USA 2000;97:8358–63.

[49] Lim SK, Hoffmann FM. Smad4 cooperates with lymphoid enhancer-binding factor 1/T cell-specific factor to increase c-myc expression in the absence of TGF-beta signaling. Proc Natl Acad Sci USA 2006;103:18580–5.

[50] Schohl A, Fagotto F. Beta-catenin MAPK and Smad signaling during early Xenopus development. Development 2002;129:37–52.