IGFBP-3 Inhibits Wnt Signaling in Metastatic Melanoma Cells

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In previous works, we have shown that insulin-like growth factor-binding protein-3 (IGFBP-3), a tissue and circulating protein able to bind to IGFs, decreases drastically in the blood serum of patients with diffuse metastatic melanoma. In agreement with the clinical data, recombinant IGFBP-3 was found to inhibit the motility and invasiveness of cultured metastatic melanoma cells and to prevent growth of grafted melanomas in mice. The present work was aimed at identifying the signal transduction pathways underlying the anti-tumoral effects of IGFBP-3. We show that the anti-tumoral effect of IGFBP-3 is due to inhibition of the Wnt pathway and depends upon the presence of CD44, a receptor protein known to modulate Wnt signaling. Once it has entered the cell, IGFBP-3 binds the Wnt signalosome interacting specifically with its component GSK-3β. As a consequence, the β-catenin destruction complex dissociates from the LRP6 Wnt receptor and GSK-3β is activated through dephosphorylation, becoming free to target cytoplasmic β-catenin which is degraded by the proteasomal pathway. Altogether, the results suggest that IGFBP-3 is a novel and effective inhibitor of Wnt signaling. As IGFBP-3 is a physiological protein which has no detectable toxic effects either on cultured cells or live mice, it might qualify as an interesting new therapeutic agent in melanoma, and potentially many other cancers with a hyperactive Wnt signaling. © 2016 The Authors. *Molecular Carcinogenesis* Published by Wiley Periodicals, Inc.

Key words: melanoma; Wnt; IGFBP-3

INTRODUCTION

Melanoma is the leading cause of death from skin diseases due to its propensity to metastasize. In 2010, 114 900 new cases of melanoma were diagnosed in the United States for, out of which 68 130 were invasive and resulted in the death of nearly 8700 individuals [1]. Activating mutations of the proto-oncogene *BRAF* have been observed in approximately 50% of malignant melanomas [2]. However, *BRAF* mutations alone are insufficient to cause malignant transformation and other triggering events are needed for melanomagenesis. Since melanoma is a highly malignant cancer with a potent capacity to metastasize distantly, an approach that decreases its metastatic ability may facilitate the development of an effective strategy for its treatment and/or prevention. Although the molecular mechanisms underlying the progression of melanoma remain unresolved, several studies have implicated constitutively active Wnt/β-catenin signaling in melanoma progression and metastasis [3]. Non-phosphorylated β-catenin accumulates in the cytoplasm; when activated, it enters the nucleus and interacts with T-cell transcription factors to control various target genes that are involved in cellular proliferation and migration. Nuclear β-catenin accumulation has been correlated with late stages of tumor progression and metastasis. The presence of mutated β-catenin is associated with aggressive tumor growth and regulates expression of various target genes that mediate cellular processes including proliferation (e.g., cyclins and c-myc), migration, and invasion (e.g., matrix metalloproteinases) [3,4]. In the canonical model of Wnt signaling, β-catenin is phosphorylated at certain key residues by glycogen synthase kinase 3-β (GSK3-β)

and casein kinase 1α (CK1α), leading to its ubiquitination and subsequent degradation [5,6]. Like cancers of other organs, the regulation of β-catenin is lost in melanoma [7–9]. β-catenin is also an important component of cell–cell adhesion, where it forms a dynamic link between E-cadherin and cytoskeleton [10,11]. However, the breaking of cell-to-cell adhesion due to activation of β-catenin and its nuclear accumulation may increase the migration potential of tumor cells. Thus, nuclear/cytoplasmic traffic of β-catenin in the cells determines their migration potential [12,13].

The insulin-like growth factor (IGF/IGF-binding protein) axis has been shown to influence the proliferation and survival of various tumors [14]. IGFBP-3 is reported to be a growth suppressor by virtue of its effect on multiple pathways [15]. However, there is also evidence that IGFBP-3 can have stimulatory effects on the growth of breast cancer cells [16,17]. In the IGF receptor-dependent pathways, IGFBP-3 binds to IGF/1-2 and suppresses their growth signal [14]. In the IGF receptor-independent pathways, IGFBP-3 mediates a wide variety of growth-controlling signals such as TGFβ, retinoic acid, tumor suppressor protein p53, vitamin D, anti-estrogens, and tumor necrosis factor-α [14,17]. Several epidemiological studies have examined the relationship between the serum concentrations of IGF/IGFBPs and cancer incidence, emphasizing the evidence that insulin-like growth factors (IGFs) and insulin-like growth factor binding proteins (IGFBPs) may represent specific tumor markers [18–20].

In previous studies, we have shown that a strong correlation exists between the serum concentration of IGFBP-3 and disease progression in melanoma patients [20]. Moreover, a low concentration of IGFBP-3 was highly significantly correlated with survival and metastatic volume, indicating that a dearth of this protein in the blood accompanies, and perhaps favors, the metastatic dissemination of melanoma. The anti-migratory and anti-invasive effect of IGFBP-3 on melanoma cells was confirmed in vitro, and evidence was also obtained that the cytokine has anti-tumoral activity in vivo [21]. An investigation of the cellular pathways involved in mediating the anti-tumoral action of IGFB-3 indicated Akt and its downstream target GSK3-β as the kinases predominantly involved. Importantly, we showed that IGFBP-3 action was independent of IGF1 and was not mediated by the IGF1 receptor [21].

In this work, we have furthered the investigation of the cellular pathways affected by IGFBP-3, obtaining evidence that it acts as an inhibitor of the Wnt pathway. IGFBP-3 interacts with the β-catenin destruction complex at the membrane level, thereby interfering with Wnt signaling and causing β-catenin degradation. A model of IGFBP-3 action on the Wnt pathway is presented.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The Wistar primary melanoma (WM793) cell lines (BRAF V600E; NRAS wt) were kindly provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA. The cell lines Me501 (BRAF wt; NRAS G10D) were established from metastases surgically excised from melanoma patients at the Istituto Nazionale dei Tumori, Milan, Italy. The LG cell line (BRAF V600E; NRAS wt) was kindly provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA [21]. The MCF7 cell line was obtained from ATCC/Wesel, Germany. All cell lines were seeded in 3-cm Petri dishes in RPMI 1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin (Life Technologies, Gaithersburg, MD), and 2 mmol/L glutamine (Life Technologies) with 10% FCS in a 5% CO_2 environment at 37°C.

Western Blot and Co-Immunoprecipitation and Reagents

To collect cytosolic, nuclear, and membrane fractions, cells were grown in 60 mm dishes and were lysed. Fractions were collected following the Subcellular protein Fractionation Kit (Thermo Scientific, Meridian Rd, Rockford, IL) protocol. All the fractions were incubated with Laemmli sample buffer containing 5% β-mercaptoethanol at 100°C for 5 min before loading onto the gel. For co-immunoprecipitation of endogenous protein, Me501 cells were seeded in 100-mm dishes and incubated for the indicated time points with IGFBP-3. Cells were washed with PBS and lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40 and mix protease inhibitor) for 15 min at 4°C. Immunoprecipitation was performed on lysates with indicated antibody and protein A Sepharose CL-4B (GE Healthcare, Little Chalfont, UK) or rabbit-IgG (IgG control, Millipore, Bedford, MA) at 4°C over night. The precipitates were washed in lysis buffer (3×) and boiled in SDS-sample buffer containing β-mercaptoethanol. Samples were subjected to Western blotting analysis. Antibodies used for Westerns are as follows: anti-β-catenin, anti-LRP6, anti-Axin1, anti-Akt (total and phosphor-ser 473), anti-βTrcp, anti-Gsk3β (S9) (Cell Signaling Technology, Danvers, MA), anti-IGFBP-3 (Acris, San Diego, CA), anti-APC (Santa Cruz, Dallas, Texas), anti-E Cadherin (BD, Franklin Lakes, NJ), anti-Tubulin (Sigma, Taufkirchen, Germany), anti-Gsk3β and anti-lamin B1 (Abcam, Cambridge, UK). For IGFBP-3 treatments, recombinant, glycosylated His-tagged IGFBP-3 was used (Sino Biological, Inc., North Wales, PA) and, when specified, IGFBP-3 recombinant, non-glycosylated (MyBioSource San Diego, CA). Wnt induction was done using recombinant Wnt3a (200 ng/mL: R&D System, Wesbaden, Germany). The PI3K inhibitor (LY294002) was purchased from Selleckchem (Munich, Germany). The Akt inhibitor (Ab142088) was purchased from Abcam.

Transfection Procedures

siRNA transfection in Me501 or LG cells was performed using the Lipofectamine LTX Plus transfection reagent (Invitrogen, Meridian Rd, Rockford, IL) according to the manufacture's protocol using 10nM of CD44 pan siRNA (Santa Cruz).

Immunoelectron Microscopy

Cells samples were fixed for 3 h at 4°C in a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6. They were dehydrated in alcohol at progressively higher concentrations and embedded in Bioacryl resin (British Biocell, Cardiff, UK) followed by UV polymerization according to standard procedures [22]. Ultrathin sections were cut and mounted on 300 mesh nickel grids. To block nonspecific binding sites, the grids were treated with a blocking buffer made of phosphate buffer saline supplemented with 0.1% Tween-20, 0.1% bovine serum albumin, and 4% normal goat serum. For double localization experiments, the grids were first incubated with anti-IGFBP-3 antibodies 4 μg/mL (Acris) and subsequently with goat anti-rabbit IgG conjugated with 10 nm colloidal gold particles (British Biocell). After washing, the grids were incubated again with CD44 Mouse Ab (156-3C11, Cell Signaling) followed by incubation with goat anti-mouse IgG conjugated with 20 nm colloidal gold particles. Sections were counterstained in uranyl acetate to display cell morphology and observed with EM TECNAI 12 (D1157).

Immunofluorescence Microscopy

Cells were fixed in 4% PBS–paraformaldehyde for 15 min, incubated in 0.1% Triton X-100 for 5 min on ice, then in 3% in PBS/BSA for 1 h, and stained for 1 h with an anti-β-catenin. DAPI (Invitrogen) was used to stain nucleic acids (1:1000). The preparations were examined under a Nikon Eclipse fluorescent microscope equipped with a 20× (0.9 NA) and a CCD camera (Nikon, Inc., Firenze, Italia). Digital images were processed with Adobe Photoshop 7 software (Adobe Systems, Mountain View, CA). The level of β-catenin was quantified and statistically analyzed using the Student's t-test.

Scratch-Repair Assay

To evaluate the effect of siRNA CD44 on cell migration, a scratch-wound assay was done on Me501 cell. The cells were transfected with control siRna or siRna against all CD44 isoforms. After the cells reached confluence (48 h), the cell monolayer was scraped with a pipette tip to generate a scratch wound. The wounded surface was washed with 1× PBS and incubated in RPMI without fetal bovine serum. Cell migration into the wound was monitored by phase microscopy using an Axiovert 200 M microscope with digital camera (Carl Zeiss, Thornwood, NY), taking pictures after 6 h. The images were captured by AxioVision 4.0 software (Carl Zeiss). The closure of the initial gap area was assessed by calculating the difference between the initial and the remaining wound area. All the experiments were repeated at least three times.

Statistical Analysis

The Student's t-test was used for comparison of two samples. Calculation of the average was performed using at least three biological replicates. P-values <0.05 were considered significant. Error bars indicate SD.

RESULTS

IGFBP-3 Treatment Lowers the Cellular Levels of β-Catenin

Previously, we showed that a likely mediator of the IGFBP-3 anti-migratory and anti-invasive capacity was the kinase GSK3-β. Since IGFBP-3 treatment of metastatic melanoma cells up-regulated tyrosinase and increased the cell's melanin content [21], we hypothesized that the cytokine caused the tumor cells to revert towards a more melanocytic phenotype, given the known activity of GSK3-β to activate the transcription of genes involved in melanin synthesis. GSK3-β, however, is also involved in phosphorylating key components of the destruction complex that regulates the cellular levels of β-catenin. As a component of the destruction complex (APC/Axin/GSK3) GSK3-β phosphorylates β-catenin, thus targeting it for ubiquitination and degradation by the proteasome. Wnt signaling is assumed to block GSK3-mediated β-catenin phosphorylation, leading to the accumulation and nuclear translocation of β-catenin [23]. We decided, therefore, to investigate whether IGFBP-3 had any effect on the amount of β-catenin in metastatic melanoma cells.

For this purpose, we began to determine the abundance of β-catenin and other components of the Wnt/APC/CK1 pathway in metastatic (Me501 and LG) and primary (WM793) melanoma cell lines. The cells were grown to semi-confluence, transferred to a serum-free medium for 24 h, lysed and analyzed. The cellular levels of the proteins of interest were determined by Western blotting. As expected (Supplemental Figure S1) the primary melanomas had APC and Axin 1 levels higher than those of the metastatic cells; the opposite was true for β-catenin which was abundant in the metastatic lines and below detection in the primary one. As noted previously, the kinase Akt was much more active in the metastatic cells than in primary ones, despite the 24 h of starvation. (Supplemental Figure S1). It should be stressed that both metastatic cell lines had similar levels of the markers under study despite their different genetic backgrounds. The LG cell line had the same mutational pattern as the primary WM793 cells (BRAF V600E; NRAS wt), while the Me501 cells were BRAF wt; NRAS G10D. Therefore, the possibility

that the different pattern of markers of the primary and the metastatic cells may be due to the different mutations in the *BRAF* and *NRAS* genes can be ruled out. Accordingly, most of the subsequent experiments were made using the Me501 cells while the LG cells were used when specified.

To calibrate the experimental system, the metastatic Me501 cells were preliminarily treated for 2 h with increasing doses of IGFBP-3, using both the untagged and the His-tagged recombinant cytokine (Figure 1). The response of the main molecular markers previously identified [21], Akt (pSer473) and GSK3-β (pSer9) was checked. As shown in Figure 1, IGFBP-3 caused a marked dephosphorylation of both target proteins already at the lowest dose (0.2 μg/mL). We note that in most of the assays presented in this work, we used IGFBP-3 concentrations in the range of 2–5 μg/mL, that is, those measured in human blood serum and used in previous experiments made in vitro and in vivo [21]. Although the tissual concentrations of the cytokine must be lower, the results in Figure 1 suggest that the molecular phenotype observed is most probably not an artefact of IGFBP-3 over-dosage. Previously [21], we also reported that IGFBP-3 does not exert any toxic effect on either cultured cells or live mice, in the latter case even at concentrations ten times as high as those used in the present work.

The experiments in Figure 1 also demonstrate that His-tagged and untagged IGFBP-3 were almost equally effective, discounting the possibility that the presence of the His-tag may impair the signaling activity of the cytokine. Accordingly, most of the subsequent experiments were performed with the His-tagged IGFBP-3, which can be distinguished from the endogenous species using the appropriate antibodies.

To determine whether treatment with IGFBP-3 affected the cellular levels of β-catenin, WM793, Me501, and LG cells were exposed to the cytokine for 24 h and 48 h, and the amount of β-catenin was checked on whole cell lysates by Western blotting. As shown in Figure 2a, treatment with IGFBP-3 drastically lowered the overall levels of β-catenin in both metastatic cell lines, while the primary WM793 cells remained negative for the presence of the protein. These observations were further checked and verified by immunofluorescence staining of Me501 cells, which clearly evidenced the reduced amounts of β-catenin after treatment with IGFBP-3 for 48 h (Figure 2c).

The analysis was further refined by fractionating the lysates of Me501 cells and measuring the levels of β-catenin in the different cellular compartments. The results revealed that both the cytosolic and nuclear β-catenin fractions were lowered by the IGFBP-3 treatment, while the membrane-bound fraction remained essentially unchanged (Figure 2b).

IGFBP-3 Decreases β-Catenin Levels via Proteasome Degradation

Several articles in the literature show that GSK3-β is able to adjust the levels of β-catenin, by proteasome dependent degradation [23]. To evaluate whether this was true in our case, the Me501 cells were treated with IGFBP-3 in the presence or in the absence of cycloeximide and of the proteasome inhibitor MG132. As shown in Supplemental Figure S2a, treatment with IGFBP-3 decreased the cellular levels of β-catenin both in the absence and in the presence of cycloeximide. The effect was, however, reverted by treatment with the proteasome inhibitor MG132, suggesting that IGFBP-3-induced β-catenin degradation depends on an active proteasome pool. This surmise was reinforced by the finding that treatment with IGFBP-3 for 48 h increased the amount of β-catenin bound to the β-transducin repeat-containing protein (β-TrCP), a component of the ubiquitin-ligase complex targeting β-catenin for proteasomal degradation β-TrCP [24] (Supplemental Figure S2b).

IGFBP-3 Treatment Increases the Cellular Levels of Axin-1

In resting cells, very low levels of free β-catenin are present in the cytosol. This pool of β-catenin is efficiently captured by the destruction complex and phosphorylated by CKI at Ser45 and by GSK3-β at Thr41, Ser 37, and Ser33 residues [23].

Axin-1 is the least abundant component and therefore the rate-limiting factor of the destruction complex. As such, it can regulate its rapid assembly and disassembly. Active GSK3-β may adjust the levels of Axin-1 by phosphorylation and the phosphorylated form of Axin-1 is more stable than the unphosphorylated form. Therefore, active GSK3-β phosphorylates both Axin-1 and β-catenin in the axin
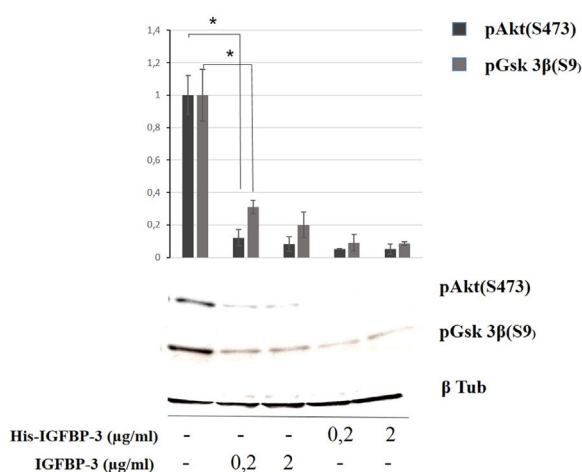

Figure 1. IGFBP-3 effect on Akt and GSK3 phosphorylation. The metastatic Me501 cells were treated for 2 h with increasing doses of IGFBP-3, using both the untagged and the His-tagged recombinant cytokine (0.2–2 μg/mL) The phosphorylation levels of Akt (p473) and GsK3β (pS9) were determined by Western blotting. Statistical significance was analyzed using the Student's t-test (*$P < 0.05$).
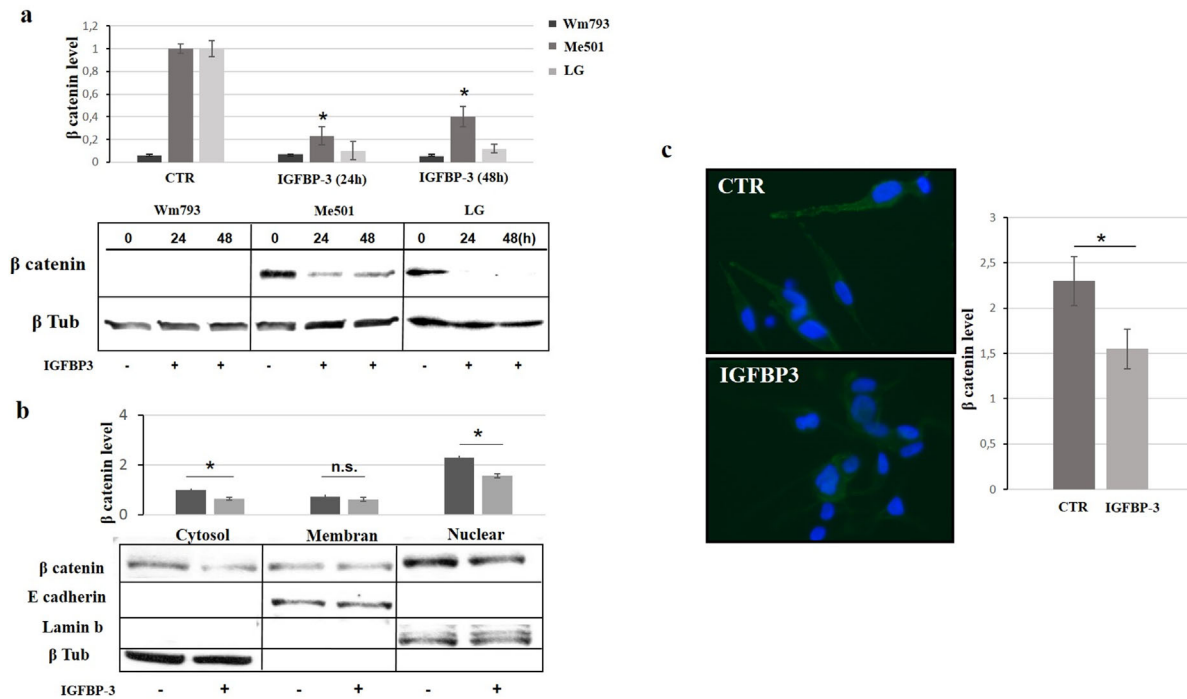
Figure 2. IGFBP-3 decreases the levels of free, but not membrane-bound, β-catenin. (a) Me501, LG, and control Wm793 cells were treated with IGFBP-3 (5 μg/mL) for 24 and 48 h. Whole cell lysates were prepared, fractionated on gels, and probed with the indicated antibodies. The lower panel presents a typical Western blot, whereas the upper one presents a quantification of the data as the mean of three independent experiments. (b) Membrane, cytosol, and nuclear fractions were obtain from Me501 cells treated with IGFBP-3 for 24 h following the Subcellular protein Fractionation Kit (Thermo Scientific) protocol. The lower panel presents a typical Western blot, whereas the upper one presents a quantification of the data as the mean of three independent experiments. (c) Me501 cells were treated with IGFBP-3 (5 μg/mL) for 48 h and were then subjected to immunofluorescence staining using antibodies against β-catenin (green). The nucleus was stained with DAPI. The right panel presents a quantification of the data as the mean of three independent experiments. Statistical significance was analyzed using the Student's t-test (*$P < 0.05$; n.s., not significant).

complex, and these phosphorylations have opposite effects: the phosphorylation of Axin results in its stabilization, whereas that of β catenin leads to its degradation [25].

Accordingly, we tested whether IGFBP-3 treatment had any effect on Axin-1 stability, and whether this effect was dependent on GSK3-β inhibition. As shown in Figure 3a (lanes 1 and 2), treatment of Me501 cells with LiCl (20 mM), a well-established inhibitor of GSK3-β, caused a marked decrease of cellular Axin-1 and a concomitant increase of β-catenin. The addition of increasing doses of IGFBP-3 reverted the effect of LiCl, suggesting reactivation of GSK3-β by this cytokine. Indeed, as shown in Figure 3b, treatment with IGFBP-3 in the presence of LiCl caused the dephosphorylation, hence activation, of GSK3-β (Ser9).

Importantly, the β-catenin-lowering effect of IGFBP-3 is not specific of melanoma cells. As shown in Figure 3c and d, treatment of breast cancer cells (MCF7) with IGFBP-3 also caused the increase of Axin-1 and the decrease of β-catenin, indicating that the same cellular pathways mediate the effect of IGFBP-3 in these cell types.

β-Catenin Downregulation by IGFBP-3 Does Not Depend on the PI3K Pathway

It is known that a main biological role of IGFBP-3 is to regulate the PI3K pathway by controlling the binding of IGF1 to its receptor [17]. In a previous paper, we showed that the anti-migratory and anti-invasive effects of IGFBP-3 on melanoma cells are not mediated by the IGF1 receptor [21]. To further exclude the possibility that the effect of IGFBP-3 on the cellular levels of β-catenin and Axin could be mediated by the PI3K pathway, we performed the following experiments.

Me501cells were treated with activators (insulin) and inhibitors (LY294002) of PI3K, and then the amounts of β-catenin and the phosphorylation levels of Akt and GSK3-β were measured by Western blotting.

As expected, insulin treatment increased, and PI3K inhibitor decreased, the phosphorylation levels of both Akt and GSK3-β (Supplemental Figure S3). However, the levels of β-catenin remained unaffected, showing that the PI3K pathway does not control them in the Me501 cells. These experiments clearly show that in our cell lines, the stability of β-catenin does not depend on the state of phosphorylation of GSK3-β
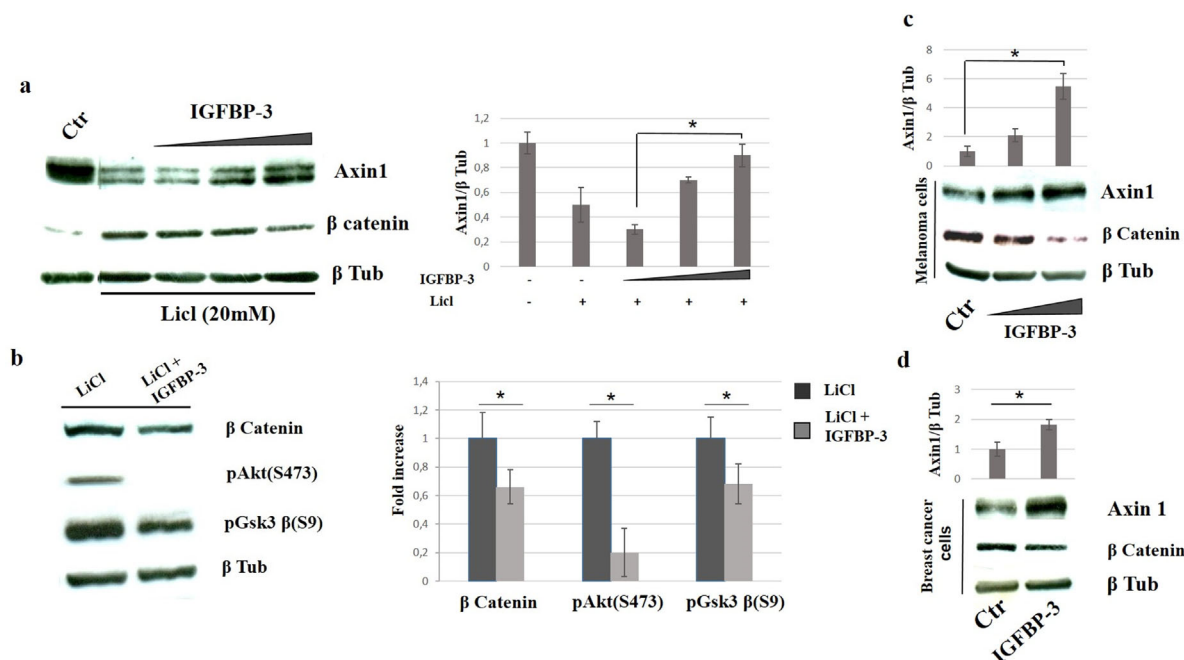
Figure 3. IGFBP-3 increases the cellular levels of Axin-1. (a) Me501 cells were treated with LiCl (20 mM) and with increasing doses of IGFBP-3 (2–5 to 10 μg/mL) for 8 h. The cell lysates were probed by Western blot using the indicated antibodies. (b) Me501 cells were treated with LiCl (20 mM) and IGFBP-3 (5 μg/mL) for 8 h. The phosphorylation levels of Akt (p473) and GsK3β (pS9) were determined by Western blotting. The right panels in (a) and (b) present a quantification of the data as the mean of three independent experiments. Statistical significance was analyzed using the Student's t-test ($^*P < 0.05$). (c) Me501 cells were treated with IGFBP-3 (1.5–3 μg/mL) for 48 h and the amounts of β-catenin and Axin1 were determined by Western blotting with the corresponding antibodies. (d) Same experiments as in (c) on MCF7 breast cancer cells (3 μg/mL).

regulated by PI3K/Akt signal transduction pathway. In turn, this suggests that IGFBP-3 stimulates the GSK3-β-induced degradation of β-catenin acting on a signal transduction pathway different from the PI3K one.

IGFBP-3 Promotes the Dissociation of GSK3-β From the LRP6 Wnt Co-Receptor

It is well described in the literature that there are two different pools of GSK3-β participating separately in the PI3K/Akt pathway or in the Wnt/Axin pathway [26].

The Axin–GSK3-β complex appears to serve two functions. First, it allows GSK3-β to target β-catenin specifically by providing docking sites for both proteins. Second, when GSK3-β is complexed to Axin, it cannot be targeted by Akt activated by PI3K signaling. This molecular arrangement allows Wnt and PI3K inputs to have independent effects on the biological outputs of the cells that receive these signals [26].

From the above results it can be surmised that IGFBP-3 may affect β-catenin stability by acting on the Wnt pathway. To test this hypothesis, we first verified whether the levels of β-catenin in our cells were sensitive to the cytokine Wnt. As shown in Figure 4a, stimulation of the Me501 cells with Wnt for 0.5 h after 16 h of starvation increased the phosphorylation of Akt (Ser473) and GSK3-β (Ser9) and raised the β-catenin level. IGFBP-3 effectively contrasted the Wnt-induced Akt and GSK3-β phosphorylation. Note that in these experiments, the reduction of the β-catenin levels is barely visible because of the short time of treatment, while it becomes evident at longer times. (Figure 4b).

It is known that Wnt treatment activates its co-receptor LRP6 by phosphorylation at multiple sites including Thr1479, Ser1490, and Thr1493 by kinases such as GSK3-β and CK1, resulting in recruitment of the scaffolding protein Axin to LRP6 and β-catenin stabilization [27]. This effect is clearly visible on the Me501 cells following treatment with Wnt (Figure 4a, lane 2). Notably, IGFBP-3, besides reverting the Wnt-induced phosphorylation of Akt and GSK3-β (Figure 4a), also caused dephosphorylation of the LRP6 protein (Figure 4a and b).

The events following the LRP6 receptor activation are still a subject of debate. However, there is a consensus that Wnt stimulation promotes the sequestration of GSK3-β by binding to the LRP6 receptor, thus reducing the availability of the cytoplasmic destruction complex and causing β-catenin accumulation [27].

With this in mind, we investigated whether IGFBP-3 affected the interaction of GSK3-β with LRP6. For this purpose, Me501 cells were treated with IGFBP-3 for 4 h, cell lysates were prepared, and the amount of GSK3-β bound to LRP6 before and after treatment was
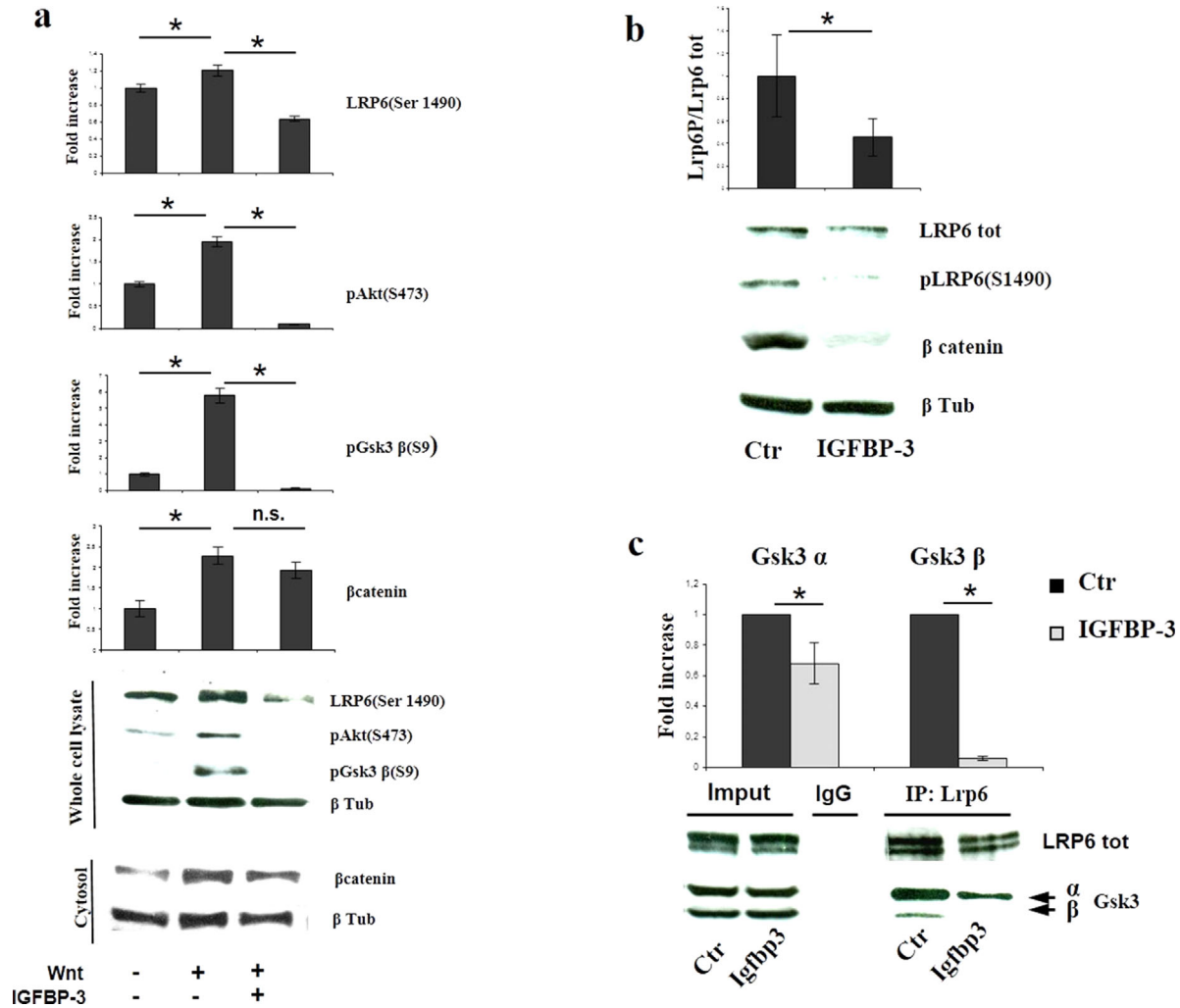
Figure 4. IGFBP-3 effects on various components of the Wnt signalosome. (a) IGFBP-3 inhibits Wnt-induced phosphorylation of Akt (Ser473) and GSK3-β (Ser9). Me501 cells, deprived of serum for 16 h, were treated with Wnt (200 ng/mL) and IGFBP-3 (2 µg/mL) for 0.5 h and the cell lysates were probed with the indicated antibodies (bottom panel). The results of three independent experiments were quantified using the ImageJ software, averaged, and plotted (upper panel). (b) IGFBP-3 induces the dephosphorylation of the Wnt LRP6 receptor. The serum-starved Me501 cells were treated with IGFBP-3 (2 µg/mL) for 3 h, lysed, and subjected to Western blot analysis with the indicated antibodies. Quantification of the data was carried out as in (a). (c) IGFBP-3 promotes the dissociation of GSK3-β from the LRP6 receptor. The Me501 cells were treated with IGFBP-3 (2 µg/mL) for 4 h, lysed, and the proteins were immunoprecipitated with the anti-LRP6 antibody. The precipitated proteins were detected by Western blotting and quantified as described in (a). Statistical significance was analyzed using the Student's t-test (*$P < 0.05$; n.s., not significant).

assayed by immunoprecipitation with anti-total LRP6 antibodies and Western blotting. The results, shown in Figure 4c, revealed that IGFBP-3 did not affect the expression levels of LRP6, but did decrease the amount of GSK, especially of its isoform β, bound to the LRP6 receptor. These data suggest that IGFBP-3 may promote the disassembly of the destruction complex from the Wnt receptor, thus enhancing the availability of GSK3-β in the cytoplasm.

IGFBP-3 Interacts With GSK3-β Below the Plasma Membrane

To assess the mechanism whereby IGFBP-3 interferes with the Wnt pathway, we hypothesized the possibility of a direct interaction with the β-catenin destruction complex. As demonstrated in the literature, IGFBP-3 is rapidly endocytosed in a complex with caveolin and clathrin [28]. Indeed, as shown in Figure 5a, recombinant IGFBP-3 administered to cultured cells appears in the whole lysates after only 15 min. Dephosphorylation of Akt and GSK is also evident at this time of treatment (see also Figure 1).

To assess the possibility of an interaction with the destruction complex, we treated the Me501 cells for 0.5 h with IGFBP-3, cell lysates were prepared, and total IGFBP-3 was immunoprecipitated with the specific antibody. As shown in Figure 5b, the Western blot analysis of the immunoprecipitates evidenced an
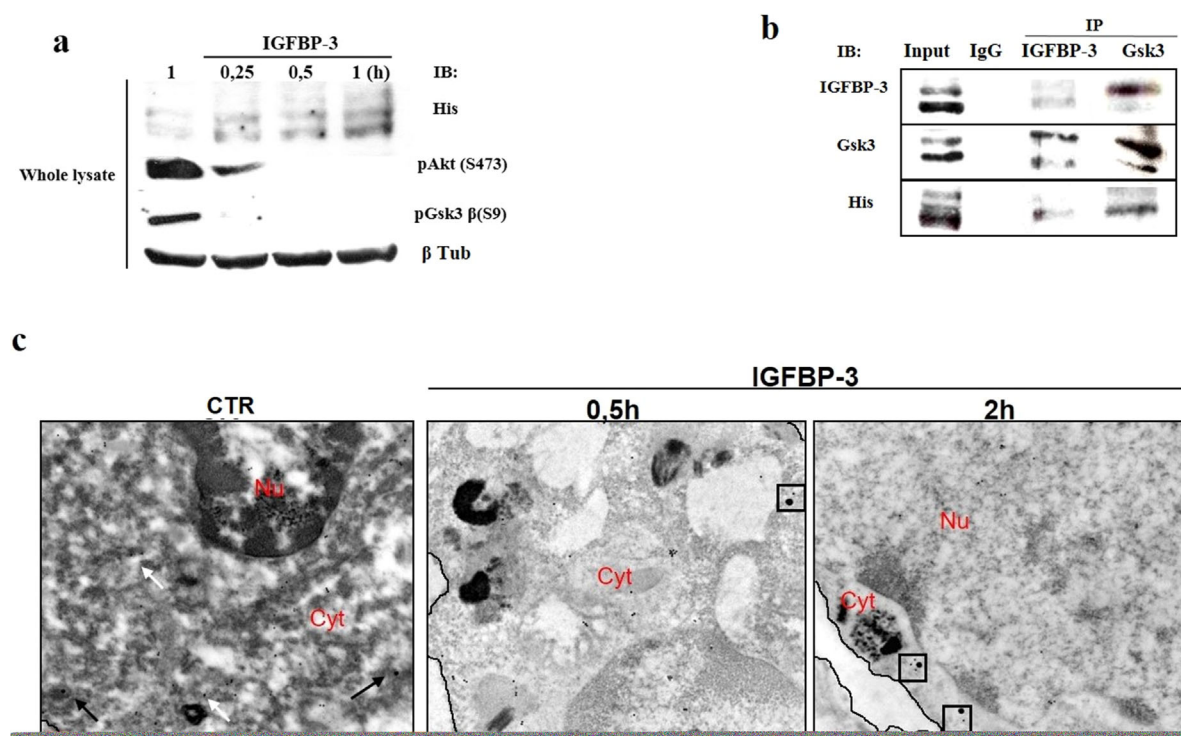
Figure 5. IGFBP-3 interacts with GSK3 under the plasma membrane. (a) The Me501 cells were treated for 0.5 h with IGFBP-3 (2 μg/mL), cell lysates were prepared, and the entrance of recombinant IGFBP-3 within the cells at the indicated times was monitored by Western blotting with an anti-histidine antibody. The phosphorylation state of Akt (S473) and GSK3-β (S9) was determined with the specific antibodies (b) The lysates of Me501 cells treated with IGFBP-3 (2 μg/mL) for 0.5 hours were immunoprecipitated with anti-IGFBP-3 and GSK3 antibodies and the precipitates were probed with the anti-GSK3, anti-IGFBP-3, and anti-Histidin antibodies. (c) Representative ultrathin sections of control Me501 cell samples (left) and samples treated with IGFBP-3 (5 μg/mL) for the indicated times (middle and right), stained for the immuno-localization of GSK3 (larger dots) and IGFBP-3 (smaller dots). Specificity controls were negative. The co-localization of the two proteins in the peripheral zone of the cytoplasm after IGFBP-3 treatment is clearly visible (black box). The continuous line shows the plasma membrane (——). Original magnifications: 30.000×. Nu, nucleus; Cyt, cytoplasm. Five images from 50 cells were analyzed and percent co-localization was estimated to be 50 and 95 at 0.5 and 2 h, respectively.

interaction of IGFBP-3 with both GSK3 isoforms. The results were confirmed by the reciprocal immunoprecipitation of IGFBP-3 with the anti-GSK antibodies. In this case, the precipitates were probed with both anti-IGFBP-3 and anti-His antibodies to be sure that GSK indeed interacted with the exogenous IGFBP3 (Figure 5b). In contrast, no interaction was observed with the Wnt co-receptor LRP6 (not shown).

Since the fraction of GSK3 involved in the Wnt pathway is that which resides on the membrane, and IGFBP-3 is associated with GSK3 already after 0.5 h of treatment, we investigated the cellular localization of IGFBP-3 and of the GSK–IGFBP-3 complex using the electron microscopy "immunogold" technique.

As shown by the image in Figure 5c, in the control, IGFBP-3 (smaller dots) and GSK3 (larger dots) are dispersed over the nucleus and the cytosol, while already after 0.5 h treatment with IGFBP-3, the two proteins co-localize just below the plasma membrane. After 2 h of treatment, IGFBP-3 and GSK3 appear to be associated in most of the samples observed (Figure 4c, last panel). No IGFBP-3 associated with the external

membrane was detected, suggesting that the internalization of the protein was rapid and efficient.

IGFBP3 Decreases the Phosphorylation at Ser473 of Axin-Linked Akt

The results presented above demonstrate that IGFBP-3 interferes with the Wnt pathway. Specifically, the cytokine is able to interact with GSK3-β soon after entering the cell, causing the dissociation of this kinase from the β-catenin destruction complex.

However, we have shown previously and in this work that a prominent early effect of IGFBP-3 treatment on Me501 cells is an almost complete dephosphorylation of Akt. As shown in Figure 3a, Akt is phosphorylated at Ser473 upon activation of the Wnt pathway. Therefore, it is reasonable to assume that Akt dephosphorylation triggered by IGFBP-3 is also transduced by the Wnt pathway.

Assessing this point is of particular interest, because a link between the Wnt pathway and Akt has been suggested in a few papers but is far from being demonstrated.

There are a few reports in the literature suggesting that Akt associates with the Axin/GSK3 complex upon Wnt stimulation [29]. We confirmed this observation by performing an immunoprecipitation with the anti-Axin antibody. As shown in Figure 6a, both GSK and Akt co-immunoprecipitate with Axin in Me501 and LG cells. The Akt in the destruction complex is phosphorylated at Ser473. Treatment with IGFBP-3 (2 h) does not change the state of association of Akt with the complex but causes the almost complete dephosphorylation of Ser473. As suggested in the literature, the IGFBP-3 induced inactivation of Akt could in turn induce the dephosphorylation, hence activation, of GSK [29].

What is the role of Akt in the destruction complex? It is probably required to maintain in a phosphorylated state (hence inactive) GSK3-β, but it could possibly act on other components of the Wnt signaling pathway. To learn more about this point, we treated the Me501 cells with the Akt1/2 inhibitor for 0.5–1 to 2 h and analyzed by Western blot the phosphorylation state of Akt (Ser473) and LRP6 (Ser1490). The results (Figure 6b) show that Akt inhibition is able to effectively dephosphorylate the Wnt co-receptor LRP6 at Ser1490.

Taken together, the above results suggest that, in melanoma cells, Akt bound to the destruction complex may have a role in transducing Wnt-pathway depending signals, keeping in a phosphorylated state both

GSK and LRP6. IGFBP-3 is apparently able to inactivate by dephosphorylation the Akt fraction bound to Axin-1, and this event is probably sufficient to dephosphorylate the LRP6 receptor and thereby inhibit the Wnt signaling pathway.

IGFBP3 Acts Through Interaction With CD44

While we were engaged in the study of the effect of IGFBP-3 on the Wnt pathway in melanoma cells, a report appeared in the literature indicating that the protein known as CD44 acts as a positive regulator of the Wnt receptor complex [30]. CD44 is a transmembrane glycoprotein involved in the control of growth, survival, differentiation, and motility. Such control is exerted through interaction with other surface receptors such as receptor tyrosine kinases (RTKs) and Wnt [31].

In addition, CD44 is listed as an interactor of IGFBP-3 in the Human Protein Reference Database (http://www.hprd.org). Altogether, these reports prompted us to investigate whether CD44 was involved in mediating the action of IGFBP-3 on the Wnt pathway.

To this end, we first analyzed the cellular localization of IGFBP-3 and CD44 by the immunogold electron microscopy technique. The Me501 cells were treated for 30 min with IGFBP-3 and processed for electron microscopy as described in the Materials and Methods Section.
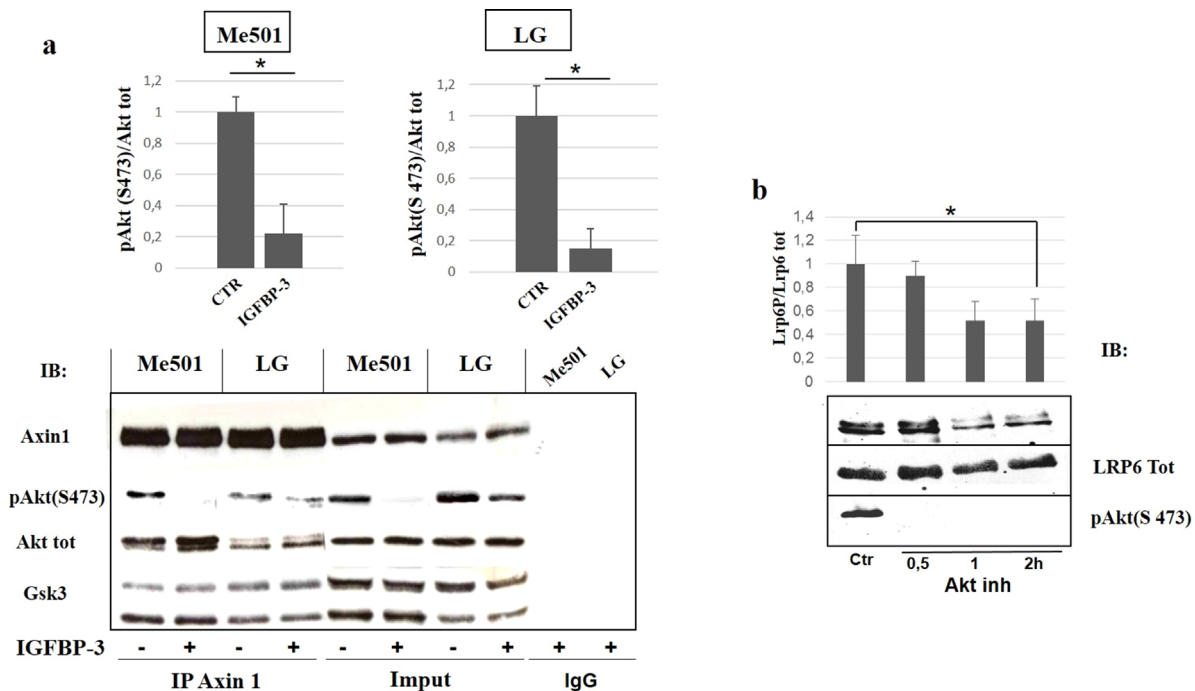

Figure 6. IGFBP-3 dephosphorylates Akt on the destruction complex and the Wnt LRP6 receptor. (a) Lysates of Me501 and LG cells treated with IGFBP-3 (2 μg/mL) for 2 h were immunoprecipitated with the anti-Axin1 antibody. The indicated proteins were detected with the appropriated antibodies (bottom panel) and their amounts were determined using the Image J software (upper panel, averaging three independent experiments). (b) Western-blotting experiments on lysates of Me501 cells treated with 20 μm Akt inhibitor (Ab142088) for 0.5–1–2 h. The dephosphorylation of both Akt (S473) and the Wnt LRP6 (S1490) receptor is clearly visible in the treated samples. Statistical significance was analyzed using the Student's t-test (*P < 0.05).

As shown in Figure 7a, in untreated cells, IGFBP-3 (white arrow) and CD44 (black arrow) were distributed between the cytoplasm and the membrane. However, after treatment with recombinant IGFBP-3, a co-localization of the two proteins below the membrane was detected, suggesting that they may interact in living cells. Interestingly, the co-localization was clearly observed 0.5 h after IGFBP-3 additions, but appeared to be entirely lost after 2 h (not shown). This may indicate that the contact between CD44 and IGFBP-3 is transient and precedes the interaction of IGFBP-3 with GSK3 in the destruction complex. These results are suggestive of a direct interaction between IGFBP-3 and CD44, but further data will be required to confirm this hypothesis.

We then asked whether CD44 was necessary to mediate the effect of IGFBP-3 on the components of the Wnt pathway. To this end, we analyzed the phosphorylation levels of Akt and GSK3 before and after treatment with IGFBP-3 in Me501 and LG cells where the expression of CD44 was attenuated by RNAi.

The results (Figure 7b) revealed that siRNA-mediated knockdown of CD44 strongly inhibited IGFBP-3-induced dephosphorylation of Akt and GSK3 in both cell lines. Note that the efficiency of siRNA suppression of CD44 expression was somewhat better in LG than in Me501 cells (Supplemental Figure S4b) but the magnitude of the observed biological effect was similar in the two lines. Also, it should be stressed that inhibition of CD44 expression per se had no effect on any of the molecular markers analyzed nor affected the amounts of total Akt or GSK3 (Supplemental Figure S4a, right panel).

Remarkably, the attenuation of CD44 expression by RNAi had by itself a strong inhibitory effect on the migratory behavior of Me501 cells (Supplemental Figure S4a). Taken together, these results suggest that the presence of CD44 is essential to mediate the action
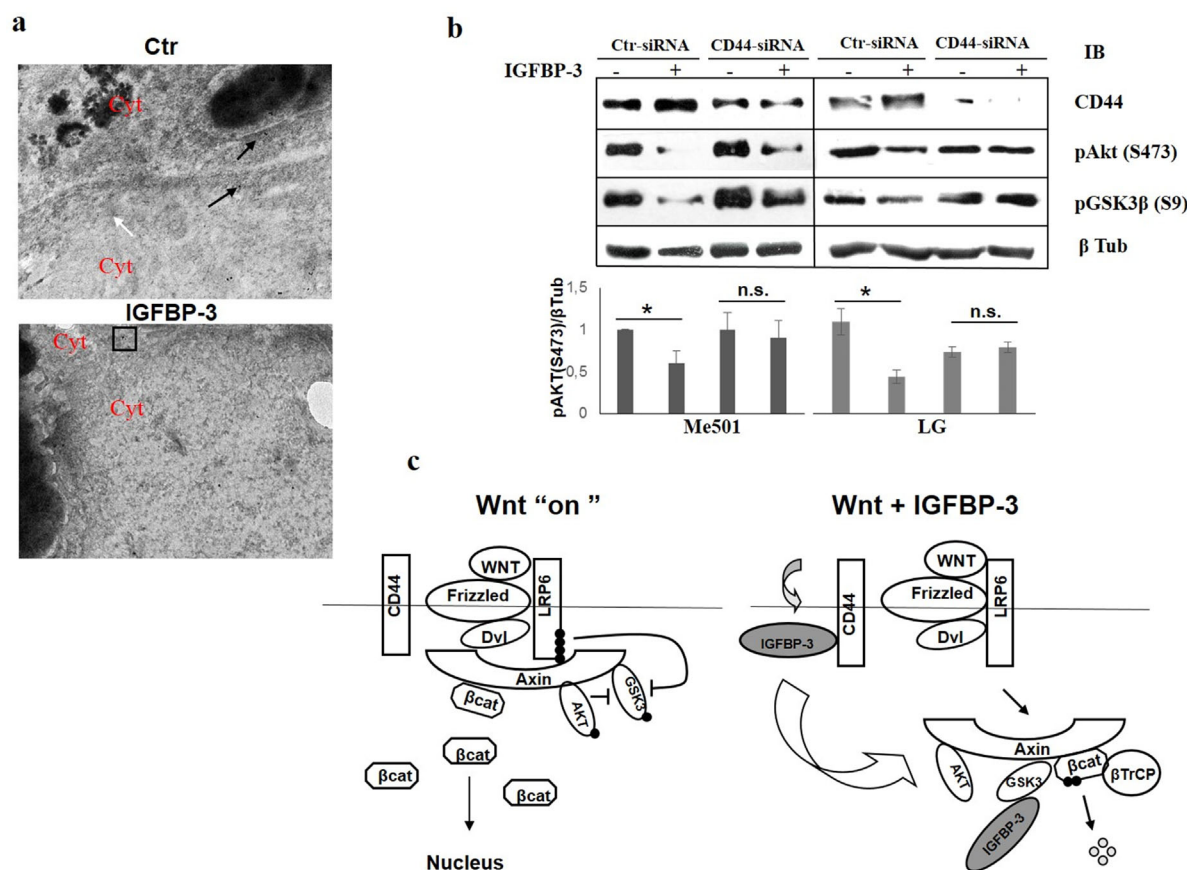

Figure 7. IGFBP-3 requires CD44 to act on the Wnt signalosome. (a) Electron micrographs showing the co-localization of CD44 (larger gold particles) and IGFBP-3 (smaller gold particles) after 0.5 h of treatment of Me501 cells with IGFBP-3 (2 μg/mL). Original magnification: 23.000×. Nu, nucleus; Cyt, cytoplasm. (b) Me501 (left panel) and LG cells (right panel) were transfected with control siRna or siRna against all CD44 isoforms for 48 h. All cells were treated with IGFBP-3 (2 μg/mL) for 1 h, lysed and analyzed by Western blot for the phosphorylation state of the indicated proteins. Statistical significance was analyzed using the Student's t-test ($^*P < 0.05$; n.s., not significant) (c) A possible model depicting the mode of action of IGFBP-3 on the Wnt signalosome. IGFBP-3 contacts the signalosome by interacting with CD44, then binds GSK3-β causing the dephosphorylation of Akt (S473), Gsk3 (S9), and Lrp6 (S1490) and the dissociation of the destruction complex from the receptorial complex. The destruction complex becomes activated and β-catenin is degraded.

of IGFBP-3 in melanoma and probably other types of cancer cells.

DISCUSSION

In previous works, we showed that IGFBP-3 is strongly involved in melanoma progression. A marked decrease of IGFBP-3 in blood serum was observed in stage IV melanoma patients, where it predicts increased metastatic dissemination of the disease and shorter life expectancy [20]. On the other hand, treatment with recombinant IGFBP-3 inhibited melanoma growth in mice and blocked the motility and invasiveness of cultured melanoma cells [21].

Although the main described role of IGFBP-3 is to modulate the activity of IGFs, mainly IGF1, the cytokine also has well-documented IGF1-independent activities [17]. This turned out to be true also for the anti-melanoma activity of IGFBP-3. As shown in a previous work [21], upon treatment of melanoma cells with IGFBP-3, the IGF1-PI3K axis remained inactive, while the main mediators of the IGFBP-3 anti-tumoral effect appeared to be AKT and GSK3-β. The underlying mechanism was not, however, investigated in detail.

In this paper, we have further studied the signal transduction pathways underlying the anti-tumoral properties of IGFBP-3 on melanoma cells. Our findings indicate that IGFBP-3 functions as a modulator of Wnt/β-catenin signaling. The involvement of IGFBPs in Wnt signaling had been suggested in a few works by other investigators [32,33] but the possible mechanism was not dealt with. Here, we show that IGFBP-3 interacts with certain molecular effectors of Wnt signaling at the level of the membrane destruction complex, causing the dissociation thereof from the Wnt receptor complex and activating β-catenin degradation.

As shown by our experiments, IGFBP-3 added to the culture medium rapidly enters the cells. It is unclear at this stage whether IGFBP-3 requires a receptor, possibly CD44, to be transported inside the cell or whether it enters independently (presumably via caveolin-clathrin-dependent endocytosis) and encounters an internal interactor, which again may be CD44. The existence of a direct interaction between CD44 and IGFBP-3 needs further experimental confirmation; however, CD44 appears to be essential to allow IGFBP-3 to act on the Wnt signaling machinery. Indeed, lowering the CD44 concentration by si-RNA interference strongly reduces IGFBP-3-induced GSK3-β and Akt dephosphorylation. Strikingly, a dearth of CD44 has by itself a potent inhibitory effect on cell motility, reinforcing the surmise that it is a key player in regulating the metastatic potential of melanoma cells.

Among the components of the Wnt signaling machinery, the principal interactor of IGFBP-3 seems to be GSK3-β. Treatment with IGFBP-3 causes the dissociation of GSK3-β (probably along with the other components of the destruction complex) from the Wnt LRP6 receptor. Concomitantly, GSK3-β becomes dephosphorylated, hence active, and thereby able to phosphorylate β-catenin targeting it for destruction. IGFBP-3 interacts directly with GSK3-β, as shown by the fact that the two proteins can be immunoprecipitated. The primary role of GSK3-β in mediating IGFBP-3 inhibition of Wnt signaling is also borne out by the fact that treatment with LiCl, a well-known inhibitor of GSK3-β, has opposite effects to those observed upon treatment with IGFBP-3. Treatment with LiCl increases the phosphorylation of GSK3-β, decreases the Axin-1 levels, and raises β-catenin ones. Administration of increasing doses of IGFBP-3 in the presence of LiCl reverses these Wnt-stimulating effects of LiCl.

In summary, cells treated with IGFBP-3 have higher amounts of active destruction complex as demonstrated by the increased degradation of β-catenin mediated by β-TrCP.

It is important to point out that PI3K signaling, although involved in other physiological activities of IGFBP-3, has nothing to do with the ability of this cytokine to decrease β-catenin levels. Indeed, treatment of melanoma cells with an inhibitor of PI3K has no effect at all on the concentration of β-catenin, albeit reducing the phosphorylation of both Akt and GSK3-β. This shows that different cellular fractions of Akt and GSK3-β are involved in PI3K and Wnt signaling, respectively, and that IGFBP-3 acts on the latter one.

As we have shown in a previous work and again in the present one, the earliest effect of IGFBP-3 treatment actually is Akt dephosphorylation. Some works in the literature implicate Akt in the regulation of the Wnt signaling pathway [29], but its precise role remains unclear. Here, we show that Akt phosphorylation at Ser 473 is indeed stimulated upon Wnt treatment, and that IGFBP-3 is able to reverse this effect. More importantly, IGFBP-3 specifically causes the dephosphorylation of the Akt fraction associated with Axin, hence with the destruction complex. The role of Akt within the Wnt signalosome warrants more detailed investigations, but our data indicate that it is probably important to keep in a phosphorylated state both GSK3-β and the LRP6 receptor. Indeed, we show that treatment with an inhibitor of Akt causes the almost complete dephosphorylation of the LRP6 receptor, indicating that Akt, in addition to its other multifarious activities, is also probably pivotal in modulating Wnt signaling.

In Figure 7c, we present a tentative model to explain the mode of action of IGFBP-3 on Wnt signaling. IGFBP-3, before or after its entrance in the cell, interacts (directly or indirectly) with CD44 bound to the Wnt signalosome. Simultaneously, or in rapid sequence, IGFBP-3 also interacts with GSK3-β. This interaction is accompanied by the dissociation of the Axin destruction complex from the Wnt receptor, and

by the dephosphorylation of Akt first and GSK3-β immediately afterwards.

Exactly how IGFBP-3 causes Akt and GSK3-β dephosphorylation is still unclear and will be the subject of future investigation. Since Akt dephosphorylation precedes that of GSK3-β, it is conceivable that GSK3-β activation by dephosphorylation is, at least in part, promoted by the lack of ongoing Akt activity. The dephosphorylation of the Akt pool linked to the destruction complex is harder to explain. A possibility is that the GSK3-β-mediated interaction of IGFBP-3 with the destruction complex increases the activity of the PP1 phosphatase, known to be associated with the complex. Another possibility would be that the binding of IGFBP-3 to the destruction complex somehow hinders the action of the kinase mTorc2, principally involved in phosphorylating Akt at Ser 473. The two possibilities are not mutually exclusive.

In conclusion, our data show that IGFBP-3 act as a novel, and strong, inhibitor of Wnt signaling. As Wnt signaling is involved in a vast series of pathologies, and pharmaceutical Wnt inhibitors are both scarce and toxic, recombinant IGFBP-3 might prove to be useful in the treatment of pathologies related to perturbations of Wnt signaling, first of all cancer. This is all the more important since IGFBP-3 is a physiological protein that showed no adverse effects when administered in vivo to mice, even at concentrations ten times the physiological one in blood serum [21].

AUTHORS' CONTRIBUTIONS

NA: conceived the study, participated in its design and coordination, and helped to draft the manuscript. ZM, SL, RR: carried out the immunoelectron microscopy experiments. PD, MM: carried out the siRNA experiments. PL: participated in its design and coordination and helped to draft the manuscript.

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