FOXP3 expression is modulated by TGF-β1/NOTCH1 pathway in human melanoma

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Abstract. Forkhead box protein 3 (FOXP3) transcription factor is expressed by immune cells and several human cancers and is associated with tumor aggressiveness and unfavorable clinical outcomes. NOTCH and transforming growth factor-β (TGF-β) protumorigenic effects are mediated by FOXP3 expression in several cancer models; however, their interaction and role in melanoma is unknown. We investigated TGF-β-induced *FOXP3* gene expression during NOTCH1 signaling inactivation. Primary (WM35) and metastatic melanoma (A375 and A2058) cell lines and normal melanocytes (NHEM) were used. FOXP3 subcellular distribution was evaluated by immunocytochemical analysis. Gene expression levels were assessed by reverse transcription-quantitative polymerase chain reaction. Protein levels were assessed by western blot analysis. The γ-secretase inhibitor (GSI) was used for NOTCH1 inhibition and recombinant human (rh)TGF-β was used for melanoma cell stimulation. Cell proliferation and viability were respectively assessed by MTT and Trypan blue dye assays. FOXP3 mRNA and protein levels were progressively higher in WM35, A375 and A2058 cell lines compared to NHEM and their levels were further increased after stimulation with rh-TGF-β. TGF-β-mediated FOXP3 expression was mediated by NOTCH1 signaling. Inhibition of NOTCH1 with concomitant rh-TGF-β stimulation determined the reduction in gene expression and protein level of FOXP3. Finally, melanoma cell line proliferation and viability were reduced by NOTCH1 inhibition. The results show that nn increase in FOXP3 expression in metastatic melanoma cell lines is a potential marker of tumor aggressiveness and metastasis. NOTCH1 is a central mediator of TGF-β-mediated FOXP3 expression and NOTCH1 inhibition produces a significant reduction of melanoma cell proliferation and viability.

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Introduction

Among all skin tumors, melanoma is the most aggressive form because of rapid metastasis and resistance to conventional radio- and chemotherapy (1-3). The advances in understanding the microenvironment of melanoma and cell biology make it obvious that the treatment needs to be multi-directional. Melanoma is a highly immunogenic tumor (4) and numerous immunotherapeutic strategies have been tested (5-8). Although it has been demonstrated that the various immune-based therapies induce an increase in circulating tumor antigen-specific T cells, these approaches have produced a poor therapy response, due to tumor-induced immune suppression and tumor evasion of anti-tumor immune responses (9).

Regulatory T cells (Tregs) are $CD4^+$ $CD25^+$ cells characterized by the forkhead box protein 3 (*FOXP3*) transcription factor expression, which is the most specific marker for Tregs (10,11). These cells exert an immunosuppressive function and *FOXP3* is a prerequisite for this suppressive activity, ultimately leading to tumor immune evasion/escape (12,13). Additionally, patients with an altered expression or function of *FOXP3* can develop serious autoimmune diseases and cancers (14,15). FOXP3, a member of the forkhead/winged-helix family of transcription factors, constitutively translocate into the nucleus where it binds to specific sequences of DNA to regulate the transcription of its target genes (16,17). Although FOXP3 protein expression was considered to be restricted to lymphocytes, recently it has been reported to be expressed in various human malignancies, such as pancreatic, lung, colon, breast, ovarian, prostate cancers, hepatocellular carcinoma, and melanoma (18-28). *FOXP3* has been also associated with an unfavorable disease course (24,25,27) and identified as an independent prognostic factor and a marker of tumor progression and metastasis (29-33). Indeed, numerous studies have

demonstrated that metastases and poor clinical response of melanoma are closely related to the large number of Tregs and high *FOXP3* expression (27,34-36).

Multiple signaling pathways, including NOTCH and transforming growth factor-β (TGF-β/Smad), are closely associated with *FOXP3* transcription (37-41). NOTCH signaling regulates essential cell processes, such as stem cell self-renewal, proliferation, differentiation and apoptosis (42-44). Previous experimental data have shown that aberrant NOTCH signaling may lead to cancer, although its effect greatly depends on tissue type and interaction with other signaling pathways (45,46). Activation of the NOTCH receptor is triggered by its interaction with NOTCH ligands (Delta-like 1, 3, 4; Jagged-1, 2) present on adjacent cells (47). Upon ligand binding, the NOTCH intracellular domain (NICD) is proteolytically cleaved and translocated into the nucleus where it interacts with its corresponding co-activators to promote the transcription of downstream target genes (48,49). Dysregulated NOTCH signaling has been involved in the development and progression of many types of cancer (50-56). Findings have shown that the upregulation of NOTCH signaling may play a role in melanoma cells transformation and progression (50-62,33).

In addition to NOTCH, TGF-β is known as a double-edged sword during cancer progression, being a tumor suppressor or a tumor promoter, depending on the context of signal activation (63-65).

TGF-β is a pleiotropic cytokine that negatively regulates the activity of immune cells, playing an important role in the control of T-cell functions, growth and differentiation (66). Moreover, TGF-β signaling is involved in Tregs differentiation being required for their *in vivo* expansion and immunosuppressive capacity (67). *In vitro* studies have shown that TGF-β may trigger FOXP3 expression in $CD4^+$ $CD25^-$ naive T cells, switching them towards a $CD4^+CD25^+$ regulatory phenotype, probably through activation of Smads, which results in a positive autoregulatory loop (68,69). Furthermore, all human tumors overproduce TGF-β, whose autocrine and paracrine actions promote tumor cell invasiveness and metastasis (70-74). TGF-β signaling can synergize with NOTCH in many processes (75-77). Previous findings have identified the bidirectional regulation of *NOTCH* and *TGF-β*, through different context-dependent mechanisms and a functional synergism in the regulation of hairy and enhancer of split 1 (*HES1*), a direct target of the NOTCH signal, has been demonstrated (78-80). It has been previously shown that the induction of FOXP3-Tregs is cooperatively regulated by NOTCH signaling and TGF-β (76,79,81-83).

Few reports have shown the association between *FOXP3* and *NOTCH* in cancers (84,85) and the cross-talk between them is unexplored in melanoma. Since TGF-β and NOTCH are involved in the regulation of the *FOXP3* gene transcription, we investigated, in melanoma *in vitro* models, the mechanisms of TGF-β1-induced *FOXP3* gene expression in relation to NOTCH signaling inactivation. For this reason, we have used a synthetic tripeptide aldehyde containing γ-secretase inhibitor (GSI), a pharmacological agent known to block NOTCH processing and activation through the inhibition of proteolysis and translocation of NIDC to the nucleus (86).

Materials and methods

Human melanoma cell lines and culture conditions. Human epithelial melanocytes (NHEM) were purchased from Lonza (Lonza Group, Ltd., Basel, Switzerland), cultured in Melanocyte Growth Medium (Lonza Group, Ltd.) and used as controls. WM35 (from primary lesion), A375 and A2058 (from metastatic lesion) melanoma cell lines, a kind gift from V. Russo (Tumor Targeting Research Unit, San Raffaele Scientific Institute, Milano, Italy) were cultured in RPMI-1640 medium (Gibco; Life Technologies, Inc., Monza, Italy), supplemented with 2 mmol/l L-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, 10% of heat-inactivated fetal calf serum (Gibco; Life Technologies, Inc.) and maintained under an atmosphere of 5% CO_2 at 37˚C. For the western blot analysis, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunocytochemical analysis (ICC), 70-80% confluent cultures were used.

Immunocytochemical analysis. A total of $1x10^5$ cells (WM35, A375 and A2058) were grown on glass slides. Cells were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde, pH 7.4 for 20 min at room temperature. The cells were permeabilized with 0.5% Triton X-100 for 4 min and after washing in PBS were treated with 1% BSA to block non-specific binding sites. FOXP3 immunodetection was performed using a primary antibody anti-FOXP3 (1:100 dilution; ThermoFisher Scientific, eBioscience, Inc., San Diego, USA) for 2 h at room temperature, revealed using the Immuno Cruz Staining System (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The cells were counterstained with hematoxylin for 30 sec. Appropriate positive and negative controls were carried out.

RNA extraction and RT-qPCR. Total RNA was extracted from WM35, A375 and A2058 cell lines stimulate or not with TGF-β1 using the Micro-to-Midi total RNA purification system (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

mRNA expression was measured using SYBR-Green RT-qPCR using the Rotor-Gene Q thermal cycler (Qiagen, Inc., Valencia, CA, USA).

Amplification reactions were performed using primers specific for FOXP3 (forward, 5'-CACAACATGCGACCCC CTTTCACC-3' and reverse, 5'-AGGTTGTGGCGGAT GGCGTTCTTC-3'), NOTCH1 and HES1 (QuantiTect® Primer Assay; Qiagen, Inc.). The PCR reaction was carried out in 25 μl buffer, containing 50 ng cDNA, 1 μM of each primer and 12.5 μl 2X RotorGene SYBR-Green PCR Master Mix (Qiagen, Inc.). The thermal cycling conditions were as follows: denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation for 10 sec at 95˚C and annealing and extension for 15 sec at 60˚C. As housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; QuantiTect Primer assay; Qiagen, Inc.) was used. Transcripts quantification was carried out utilizing the software supplied with Rotor-Gene Q. The experiments were repeated three times.

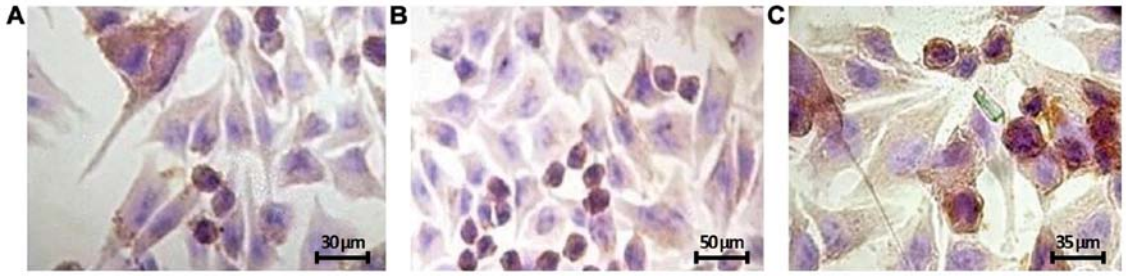

Figure 1. Immunocytochemical analysis of FOXP3 expression in the melanoma cell lines. (A) A moderate nuclear/cytoplamic Foxp3 staining in WM35 was observed. (B and C) Moderate to strong nuclear/cytoplasmic staining of FOXP3 protein is detected in the A375 and A2058 cell lines. FOXP3, forkhead box protein 3.

Protein extraction and western blot analysis. Cells were lysed in RIPA buffer (Thermo Fisher Scientific, Inc.) in the presence of 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Thermo Fisher Scientific, Inc.) and incubated on ice for 30 min. Protein concentration was determined by Bradford assay, using the Quick Start Bradford kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins were fractionated using SDS-PAGE (Invitrogen; ThermoFisherScientific,Inc.)andtransferredontonitrocellulose membranes (Trans-Blot Transfer Medium Pure Nitrocellulose Membrane 0.45 μm; Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% non-fat dried milk in TBS buffer containing (20 mM Tris, 500 mM NaCl, and 0.05% Tween-20) and incubated overnight with the appropriate primary monoclonal antibody: Anti-NOTCH1-NICD (dilution 1:1,000; cat. no. 14-5785-81; ThermoFisher Scientific, eBioscience, Inc.), anti-FOXP3 (dilution 1:1,000; cat. no. 14-5773-80; ThermoFisher Scientific, eBioscience, Inc.), anti-HES1 (dilution 1:500; cat. no. AB5702 ; EMD Millipore, Billerica, MA, USA), anti-Smad3 and phospho-Smad3 (dilution 1:1,000; cat. nos. 9513S and 9520S, respectively; Cell Signaling Technology, Inc., Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG was used as the secondary antibody and the protein bands were detected using the enhanced chemiluminescence detection system (ECL detection system; Bio-Rad Laboratories, Inc.). Protein levels were determined using laser densitometry and normalized to GAPDH (Calbiochem; Merck KGaA, Darmstadt, Germany) levels in each sample.

GSI and TGF-β treatment. A synthetic tripeptide aldehyde inhibitor, GSI (Calbiochem; Merck KGaA), a potent GSI, was used to block NOTCH1-mediated signal transduction in melanoma cell lines. Cells in the logarithmic growth phase were seeded at densities of $4x10^5$ cells/ml (WM35) and $2x10^5$ cells/ml (A375 and A2058) and treated or not with GSI, at different concentrations (5, 10 and 20 μM) for the desired period of time. The cells were then stimulated with recombinant human (rh)TGF-β (5 ng/ml; Gibco; Life Technologies, Inc.) for 48 h. Control cells were treated with an equal volume of dimethyl sulfoxide (DMSO). Expression of FOXP3 and NOTCH signaling, cell growth proliferation and inhibition of melanoma cell lines were analyzed.

Cell proliferation-cytotoxicity assay. GSI effects on cell proliferation was measured using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); Sigma-Aldrich, St. Louis, MO, USA] colorimetric assay as described by Cardile *et al* (87). In brief, melanoma cell lines ($1\text{-}2x10^4$ cells/well) were grown overnight in 24-well plates and then treated with vehicle alone or different concentrations of GSI. After 24, 48 and 72 h treatment, the cells were incubated with 20 μl of 0.5% MTT in PBS for 4 h at 37˚C in a humidified 95% air/5% CO_2; supernatant was removed and 100 μl of DMSO was added to each well. Optical density was measured at 550 nm (Titertek Multiskan; DAS). Cell viability was expressed as a percentage of treated cells with respect to appropriate controls. Trypan blue dye exclusion assay was used to evaluate the percentage of dead cells with respect to the total number of cells.

Statistical analysis. Differences between TGF-β1 stimulated or not were compared by Student's t-test. Differences among multiple groups were compared by analysis of variance (ANOVA test) and a post hoc test for multiple comparisons (Tukey's test). Data are presented as mean ± SD. P-values <0.05 were considered statistically significant.

Results

Upregulation of FOXP3 expression on human melanoma cell lines by TGF-β1. We used different approaches to examine the baseline gene expression of *FOXP3* as well as the protein levels, in human melanoma cell lines at different stages, primary (WM35) versus metastatic (A375 and A2058) cells. Normal human epidermal melanocytes (NHEM) were used as a control.

First, we observed by immunocytochemistry that *FOXP3* was mainly localized in the nucleus and, was less evident, in the perinuclear region and cytoplasm. Moreover, the three melanoma cell lines showed different staining intensity, being the A2058 cell lines that were mostly expressed (Fig. 1), while NHEM *FOXP3* staining was undetectable (data not shown).

Next, we examined the relative mRNA level of *FOXP3* in WM35, A375 and A2058 melanoma cells. As expected, *FOXP3* transcriptional levels, assessed by RT-qPCR, were higher in A2058 cells compared to A375 and WM35 cells, and very low in NHEM (Fig. 2A). In accordance with the RT-qPCR data, western blot analysis revealed that FOXP3 protein level expression was higher in A2058 compared to A375 and WM35 cells, whereas NHEM cells had a very weak expression (Fig. 2B). Moreover, since TGF-β1

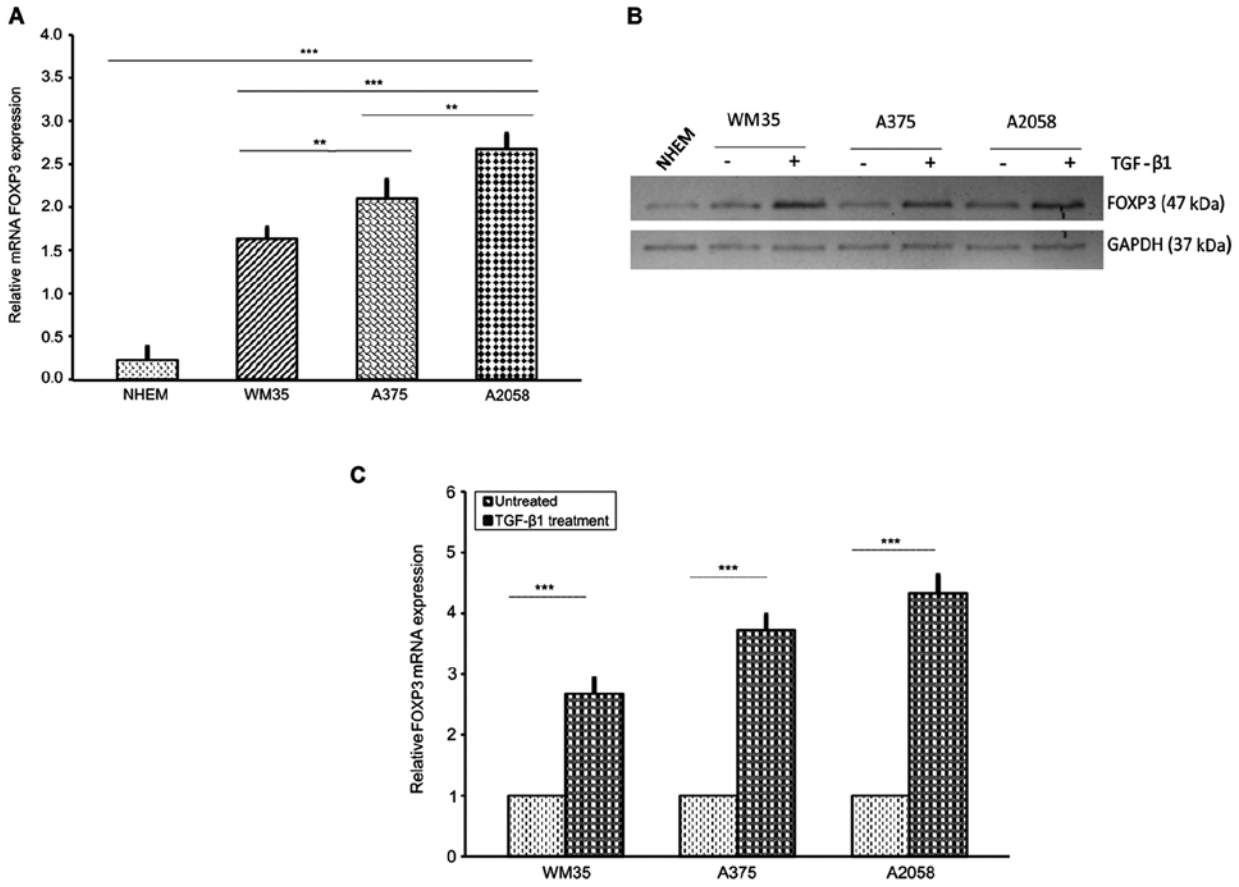

Figure 2. FOXP3 expression in human melanoma cell lines at different stages. (A) RT-qPCR of FOXP3 in melanocytes (NHEM), primary (WM35) and metastatic (A375 and A2058) melanoma cells. The melanoma cell lines expressed FOXP3 mRNA. Melanocytes served as a control. A375 and A2058 cells showed the highest levels of *FOXP3* gene expression. (B and C) Effect of TGFβ-1 treatment on protein and FOXP3 mRNA levels in melanoma cell lines. Treatment with rhTGF-β1 (5 ng/ml) for 48 h induced a higher increase of FOXP3 mRNA and their own protein levels in WM35, A375 and A2058 melanoma cells. As an internal control, *GAPDH* was used for normalization. Data are shown as mean ± SD of three independent experiments. The comparison of multiple groups was performed by ANOVA and Tukey's test. High significance (***P<0.0001) was found between TGF-β1 stimulated or not in the three melanoma lines (Student's t-test). FOXP3, forkhead box protein 3; RT-qPCR, reverse transcription-quantitativepolymerase chain reaction; NHEM, normal human epidermal melanocytes; TGF-β, transforming growth factor-β; rh, recombinant human; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

regulates T-cell function through FOXP3 (69), we examined the effect of this cytokine on *FOXP3* expression in melanoma cells. Stimulation with rhTGF-β1 (5 ng/ml) for 48 h significantly increased the mRNA expression of *FOXP3*, approximately of 1.68-, 2.74- and 3.3-fold in WM35, A375 and A2058 cells, respectively, compared to untreated cells (Fig. 2C). Shorter treatments did not induce any appreciable change in FOXP3 expression. Western blot analysis confirmed the upregulation of TGF-β1-induced FOXP3 protein levels (Fig. 2B). Altogether, our results show a very high expression of the transcription factor FOXP3 in human metastatic melanoma cells, suggesting that FOXP3 could be considered a biological marker of melanoma progression, probably contributing to metastasis, as described by other authors (29,34-36).

Modulation of FOXP3 expression through the NOTCH signaling pathway. It has been shown that NOTCH signaling may be involved in the activation of *FOXP3* promoter (39,85). To analyze the potential role of NOTCH in modulating the *FOXP3* expression in melanoma cells, we used the GSI, a GSI, responsible for inhibition of NOTCH cleavage into the active NICD (47,86).

First, we tested, by RT-qPCR, the efficacy of GSI in *FOXP3* expression reduction. WM35, A375 and A2058 cells were treated with DMSO, as a control, or increasing concentrations of GSI (5, 10 and 20 μM) for 24, 48 and 72 h. *FOXP3* mRNA expression decreased in a concentration- and time-dependent manner in all cell lines (Fig. 3A and B). GSI in low doses (5, 10 μM) for 24 and 48 h did not show any significant modification of *FOXP3* gene expression (data not shown). At 72 h (Fig. 3A), we found a *FOXP3* mRNA reduction of 40.7, 52.9%; 40.1, 62.2 and 53%, 62.6% in WM35, A375 and A2058 cells treated with 20 μM GSI compared to low doses (5, 10 μM), respectively. Furthermore, at 20 μM GSI, a modest reduction of *FOXP3* mRNA was observed in the three melanoma cell lines at 24 and 48 h, while a strong and significant reduction was observed at 72 h (Fig. 3B). Thus, 20 μM GSI for 72 h was the proper concentration and time for treating melanoma cells. We also verified whether *FOXP3* was regulated at the translation level. In agreement with the RT-qPCR data, we observed a decrease of FOXP3 protein levels, in a dose-dependent manner, in the three melanoma cell lines after 72 h of GSI treatment. Fig. 3C shows the protein levels in the WM35 cell line treated with 5, 10 and 20 μM GSI at 72 h.

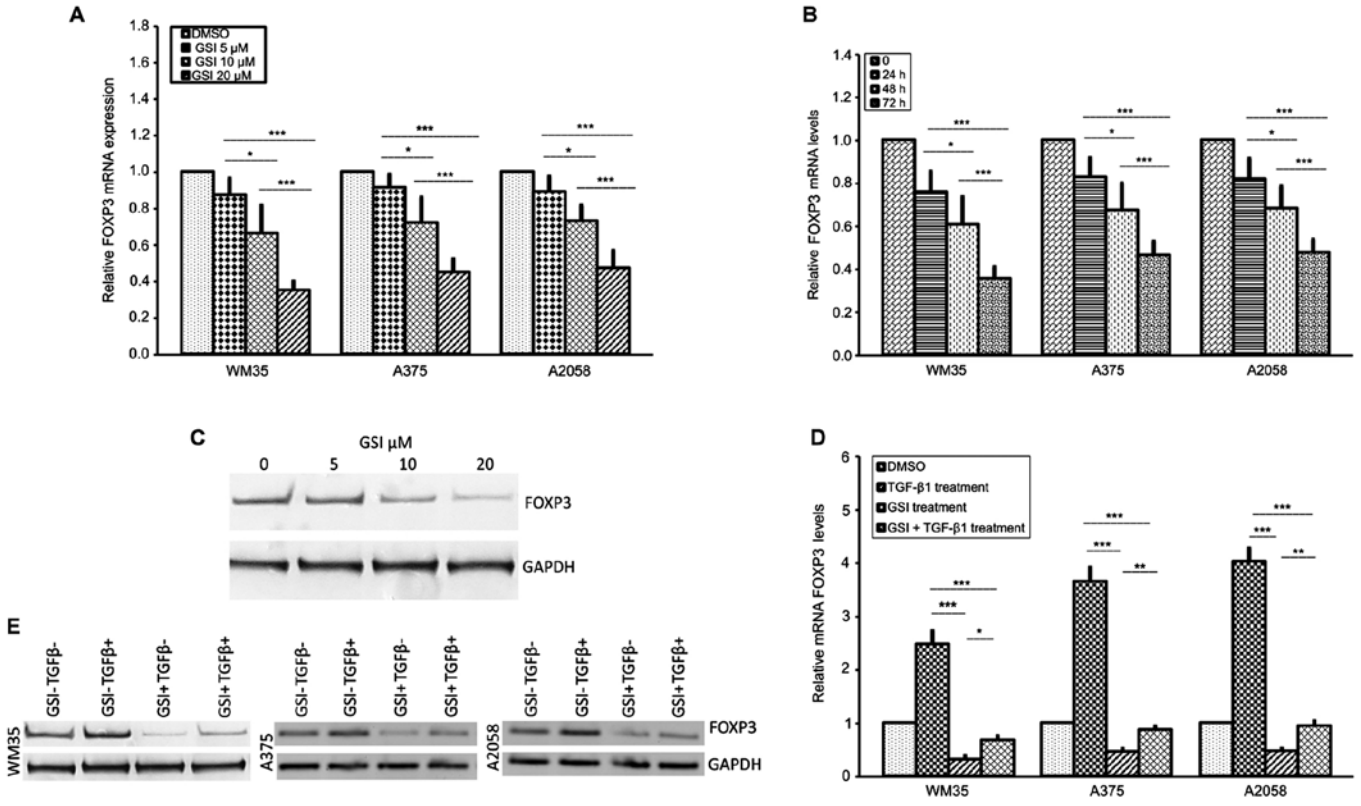

Figure 3. Effect of GSI on FOXP3 expression in melanoma cell lines. (A) Inhibition of FOXP3 mRNA is shown after 72 h of 5-, 10- and 20 μM GSI treatment in melanoma cells. RT-qPCR shows that FOXP3 mRNA levels were downregulated in GSI-treated WM35, A375 and A2058 melanoma cells in a dose-dependent manner. Maximum inhibition of FOXP3 was observed at 20 μM of GSI. (B) Inhibition of FOXP3 mRNA after 24, 48 and 72 h with 20 μM/GSI treatment in WM35, A375 and A2058 melanoma cell lines. A statistically significant time-dependent decrease in FOXP3 mRNA level was observed in each melanoma cell line. (C) Inhibition of FOXP3 protein expression after 72 h of 5-, 10- and 20 μM GSI treatment in WM35 melanoma cells. Western blot analysis showed that the protein levels of FOXP3 were downregulated in GSI-treated WM35 cells in a dose-dependent manner. GAPDH expression was used as a loading control. (D and E) Effect of GSI/TGF-β1 treatment on FOXP3 mRNA and protein expression in melanoma cell lines. Inhibition of FOXP3 mRNA and protein levels are shown after 72 h of GSI treatment in WM35, A375 and A2058 melanoma cells. *In vitro* GSI treatment downregulated TGF-β1-induced FOXP3 mRNA and protein levels in all the melanoma cell lines. As an internal control, *GAPDH* was used for normalization. Data are shown as mean ± SD of three independent experiments. The comparison of mRNA FOXP3 expression in multiple groups was performed by ANOVA and Tukey's test. GSI, γ-secretase inhibitor; FOXP3, forkhead box protein 3; TGF-β, transforming growth factor-β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *P<0.01; **P<0.001; ***P<0.0001.

To validate the possible relationship between NOTCH1/TGF-β1 in regulating *FOXP3* expression, we also examined whether GSI, a NOTCH signaling inhibitor, influenced the TGF-β1-dependent *FOXP3* upregulation. WM35, A375 and A2058 cell lines were pre-treated overnight with 20 μM GSI, to block NOTCH activation, using an equal volume of DMSO as control, and the cells were subsequently stimulated with rhTGF-β1 for 48 h. Fig. 3D shows that 20 μM GSI significantly decreased FOXP3 mRNA levels in WM35, A375 and A2058 cell lines treated with TGF-β1, although the downregulation did not reach the levels observed in cells treated with GSI alone. Data were confirmed by western blot analysis (Fig. 3E).

Taken together, these data indicate that upregulation of *FOXP3* by TGF-β1 may require input from the NOTCH signaling pathway.

Activation of NOTCH1 pathway by TGF-β1 in human melanoma cell lines. Since TGF-β1 upregulates *FOXP3* levels and in considering that NOTCH/TGF-β signaling is involved in the tumorigenic process of cancers (50,65,88), including melanoma (62,89,90), we investigated whether TGF-β1 was able to affect the NOTCH signaling in melanoma cell lines. We tested NOTCH1^{NICD} and the NOTCH-specific target gene *HES1* expression in WM35, A375, A2058 and NHEM cells by RT-qPCR and western blot analysis. Generally, NICD levels reflect the activation status of NOTCH signaling.

Our results showed that NOTCH1^{NICD} and *HES1* expression were significantly increased in the three melanoma cell lines compared to NHEM cells at both mRNA and protein levels (Fig. 4A and D). This increase was more evident in WM35 compared to A375 and A2058 cells. Next, to confirm the association between TGF-β1 and NOTCH1, we stimulated WM35, A375 and A2058 cells with rhTGF-β1 (5 ng/ml) for 48 h. We found higher mRNA and protein levels of NOTCH1^{NICD} and HES1, in WM35, A375 and A2058 cells, after stimulation with rhTGF-β1, compared to untreated cells (Fig. 4B-D), confirming earlier studies carried out in other cancer types (78,79,88,91).

GSI prevents TGF-β1-dependent NOTCH1 activation pathway. In parallel, we explored whether NOTCH signaling inhibition could prejudice NOTCH/TGF-β axis. Western blot analysis revealed that NOTCH-1 protein and its downstream effector *HES1* were downregulated in GSI-treated melanoma

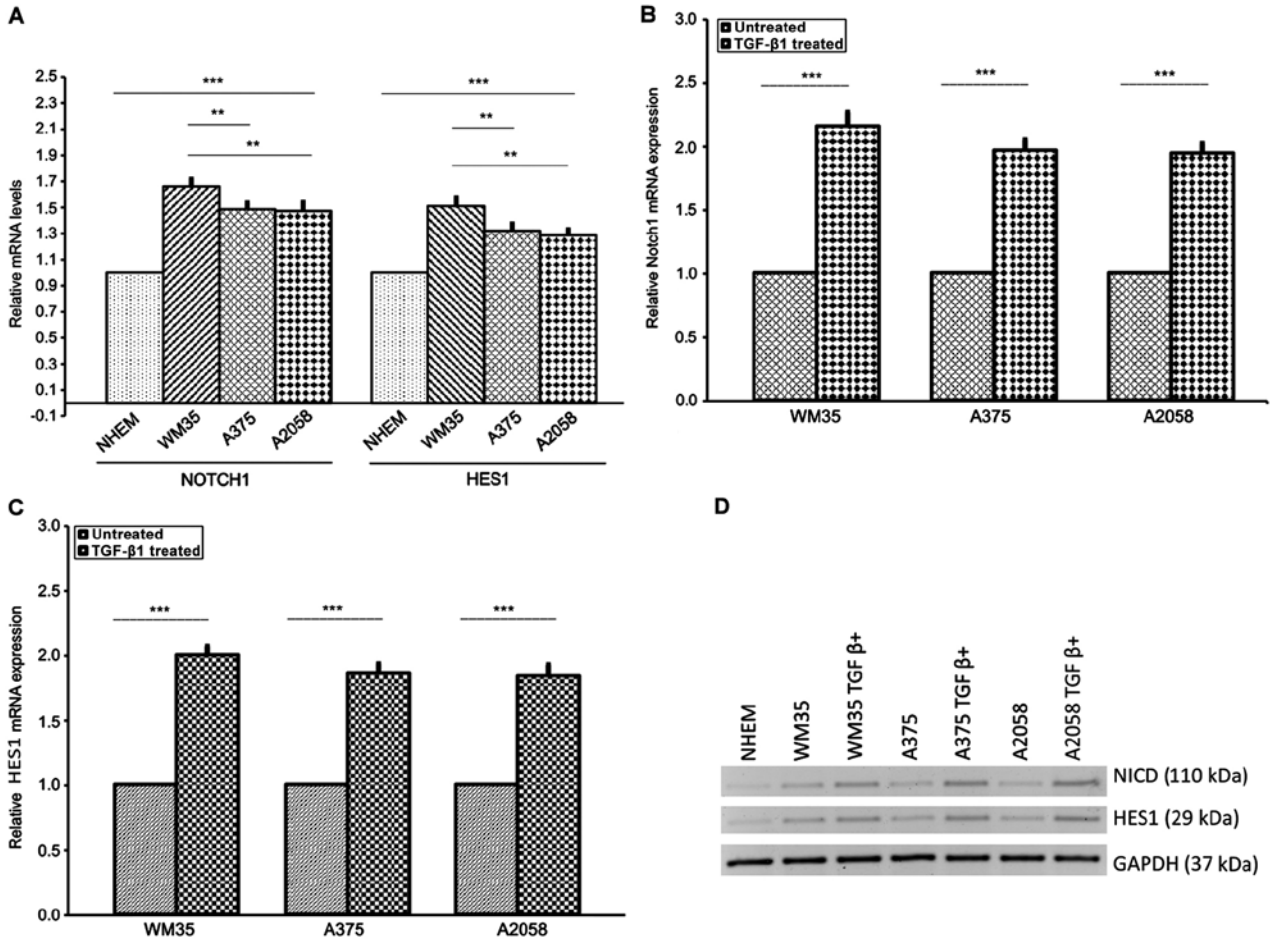

Figure 4. Expression of NOTCH1^{NICD} and NOTCH-specific target gene *HES1* in melanoma cell lines. (A) mRNA of NOTCH1^{NICD} and HES1 was measured by RT-qPCR in NHEM, WM35 and A375 and A2058 melanoma cells. Melanocytes served as the control. WM35 showed a higher level of NOTCH1^{NICD} mRNA and HES1 mRNA than A375 and A2058 cells. (B) Protein level of NOTCH1^{NICD} and HES1 was measured by western blot analysis in WM35, A375 and A2058 melanoma cell lines. All of the melanoma cell lines positively expressed NOTCH1^{NICD} and HES1. (B-D) Effect of TGFβ-1 treatment on NOTCH1^{NICD}, HES1 mRNA and protein levels in melanoma cell lines. Treatment with rhTGF-β1 (5 ng/ml) for 48 h induced a higher increase of NOTCH1^{NICD} and HES1 mRNA and their own protein levels in WM35, A375 and A2058 melanoma cells. As an internal control, GAPDH was used for normalization. Data are shown as mean ± SD of three independent experiments. The comparison of mRNA NOTCH-1 and HES1 expression in multiple groups was performed by ANOVA and Tukey's test. HES1, hairy and enhancer of split 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGF-β, transforming growth factor-β; rh, recombinant human; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. **P<0.001; ***P<0.0001.

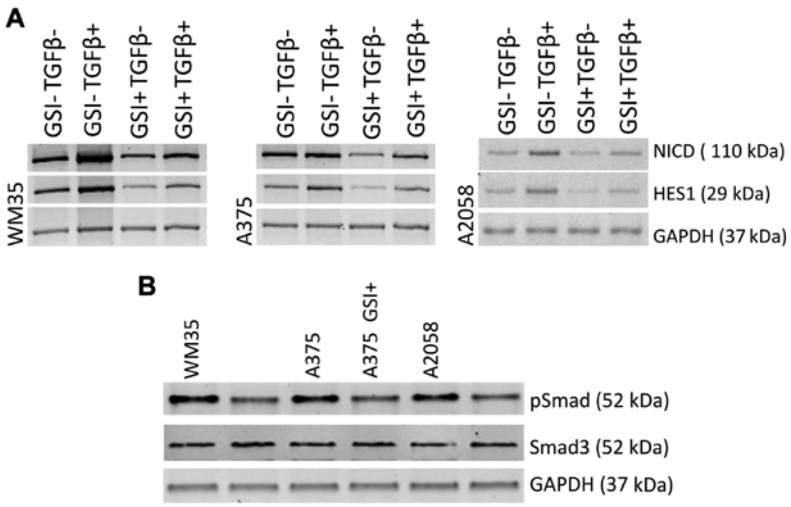

Figure 5. GSI effect on NOTCH1^{NICD}, NOTCH-specific target gene *HES1* expression and on TGF-β/Smad signaling in melanoma cell lines. (A) Inhibition of NOTCH1^{NICD} and NOTCH-specific target gene *HES1* is illustrated after 72 h of 20 μM GSI treatment in WM35, A375 and A2058 cells. Western blot analysis showed that GSI suppressed NOTCH1^{NICD} and HES1 protein levels and downregulated TGFβ-1- induced NOTCH1^{NICD}, HES1 protein levels in melanoma cell lines. (B) GSI treatment consistently decreased pSMAD3 levels in all melanoma cell lines. GAPDH served as loading control. GSI, γ-secretase inhibitor; HES1, hairy and enhancer of split 1; TGF-β, transforming growth factor-β; pSMAD3, phosphorylated Smad3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

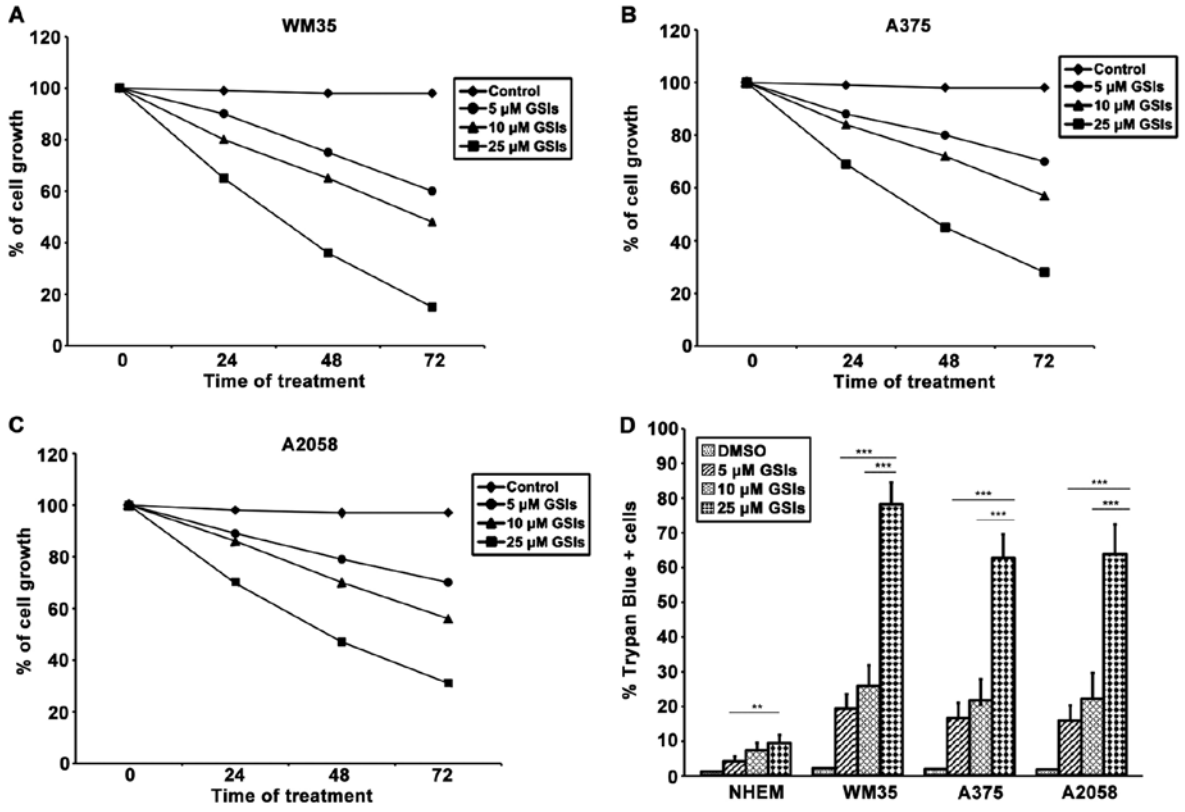

Figure 6. The effect of GSI on human melanoma cell line proliferation. (A) WM35, (B) A375 and (C) A2058 cell viability following 24-, 48- and 72-h GSI treatment. Cells were incubated with GSI at 5-, 10- and 20 μM. (A-C) GSI 20 μM induces a significant decrease in cell proliferation. (D) Trypan blue exclusion test was performed in NHEM, WM35, A375 and A2058 cell lines. High cytotoxicity was observed with 20 μM GSI only in melanoma cell lines. Data are expressed as percentage of cell viability/cytotoxicity with respect to vehicle control as described in the Materials and methods. Results are the mean ± SD from three separate experiments. The comparison of multiple groups was performed by ANOVA and Tukey's test. GSI, γ-secretase inhibitor; NHEM, normal human epidermal melanocytes. ***P<0.0001.

cells compared with vehicle control (Fig. 5A), confirming the ability of the drug to affect the NOTCH signaling pathway (61). Furthermore, to corroborate the effects of the NOTCH inhibition on TGF-β1-induced NOTCH1^{NICD} activation, WM35, A375 and A2058 cell lines were treated with TGF-β1 alone or combined with GSI. Notably, we found that the upregulation of TGF-β1-induced of the NOTCH1^{NICD} protein level was strongly decreased but not completely abolished by concomitant GSI treatment in WM35, A375 and A2058 cells. The same trend was evident for downstream target genes *HES1* (Fig. 5A).

Finally, to confirm the effect of NOTCH on TGF-β/Smad signaling, we treated WM35, A375 and A2058 cells with and without GSI for 72 h. Phosphorylated Smad3 (pSMAD3), a marker of constitutive TGF-β1 receptor activity, was detected in WM35, A375 and A2058 cells; GSI treatment consistently decreased pSMAD3 levels in all melanoma cell lines (Fig. 5B), without interfere with the unphosphorylated Smad3 protein levels. These data highlighted the effect of NOTCH on TGFβ/Smad signaling in melanoma cell lines.

Cytotoxic and anti-proliferative effects of GSI on melanoma cells. To further investigate whether GSI could be an effective therapeutic target for melanoma, we first tested the effect of the drug on cell proliferation in WM35, A375 and A2058 after treatment to 0, 5, 10 and 20 μM of GSI for 24, 48 and 72 h. MTT analyses showed that melanoma cell lines, without GSI treatment, exhibited a linear growth up to confluence. In these experiments, DMSO, which was used as a vehicle control, did not affect cell growth, while GSI treatment induced a marked cell growth inhibition, in a dose- and time-dependent manner. The concentrations of 5 and 10 μM showed a weak growth inhibition, but at 20 μM, GSI significantly inhibited proliferation at each incubation time for all the cell lines, the maximum inhibition was attained at 72 h (Fig. 6A-C). However, GSI effects were significantly greater in WM35 cells rather than in A375 and A2058; no difference was noted between A375 and A2058 cells. MTT assay did not discriminate if the decrease of proliferation rate was attributed to growth arrest or cell death, since both mechanisms induce a decrease in cell numbers and an apparent loss of viability. To establish this, a Trypan blue exclusion test was performed on melanoma cell lines. As shown in Fig. 6D, GSI exerted a significantly more toxic effect on the melanoma cells compared to DMSO. The cytotoxicity of GSI on WM35 cells was significantly higher than that of A375 and A2058 cells. GSI showed very low toxicity in normal melanocytes.

Discussion

Many studies have shown that *FOXP3* is expressed not only by Tregs, but also in a variety of tumor cells, including melanoma (18-20,25,27,28,33). Expression of *FOXP3* by cancer cells may cause the inhibition of tumor directed T-cell responses and may favor tumor cells immune-evasion

mechanisms (22,23,27,30,31,33). By contrast, other studies have suggested that FOXP3 plays a critical role in suppressing the development of several types of tumors, such as ovarian, prostate and breast cancer, through the inhibition of cell proliferation, migration and invasion or by modulating the expression of oncogenes or tumor suppressor genes (29,91-93). Thus, the *FOXP3* gene or protein exerts different functions in different types of tumors. In any case, the role of FOXP3 in carcinogenesis is intriguing and remain controversial.

Multiple signaling pathways, including NOTCH and TGF-β/Smad pathways, are involved in FOXP3 transcription regulation (17,37,38,41,76,83,95). Few studies have shown the association between NOTCH/FOXP3 in cancers (84,85) and to the best of our knowledge there are no reports investigating directly the relationship between NOTCH/TGF-β signaling and FOXP3 transcription factor in melanoma.

In the present study, we investigated the involvement of NOTCH/TGF-β1 signaling pathways in regulating the FOXP3 transcription factor and demonstrated, for the first time, that FOXP3 expression was modulated by NOTCH/TGF-β1 pathways in primary and metastatic melanoma cell lines. The subcellular localization of *FOXP3* in human melanoma cell lines at different stages of cancer progression was studied by immunocytochemistry. Our results showed that the intensity of *FOXP3* expression in melanoma metastatic cells lines (A375 and A2058) was higher than that in primary melanoma cells (WM35), while FOXP3 staining was undetectable in melanocytes. These results underline that FOXP3 staining gradually increase from the primary to the metastatic melanoma cell lines.

This result suggested that FOXP3 expression may be associated with metastatic spread. These data partially confirm the study by Quaglino *et al* (32), which demonstrated a significant association between FOXP3 expression in primary melanomas and development of visceral metastases. Our study also showed an heterogeneous subcellular localization of FOXP3 mainly in the nucleus, less in cytoplasm and in perinuclear region. Metastatic melanoma cell lines exhibited strong FOXP3 positive staining in the nucleus and weak staining in the cytoplasm. Similar to our results, Brody *et al* (96) reported a nuclear FOXP3 expression in Tregs localized at the primary melanomas and at the interface of metastasis with the lymph node parenchyma. Chen *et al* (97) showed that activation of $CD4^+$ $CD25^+$ Treg induced a shift in the subcellular localization of FOXP3 from a primarily cytoplasmic/perinuclear pattern, in most cells, to a nuclear pattern, suggesting that the change in the FOXP3 expression pattern may be a result of post-translational modifications. Similar results were obtained by Niu *et al* (27) and subcellular staining of FOXP3 was demonstrated in other types of cancer (19-21,24) due to post-translational modification and types of cancer (97). However, the exact involvement of this variable expression of FOXP3 remains unclear.

Subsequently, we confirmed *in vitro*, by RT-qPCR and western blot analysis, *FOXP3* expression in melanoma cell lines. Our study has revealed that *FOXP3* was strongly expressed in metastatic melanoma cell lines. A higher *FOXP3* at mRNA and protein levels was more evident in the metastatic melanoma cell line A2058, compared to A375, cell lines derived from the dermis of a malignant melanoma. In addition, *FOXP3* expression was slightly lower in the primary melanoma cells (WM35), but was still significantly higher than that in the melanocytes.

This result suggests that *FOXP3* is a biological marker of melanoma progression and may contribute to metastasis. These results are particularly noteworthy and confirm those reported by other studies that associated high *FOXP3* levels with metastasis in several tumors, including melanoma (22,31,33,98,99).

Since the potential role of FOXP3 has been demonstrated in various cancer types on immune surveillance (20), we examined the effects of TGF-β1 on the induction of FOXP3 in melanoma cell lines. We showed that TGF-β1 treatment upregulated *FOXP3* expression at the transcriptional and post-translational level, more in A2058 and A373 cells than in WM35 cells.

Overall, our data strongly emphasize the role of TGF-β1 and FOXP3 in promoting melanoma progression.

Melanoma is a type of highly immunogenic cancer and is a rich source of TGF-β (65). It is possible that TGF-β1, one of the many factors present in the tumor microenvironment, can induce FOXP3 and the regulatory activity in Treg cells (27,28,68,69,100). FOXP3-expressing melanoma cells may have Treg-like activity, thus suppressing effector T-cell activity (28,34). It is possible that FOXP3 immunosuppressive function in the FOXP3-driven metastatic process requires a crosstalk between tumor cells and the microenvironment (34).

It has been demonstrated that, for the majority of the Treg cells, *FOXP3* expression is transient and its persistence is highly dependent on the TGF-β exposure present in tumoral microenvironment (101).

The identification of regulatory mechanisms that potentially lead to a decreased expression of *FOXP3* may offer insight into the control of tumor cell proliferation and progression in melanoma and provide new perspectives to develop potential therapeutic targets. Although numerous cell surface molecules could mediate this condition, we focused on NOTCH signaling because it was shown that NOTCH may be involved in the activation of *FOXP3* promoter through RBP-J- and HES1-dependent mechanisms (39). In addition, emerging evidence indicated that TGF-β1 and NOTCH act in concert to regulate the transcription of target genes (102-104). For example, TGF-β1, through effector Smad3, and $NOTCH^{NICD}$ physically interact to coordinately regulate the transcription of *Hes1* and *FOXP3* (76-79). Further findings have shown that the NOTCH ligand Jagged2 promotes Treg cell proliferation, leading to an increase in TGF-β production (105).

NOTCH/TGF-β1 pathways are important regulators of many fundamental processes of cancer cell biology, such as tumor growth, angiogenesis, invasion and tumor progression (72,78,79,80,88,91,106,107).

Aberrant expression of TGF-β1 and NOTCH pathway has been demonstrated previously in melanoma (57,61,74,90,108).

In accordance with the above reported studies, we found that both the $NOTCH^{NICD}$ and Hes1 mRNA and protein levels were higher in primary melanoma (WM35) compared to metastatic melanoma cells (A375 and A2058). Moreover, TGF-β1 treatment induced upregulation of $NOTCH^{NICD}$ and Hes1 in all the melanoma cell lines. The increase of Hes1 by TGF-β1

is consistent with earlier studies, one of which demonstrated a cooperative interaction between Smad3 and NOTCH on CSL binding elements (79,91,109). *Hes1* is the most well characterized target gene of NOTCH, and its upregulated expression symbolizes the activated NOTCH signaling.

Consistent with our data, other reports have found that NOTCH signaling are upregulated in primary lesions of human malignant melanoma (61,62,89).

This suggests that NOTCH signaling may be involved in melanomagenesis, by activating MAPK/PI3K/AKT signaling pathways, and corroborate the idea that it has less effect on metastatic cell lines, suggesting its primary function in early transformation events (57,60,110). In contrast to oncogenetic role in melanoma, NOTCH pathway is downregulated in other cancers, including skin carcinoma (111-113). A recent study of Banerjee *et al* (114) showed that inhibition of NOTCH causes pathologic activation of liver stromal cells, promoting angiogenesis and growth of hepatic metastases. In a later study, Talora *et al* (115) showed that in HPV-positive cervical cancer cells, activated NOTCH causes growth suppression. The involvement of NOTCH in cancer development is complex, since NOTCH can function as an oncogene or a tumor suppressor depending on the tissue type, different cell context and on crosstalk with other signaling pathways (45,46). Previous findings have shown that NOTCH signaling is largely regulated by γ-secretase activity, responsible for cleavage of NOTCH into its active intracellular transactivator NICD and for its translocation to the nucleus, where it induces the transcription of target genes (47,86). Recently, there has been an increased interest in targeting the NOTCH pathway using GSIs as a new target therapy for those tumors with NOTCH activation (86,116,117). Thus, when we treated the melanoma cell lines with the GSI, a strong effect on both growth inhibition and cellular death in WM35, A375 and A2058 cells was evident.

We have observed that low GSI doses did not induce cell growth inhibition or cell death, as shown by MTT assay and dye test. Instead, a high dose of GSI, led to inhibition of the proliferation rate, with concomitant induction of death of WM35, A375 and A2058 cells due to a strong NOTCH inhibition. Thus, the level of treatment operating on NOTCH signaling appears to be critical for the proliferation outcome.

The role of GSI in inhibiting the growth of melanoma cell lines is consistent with the previous findings in other cancers (110,118-120). Notably, some research has shown an opposite outcome; the overexpression of NOTCH signaling can inhibit the growth of cancer cells through induction of cell cycle arrest (121,122).

Our results underline the role of NOTCH as an oncogene in melanoma because its downregulation causes inhibition of cell growth and induction of cellular death in all the melanoma cell lines.

Based on the data presented herein and in consideration that FOXP3 signaling may function as a potential oncogenic factor in melanoma (28,32,33,99,123), we hypothesized that the pharmacological inhibition of NOTCH by GSI, could reduce the tumorigenic activity that NOTCH exerts through several signaling pathways in melanoma cells, such as FOXP3 pathways and TGF-β/Smad3 signaling.

Of note, we have found that GSI treatment strongly decreased *FOXP3* expression at the transcriptional and translational level in WM35, A375 and A2058 cells in a dose- and time-dependent manner. Consistent with our results, prior studies have shown that blockade of the NOTCH1 inhibited *FOXP3* expression and Treg suppressor function (76,82,85,124). This finding emphasizes the role of NOTCH signaling in Treg differentiation and *FOXP3* transcription. In addition, we found that GSI reduced the upregulation of TGF-β1-mediated *FOXP3* gene and protein in primary and metastatic melanoma cells. In this regard, it is interesting to note that various reports have shown cell-type specific effect of TGF-β1, as a mediator of FOXP3 and NOTCH signaling pathways (76-78). In other cases, however, the $NOTCH^{NICD}$ signaling blocks TGF-β1 signaling by mutually interfering with the Smad3 (125-127). This emphasizes the complexity of the interaction between FOXP3 and NOTCH/TGF-β signaling that may produce different signaling outcomes depending on other signaling pathways.

This study further confirms the functional integration between NOTCH and TGF-β1 signaling pathways and underlines the synergistic effect of NOTCH on a subset of Smad3-inducible genes. We found that GSI markedly induced both a decreased level of $NOTCH^{NICD}$ protein, that of its downstream gene *Hes1*, and attenuated strongly the levels of TGF-β1-induced $NOTCH^{NICD}$ and Hes1 protein in WM35, A375 and A2058 melanoma cell lines. In addition, we detected that the downregulation of $NOTCH^{NICD}$ by GSI decreased the pSmad3 protein, a downstream transcription factor of TGF-β1. $NOTCH^{NICD}$, not only interacts with pSmad3, facilitating its nuclear translocation (91,128), but also remains bound with pSmad3 in the nucleus where they cooperatively upregulate the transcription factor FOXP3 (76, 81,129). Tone *et al* (130), have demonstrated that the mechanism underlying TGF-β-driven of *FOXP3* expression involves the induction of activated Smad3 (pSmad3), which acts as a powerful transcription factor for the *FOXP3* gene.

Taken together, our results sustain the role of NOTCH signaling in mediating the *FOXP3* expression in melanoma cells by a dual mechanism: direct modulation of *FOXP3* transcription and cooperative interaction with the TGF-β1 pathway in the modulation of *FOXP3* expression. Our data suggest a possible crosstalk between NOTCH1/TGF-β1 and *FOXP3* pathways in melanoma cells.

Moreover, our data show that $NOTCH^{NICD}$ activation has an effect on TGF-β/Smad signaling and confirm that the NOTCH and TGF-β1 pathways are intertwined to regulate FOXP3 transcription factor in melanoma cell lines. Nevertheless, future studies are needed to validate our data. *In vivo* experiments are required to explore the role of NOTCH/TGF-β pathway in the regulation of FOXP3 transcription factor in melanoma.

Finally, this study may provide a double additional rationale for targeting the NOTCH and FOXP3 signaling pathways for treatment of melanoma.

Despite various advances on the comprehension of the signal transduction pathways that modulate FOXP3 transcriptional activity, there are still many uncertainties.

A growing body of evidence suggests a connection between FOXP3 and NOTCH/TGF-β signaling pathways and their link with cancer recurrence, metastasis, and patient prognosis (76,85). Taken together these studies highlight the need of a more detailed understanding of how the

NOTCH/TGF-β/FOXP3 signals interact with other pathways in order to design rationally oriented targeted therapy experiments and trials.

The problem of secondary resistance to targeted therapy is a common problem in oncology. Thus, the use of drugs specifically targeting NOTCH, such as GSI, combined with other drugs, either standard chemotherapeutic agents or selective pathway-specific inhibitors, such as TGF-β1 or Braf inhibitors, could offer a potential strategy for therapeutic investigations in melanoma. Therefore, our study not only corroborated some of these findings, but also identified a novel interaction between NOTCH and TGF-β1 in modulating *FOXP3* expression in melanoma cells. Although further studies are needed to clarify the role and molecular mechanisms that govern the association between FOXP3 and NOTCH/TGF-β signaling in the progression of melanoma, the current study provides new insight into the carcinogenesis of melanoma.

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Availability of data and materials

All data and materials are available at the Department of Biomedical and Biotechnological Science of the University of Catania and the Department of Clinical and Experimental Medicine, Section of Dermatology of the University of Messina.

Authors' contributions

ES, VB, FDA and AR have performed all the experiments. GM and CG conceived the study and wrote the manuscript. DAS and AS have revised the final version of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to publication.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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