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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Latent-Transforming Growth Factor β-Binding Protein 1/Transforming Growth Factor β1 Complex Drives Antitumoral Effects upon ERK5 Targeting in Melanoma

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Melanoma is the deadliest skin cancer, with a poor prognosis in advanced stages. While available treatments have improved survival, long-term benefits are still unsatisfactory. The mitogen-activated protein kinase extracellular signal-regulated kinase 5 (ERK5) promotes melanoma growth, and ERK5 inhibition determines cellular senescence and the senescence-associated secretory phenotype. Here, latent-transforming growth factor β-binding protein 1 (LTBP1) mRNA was found to be up-regulated in A375 and SK-Mel-5 BRAF V600E melanoma cells after ERK5 inhibition. In keeping with a key role of LTBP1 in regulating transforming growth factor β (TGF-β), TGF-β1 protein levels were increased in lysates and conditioned media of ERK5-knockdown (KD) cells, and were reduced upon LTBP1 KD. Both LTBP1 and TGF-β1 proteins were increased in melanoma xenografts in mice treated with the ERK5 inhibitor XMD8-92. Moreover, treatment with conditioned media from ERK5-KD melanoma cells reduced cell proliferation and invasiveness, and TGF-β1—neutralizing antibodies impaired these effects. *In silico* data sets revealed that higher expression levels of both LTBP1 and TGF-β1 mRNA were associated with better overall survival of melanoma patients. Increased LTBP1 or TGF-β1 expression played a beneficial role in patients treated with anti-PD1 immunotherapy, making a possible immunosuppressive role of LTBP1/TGF-β1 unlikely upon ERK5 inhibition. This study, therefore, identifies additional desirable effects of ERK5 targeting, providing evidence of an ERK5-dependent tumor-suppressive role of TGF-β in melanoma. *(Am J Pathol 2024, 194: 1581—1591; https://doi.org/10.1016/j.ajpath.2024.03.015)*

Malignant melanoma is one of the most aggressive skin cancers, and its incidence is increasing worldwide. While early stage disease can be cured in the majority of cases by surgical excision, late-stage melanoma is still a highly lethal disease.[1] Common genetic alterations associated with melanoma include mutations in *BRAF* (50% to 60%), *NRAS* (20% to 25%), and *NF1* (14%), which hyperactivate the mitogen-activated protein kinase extracellular signal-regulated kinase (ERK)1/2, thus supporting sustained cell proliferation.[2] Development of serine/threonine-protein kinase B-raf (BRAF)— and MAPK/ERK kinase (MEK)

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1/2−targeting drugs and immunotherapy have increased the survival of patients with melanoma.[3] However, intrinsic or acquired resistance to the former as well as the lack of responsiveness to the latter limit the benefits of available therapies.[4,5]

ERK5 (also referred to as big mitogen-activated protein kinase 1), the last discovered member of conventional mitogen-activated protein kinases, is involved in cell survival, proliferation, and differentiation of several cell types,[6] and plays a relevant role in the biology of cancer, including melanoma.[7−9] ERK5 activation is achieved through MEK5-dependent or MEK5-independent phosphorylation, which stimulates ERK5 nuclear translocation, a key event for cell proliferation.[10,11] On the other hand, upon ERK5 inhibition, melanoma cells undergo cellular senescence, and produce a number of soluble mediators [namely CXCL1, CXCL8, and C-C motif chemokine 20 (CCL20)] typically involved in the senescence-associated secretory phenotype that slows down the proliferation of melanoma cells.[9,12]

Accumulating evidence points to the involvement of transforming growth factor β (TGF-β) in cellular senescence.[13] TGF-β secretion and activation is regulated by its association to latent-transforming growth factor β-binding protein 1 (LTBP1).[14,15] Typically, TGF-β controls proliferation, differentiation, and other functions in most cells. These roles are highly context-dependent, and TGF-β may induce even opposite effects in different contexts.[16] Regarding melanoma in particular, the role of tumor suppression versus tumor promotion of TGF-β has been scarcely addressed.[17] This article identifies a tumor-suppressive role for LTBP1/TGF-β among the antitumoral outcomes of ERK5 inhibition that could be exploited for future therapeutic strategies in melanoma.

Materials and Methods

Cells and Cell Culture

A375^{BRAFV600E} (Research Resource Identifier: CVCL_0132)[18] and SK-Mel-2^{NRASQ61R} (Research Resource Identifier: CVCL_0069CVCL_0069)[19] melanoma cells were obtained from ATCC (Manassas, VA); SK-Mel-5^{BRAFV600E} (Research Resource Identifier: CVCL_0527) melanoma cells[19] were kindly provided by Dr. Laura Poliseno (Core Research Laboratory, Institute for Cancer Research and Prevention, Pisa, Italy); SSM2c melanoma cells have been described elsewhere.[20] Cells were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin (Euroclone, Milan, Italy). Cell lines are authenticated yearly (BMR Genomics, Padua, Italy) by short tandem repeat profiling using the Promega PowerPlex Fusion System Kit (Promega Corporation, Madison, WI). The presence of *Mycoplasma* was tested periodically by PCR.

Drugs

ERK5 inhibitors XMD8-92[21] and JWG-071[22] were from MedChemExpress (Monmouth Junction, NJ). Cell-cycle inhibitor L-mimosine was from Sigma-Aldrich (St. Louis, MO).

Cell Lysis and Western Blot

Total cell lysates were obtained using Laemmli buffer or radioimmunoprecipitation assay buffer as reported previously.[23] Immunoprecipitation was performed by incubating 2 mg conditioned media proteins with the anti−TGF-β antibody and 20 μL Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 24 hours at 4°C. Immunocomplexes then were washed three times and proteins were eluted using Laemmli buffer. Proteins were separated by SDS-PAGE and transferred onto Amersham Protran nitrocellulose membranes (GE Healthcare, Chicago, IL) by electroblotting. Infrared imaging (Odissey, Li-Cor Bioscience, Lincoln, NE) was performed. Images were quantified with ImageJ software version 1.53k (NIH, Bethesda, MD; *https://imagej.net/ij*). The list of antibodies is shown in Table 1.

RNA Interference

A375 and SK-Mel-5 cells were transduced with negative control nontargeting shRNA (shNT) or ERK5-specific shRNAs (shERK5-1 and shERK5-2) (Table 2) as previously reported.[23] Transduced cells were selected with 2 μg/mL puromycin for at least 72 hours. Fourteen days after lentiviral transduction, medium was replaced with Dulbecco's modified Eagle's medium/10% fetal bovine serum, and conditioned media (CM) were harvested after 72 hours. For siRNA inhibition studies, the cells were transfected with human LTBP1 siRNAs (SASI_Hs01_00187276 and SASI_Hs01_00168991) or negative control nontargeting siRNA (SIC001) from Sigma-Aldrich at a final concentration of 100 nmol/L using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. Seventy-two hours after transfection, cells were harvested for protein extraction and additional analysis.

Measurement of Cell Viability, Cell-Cycle Phase Distribution, and Cell Death

The number of viable cells in culture was evaluated by counting Trypan blue−positive and −negative cells with a hemocytometer. Cell-cycle phase distribution (propidium iodide staining) was estimated by flow cytometry using a FACSCanto (Becton Dickinson, San Jose, CA) as previously reported.[24] Dead cells were evaluated by flow cytometry using a FACSCanto (Becton Dickinson). Annexin-V−positive and Annexin-V−negative/propidium

Table 1 List of Antibodies Used and Their Application

Protein	Application	Species	Catalog no.	Supplier
ERK5	WB	Rabbit polyclonal	3372	Cell Signaling Technology
LTBP1	WB, IHC	Mouse monoclonal	sc-271140	Santa Cruz Biotechnology
α-Tubulin	WB	Mouse monoclonal	sc-32293	Santa Cruz Biotechnology
TGF-β	WB	Rabbit polyclonal	3711	Cell Signaling Technology
TGF-β1	N, IHC	Mouse monoclonal	69012-1-Ig	Proteintech Group, Inc. (Rosemont, IL)
KLF-2	WB	Rabbit monoclonal	15306	Cell Signaling Technology
IgG1	N	Mouse monoclonal	MAB002	R&D Systems (Minneapolis, MN)

ERK5, extracellular signal-regulated kinase 5; IHC, immunohistochemistry; KLF-2, Krueppel-like factor 2; LTBP1, latent-transforming growth factor β-binding protein 1; N, neutralization; TGF-β, transforming growth factor β; TGF-β1, transforming growth factor β1; WB, Western blot.

iodide−positive cells were measured using the Annexin-V−FLUOS Staining Kit (Sigma-Aldrich), as previously reported.[24]

Transcriptomic Analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and mRNA expression was evaluated with the Affymetrix (Santa Clara, CA) Clariom-S Human Genechip following the manufacturer's instructions. Applied Biosystems Transcriptome Analysis Console software version 4.0.2.15 (Thermo Fisher Scientific) was used (fold change, >1.5/<1.5 and $P \leq 0.05$) to identify differentially expressed genes.

Patients

Analysis of the relationship between LTBP1 and TGF-β1 mRNA expression and overall survival (OS) of melanoma patients was performed using the publicly available Skin Cutaneous Melanoma data set from The Cancer Genome Atlas (PanCancer Atlas) on cBioPortal for Cancer Genomics (https://www.cbioportal.org).[25,26] The same database was used to verify the correlation between LTBP1 and TGF-β1 mRNA. Analysis of the relationship between LTBP1 and TGF-β1 mRNA expression and OS and disease-free survival outcomes of melanoma patients treated with anti-PD1 therapy was performed using the open-access database Kaplan-Meier plotter (http://www.kmplot.com).[27] Expression of LTBP1 in normal, primary, and metastatic tumors was obtained from The Cancer Genome Atlas data set on TNMplot database (http://www.tnmplot.com).[28]

Quantitative Real-Time PCR

Total RNA was isolated using TriFast II (Euroclone). cDNA synthesis was performed using the ImProm-II Reverse Transcription System, and quantitative real-time PCR (qPCR) was performed using GoTaq qPCR Master Mix (Promega Corporation). qPCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Expression levels were determined by qPCR with the following primers: LTBP1: forward: 5′-TGAATGCCAGCACCGTCATCTC-3′ and reverse: 5′-CTGGCAAACACTCTTGTCCTCC-3′. mRNA expression was normalized as follows: glyceraldehyde-3-phosphate dehydrogenase mRNA: forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′ and reverse: 5′-ACCACCCTGTTGCTGTAGCCAA-3′; and 18S mRNA: forward: 5′-ACCCGTTGAACCCCATTCGTGA-3′ and reverse: 5′- GCCTCACTAAACCATCCAATCGG-3′.

Cell Viability Assay

Cell viability was measured by MTT assay. Cells were seeded in 96-well plates in Dulbecco's modified Eagle's medium/10% fetal bovine serum. After 24 hours, medium was replaced with CM and cells were incubated further for 72 hours. MTT (0.5 mg/mL) was added during the last 4 hours. Plates were read at 595 nm using microplate

Table 2 List and Sequences of shRNA

Gene	Sequence reference*	shRNA	Clone ID	Sequence
None	None	shNT	SHC202	5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3′
MAPK7	NM_139032.X	shERK5-1	TRCN0000010262	5′-CCGGGCTGCCCTGCTCAAGTCTTTGCTCGAGCAAAGACTTGAGCAGGGCAGCTTTTT-3′
MAPK7	NM_139032.X	shERK5-2	TRCN0000010275	5′-CCGGGCCAAGTACCATGATCCTGATCTCGAGATCAGGATCATGGTACTTGGCTTTTT-3′

*Sequence reference from https://www.ncbi.nlm.nih.gov/gene/5598.
shERK5, extracellular signal-regulated kinase 5−specific shRNA; shNT, control nontargeting shRNA.

reader-550 (Bio-Rad). For neutralization experiments, control isotype IgG or neutralizing antibodies (Table 1) were added to CM before administration to cells.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections from archival xenografts established with A375 cells from 25 mg/kg XMD8-92−treated or vehicle-treated (2-hydroxypropyl-β-cyclodextrin 30%) mice were used.[8] Experiments were approved by the Italian Ministry of Health (authorization 213/2015-PR) and were in accordance with Italian ethics guidelines and regulations. Sections (3-μm thick) were deparaffinized and incubated overnight at 4°C with primary antibodies (Table 2) and 3,3′-diaminobenzidine (Thermo Fisher Scientific) used as a chromogen. Sections were counterstained with hematoxylin and the percentage of stained area was evaluated with ImageJ software version 1.53k.

Invasion Assay

A375 or SK-Mel-5 melanoma cells (1×10^4 cells/well) were seeded in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum albumin in the presence or absence of neutralizing antibodies onto the top chamber of 48-well Transwell plates equipped with 8-μm polycarbonate nucleopore filters (Neuro Probe, Gaithersburg, MD) precoated with Matrigel (Sigma-Aldrich). The bottom chamber was supplemented with CM obtained as described in *RNA Interference*. After 24 hours of incubation, cells that had not migrated were removed with a cotton swab from the upper surface of filters and cells that had migrated to the lower surface of the membrane were subjected to Diff-Quick staining (Medion Diagnostics AG, Dudingen, Switzerland) and observed with a light microscope. The number of cells per well was evaluated by counting cells in 5 randomly chosen microscope fields (magnification, ×20).

Statistical Analysis

Data represent means ± SD values calculated on at least three independent experiments. *P* values were calculated using the *t*-test or one-way analysis of variance (multiple comparisons). $P < 0.05$ was considered statistically significant.

Results

ERK5 Inhibition Increases LTBP1

CXCL1, CXCL8, and CCL20 are among the senescence-associated secretory phenotype−related soluble mediators responsible for the reduced proliferation in BRAF V600E melanoma cells undergoing cellular senescence after ERK5 knockdown (KD).[9] In view of the exploitation of ERK5 targeting for the treatment of melanoma, further characterization of the secretome of BRAF V600E melanoma cells upon ERK5 inhibition was performed, taking advantage of a previously performed transcriptomic analysis in A375 and SK-Mel-5 ERK5-KD cells.[9] ERK5 KD up-regulated LTBP1 mRNA levels when compared with control nontargeting shRNA (Supplemental Figure S1). qPCR confirmed the increased expression of LTBP1 mRNA upon ERK5 KD in both A375 and SK-Mel-5 BRAF V600E cells (Figure 1A). Interestingly, the publicly available Skin Cutaneous Melanoma data set from The Cancer Genome Atlas on TNMplot[28] provided evidence that LTBP1 mRNA levels are lower in primary and metastatic melanomas than in normal tissues from noncancer patients (Figure 1B). However, the same data set did not provide evidence of changes in ERK5 mRNA along melanoma progression (not shown), in keeping with a previous report showing consistent activation of the MEK5/ERK5 pathway without appreciable ERK5 overexpression in melanoma patients.[8] More importantly, the same data set used on the cBioPortal for Cancer Genomics indicated that higher expression levels of LTBP1 mRNA are associated with a better OS (Figure 1C) of melanoma patients, pointing to a possible tumor-suppressive role of LTBP1 in melanoma. ERK5 KD resulted in increased levels of LTBP1 protein (Figure 1D), and the same effects were recapitulated by pharmacologic inhibition of ERK5 using XMD8-92[21] and the more specific JWG-071[22] small-molecule inhibitors (Figure 1E). Effectiveness of the ERK5 inhibitors was confirmed by the reduced protein level of the downstream target Krueppel-like factor 2.[29] Of note, AX15836, which inhibits the catalytic function of ERK5 but paradoxically stimulates its transactivation function,[30,31] did not elicit the same effects (not shown). Taken together, the data in Figure 1 indicate that ERK5 negatively regulates LTBP1, whose expression correlates with a better outcome in melanoma patients.

ERK5 Inhibition Promotes an LTBP1-Dependent Increase of TGF-β1, Leading to a Better Prognosis in Melanoma

Because LTBP1 is involved in the stabilization and activation of TGF-β, which plays a role in cancer onset and progression,[15] the impact of ERK5 KD on TGF-β protein levels was analyzed. Increased protein levels of both mature (Figure 2A) and latent forms (Supplemental Figure S2A) of TGF-β1 were found, in CM and whole cell lysates of ERK5-KD A375 and SK-Mel-5 cells, respectively. Of note, mRNA levels of TGF-β1 were not affected consistently (ie, were not increased in both cell lines upon ERK5 KD), pointing to post-transcriptional effects of LTBP1 on TGF-β upon ERK5 KD (Supplemental Figure S2B), at least in these experimental models. Interestingly, in line with the fact that LTBP1-dependent regulation of TGF-β could impact the activity of transcription factors (eg, SMAD proteins, activator protein 1, NF-kB, and transcription factor Sp1[32−34]) known to be regulated by TGF-β itself that are, in

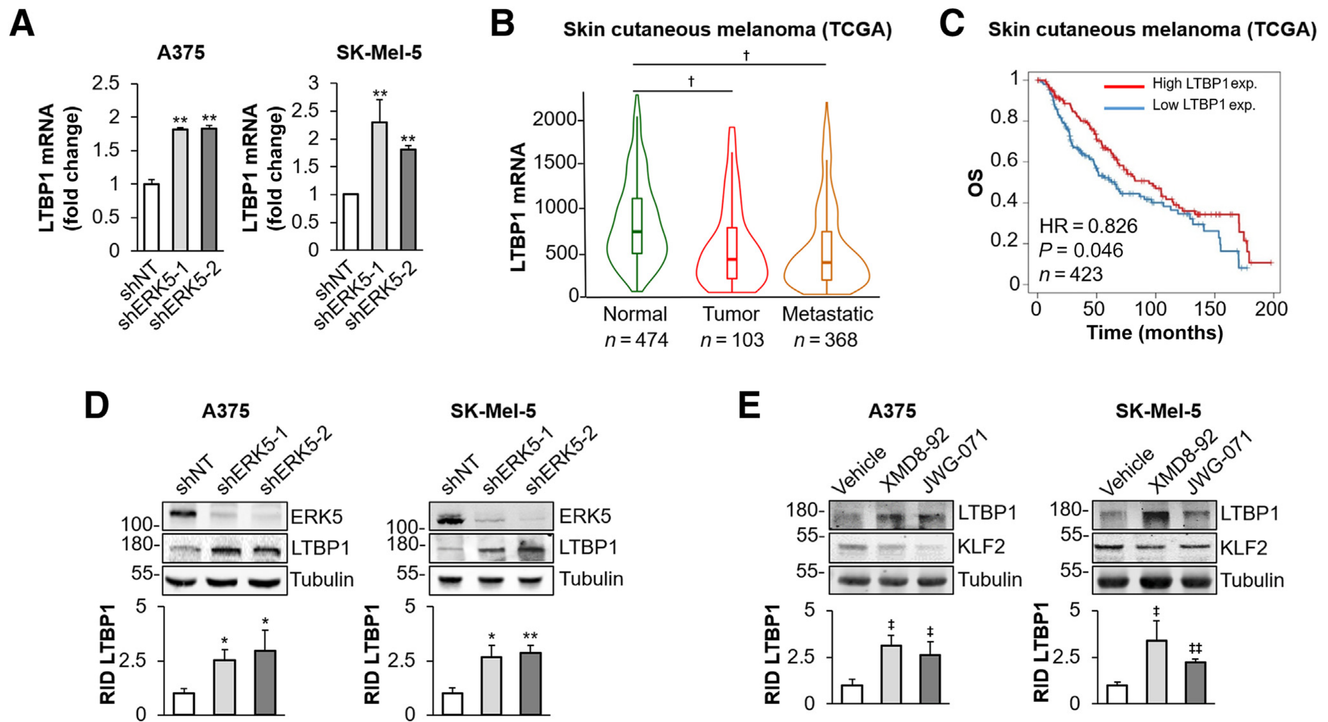

Figure 1 Effects of extracellular signal-regulated kinase 5 (ERK5) inhibition on the expression of latent-transforming growth factor β-binding protein 1 (LTBP1) in melanoma cells. **A:** A375 and SK-Mel-5 cells transduced with lentiviral vectors harboring control nontargeting shRNA (shNT) or ERK5-specific shRNAs (shERK5-1 and shERK5-2) were lysed after 72 hours, and LTBP1 mRNA levels were determined by quantitative real-time PCR. Data shown are means (±SD) from three independent experiments. **B:** Violin plots show the LTBP1 gene expression profile in normal skin (Normal), and primary (Tumor) and metastatic (Metastatic) melanoma obtained by the Skin Cutaneous Melanoma (SKCM) data set [The Cancer Genome Atlas (TCGA)] on TNMplot. **C:** Kaplan-Meier analysis of the relationship between LTBP1 expression and overall survival (OS) in melanoma patients using the SKCM data set on cBioPortal. Patients were stratified according to low or high LTBP1 expression. The median LTBP1 expression value was used as the cut-off value. To reduce noise, 5% of samples above and below the cut-off value were excluded from the analysis. A hazard ratio (HR) <1 indicates a reduced hazard of death. **D:** A375 and SK-Mel-5 cells transduced with control shNT or shERK5 (shERK5-1 and shERK5-2) were lysed after 72 hours. Western blot was performed with the indicated antibodies. Images are representative of three independent experiments showing similar results. Migration of molecular weight markers is indicated on the left (in kilodaltons). The graphs show the average relative integrated density (RID) ± SD of ERK5 protein levels normalized for tubulin content from three independent experiments. **E:** A375 and SK-Mel-5 cells treated with XMD8-92 (5 μmol/L) or JWG-071 (5 μmol/L) for 72 hours were lysed. Western blot was performed with the indicated antibodies. Images are representative of three independent experiments showing similar results. Migration of molecular weight markers is indicated on the left (in kilodaltons). The graphs show the average RID ± SD of LTBP1 protein levels normalized for tubulin content from three independent experiments. $n = 423$, with $n = 223$ and $n = 200$ in the low/high group, respectively (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$ versus shNT; $^{\dagger}P < 0.0001$; $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ versus vehicle. exp., expression.

turn, able to regulate TGF-β expression, the Skin Cutaneous Melanoma data set from The Cancer Genome Atlas on cBioPortal provided evidence of a positive correlation (Spearman, 0.32; $P = 2.82\text{e-}12$) between LTBP1 mRNA and that of TGF-β1 (Figure 2B). More importantly, the same data set provided a positive association between higher levels of TGF-β1 expression and a better prognosis in melanoma patients (Figure 2C). To show that LTBP1 participates in the regulation of TGF-β1 protein level in melanoma cells, LTBP1 was knocked down using two different siRNAs (Figure 2D). LTBP1 KD determined a marked decrease of TGF-β1 protein in both A375 and SK-Mel-5 cells (Figure 2E), and prevented the increase of TGF-β1 upon pharmacologic inhibition of ERK5 (Supplemental Figure S2C). Importantly, both LTBP1 and TGF-β1 protein levels were increased upon ERK5 inhibition *in vivo*. Indeed, administration of the ERK5 inhibitor XMD8-92, which reduces melanoma tumor growth similar to ERK5 KD,[8] induced a robust increase of both LTBP1 and TGF-β1 in A375 xenografts, with respect to vehicle-treated mice (Figure 2F).

TGF-β1 Exerts an Antiproliferative Effect in Melanoma Cells upon ERK5 KD

ERK5 KD results in the increased production of CXCL1, CXCL8, and CCL20 in melanoma cells, which are responsible for reduced viability of melanoma cells.[9] TGF-β is involved in cellular senescence and exerts potent growth-inhibitory activities in various cell types and in different contexts, including cancer cells.[13] Similarly, TGF-β1 emerges as the soluble factor responsible for reduced viability of melanoma cells upon ERK5 KD. Indeed, TGF-β1−neutralizing antibodies prevented the antiproliferative effect induced by CM harvested from ERK5-KD A375 (Figure 3A) or SK-Mel-5 (Figure 3B) cells in a

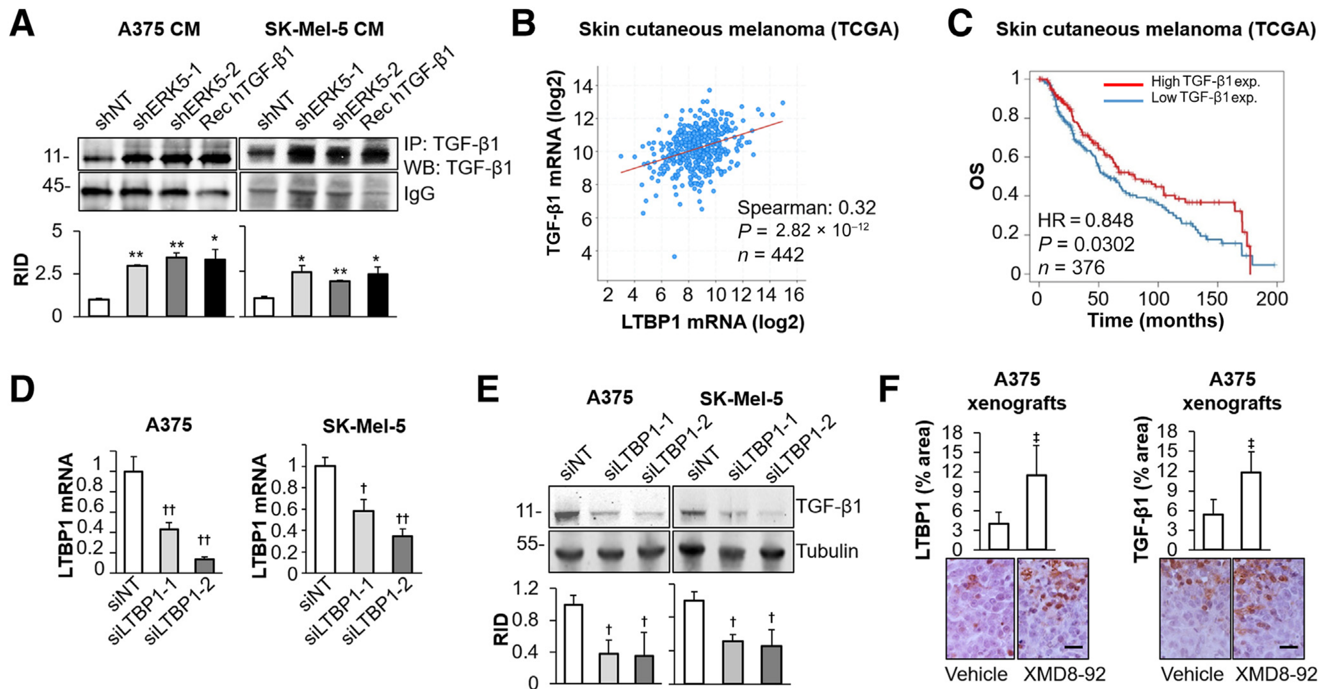

Figure 2 Effects of extracellular signal-regulated kinase 5 (ERK5) inhibition on transforming growth factor β1 (TGF-β1) expression in melanoma cells and in xenografts. **A:** TGF-β1 immunoprecipitation was performed in 72-hour-conditioned media (CM) from A375 or SK-Mel-5 cells transduced with control nontargeting shRNA (shNT) or ERK5-specific shRNAs (shERK5) (shERK5-1 or shERK5-2) lentiviral vectors. Human recombinant TGF-β1 (100 ng/mL) was used as positive control. Western blot was performed with the indicated antibodies. Images are representative of three independent experiments showing similar results. Migration of molecular weight markers is indicated on the left (in kilodaltons). The graphs show the average relative integrated density (RID) ± SD of TGF-β1 protein levels normalized for IgG content from three independent experiments. **B:** Expression levels of TGF-β1 and latent-transforming growth factor β-binding protein 1 (LTBP1) mRNA from the Skin Cutaneous Melanoma (SKCM) data set [The Cancer Genome Atlas (TCGA)] on cBioPortal. **C:** Kaplan-Meier analysis of the relationship between TGF-β1 expression and overall survival (OS) in melanoma patients using the SKCM data set on cBioPortal. Patients were stratified according to low or high TGF-β1 expression, using the median TGF-β1 expression value as the cut-off value. To reduce noise, 10% of samples higher and lower than the cut-off value were excluded from the analysis. A hazard ratio (HR) <1 indicates a reduced hazard of death. **D:** Quantitative real-time PCR of LTBP1 mRNA from LTBP1-knockdown (KD) A375 and SK-Mel-5 cells after treatment with siRNA targeting LTBP1 (siLTBP1-1 or siLTBP1-2) or control nontargeting siRNAs (siNT) for 72 hours. Data shown are means (±SD) of three independent experiments. **E:** Western blot showing TGF-β1 protein levels in LTBP1-KD cells 72 hours after transfection with siLTBP1-1 or siLTBP1-2 or siNT. Migration of molecular weight markers is indicated on the left (in kilodaltons). The graphs show the average RID ± SD of TGF-β1 protein levels normalized for tubulin content from three independent experiments. **F:** Immunohistochemistry detection of LTBP1 (**left**) or TGF-β1 (**right**) in XMD8-92-treated (25 mg/kg) or vehicle-treated (2-hydroxypropyl-β-cyclodextrin 30%) mice.[8] Hematoxylin counterstaining was performed. Bar plots of the percentage of LTBP1 or TGF-β1—positive cells are shown. The percentage of positive cells was calculated from six different magnified fields from three randomly chosen vehicle-treated and XMD8-92—treated tumors. Representative images are shown. $n = 376$, with $n = 195$ and $n = 181$ in the low/high group, respectively (**C**). $^{*}P < 0.05$, $^{**}P < 0.01$ versus shNT; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus siNT; $^{\ddagger}P < 0.05$ versus vehicle. Scale bars = 100 μm (**F**). Original magnification, ×40 (**F**). exp., expression; IP, immunoprecipitation; Rec hTGF-β1, human recombinant TGF-β1; WB, Western blot.

dose-dependent manner. The effects observed in Figure 3, A and B, were not restricted to BRAF V600E—mutated melanoma cells. Indeed, in both *NRAS*-mutated SK-Mel-2 and triple wild-type SSM2c melanoma cells, TGF-β1—neutralizing antibodies reverted the reduction of cell proliferation elicited by the ERK5-KD—derived CM (Supplemental Figure S3A). Moreover, in keeping with the biological evidence, both pharmacologic and genetic inhibition of ERK5 determined an increase of TGF-β1 in these cell lines (Supplemental Figure S3B). To confirm that TGF-β1 exerts an antiproliferative effect in melanoma cells, A375 and SK-Mel-5 cells were treated with human recombinant TGF-β1. This cytokine reduced the number of viable cells in culture in a dose-dependent manner in both cell lines (Figure 3C). This effect was maximal with 100 ng/mL TGF-β1, a concentration in line with previous reports.[35,36] To better understand how TGF-β1 affects cell growth, cell-cycle analysis was performed and treatment with TGF-β1 significantly increased the fraction of cells in G0/G1 phase (Figure 3D). In the same experimental settings, TGF-β1 determined the increase of the cyclin-dependent kinase inhibitor p21 (Supplemental Figure S3C). In further support of a possible involvement of p21 in the antiproliferative effects of TGF-β1 upon ERK5 inhibition, treatment with TGF-β1—neutralizing antibodies reduced the increase of p21 elicited by CM harvested from ERK5-KD (A375) cells in both A375 and SK-Mel-5 cell lines (Supplemental Figure S3D). The reduction in cell number observed in melanoma cells treated with TGF-β1 was attributed, in part, to increased cell death (Figure 3E). On the whole, data in

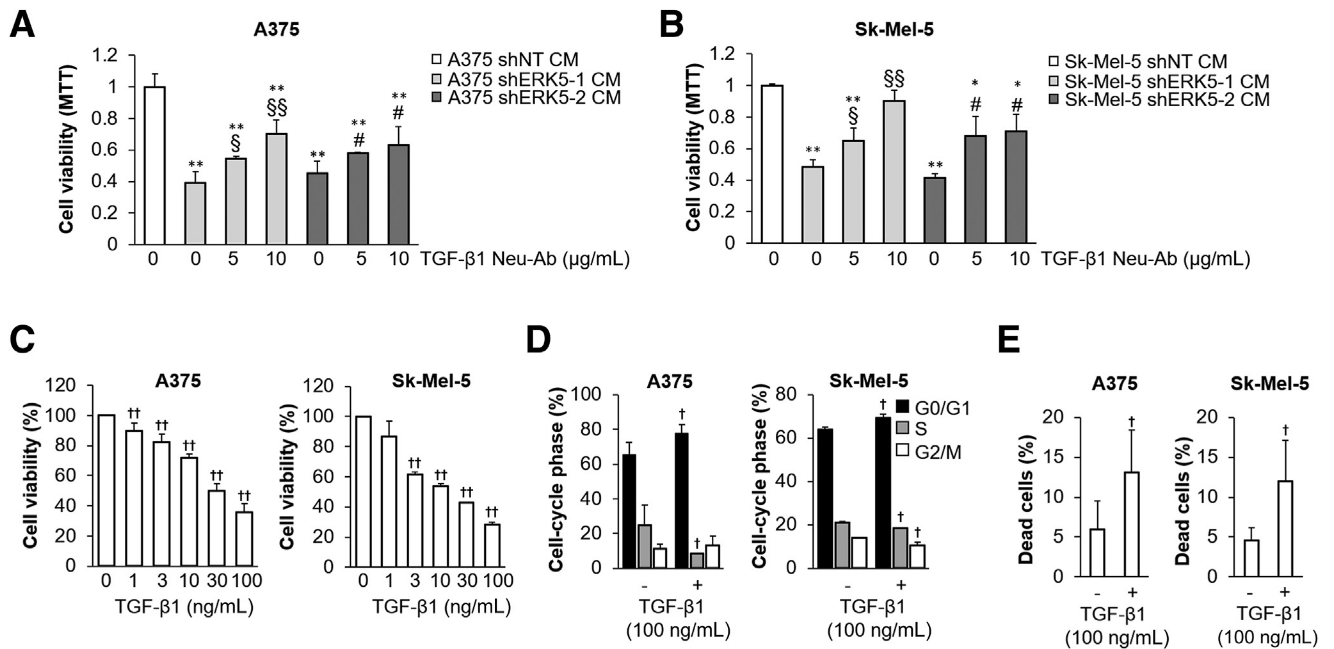

Figure 3 Involvement of transforming growth factor β1 (TGF-β1) in the antiproliferative outcome of extracellular signal-regulated kinase 5 (ERK5) inhibition in melanoma cells. **A** and **B:** MTT performed in A375 and SK-Mel-5 cells treated for 72 hours with 72-hour conditioned media (CM), obtained from A375 or SK-Mel-5 cells transduced with control nontargeting shRNA (shNT) or ERK5-specific shRNAs (shERK5) (shERK5-1 or shERK5-2) lentiviral vectors, alone or in combination with the indicated concentrations of transforming growth factor β1 neutralizing antibodies (TGF-β1 Neu-Ab). Data shown are means (±SD) from three independent experiments. **C:** Cells were treated with the indicated concentrations of TGF-β1 for 72 hours, and the number of viable cells was counted. Histograms represent means (±SD) from three independent experiments. **D:** Cells were treated or not with 100 ng/mL human recombinant TGF-β1 for 72 hours, and cell-cycle phase distribution then was determined. Data shown are means ± SD from three independent experiments. **E:** Dead cells (Annexin-V-positive and Annexin-V—negative/propidium iodide—positive cells) were evaluated after treating A375 or SK-Mel-5 melanoma cells with or without 100 ng/mL of human recombinant TGF-β1. Histograms represent mean percentages ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus shNT CM; $^{§}P < 0.05$, $^{§§}P < 0.01$ versus shERK5-1 CM; $^{\#}P < 0.05$ versus shERK5-2 CM; $^{†}P < 0.05$ versus untreated, $^{††}P < 0.01$ versus untreated.

Figure 3 provide evidence that TGF-β1 is among the soluble mediators that increase upon ERK5 inhibition, and is responsible for reduced proliferation.

TGF-β1 Produced upon ERK5 Inhibition Reduces the Invasive Ability of Melanoma Cells

The possible impact of the secretome of ERK5-KD cells on melanoma cell invasiveness was tested. CM from ERK5-KD cells markedly reduced the invasive ability of A375 and SK-Mel-5 cells (Supplemental Figure S4A) in the presence of mimosine, a DNA replication inhibitor used at a concentration able to completely prevent changes in the number of cells throughout the duration (ie, 24 hours) of the invasion assays Supplemental Figure S4, B and C). To shed light on the possible role of TGF-β1 in the regulation of this biological process upon ERK5 KD, the effect of TGF-β1—neutralizing antibodies on cell invasion ability was evaluated. TGF-β1—neutralizing antibodies restored the invasion ability of A375 (Figure 4A) and SK-Mel-5 (Figure 4B) that was reduced by CM harvested from ERK5-KD A375 or SK-Mel-5 cells, whereas control IgG did not. To confirm that TGF-β1 reduced melanoma cell invasiveness, A375 and SK-Mel-5 cells were treated with increasing doses of this cytokine. TGF-β1 dose-dependently decreased the invasive ability of both A375 and SK-Mel-5 cells (Figure 4, C and D). Altogether, the data in Figure 4 indicate that TGF-β1 reduces the invasive propensity of melanoma cells, at least *in vitro*.

Increased TGF-β1 and LTBP1 Expression Positively Affects the Impact of Immunotherapy in Patients With Melanoma

As reported in Figures 1C and 2C, higher levels of both TGF-β1 and LTBP1 mRNA correlate with a better OS. Moreover, OS and disease-free survival of patients treated with anti-PD1 therapy (ie, nivolumab or pembrolizumab) were significantly higher in patients with high TGF-β1 expression than in those with lower expression (Figure 5, A and B). This positive association was also detected with high levels of LTBP1 expression and better OS and disease-free survival in patients with melanoma treated with anti-PD1 therapy (Figure 5, C and D), pointing to additional desirable effects of ERK5 inhibition in melanoma.

Discussion

TGF-β controls a wide spectrum of cellular functions and deregulated TGF-β signaling is linked to several human

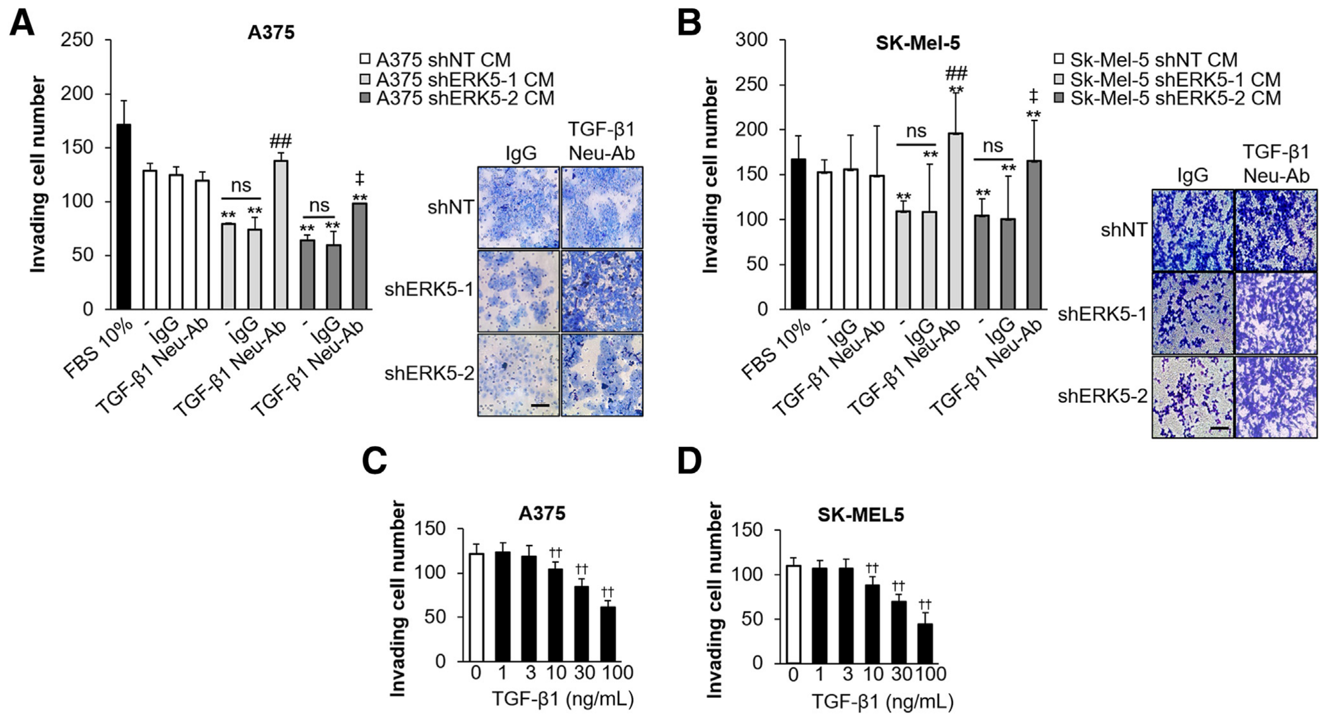

Figure 4 Involvement of transforming growth factor β1 (TGF-β1) in the anti-invasive effect of extracellular signal-regulated kinase 5 (ERK5) inhibition in melanoma cells. **A** and **B:** Invasion assays were performed for 24 hours in A375 (**A**) and SK-Mel-5 (**B**) cells in the presence of 72-hour conditioned media (CM), obtained from A375 or SK-Mel-5 cells transduced with control nontargeting shRNA (shNT) or ERK5-specific shRNAs (shERK5) (shERK5-1 or shERK5-2) lentiviral vectors, alone or with TGF-β1−neutralizing antibodies (TGF-β1 Neu-Ab, 10 μg/mL) or control IgG. Histograms represent means (±SD) from three independent experiments. Representative images of wells are included. **C** and **D:** A375 and SK-Mel-5 cells were exposed for 24 hours at increasing concentrations of human recombinant TGF-β1. Histograms represent means (±SD) from three independent experiments. $^{**}P < 0.01$ versus shNT CM/0, $^{\#\#}P < 0.01$ versus shERK5-1 CM/0, $^{\ddagger}P < 0.01$ versus shERK5-2 CM/0; $^{\dagger\dagger}P < 0.01$ versus NT. Scale bars = 150 μm (**A** and **B**). FBS, fetal bovine serum; ns, not significant.

diseases, including cancer.[37] In particular, TGF-β may play the role of a double-edged sword in tumor progression,[38,39] acting as a tumor suppressor during the early stage of the tumor, because inhibition of TGF-β signaling results in the disruption of the normal homeostatic process and subsequent carcinogenesis, while behaving as a tumor promoter at later stages.[40] Understanding how TGF-β1 can coordinate its effects in melanoma is a key issue in the biology of this cancer.

ERK5 is involved in melanoma growth,[8] and ERK5 inhibition induces marked cellular senescence and production of several soluble mediators involved in the senescence-associated secretory phenotype in both BRAF-mutated and wild-type melanoma cells and xenografts.[9] In this study, ERK5 inhibition evoked increased expression of LTBP1, which is known to modulate the availability of TGF-β1.[15] Besides increased LTBP1 expression, increased TGF-β1 protein levels were observed in ERK5-KD melanoma cells and in A375 xenografts from XMD8-92−treated mice in the current study. LTBP1 was found to be responsible for the regulation of TGF-β1 protein levels, likely through a post-transcriptional regulation, and also to support the increase of TGF-β1 upon ERK5 inhibition. This work also identified an antiproliferative and anti-invasiveness ability of TGF-β1 in melanoma cells, providing evidence that the increase in the LTBP1/TGF-β1 complex could be an additional desirable effect obtained by ERK5 inhibition.

TGF-β is a potent inhibitor of cell proliferation, which is a result of its ability to induce G1 cell-cycle arrest.[41] In line with this, the data provided in this work indicate that TGF-β1 is among the soluble factors responsible for the reduction of melanoma cell proliferation induced by the secretome of ERK5-KD melanoma cells. In fact, this event is restored in part by TGF-β1−neutralizing antibodies. Moreover, in BRAF V600E−expressing cells, TGF-β1 slows down cell-cycle progression with the accumulation of cells in the G0/G1 phase, and is able to increase cell death. Despite these effects being elicited at relatively high TGF-β1 concentrations, the latter are in line with previous reports,[35,36] and are consistent with the amount contained in the CM of ERK5-KD melanoma cells. The observed antiproliferative effects are consistent with the results obtained in other studies, which demonstrated that cell-cycle arrest was induced upon treatment with TGF-β1 via SMAD2/3 in proliferating melanoma cells *in vitro* and *in vivo*.[42−44] Moreover, in another report, the activation of TGF-β1 led to the up-regulation of plasminogen activator inhibitor 1 expression that resulted in tumor growth inhibition in murine melanoma.[45] The results reported in this paragraph, including those described in this article, do not support a

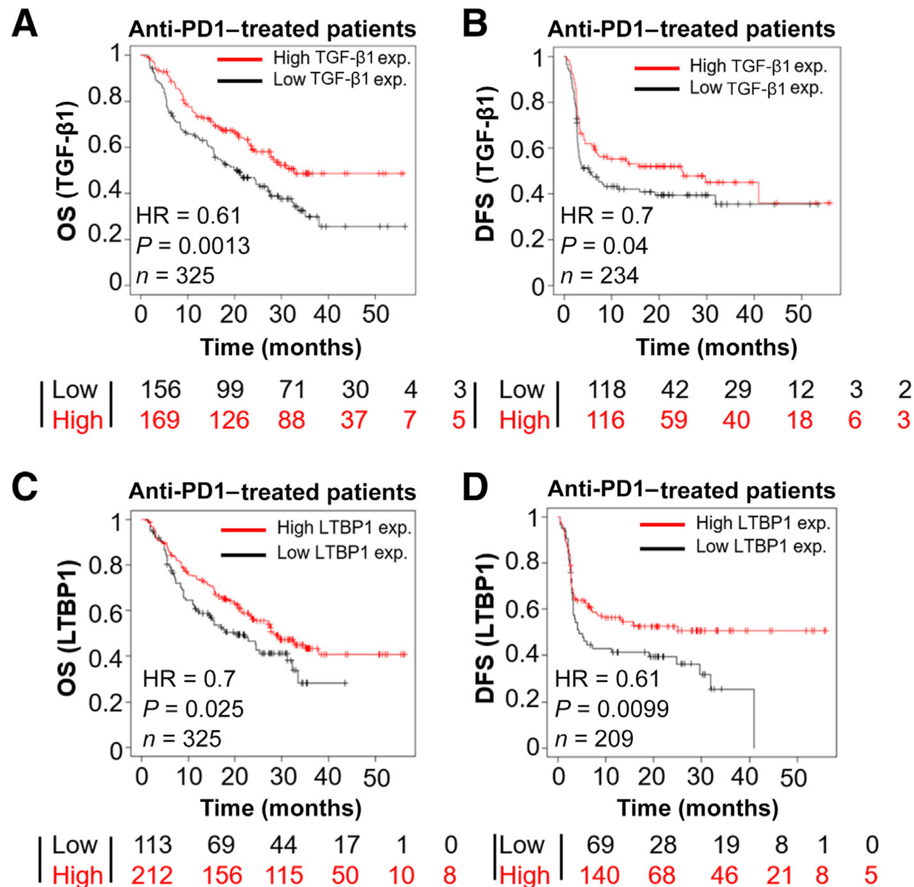

Figure 5 Impact of latent-transforming growth factor β-binding protein 1 (LTBP1) and transforming growth factor β1 (TGF-β1) expression on the overall survival (OS) and disease-free survival (DFS) in anti-PD1—treated melanoma patients. **A:** Sixty-month follow-up Kaplan-Meier analysis of the relationship between TGF-β1 expression and OS in anti-PD1—treated melanoma patients from the Kaplan-Meier plotter database. Patients were stratified according to low or high TGF-β1 expression. The number of patients at risk in the low- and high-expression groups are indicated. **B:** Sixty-month follow-up Kaplan-Meier analysis of the relationship between TGF-β1 expression and DFS in melanoma anti-PD1—treated patients calculated as described in panel **A**. **C:** Sixty-month follow-up Kaplan-Meier analysis of the relationship between LTBP1 expression and OS in melanoma anti-PD1—treated patients calculated as described in panel **A**. **D:** Sixty-month follow-up Kaplan-Meier analysis of the relationship between LTBP1 expression and DFS in melanoma anti-PD1—treated patients calculated as described in panel **A**. $n = 325$ (**A** and **C**); $n = 234$ (**B**); $n = 200$ (**D**). exp., expression.

previous report showing that inhibition of canonical TGF-β signaling inhibits tumor growth in melanoma.[46] Despite this study showing that there is clearly a tumor suppressing role of TGF-β1 in melanoma cells upon ERK5 inhibition, the molecular mechanism underlying this connection remains to be established. However, TGF-β1 increased the expression of the cyclin-dependent kinase inhibitor p21, a previously established ERK5-regulated protein[8,9] that is a downstream mediator of the antiproliferative effects of TGF-β, including in melanoma cells.[38,47]

Another interesting finding of this study was that ERK5-KD melanoma cells produced TGF-β1, which is anti-invasive. These results, together with the identified anti-proliferative effect, are in line with the evidence reported here that patients with melanoma with higher expression of TGF-β1 have a better prognosis. On the other hand, this is at odds with the established notion that, at least in the advanced stages, TGF-β acts as a tumor promoter by stimulating invasiveness along the epithelial to mesenchymal transition.[48] Of note, A375 and SK-Mel-5 cell lines used as models for this study were derived from metastatic melanoma.[18,19] Moreover, increased expression levels of TGF-β1 are associated with melanoma progression *in vivo*, and TGF-β1—elicited signals stimulate melanoma cell dissemination from primary tumors.[49,50]

From a clinical point of view, the possibility of eliciting an increase in LTBP1 and TGF-β1 expression after ERK5 inhibition seems to have positive therapeutic implications in patients with melanoma. Indeed, LTBP1 expression is lower in primary and metastatic melanoma compared with healthy tissues, and patients with melanoma with higher expression of LTBP1 or TGF-β1 have a better prognosis (OS) with respect to those with lower expression of LTBP1 or TGF-β1. On the other hand, *in silico* data analysis revealed that among patients with melanoma who have received anti-PD1 antibodies, those with higher expression of LTBP1 or TGF-β1 showed improved OS or disease-free survival compared with those with low expression of LTBP1 or TGF-β1. This

is of relevance, since TGF-β affects multiple components of the immune system, exerting systemic immune suppression most of the time.[51] Furthermore, the first-line therapeutic approach for advanced melanoma consists of immunotherapy with anti-PD1 antibodies (nivolumab/pembrolizumab) or targeted therapy with BRAF and MEK inhibitors, and their combination is under study.[52] Targeting ERK5 is also expected to boost the efficacy of immunotherapy in melanoma patients, adding value to the possible targeting of ERK5 in this cancer, taking into consideration that ERK5 inhibition reduces melanoma growth and improves BRAF targeting *in vivo*,[8] and that ERK5 activation is among the resistance mechanisms in RAF-MEK1/2-ERK1/2−directed therapies.[9]

Author Contributions

A.T. curated and analyzed data, performed experiments, and wrote, reviewed, and edited the manuscript; A.M. and Z.L. curated and analyzed data, performed assays, and reviewed the manuscript; I.T. curated and analyzed data, and edited the manuscript; T.G. analyzed data and performed assays; A.E.-O., A.P., and B.S. procured resources and edited the manuscript; and E.R. conceptualized and supervised the study, procured resources and funding, curated and analyzed data, and wrote, reviewed, and edited the manuscript.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2024.03.015*.

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