HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Estrogen-Sensitive PTPRO Expression Represses Hepatocellular Carcinoma Progression by Control of STAT3

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Protein tyrosine phosphatase receptor type O (PTPRO), one of the receptor types of phosphotyrosine phosphatases (PTP), was recently described as a tumor suppressor in various kinds of cancers. We aimed to clarify the role of PTPRO in hepatocellular carcinoma (HCC). It was demonstrated in 180 pairs (120 male and 60 female) of clinical HCC specimens that the PTPRO level was significantly reduced, as compared with adjacent tissue, and the PTPRO level in male adjacent tissue was lower than in female. We further found that estrogen receptor alpha (ERα) could up-regulate PTPRO expression as a transcription factor. Moreover, an *in vitro* study showed that cell proliferation was inhibited and apoptosis was promoted in PTPRO-transduced HCC cell lines, whereas an *in vivo* study represented that tumor number and size was increased in *ptpro*$^{-/-}$ mice. As a result of its tumor-suppressive position, PTPRO was proved to down-regulate signal transducers and activators of transcription (STAT3) activity dependent on Janus kinase 2 (JAK2) and phosphoinositide 3-kinase (PI3K) dephosphorylation. Conclusions: PTPRO expression results in pathological deficiency and gender bias in HCC, which could be attributed to ERα regulation. The suppressive role of PTPRO in HCC could be ascribed to STAT3 inactivation. (HEPATOLOGY 2013;57:678-688)

Protein tyrosine phosphatase receptor type O (PTPRO), one of the receptor types of phosphotyrosine phosphatases (PTP), was initially discovered in human renal glomerulus.[1] It contains six isoforms; the full-length type is expressed in kidney, brain, lung, liver, and breast, whereas the truncated types are expressed in macrophages and B lymphocytes.[2] PTPRO is a transmembrane protein; its intracellular region contains a PTP domain that catalyzes the dephosphorylation of tyrosine peptides. This critical function of PTPs is involved in numerous intracellular signaling events that serve various biological behaviors, such as cell proliferation, differentiation, apoptosis, and so forth.[3,4] Recently, an accumulation of evidence has enriched the understanding of cancer biology, and it has been observed that PTPRO exhibits important

Abbreviations: AP-1, activator protein 1; Bcl-2, B-cell lymphoma 2; bp, base pairs; BrdU, bromodeoxyuridine; DEN, diethylnitrosamine; E2, 17β-estradiol; EGF, epidermal growth factor; ERs, estrogen receptors; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; EREs, estrogen-responsive elements; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; IFN-γ, interferon-gamma; IHC, immunohistochemistry; IL-6, interleukin-6; IOD, integrated optical density; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; MTT, tetrazolium; PCR, polymerase chain reaction; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; p-JAK2, phosphorylated JAK2; p-STAT3, phosphorylated STAT3; PTEN, phosphatase and tensin homolog; PTP, phosphotyrosine phosphatase; PTPRO, protein tyrosine phosphatase receptor type O; S727, serine 727; SHP, SHATTERPROOF; STAT3, signal transducer and activator of transcription 3; WT, wild type; Y705, tyrosine 705.

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Received May 29, 2012; accepted July 8, 2012.

This work was supported by grants from the National Natural Science Foundation (81072029 and 91029721; to B.S.), the National Basic Research Program of China (2012CB910800; to B.S.), and the New Century Excellent Talents in University, Ministry of Education (NCET-09-0160; to B.S.). The work was also supported, in part, by the Program for Development of Innovative Research Teams in the First Affiliated Hospital of Nanjing Medical University and the Priority Academic Program of Jiangsu Higher Education Institutions.

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aspects concerning tumor suppression. Initially, it was discovered that overexpression of PTPRO enhances apoptosis of the terminally differentiated leukemic cell line, U937, in the presence of 12-*O*-tetradecanoylphorbol-13-acetate.[5] Subsequently, the PTPRO level was shown to be statistically weakened; the promoter region of the gene, *ptpro*, is frequently methylated in human chronic leukemia, lung cancer, breast cancer, hepatocellular carcinoma (HCC), and so forth.[6-10] In support of the role of PTPRO as a tumor suppressor, it was demonstrated that PTPRO could inhibit cell proliferation in lung cancer cell line A549.[8] Additionally, it has been revealed that PTPRO expression can be suppressed by estrogen receptor β (ERβ) in breast cancer. Using an *in vitro* study, it was demonstrated that ERβ, conjugated with 17β-estradiol (E2), functions at the AP-1 (activator protein 1) site located in the promoter region of *ptpro*, giving rise to the separation of c-jun and c-fos from AP-1 and leading to the inhibition of *ptpro* transcription.[9]

Human HCC, one of the most malignant cancers in the world, is closely associated with a history of chronic hepatitis caused by hepatitis B or C virus (HBV or HCV).[11,12] The global incidence of clinical HCC exhibits a striking gender disparity and occurs much more frequently in male patients.[13] Aside from estrogen itself, the role of estrogen receptors (ERs) has garnered considerable attention and provided new insight into HCC research. As previously reported, ERs, which consist of ERα and ERβ, exist not only in female endocrine cells, but also in many types of epithelial cells, including hepatocytes in healthy, cirrhotic, or carcinomatous liver tissue.[14-19] ERs in hepatocytes mediate estrogen-responsive biological effects through either DNA binding or in a DNA-independent manner.[20] Regarding nongenomic estrogen signaling, Naugler et al. reported, in a murine model, that ERα interferes with interleukin-6 (IL-6)-associated HCC genesis.[21] Alternatively, ER acts as a hormone-dependent nuclear receptor and DNA-binding transcription receptor and regulates gene expression in a similar manner as breast cancer, in which ERβ represses the transcriptional activity of the *ptpro* promoter.

Signal transducer and activator of transcription 3 (STAT3) mediates diverse cellular processes initiated by extracellular signals and plays a central role in HCC progression.[22] Subsequent to dimerization and nuclear translocation, STAT3 acts as a transcription factor and promotes cancer cell proliferation by up-regulation of cyclin D, c-Myc, and so forth and reduces apoptosis by up-regulation of BCL-2 (B-cell cell/lymphoma-2), BCLXL (B-cell lymphoma-extra large), and so forth.[23] Concerning STAT3 activation, tyrosine phosphorylation plays an essential role in the overall process of intracellular signal transduction. Tumor cells undergo sustained stimulation from a variety of cytokines and growth factors, such as IL-6, IFN-γ (interferon-gamma), EGF (epidermal growth factor), FGF (fibroblast growth factor), HGF (hepatocyte growth factor), and so forth. Their homologous receptors recruit and activate JAK2 (Janus kinase 2) in a tyrosine-phosphorylation–dependent manner, which also potentially leads to the activation of its substrate, STAT3.[24-27] Moreover, another well-known tyrosine kinase, c-Src, is activated and contributes to STAT3 activation by phosphorylation of both serine 727 (S727) and tyrosine 705 (Y705) by JNK (c-Jun N-terminal kinase), MAPK (mitogen-activated protein kinase) p38, or ERK (extracellular signal-regulated kinase) pathways. Additionally, phosphoinositide 3-kinase/mammalian target of rapamycin (PI3K-mTOR), a bypassing pathway positively regulated by JAK2 and c-Src, directly contributes to STAT3 S727 phosphorylation.[28,29] It is well understood that these pathways are all up-regulated during HCC progression.[30-34] Therefore, molecular agents or proteins that attenuate STAT3 activity or block upstream phosphorylation cascades can potentially suppress HCC. It has been previously reported that PTPs, such as PTP1B, CD45 (also known as PTPRC), PTPN2, and PTPN11, could potentially serve as inhibitors of STAT3 activation.[35-38]

Given the gender disparity of HCC, and the potential relationship between PTPRO and ER, in this study, we investigated whether PTPRO level was markedly decreased in male HCC patients and whether ER affected its expression level. To determine its suppressive effect in cancer, we performed supplementary experiments in HCC by *in vitro* and *in vivo* studies. However, the molecular mechanisms underlying the role of PTPRO as a tumor suppressor remain unclear. Regarding the potential function of PTP, we hypothesized that PTPRO was able to counterbalance oncogenic tyrosine kinase signaling. In this study, we aimed

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DOI 10.1002/hep.25980

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

to investigate the tumor-suppression ability of PTPRO with regard to STAT3 activation.

Patients and Methods

Patients. HCC and adjacent tissues were obtained from 120 male and 60 female patients at the time of surgical resection at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between January 2008 and August 2010. Informed consent for gene-expression analysis of tissue was obtained from each patient before surgery, and the study was approved by our institutional ethics committee. HCC staging was performed according to the tumor node metastasis staging system. Adjacent tissue was located within 1 cm of the tumor margin and was confirmed to be nontumor tissue by pathological examination. Detailed patient information is listed in Supporting Table 1.

Detailed information regarding animal model, lentivirus production and transduction, quantitative real-time polymerase chain reaction (PCR), western blotting, immunohistochemistry (IHC), cloning of *ptpro* promoter and mutagenesis, luciferase reporter assay, cell culture, cell-proliferation assay, cell-apoptosis assay, and statistical analysis is provided in the Supporting Materials.

Results

PTPRO and ERα Expression Levels Were Reduced in HCC Specimens. We investigated 180 pairs of HCC and adjacent patient tissue specimens using real-time PCR and IHC; both HCC and adjacent tissues were grouped by gender. Messenger RNA (mRNA) level of PTPRO was significantly decreased in human HCC tissue ($P < 0.001$; Fig. 1A). Additionally, in female adjacent tissues, PTPRO was expressed much more highly, compared to male samples ($P < 0.01$). PTPRO expression levels in male HCC tissues were negative in 37 cases and weak in 83 cases, according to IHC staining analysis, whereas 18 cases were negative and 42 weak in female HCC tissue samples. We also found greater levels in female adjacent tissues and lower levels in male adjacent tissues (Fig. 1E). Statistical analysis of integrated optical density (IOD) values of 180 slides stained with PTPRO is shown in the histogram (Fig. 1C; $P < 0.01$).

Additionally, our findings indicated that PTPRO expression level was associated with tumor multiplicity and tumor size in the 180 HCC patients ($P < 0.01$). However, PTPRO expression levels appeared to have only a slight association with age and Edmondson's stage (Supporting Table 1). Taken together, these findings suggest that the decreased expression of PTPRO was associated with the generation or progression of HCC; moreover, our findings suggest that PTPRO expression level is potentially mediated by estrogen regulation.

Based on the gender disparity of PTPRO expression and the previous findings in breast cancer, we hypothesized that the decreased PTPRO level in HCC could be the result of ERs. As suggested by our recent report, ERα was distinctly down-regulated in male HCC cases, and this finding correlated with its defensive potential against the development of HCC.[39] In this study, we identified the gender difference in ERα expression in 180 pairs of HCC specimens using real-time PCR (Fig. 1B) and IHC analysis (Fig. 1E; $P < 0.001$). We randomly analyzed correlations between PTPRO and ERα expression in 180 HCC specimens and found that they were positively correlated in adjacent tissues (Fig. 1F; $r^2 = 0.342$, $P < 0.001$). Additionally, we investigated ERβ levels, but found no significant differences between HCC and adjacent tissues. Thus, of the two types of ERs, our findings demonstrated that ERα was principally correlated with PTPRO expression in HCC.

PTPRO and ERα Levels Were Decreased in Diethylnitrosamine-Treated Male Mice. To confirm the pathological deficiency and gender bias of PTPRO expression, we collected liver specimens from diethylnitrosamine (DEN) treated and healthy mice and detected the expression of ERα and PTPRO by immunochemical staining. Gender disparity of ERα and PTPRO expression was evident in healthy C57BL/6 mice (Fig. 2). Subsequently, in mouse HCC, we found a significant decrease in ERα and PTPRO levels in male mice ($P < 0.001$), in contrast to the constant expression levels found in female mice that failed in HCC generation.

***ERα Escalated PTPRO Expression* In Vitro.** To examine the potential relationship between ERα and PTPRO expression levels, we utilized lentivirus to derive ERα-overexpressing HCC cell lines Huh-7-ERα and SMCC-7721-ERα and determined the fluctuations of PTPRO levels. Expression level of PTPRO was elevated by ERα in the presence of E2 (Fig. 3A). Moreover, we verified by real-time PCR that PTPRO mRNA was up-regulated (Fig. 3B; $P < 0.001$), suggesting that transcriptional regulation is the potential underlying internal mechanism.

Both *in vivo* and *in vitro* phenotypes suggested the potential role of ERα as a transcriptional regulator of *ptpro*; thus, we sought genomic evidence to determine its exact position. As predicted by online transcriptional factor prediction tool PROMO, the promoter

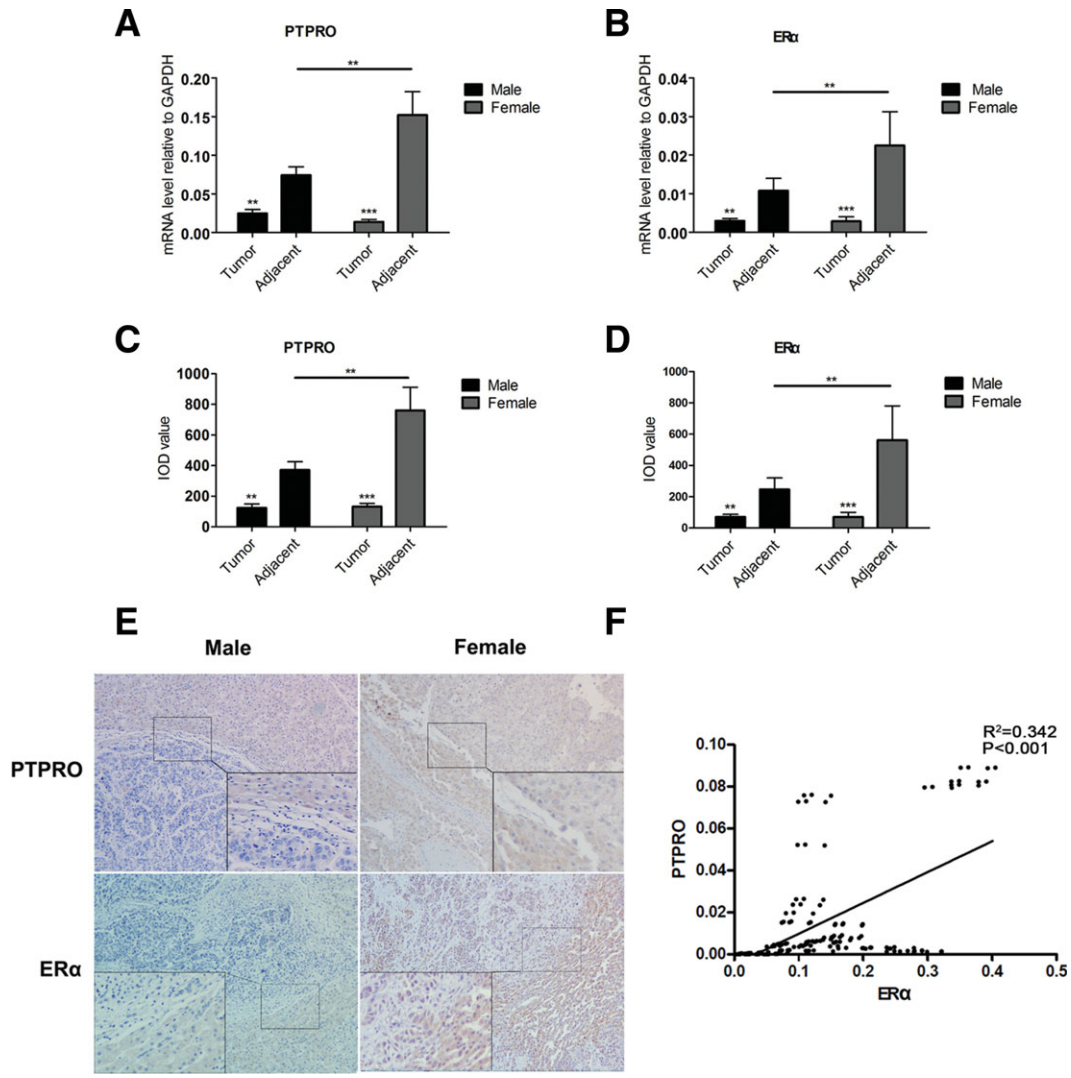

Fig. 1. PTPRO and ERα levels were reduced in human HCC specimens. (A and B). mRNA levels of PTPRO and ERα in HCC ($n_{male} = 120$; $n_{female} = 60$) and adjacent ($n_{male} = 120$; $n_{female}=60$) specimens grouped by gender, as detected by real-time PCR. PTPRO and ERα in HCC represented lower levels than adjacent specimens; levels in male adjacent tissue were lower, compared to female levels. (C and D) Average value of IOD was obtained by analyzing five fields per slide with Image-Pro Plus software (v. 5.0) and recorded in the histograms, demonstrating the pathological deficiency and gender bias of PTPRO and ERα expression according to real-time PCR. (E) IHC staining of PTPRO and ERα in HCC ($n_{male} = 120$; $n_{female} = 60$) and adjacent ($n_{male} = 120$; $n_{female} = 60$) specimens grouped by gender. (F) Pearson's correlation analysis of PTPRO and ERα level (ΔCT value) in human HCC. $r^2 = 0.342$. Data are expressed as mean ± standard error of the mean. $^{**}P < 0.01$; $^{***}P < 0.001$.

region of *ptpro* contains three estrogen-responsive elements (EREs), separately located at positions −731, −678, and −350 base pairs (bp) with respect to the initiation codon (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). It has been demonstrated that the *ptpro* CpG island is −208 to +236 bp[8]; thus, latent methylation may not affect transcriptional regulation upon EREs. We amplified the *ptpro* promoter region from −1,000 to −168 bp, designated as PP-WT, then constructed four mutants that encompassed point mutations at different EREs, designated as PP-ΔABC, PP-ΔA, PP-ΔB, and PP-ΔC (Fig. 3C). After subcloning the above sections into plasmid pGL3-Basic and after transduction into Huh-7-ERα and SMCC-7721-ERα, the luciferase reporter assay was performed. The results indicated that the promoter activity was decreased when ERE A and C in the *ptpro* promoter were mutated (Fig. 3D; $P < 0.01$), which further confirms the fact that ERα effectively promotes the expression of PTPRO in a transcriptional manner.

***PTPRO Expression Inhibited HCC Progression* In Vitro *and* In Vivo** Because PTPRO was expressed at low levels in HCC, we investigated whether PTPRO possesses the potential to inhibit HCC progression.

To determine whether PTPRO regulates HCC cell growth *in vitro*, PTPRO overexpression was analyzed using cell lines Huh-7 and SMCC-7721 by lentivirus-

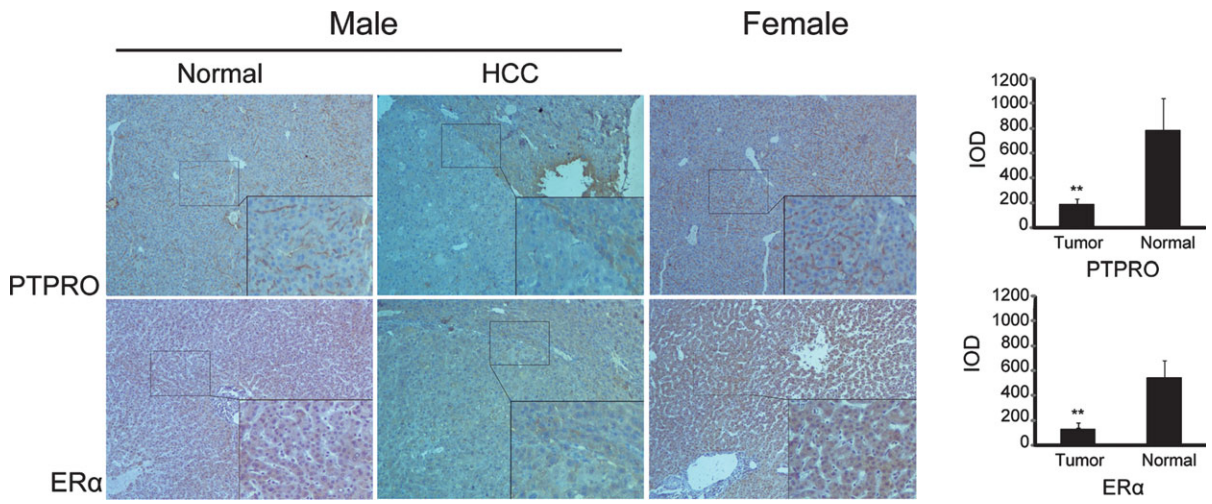

Fig. 2. PTPRO and ERα levels were decreased in DEN-treated male mice. IHC staining of ERα and PTPRO in healthy ($n_{male} = 6$; $n_{female} = 6$) and DEN-treated ($n_{male} = 6$; $n_{female} = 6$) mouse livers. Average value of IOD was obtained as described above, showing the pathological deficiency and gender bias of PTPRO and ERα expression. Data are expressed as mean ± standard error of the mean. $^{**}P < 0.01$.

mediated transduction. Tetrazolium (MTT) proliferation assays indicated that up-regulation of PTPRO did indeed arrest HCC cell growth, in contrast to the control cell group (Fig. 4A; $P < 0.01$). Moreover, these findings were confirmed by the bromodeoxyuridine (BrdU) assay, which showed that PTPRO could inhibit

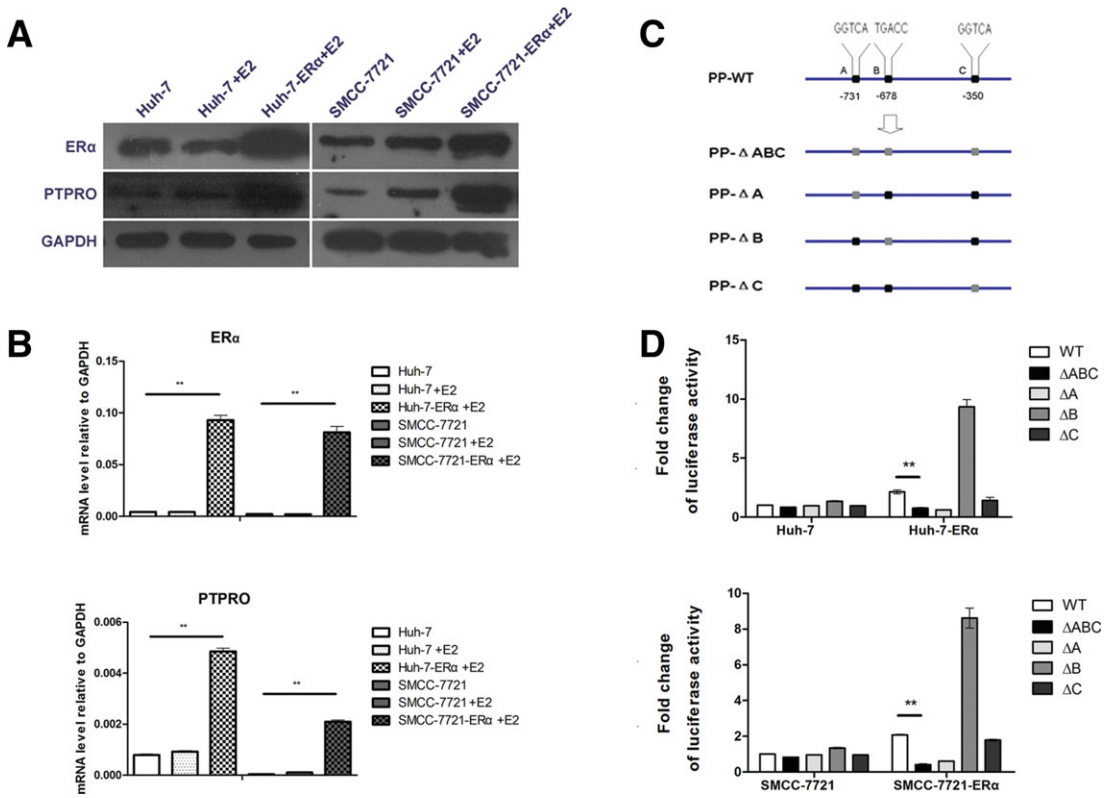

Fig. 3. ERα escalated PTPRO expression *in vitro*. (A) PTPRO levels in ERα-transduced HCC cell line Huh-7 and SMCC-7721 detected by western blotting analysis. PTPRO protein expression levels were significantly up-regulated in transgenic overexpressing Huh-7 and SMCC-7721 cell lines. (B) PTPRO levels in Huh-7-ERα and SMCC-7721-ERα detected by real-time PCR. PTPRO mRNA expression was significantly up-regulated. (C) *Ptpro* promoter cloning and mutagenesis construction. Three EREs localized to the *ptpro* promoter region: A (−731), B (−678), and C (−350). EREs were separately mutated by molecular cloning, and mutant PP-ΔABC, PP-ΔA, PP-ΔB, and PP-ΔC were generated. (D) *Ptpro* promoter activity in Huh-7-ERα and SMCC-7721-ERα detected by luciferase assay. In the presence of ERα and E2, promoter activity of PP-ΔABC was significantly reduced, compared to PP-WT. Individually, PP-ΔA and PP-ΔC represented lower activity than PP-WT, whereas PP-ΔB was higher. Data are expressed as mean ± standard error of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

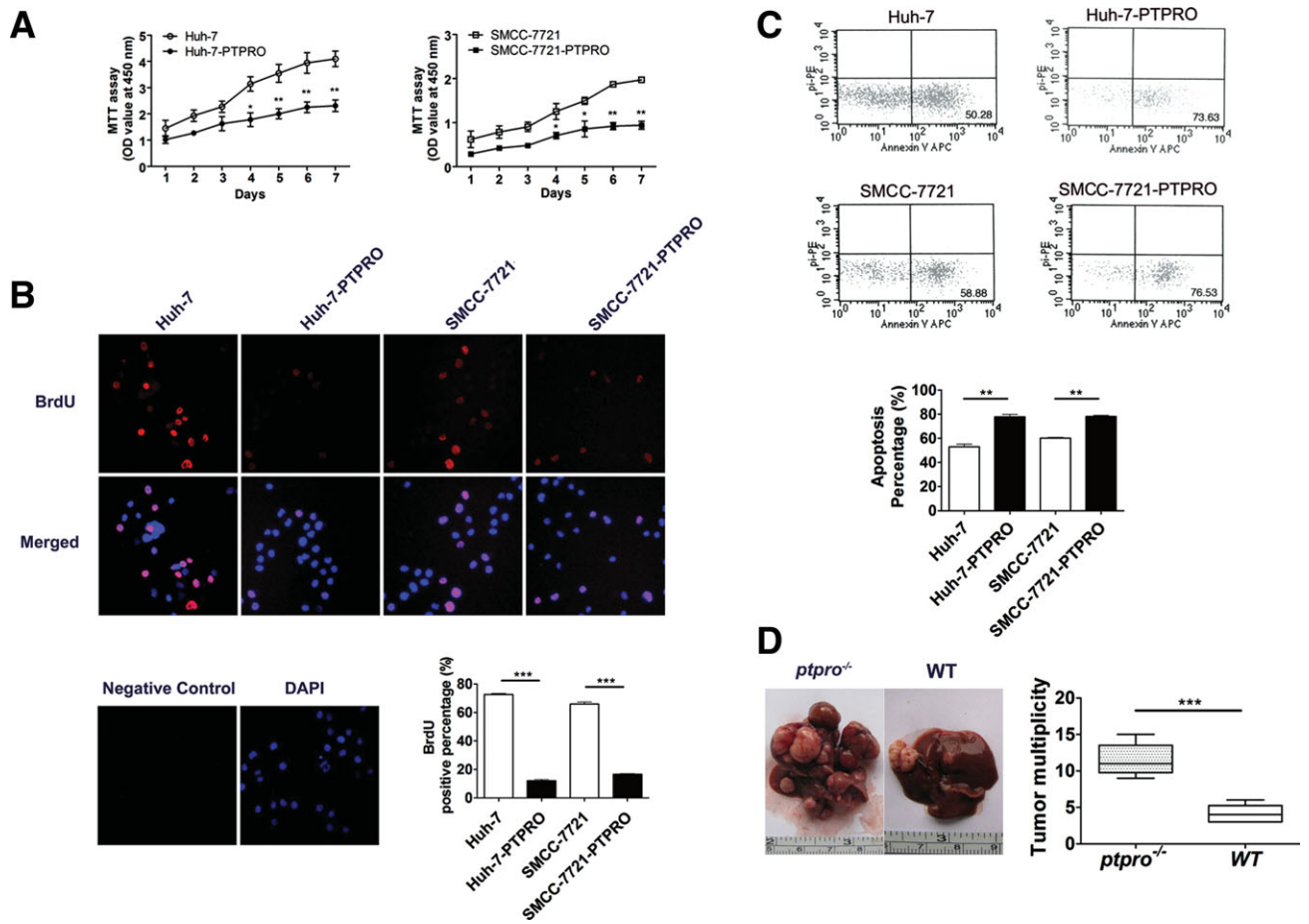

Fig. 4. PTPRO expression inhibited HCC progression *in vitro* and *in vivo*. (A) Cell proliferation of PTPRO-overexpressing HCC cell lines detected by MTT assay. Huh-7-PTPRO and SMCC-7721-PTPRO exhibited lower levels of proliferation, compared to control cells. (B) Immunofluorescence staining of BrdU in green and 4′,6-diamidino-2-phenylindole (DAPI) in blue, visualized by fluorescent microscopy (×200). The PTPRO-overexpressing group exhibited markedly less cell division. (C) Annexin V/PI assay of HCC cell apoptosis induced by peroxide and detected by flow cytometry. The PTPRO-overexpressing group represented greater cell death. (D) Tumorigenesis of $ptpro^{-/-}$ mice. Compared to WT mice (n = 6), $ptpro^{-/-}$ mice (n = 6) exhibited markedly larger tumor size and number. Data are expressed as mean ± standard error of the mean. $^{**}P < 0.01$; $^{***}P < 0.001$.

the frequency of cell division (Fig. 4B; $P < 0.001$). In addition, cell apoptosis was assessed in the above cell lines. Results from the Annexin V/propidium iodide (PI) assay demonstrated that peroxide could induce greater cell death in PTPRO-transduced cells (Fig. 4C; $P < 0.01$). The *in vitro* data confirmed the suppressive function of PTPRO in HCC.

Besides the *in vitro* study described above, we constructed a DEN-induced HCC model with C57BL/6 mice, comprised of 6 $ptpro^{-/-}$ and 6 wild-type (WT) mice. Eight months after DEN treatment, livers of each group of mice were separated and tumor number and size were recorded. As observed in our previous study, no tumors were found in female mice, including both $ptpro^{-/-}$ and WT groups. On the other hand, all male mice presented tumor growth, among which $ptpro^{-/-}$ exhibited markedly larger tumor number and size (Fig. 4D; $P < 0.001$). Taken together, our findings strongly indicate that PTPRO deficiency promotes HCC development.

PTPRO Expression Attenuated STAT3 Activity. To determine the potential molecular mechanisms underlying HCC-suppressive phenotypes, we investigated STAT3 activation between PTPRO-overexpressing and control HCC cell lines by western blotting analysis. Phosphorylation of both Y705 and S727 residues was reduced in PTPRO-overexpressing cells, indicating that PTPRO expression inhibited STAT3 activity (Fig. 5A). Moreover, STAT3 Y705 and S727 phosphorylation was detected in tissue proteins from $ptpro^{-/-}$ and WT mice. Both western blotting and IHC staining exhibited escalated phosphorylation levels of Y705 and S727 (Fig. 5B,C). In addition, cyclin D1 and Bcl-2 were found to be down-regulated in PTPRO-overexpressing HCC cells; these findings serve to explain the modified cell proliferation and apoptosis.

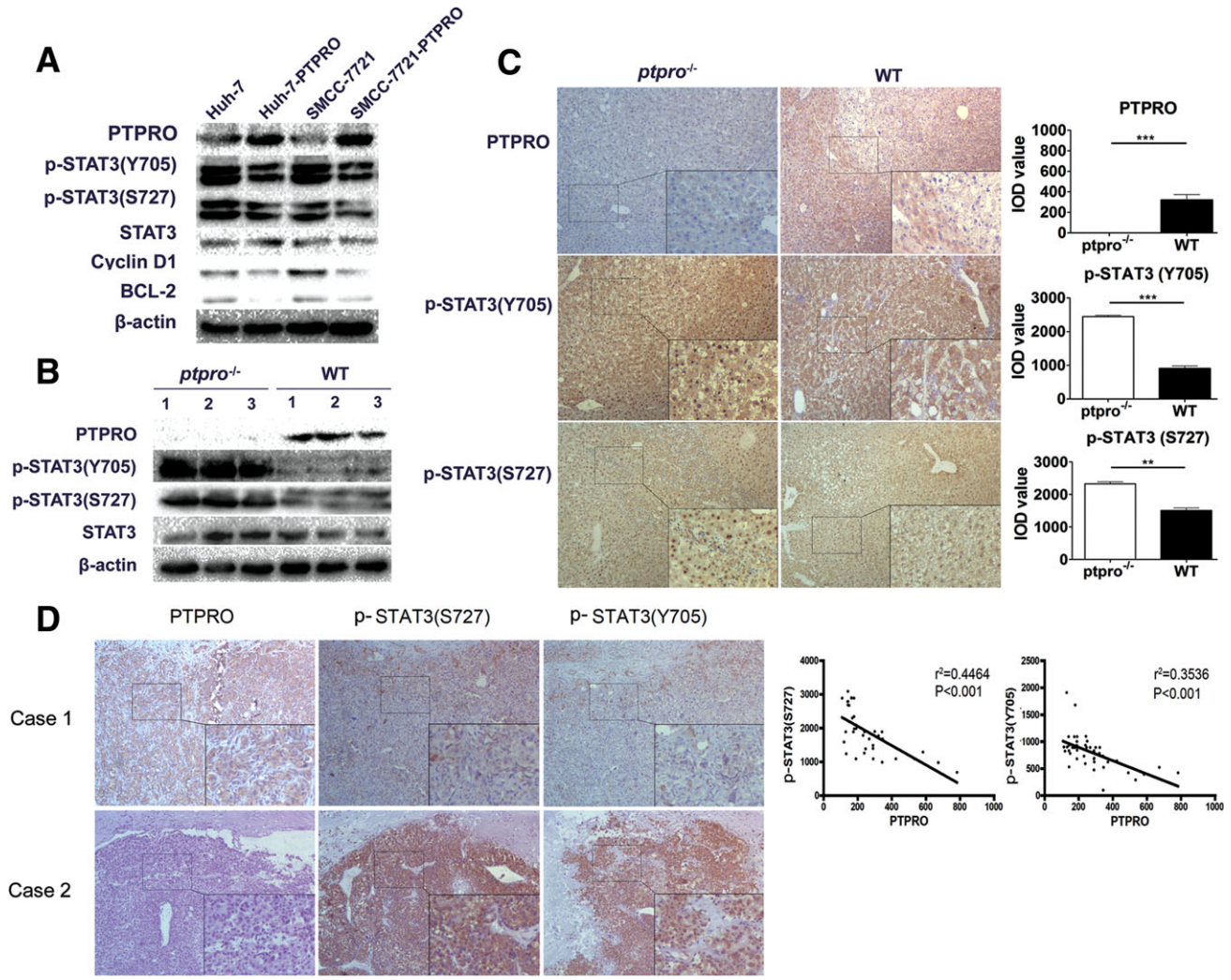

Fig. 5. PTPRO expression attenuated STAT3 activity. (A) Western blotting analysis of p-STAT3 in HCC cells. PTPRO-overexpressing HCC cells exhibited expression disparity of PTPRO, p-STAT3 (Y705), p-STAT3 (S727), cyclin D1, and Bcl-2 levels, all normalized to β-actin. (B) Western blotting analysis of p-STAT3 in mouse livers. *Ptpro*$^{-/-}$ mice exhibited expression disparity of PTPRO, p-STAT3 (Y705), and p-STAT3 (S727) levels, all normalized to β-actin. (C) IHC staining of PTPRO, p-STAT3 (Y705), and p-STAT3 (S727) in mouse HCC. The average value of integrated optical density (IOD) was obtained, as described above, and demonstrated that the p-STAT3 level in *ptpro*$^{-/-}$ mice was significantly up-regulated. Data are expressed as mean $\pm$ standard error of the mean. $^{**}P < 0.01$; $^{***}P < 0.001$. (D) IHC staining of PTPRO, p-STAT3 (Y705), and p-STAT3 (S727) in human HCC: case 1 (with weak PTPRO expression) and case 2 (with negative PTPRO expression). p-STAT3 levels were markedly up-regulated in case 2. (E) Pearson's correlation analysis of p-STAT3 and PTPRO levels in human HCC. Inverse linear relationships between p-STAT3 and PTPRO were identified (PTPRO and p-STAT3 [Y705]: $r^2 = 0.3536$, $^{***}P < 0.001$; PTPRO and p-STAT3 [S727]: $r^2 = 0.4464$, $^{***}P < 0.001$).

We further evaluated the correlation between PTPRO level and STAT3 activity with 50 paraffin-embedded human HCC tissue slices. Two cases of HCC with different PTPRO expression levels are shown in Fig. 5D: case 1 (weak positive) and case 2 (negative). phosphorylated STAT3 (p-STAT3) levels were extensively down-regulated in case 1. Pearson's correlation analysis demonstrated the inversely linear relationship between p-STAT3 and PTPRO levels in HCC (Fig. 5E; PTPRO and p-STAT3 [Y705]: $r^2 = 0.3536$, $P < 0.001$; PTPRO and p-STAT3 [S727]: $r^2 = 0.4464$, $P < 0.001$). Taken together, these findings indicated that PTPRO suppressed HCC by control of STAT3 activation.

PTPRO Expression Contributed to JAK2 and PI3K Dephosphorylation. Because PTPRO exhibited an effective role in STAT3 inactivation, we further investigated PTPRO-mediated signaling, through which STAT3 phosphorylation was directly regulated. Published data indicated that JAK2 played the role of a typical activator of STAT3; because p-JAK2 (Y1007) phosphorylation has been demonstrated to be associated with JAK2 activity, we assessed its expression in HCC cells and mice using western blotting and IHC staining. p-JAK2 level was decreased in PTPRO-overexpressing HCC cells and increased in *ptpro*$^{-/-}$ mice (Fig. 6A,D). We then treated HCC cells with JAK2

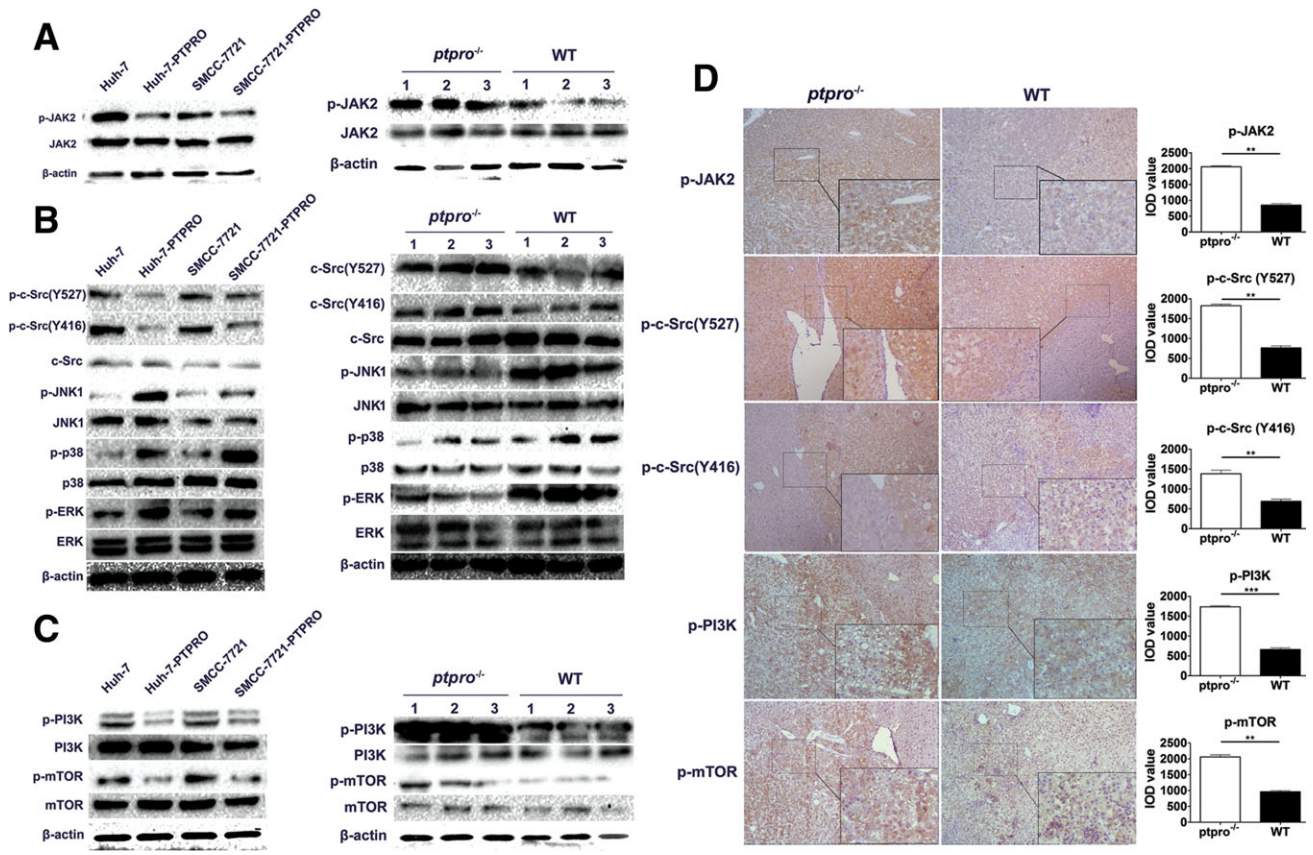

Fig. 6. PTPRO expression contributed to JAK2 and PI3K dephosphorylation. (A-C) Western blotting analysis of p-JAK2, p-c-Src, p-JNK1, p-p38, p-ERK, p-PI3K, and p-mTOR in HCC cells and mice. PTPRO-overexpressing HCC cells exhibited lower p-JAK2, p-c-Src, p-PI3K, and p-mTOR levels and higher p-JNK1, p-p38, and p-ERK levels, normalized to total JAK2, c-Src, PI3K, mTOR, JNK1, p38, ERK, and β-actin levels. *Ptpro*$^{-/-}$ mice exhibited markedly increased p-JAK2, p-c-Src, p-PI3K, and p-mTOR levels and decreased p-JNK1, p-p38, and p-ERK levels, normalized to total JAK2, c-Src, PI3K, mTOR, JNK1, p38, ERK, and β-actin levels. (D) IHC staining of p-JAK2, p-c-Src, p-PI3K, and p-mTOR in mouse HCC. The average value of integrated optical density (IOD) was obtained, as described above, demonstrating that the level of p-JAK2, p-c-Src, p-PI3K, and p-mTOR in *ptpro*$^{-/-}$ mice was significantly up-regulated. Data are expressed as mean ± standard error of the mean. $^{**}P < 0.01$.

inhibitor (AG490)[40] and found that PTPRO-overexpressing HCC cells exhibited a higher level of Y705 phosphorylation and a lower level in S727 (Fig. 7B). This finding suggested that PTPRO controlled STAT3 Y705 phosphorylation through JAK2.

Because STAT3 Y705 dephosphorylation was potentially the result of inactivated JAK2, we were intent to identify the pathway of S727 dephosphorylation regarding PTPRO regulation. Because c-Src-mediated JNK, MAPK p38, and ERK pathways activated STAT3 at both the Y705 and S727 sites, we determined the level of p-c-Src (Y527), p-c-Src (Y416), p-JNK1, p-p38, and p-ERK in PTPRO-overexpressing cells and *ptpro*$^{-/-}$ mice. p-c-Src (Y527) and p-c-Src (Y416) levels were both decreased in PTPRO-overexpressing HCC cells and increased in *ptpro*$^{-/-}$ mice (Fig. 6B,D). However, our findings confirmed that Y527 and Y416 phosphorylation levels were divergent in terms of kinase activity. Results of p-JNK1, p-p38, and p-ERK levels exhibited an increase in PTPRO-overexpressing HCC cells and a decrease in *ptpro*$^{-/-}$ mice (Fig. 7A), thus indicating that c-Src activity was enhanced in the presence of PTPRO. Furthermore, we found decreased Y705 and S727 phosphorylation in PTPRO-overexpressing HCC cells treated with c-Src inhibitor (PD180970)[41] (Fig. 7C). Therefore, these findings indicate that PTPRO-associated STAT3 S727 dephosphorylation is not attributed to the c-Src pathway.

Because mTOR was also an important activator of STAT3 S727, we investigated whether the PI3K/mTOR pathway was regulated by PTPRO. p-PI3K, mTOR, and p-mTOR levels were decreased in PTPRO-overexpressing HCC cells and were increased in *ptpro*$^{-/-}$ mice (Fig. 6C,D). To confirm the role of PI3K/mTOR, PI3K inhibitor (LY294002)[42] was utilized to treat PTPRO-overexpressing HCC cells, which then exhibited a lower Y705 phosphorylation level and a higher S727 level (Fig. 7D). Thus, PTPRO controlled STAT3 S727 phosphorylation through PI3K inactivation.

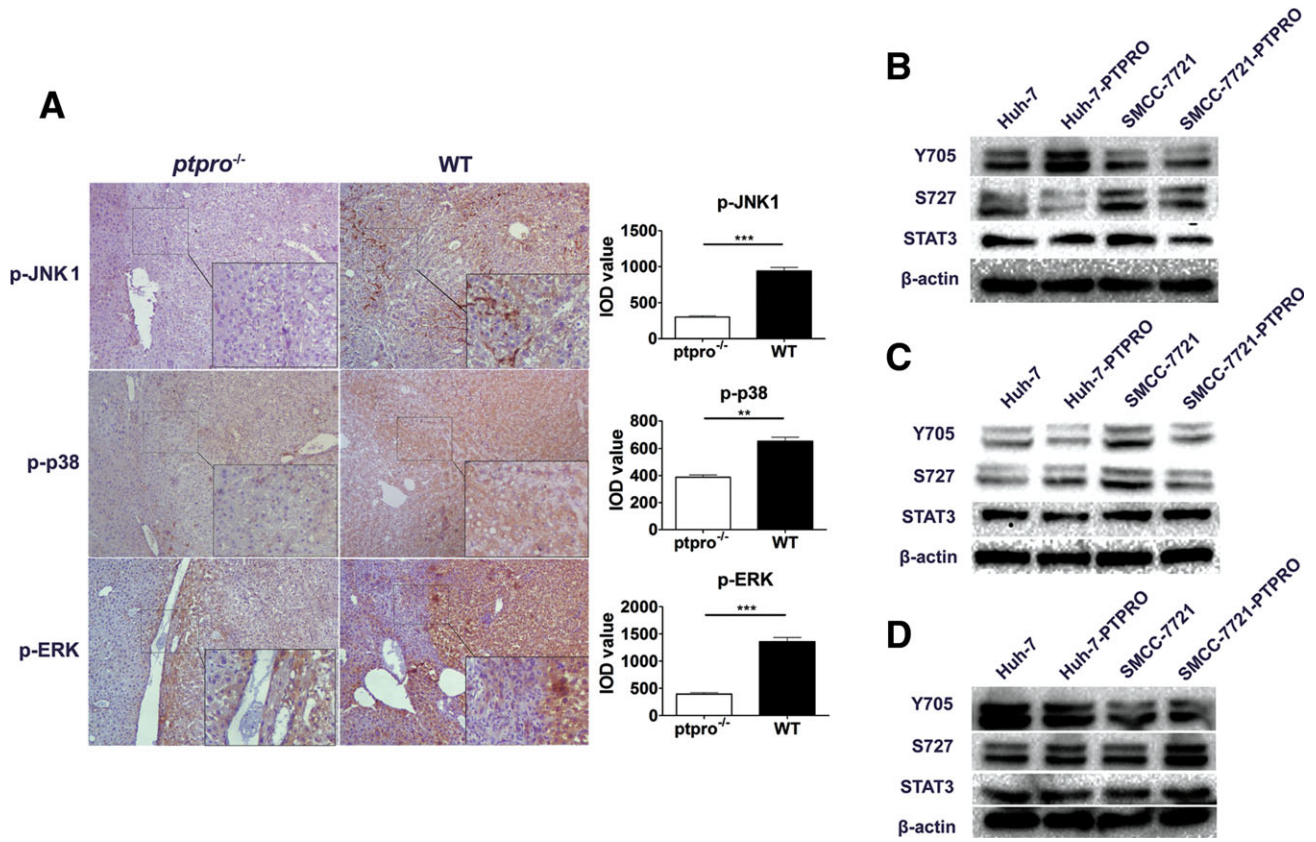

Fig. 7. PTPRO expression contributed to JAK2 and PI3K dephosphorylation. (A) IHC staining of p-JNK1, p-p38, and p-ERK in mouse HCC. The average value of IOD was obtained as described in text, showing that p-JNK1, p-p38, and p-ERK levels in *ptpro*$^{-/-}$ mice were significantly down-regulated. Data are expressed as mean ± standard error of the mean. $^{**}P < 0.01$. (B-D) p-STAT3 levels in PTPRO-overexpressing HCC cells separately treated with JAK2, c-Src, and PI3K inhibitors.

Taken together, our findings suggest that PTPRO-regulated intracellular signals are incorporated into STAT3 inactivation as a result of the down-regulation of JAK2-dependent Y705 phosphorylation and PI3K-responsive S727 phosphorylation. By contrast, mechanical regulation of PTPRO inhibited c-Src Y527 dephosphorylation, leading to increased c-Src pathway activity and limiting terminal STAT3 inactivation.

Discussion

Over the past several decades, investigators have paid close attention to the sexual disparity observed in HCC, and expression of ERs has been gradually identified in HCC specimens. In this study, we demonstrated that the ERα level was markedly reduced in the tumor region, but ERβ level exhibited no significant difference; thus, we focused on the role of ERα. Recently, it was reported that ERα may include a truncated variant (ERα 36) that lacks transcription activation domains; hence, it was not included in this study.[43] In the progression of HCC, typical ERα plays a central role in the regulation of estrogen-sensitive genes, including oncogenes and tumor suppressors, exerting a positive or negative effect; it has been suggested by a recent study that this function of ERα was dependent on Foxa1/2.[44] Our recent report demonstrates that ERα was able to inhibit the transcription of IL-1α in HCC.[39] According to a previous study, ERα not only binds to EREs, but also interacts with other transcription factors, such as AP-1, specificity protein 1, and NF-κB.[45] Unlike the indirect transcriptional regulation of the AP-1 site in breast cancer cells, we confirmed in HCC that ERα binds to the three EREs located in the promoter region of *ptpro*. ERα enhanced *ptpro* transcription at ERE A and C and repressed transcription at ERE B; however, the effect still led to significantly increased transcription activity (Fig. 3D). Therefore, reduced ERα expression in male HCC directly leads to the reduction of PTPRO expression levels.

Reduced PTPRO expression concerned with hypermethylation in the *ptpro* promoter has been demonstrated in various cancer types.[6-10] It has been shown that PTPRO overexpression in lung cancer cell line A549 leads to reduced cell proliferation. In addition to our *in vitro* findings that PTPRO inhibits cell proliferation and promotes apoptosis in HCC cell lines, we

also demonstrated in a DEN-induced mouse model in which PTPRO gene deficiency potentially causes increased tumorigenesis and accelerated tumor growth. It has been previously demonstrated that PTP1B, CD45, PTPN2, and PTPN11 potentially serve as a negative regulator of the JAK/STAT pathway.[35-38] In this study, we demonstrated that PTPRO/STAT3 signaling was responsible for the tumor-suppressive effect of PTPRO. Based on *in vitro* and *in vivo* evidence, we further demonstrated that PTPRO controls STAT3 activation by restricting tyrosine phosphorylation of JAK2. In the presence of JAK2 inhibitor AG490, PTPRO-overexpressing HCC cells failed to regulate STAT3 Y705 phosphorylation; this indicated that PTPRO-mediated STAT3 Y705 dephosphorylation was dependent of JAK2.

Moreover, we demonstrated that S727 phosphorylation, which is required for full transcriptional activity of STAT3, was extensively regulated by PTPRO. Unexpectedly, both *ptpro*$^{-/-}$ mice and PTPRO-overexpressing HCC cells demonstrated that PTPRO did not inhibit the activity of JNK1, p38, or ERK, but rather enhanced the activity. PTPRO inhibited tyrosine phosphorylation of c-Src at both the Y527 and Y416 sites; however, the greater Y527 dephosphorylation has been shown to promote c-Src activity.[46] In fact, c-Src was also identified to be positively regulated by a variety of PTPs, such as CD45, PTP1B, SHATTERPROOF (SHP)1, SHP2, PTPRα, PTPRε, and PTPRλ, for the mechanical function of PTP.[47] Therefore, purely regarding the c-Src pathway, PTPRO functions in a hostile manner. However, with the cooperation of corresponding inhibitors, PTPRO can amplify its suppressive effect in HCC.

Phosphatase and tensin homolog (PTEN), the most well-known PTP identified as a critical tumor suppressor in various kinds of cancers, has been demonstrated to attenuate STAT3 S727 phosphorylation by inhibiting the PI3K-mTOR pathway.[42,48] When we sought the source of STAT3 S727 dephosphorylation among PTPRO-mediated signals, we investigated the PI3K-mTOR pathway. Surprisingly, PTPRO was found to possess the same regulatory function as PTEN. We confirmed, using *in vivo* and *in vitro* experiments, that under PTPRO regulation, PI3K activity was decreased and mTOR failed to effectively phosphorylate STAT3 S727. PI3K has been shown to be positively regulated by JAK2 and c-Src; however, in the presence of PTPRO, the cross-talk from these pathways appeared to be neutralized. When HCC cells were treated with PI3K inhibitor, the S727 phosphorylation level in the PTPRO-overexpressing group appeared higher, compared to the control, indicating that PI3K signaling is essential for PTPRO-mediated negative regulation of STAT3 S727.

In this study, for the first time, we have reported the pathological deficiency and gender bias of PTPRO expression in HCC, and we have demonstrated the involvement of ERα. Additionally, we have, for the first time, elucidated the molecular mechanisms underlying PTPRO-mediated STAT3 inactivation and clarified the responsibility of each signal involved in the tumor-suppressive ability of PTPRO. In this study, PTPRO presented similar regulating functions to other PTPs and was implicated in three pathways linked to STAT3 activation. We not only separately analyzed the modified signaling under negative or positive regulation of PTPRO, but also systematically investigated the terminal status of STAT3, including Y705 and S727 phosphorylation, essential for STAT3 activation, which shapes the suppressive position of PTPRO in HCC progression.

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