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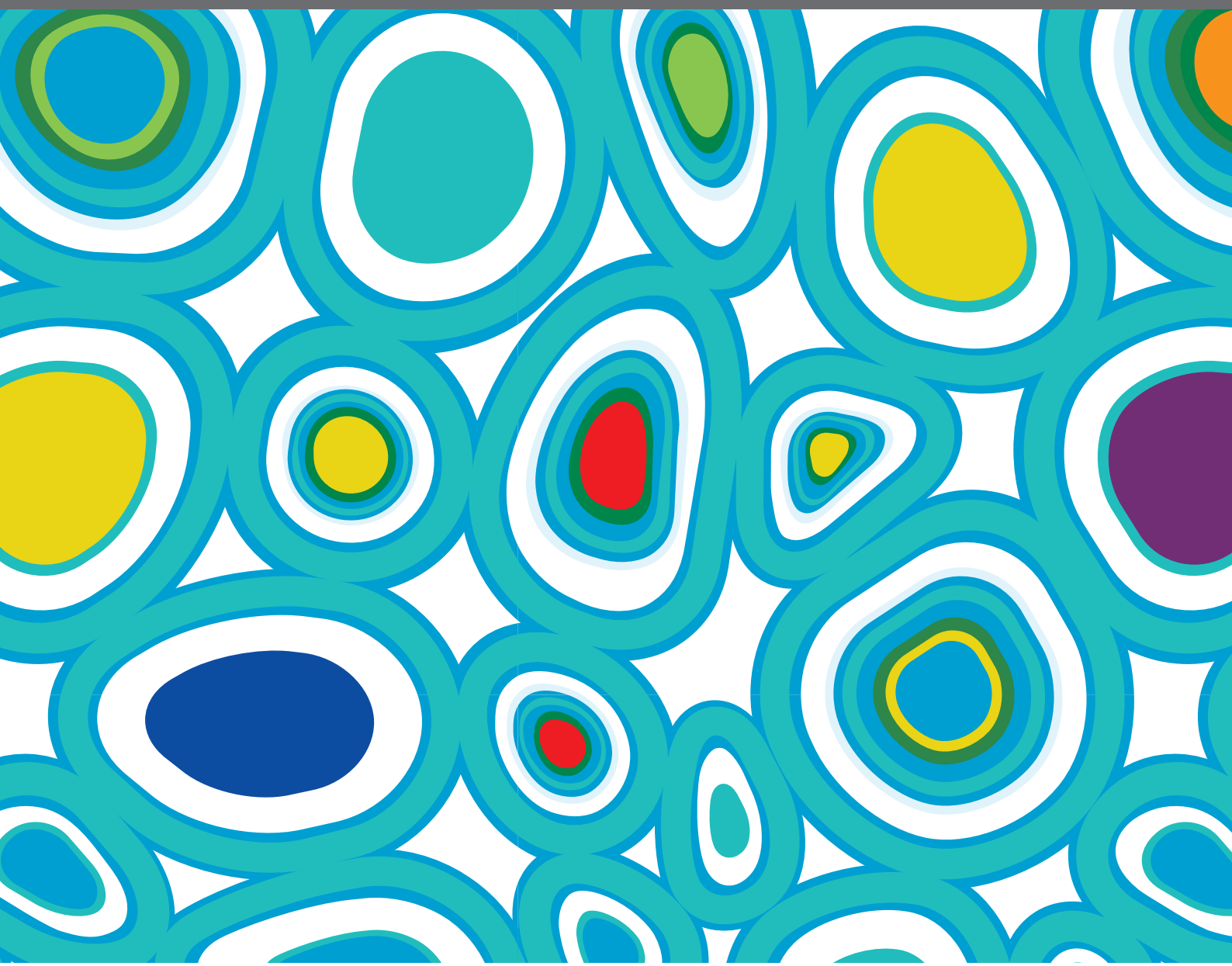

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THE ROLES OF ONCOGENIC PHOSPHATASE/KINASE IN TUMORS

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EDITORIAL
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Editorial: The Roles of Oncogenic Phosphatase/Kinase in Tumors

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Keywords: cancer, tumor, protein kinase, protein phosphatase, signaling

Editorial on the Research Topic

The Roles of Oncogenic Phosphatase/Kinase in Tumors

Protein kinases and phosphatases are antagonistic mediators of signal transduction pathways, which function through the phosphorilation/dephosphorylation, respectively, of their specific downstream targets. The proper balancing of protein kinases/phosphatases activity is crucial to maintain cell homeostasis and assure the correct timing, amplitude and duration of environmental signals underlying biological process such as cell growth, differentiation, migration, metabolism, survival and death.

Dysregulation of the complex network of protein kinase/phosphatase activity has long been recognized as an intrinsic hallmark of tumor transformation and progression, either as primary cause or consequence. Decades of research on this topic have allowed to identify hundreds of kinases and phosphatases in human genome (Hooft van Huijsduijnen, 1998) and the abnormal activity of many of them have been shown to underly tumorigenesis in multiple types of cancer (Stebbing et al., 2014).

In this research topic, experts in the field contributed with either original research or review articles to move forward our knowledge on interesting aspects of the complex role of kinases and phosphatases in tumorigenesis.

Dong et al. summarized how the gain-of-function mutation of Src homology region 2 protein tyrosine phosphatase 2 (SHP2) might contribute to promote cancer progression both through cell-autonomous and non-cell-autonomous mechanisms. They also discussed the role of SHP2 mutations in drug resistance and the potential therapeutic use of small molecule SHP2 inhibitors in anti-cancer therapies.

Huang et al. showed how the cyclin-dependent kinase 9 (CDK9) inhibitors might induce the apoptosis of B-cell acute lymphocytic leukemia (B-ALL) by inhibiting c-Myc-mediated glycolitic metabolism.

Ciummo et al. showed that the chemokine C-X-C motif ligand-1 (CXCL-1) functions as an autocrine growth factor which promotes immune escape and sustains breast cancer stem cell phenotype and epithelial-to-mesenchymal transition, two intrinsically interrelated aspects of the most aggressive breast cancers.

Turdo et al. reviewed the major protein kinase and phosphatase pathways which impact on the capability of CSCs to evade normal physiological constraints on survival, growth, and invasion. They also discussed the potential use of phosphatase/kinase inhibitors in counteracting CSCs expansion during cancer development and progression.

Centoze et al. reviewed 15 years of research about the role of the protein p130Cas to function as adaptor multiprotein signaling complexes which sustain breast cancer progression through pleiotropic effects on cell motility, cell adhesion, cytoskeleton remodeling, invasion, survival, and

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proliferation. They also discussed the p130Cas-antagonistic role of p140Cap, which associate with p130Cas through interaction with the Src kinase and display well established anti-tumor effects in breast cancer and neuroblastoma.

Hao et al. reviewed the controversial role in leukemia of two protein tyrosine phosphatases, SH2 domain-containing phosphatases 1 and 2 (SHP-1 and SHP-2) and of the phosphatase inhibitor SH2-domain-containing inositol phosphatase.

Yao et al. provided the transcriptional profiling of tumor-associated protein kinases and phosphatases and other phosphorylation-related genes in samples of human hepatocellular carcinoma, thus identifying the overexpression of a set of protein kinases and phosphorylation-related genes that were associated to cancer stem cell phenotype and poor clinical outcome.

Li et al. identified 6-Phosphogluconolactonase overexpression as a marker of poor prognosis in hepatocellular carcinoma (HCC) and showed that the downregulation of this gene was able to impair cell proliferation, migration and invasion capability of HCC likely inhibiting ROS-mediated apoptosis.

Boni and Sorio discussed the role of protein tyrosine phosphatase gamma (PTPRG) as natural counterpart of tyrosine kinases, and reviewed how its loss-of-function has been reported in many types of cancers, such as Lymphoma and Leukemia, colorectal, nasopharyngeal, ovarian, breast, lung, gastric cancer.

The understanding of the effects of kinases/phosphatases in cancer and their molecular mechanisms of action has been greatly increased over the last decades and hold promise in therapy (Vainonen et al., 2021). Nevertheless, a deeper understanding of this complex network of cancer regulators is still needed to design more effective and specifically targeted strategies of treatment which might contribute to eradicate the infinite variants of this pandemic disease that disseminates death all over the world.

AUTHOR CONTRIBUTIONS

MG conceived and wrote the manuscript.

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ORIGINAL RESEARCH
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CDK9 Inhibitor Induces the Apoptosis of B-Cell Acute Lymphocytic Leukemia by Inhibiting c-Myc-Mediated Glycolytic Metabolism

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B-cell acute lymphocytic leukemia (B-ALL), a common blood cancer in children, leads to high mortality. Cyclin-dependent kinase 9 inhibitor (CDK9i) effectively attenuates acute myeloid leukemia and chronic lymphoblastic leukemia by inducing apoptosis and inhibiting cell proliferation. However, the effect of CDK9i on B-ALL cells and the underlying mechanisms remain unclear. In this study, we showed that CDK9i induced the apoptosis of B-ALL cells *in vitro* by activating the apoptotic pathways. In addition, CDK9i restrained the glycolytic metabolism of B-ALL cells, and CDK9i-induced apoptosis was enhanced by co-treatment with glycolysis inhibitors. Furthermore, CDK9i restained the glycolysis of B-ALL cell lines by markedly downregulating the expression of glucose transporter type 1 (GLUT1) and the key rate-limiting enzymes of glycolysis, such as hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA). Moreover, cell apoptosis was rescued in B-ALL cells with over-expressed c-Myc after treatment with CDK9i, which is involved in the enhancement of glycolytic metabolism. In summary, our findings suggest that CDK9 inhibitors induce the apoptosis of B-ALL cells by inhibiting c-Myc-mediated glycolytic metabolism, thus providing a new strategy for the treatment of B-ALL.

Keywords: CDK9 inhibitors, cell apoptosis, glycolysis, c-Myc, B-cell acute lymphocytic leukemia

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INTRODUCTION

B-cell acute lymphoblastic leukemia (B-ALL) is one of the most frequently occurring malignancies in children, with a peak incidence between 1 and 4 years of age. Considering the improvements in multimodal chemotherapy regimens over the past few decades, the 5-year survival rate for pediatric B-ALL is now close to 90% (Malard and Mohty, 2020). However, a proportion of patients still shows

no response to existing therapeutic drugs and suffered from the side-effects of long-term multi-drug treatment. In addition, existing therapeutic drugs cannot further improve the prognosis of refractory and relapsed B-ALL (Kuhlen et al., 2019). Therefore, new strategies for the treatment of B-ALL should be identified.

The inhibition of the cell cycle is one of the key mechanisms in the development of drugs for leukemia treatment (Ghelli Luserna di Rora et al., 2017). Therefore, chemotherapeutic drugs mainly interfere with DNA synthesis and inhibit cell cycle on leukemic cells. Cyclin-dependent kinases (CDKs) are one family of serine/threonine protein kinases and regulate the cell cycle division and gene transcription. CDKs can be divided into two categories according to their function, namely, CDKs that regulate cell cycle and CDKs that modulate gene transcription (Malumbres, 2014; Lemmens and Lindqvist, 2019). Cyclin-dependent protein 9 (CDK9) belongs to the CDK cyclin family, which includes in CDK4, CDK6, and CDK7. CDK9 modulates the transcription elongation and mRNA maturation of genes but does not regulate cell cycle of cells (Asghar et al., 2015). CDK9 phosphorylates Ser-2 and Ser-5 of the carboxyl terminal domain (CTD) of RNA polymerase II (RNA Pol II), which is involved in transcription elongation (Laitem et al., 2015; Gressel et al., 2017). CDK9 participates in the development and progression of many types of tumors by recruiting p-TEFb to the promoters of oncogenes in a BRD4-dependent manner (Franco et al., 2018). Therefore, CDK9 could serve as a potential therapeutic target in most of malignant tumors (Sonawane et al., 2016). CDK9 inhibitors have a significant inhibitory effect on acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) (Yin et al., 2014; Boffo et al., 2018). SNS-032, a CDK9 selective inhibitor, has entered clinical trials for the treatment of AML, CLL, and multiple myeloma (Tong et al., 2010; Walsby et al., 2011). AZD4573, another highly selective inhibitor of CDK9, has been validated in hematological malignancies (Cidado et al., 2020). However, the effect of CDK9 inhibitors on B-ALL cells and the underlying mechanism remain unknown.

Tumor cells favor anaerobic glycolysis as energy source even under sufficient oxygen condition, which is known as the Warburg effect. As the initial step in glucose metabolism, glycolysis consists of several reactions that are involved in several key rate-limiting enzymes, such as hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) (Counihan et al., 2018). CDK6 links the cell cycle and cell metabolism of tumors by phosphorylating two key enzymes, 6-phosphofructokinase (PFK1), and pyruvate kinase M2 (PKM2) and leads to the inhibition of glycolytic pathway and fuels the pentose phosphate (PPP) and serine pathways (Wang et al., 2017). CDK9 inhibition stops the gene transcription and results in the downregulated expression of a large proportion of genes, such as c-Myc and Mcl-1 (Boffo et al., 2018). The oncogene c-Myc controls many aspects of cell biological processes, such as cell growth, proliferation, differentiation, and apoptosis (Garcia-Gutierrez et al., 2019). As a metabolic sensor, c-Myc stimulates the glycolysis, mitochondrial biogenesis and glutamine metabolism by directly modulating the expression of metabolism-related genes in tumor cells (Stine et al., 2015; Dejure and Eilers, 2017). However, whether CDK9 inhibitors induce cell apoptosis in leukemia by suppressing c-Myc-mediated glycolysis is largely unknown.

The oncogene c-Myc encodes a transcription factor c-Myc, which links altered cellular metabolism to tumorigenesis. c-Myc regulates genes involved in the biogenesis of ribosomes and mitochondria, and regulation of glucose and glutamine metabolism.

In the study, we discovered that CDK9 inhibitors induced the apoptosis of B-ALL cells by restraining glycolysis, which was enhanced by co-treatment with glycolysis inhibitors *in vitro*. Moreover, cell apoptosis was reversed in B-ALL cells with overexpressed c-Myc after treatment with CDK9 inhibitors, which are involved in the enhancement of glycolytic metabolism. Therefore, these findings provide a potential treatment strategy for B-ALL in the clinic.

MATERIALS AND METHODS

Clinical Samples

Bone marrow samples from patients with childhood B-ALL were collected in Shanghai Children's Medical Center (SCMC). Sample usage and protocols were approved and supervised by the SCMC Ethics Committee. All the samples were analyzed in a blind manner and stored in SCMC. B-ALL cells were seeded at a density of 10^6 cells/ml in STEMSPAN (Gibco) medium supplemented with 20 ng/ml recombinant human IL3 (rhIL3), 10 ng/ml rhIL7, 10 ng/ml rhIL6, 10 ng/ml rhIL2, 10 ng/ml rhIGF-1, 20 ng/ml rhFlt3L, and 10 ng/ml rhVcam1. B-ALL cells were treated with or without 1 μM of SNS-032 for 24 h, and the percentage of apoptosis was analyzed by flow cytometry.

Culture of Cell Lines

Human B-ALL cell lines, SEM, RS4;11, NALM6, and REH, were purchased from the American Type Culture Collection (Manassas, VA, United States) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco). Cell lines were routinely detected by mycoplasma contamination test and were assessed using short tandem repeat (STR) DNA profiling.

Drug Sensitivity Assay

A total number of 12,000 cells per well were seeded in a 96-well plate and then treated with different concentration of drugs (SNS-032 and AZD4573, obtained from Selleck, Houston, TX, United States) for 72 h. Cell viability was evaluated using CTG (Promega CellTiter-Glo^{TM} Luminescent Cell Viability Assay Kit) according to the manufacturer's protocol. The absorbance optical density of 405 nm was recorded using a microplate reader (Synerge2; BioTek Instruments, Winooski, VT, United States), and the half

maximal inhibitory concentration (IC50) was calculated using GraphPad Prism.

Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using the TRIzol reagent (Life technologies). qRT-PCR was performed according to the protocol of QIAGEN SYBR Green PCR. The primers are listed as follows: β-actin: forward (F): 5′-TGCCGACAGGATGCAGAAG-3′ and reverse (R): 5′-GCCGATCCACACGGAGTACT-3′; HK2: F: 5′-CTCTCTGCAACCAGTTCTCTG-3′ and R: 5′-CCAGGCATTCGGCAATGTG-3′; LDHA: F: 5′-ATGGCAACTCTAAAGGATCAGC-3′ and R: 5′-CCAACCCCAACAACTGTAATCT-3; BCL2: F: 5′-AAGATTGATGGGATCGTTGC-3′ and R: 5′-TGTGCTTTGCATTCTTGGAC-3′; GLUT1: F: 5′-CAGTTTGTGCTTTGCTGGCTACAACACTGGAGT-3′ and R: 5′ATAGCGGTGGACCCATGTCT-3′; MCM4: F: 5′-CCTCATTGGTAAAGGGCTAGAG-3′ and R: 5′-TAGCCAGGGTGACAGAGTAA-3′; MCM7: F:5′-CCAGGAGATGAAGATGCAAGAA-3′ and R: 5′-GGGCAATCCTTGTGTTCTCT-3′; BCL2L: F: 5′-TCAGGCTGCTTGGGATAAAG-3′ and R: 5′-AGGCTTCTGGAGGACATTTG-3′.

RNA-Seq Analysis

Total cellular RNA was isolated using the TRIzol reagent. Briefly, mRNA was reversed to cDNA for constructing the library. Then, the cDNA library was measured by RNA sequencing. The raw reads were filtered, and clean reads were mapped using Bowtie2 and HISAT. The gene expression level (FPKM) was calculated according to the RSEM, and the data were analyzed.

Western Blot

B-cell acute lymphocytic leukemia cell lines were seeded at a density of 10^6 cells/ml and treated with drugs. Total cells were collected and lysed in SDS sample buffer. The primary antibodies used were as follows: ACTIN, GAPDH, TUBULIN as internal control (1:5000; Hua An Biotechnology, Hangzhou, China), CDK9 (1:1000; CST), Phospho-Rpb1 CTD (Ser2) (#13499, 1:1000; CST), Phospho-Rpb1 CTD (Ser5) (#13523, 1:1000; CST), Rpb1 CTD (1:1000; AM39097), BCL2 (#3212; 1:1000; Abcam), caspase 3 (ab13847, 1:1000; Abcam), cleaved caspase 3 (#9661, 1:1000; CST), GLUT1 (AF1015, 1:500; Beyotime), HK2 (#2867, 1:1000; CST), LDHA (#3582, 1:1000; CST), c-Myc (#5605, 1:1000; CST, Danvers, MA, United States), and Flag (0912-1, 1:1000; Hua An Biotechnology, Hangzhou, China). After incubation with the fluorescence-labeled secondary antibody, fluorescence signals were analyzed using the Odyssey system (LI-COR Biosciences, Lincoln, NE, United States).

Apoptosis Analysis

Cell apoptosis was measured using the Annexin-V apoptosis detection kit (BD Bioscience, San Jose, CA, United States) according to the manufacturer's protocol. The percentage of Annexin-V positive cells were detected by flow cytometry (BD Biosciences), and the data were analyzed using the FlowJo Version 10.0 software.

Cell Proliferation Analysis

After the cells were treated with drugs for 24 h, EdU was added into the cells and incubated for 2 h. Cell proliferation was conducted using the Click-iT EdU flow cytometry assay kit (Beyotime) according to the manufacturer's protocol. Then, the stained cells were analyzed by flow cytometer.

Glucose Uptake Assay

The glucose uptake ability of the cells was detected by incubating with 2-NBDG (Invitrogen). Briefly, the cells were harvested and washed with PBS. Then, fluorescent 2-NBDG was added to the cells, which were then incubated at 37°C for 30 min in 5% CO2 incubator. After centrifugation, all media were removed and washed with PBS once, and the samples were analyzed using a flow cytometer.

Extracellular Acidification Rate (EACR)

Metabolic flux analysis with a XF Glycolytic Stress Test Kit (#103017-100, Seahorse Bioscience) was performed using a Seahorse XF 96 instruments (Seahorse Bioscience). An equal number of REH cells was plated and treated with inhibitor for 24 h. Cartridge was equilibrated overnight prior to the assay day. Exactly 5×10^5 cells were changed to base media supplemented with 10 mM glucose, 1 μM oligomycin, and 50 mM 2-DG. Results were analyzed using GraphPad Prism. Basal glycolytic rate and glycolytic capacity were calculated according to the manufacturer's instructions.

Lactate Concentration Assay

Lactate was quantified using Glycolysis cell-based Assay Kit (Cayman) according to the manufacturer's protocol. Briefly, 5×10^4 cells were cultured in RPMI-1640 medium supplemented with 0.25% fetal bovine serum (Gibco) for 24 h in 96 wells, and then treated with drugs for 24 h. Exactly 10 μl of culture supernatant was added to the lactate assay buffer. The reaction was incubated for 30 min at room temperature. The absorbance at 490 nm was assessed using a microplate reader.

Measurement of Metabolic Indicators

Cell lines were seeded in RPMI 1640 complete medium with drugs. Cells were incubated with metabolic dyes, such as mitochondrial membrane potential probe MitoTrackerTM orange (Life Technologies, M7511), total ROS probe DCFDA (Life Technologies, C369). The samples were analyzed by flow cytometry.

Metabolite Analysis

A total number of 2×10^6 cells were harvested and washed with pre-cooled PBS. Cells were cracked in ice-cold 80% methanol and centrifuged at 1,500 rpm for 10 min to obtain the supernatant. Finally, the supernatant was detected by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Construction of Overexpression Stable Cell Lines

The c-Myc overexpressed and vector plasmids were gifted from Dr. Li T (SCMC, China). The package and concentration of virus were conducted as previously reported (Wu et al., 2018). REH cells were cultured with the enriched viral medium for 48 h, and then selected with puromycin to construct the stable cell lines for 48 h. The overexpression efficiency was verified by Western blot analysis.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 7.0 (GraphPad Software). Data were presented as mean ± SD of three independent experiments. Differences between samples were analyzed using two-tailed Student's *t*-test. Results with values of $P < 0.05$ were considered statistically significant.

RESULTS

CDK9 Inhibitor SNS-032 Induces the Apoptosis of B-ALL Cell Lines *in vitro*

To determine the cytotoxic effects of CDK9 inhibitor (CDK9i) on B-ALL cells, we detected the cell viability in B-ALL cell lines after treatment with a gradient concentration of SNS-032 for 72 h. The IC50 values of NALM6, REH, SEM, and RS411 were 200, 200, 350, and 250 nM, respectively (**Figure 1A**). We then measured the cell proliferation by staining with EdU and found that EdU-positive B-ALL cells dramatically decreased after treatment with SNS-032 for 24 h (**Figure 1B**), and this result is consistent with a previous report in AML (Wang et al., 2019). Then, we detected the apoptosis of B-ALL cells after SNS-032 treatment for 24 and 48 h and found that the apoptotic rates significantly increased in all of B-ALL cell lines (**Figures 1C,D**), indicating that SNS-032 induces the cell death of B-ALL. We also determined the cell apoptosis in samples from patients with B-ALL and confirmed that the apoptotic rates increased in SNS-032-treated sample compared with that of DMSO-treated sample (**Figures 1E,F** and **Supplementary Table 1**). Additionally, the cell cycle and apoptosis of human normal peripheral blood mononuclear cells (PBMCs) were measured by flow cytometry, and the results showed that SNS-032, to a certain extent, induced apoptosis of PBMC, but did not affect the cell cycle of PBMC (**Supplementary Figures 1A,B**), indicating that SNS-032 may lead to side effects, such as myelosuppression. Furthermore, we used qRT-PCR to detect the expression of cell proliferation- and apoptosis-related genes. SNS-032 remarkably downregulated the expression of cell proliferation genes, such as MCM4 and MCM7, and the expression of anti-apoptosis genes, such as Bcl2 and BCL2L (**Figures 1G,H**). Moreover, Western blot was used to evaluate the protein expression of Bcl-2 and cleaved Caspase 3. The data displayed that Bcl-2 was down-regulated, and cleaved caspase 3 was markedly up-regulated in SNS-032-treated B-ALL cells (**Figures 1I,J**), indicating that SNS-032 activates the cell apoptosis signal pathway of B-ALL. The cytotoxic effects of SNS-032 were confirmed through degraded CDK9 protein, which inhibited phosphorylating serine 2 and 5 in the CTD of RNA Pol II in B-ALL cells (**Supplementary Figure 1C**). Taken together, these data suggested that CDK9i induces cell apoptosis and suppresses cell proliferation of B-ALL *in vitro*.

SNS-032 Perturbs the Cellular Metabolic Pathways of B-ALL Cells *in vitro*

Tumor cells reprogram their metabolism from catabolism to anabolism to prompt cells enter the cell cycle and fuel cell proliferation (Faubert et al., 2020). CDK9 inhibition promotes prostate cancer cells switch to fatty acid oxidation by inducing metabolic stress (Itkonen et al., 2019). However, the inhibitory effect of CDK9i on the energy metabolism of B-ALL cells remains unclear. To address this question, RNA sequencing (RNA-seq) was performed in B-ALL cells after treated with SNS-032 or DMSO for 24 h. The SNS-032- and DMSO-treated cell populations were clustered by correlation analysis and principal component analysis (**Figures 2A,B**). The transcript profile of SNS-032-treated cells was globally changed, where 1,294 genes were upregulated and 545 genes were downregulated compared with those of DMSO-treated cells (**Figures 2C,D**). In addition, the up- and downregulated genes were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. We found that SNS-032 significantly down-regulated the mRNA expression of p53 signaling pathway, PI3K-Akt signaling pathway, and metabolic pathways (**Figure 2E**). We further analyzed the metabolic pathways and found that pyruvate metabolism, glycolysis/gluconeogenesis, purine/pyrimidine metabolism and oxidative phosphorylation were greatly changed after treatment with SNS-032 (**Figures 2F–H**). Altogether, these findings indicated that CDK9i perturbs the glycolytic metabolism of B-ALL cells *in vitro*.

SNS-032 Prompts the Apoptosis of B-ALL Cells by Inhibiting Glycolysis

To clarify the effects of CDK9i on the glycolytic metabolism of B-ALL cells, we detected the glucose uptake of B-ALL cells by incubating fluorescence-labeled 2-deoxy-glucose analog (2-NBDG). SNS-032 treatment remarkably reduced the glucose uptake activities in B-ALL cells (**Figures 3A,B**). In addition, the mitochondrial membrane potential (MMP), ATP content, total reactive oxygen species (ROS) and intracellular lactate concentration were markedly decreased in all four cell lines after SNS-032 treatment (**Figures 3C–F**). The decreased of glucose uptake, MMP and total ROS were confirmed in SNS-032-treated primary B-ALL cells (**Figures 3G–I**). To directly determine the glycolytic capacity of B-ALL cells, the extracellular acidification rate (ECAR) was measured by Seahorse. We uncovered that SNS-032 treatment dramatically inhibited the glycolysis of B-ALL cells (**Figure 3J**). To further prove whether SNS-032 suppresses glycolysis, we used SoNar, a metabolic sensor, to monitor the dynamic of metabolic change and found that SoNar-high cells prefer glycolysis (Zhao et al., 2015; Zou et al., 2018). The alteration of the ratios in SoNar B-ALL cells was easily tested by flow cytometry. The results displayed that the ratios of SoNar-high cells notably decreased in SNS-032-treated cells (**Figure 3K**).

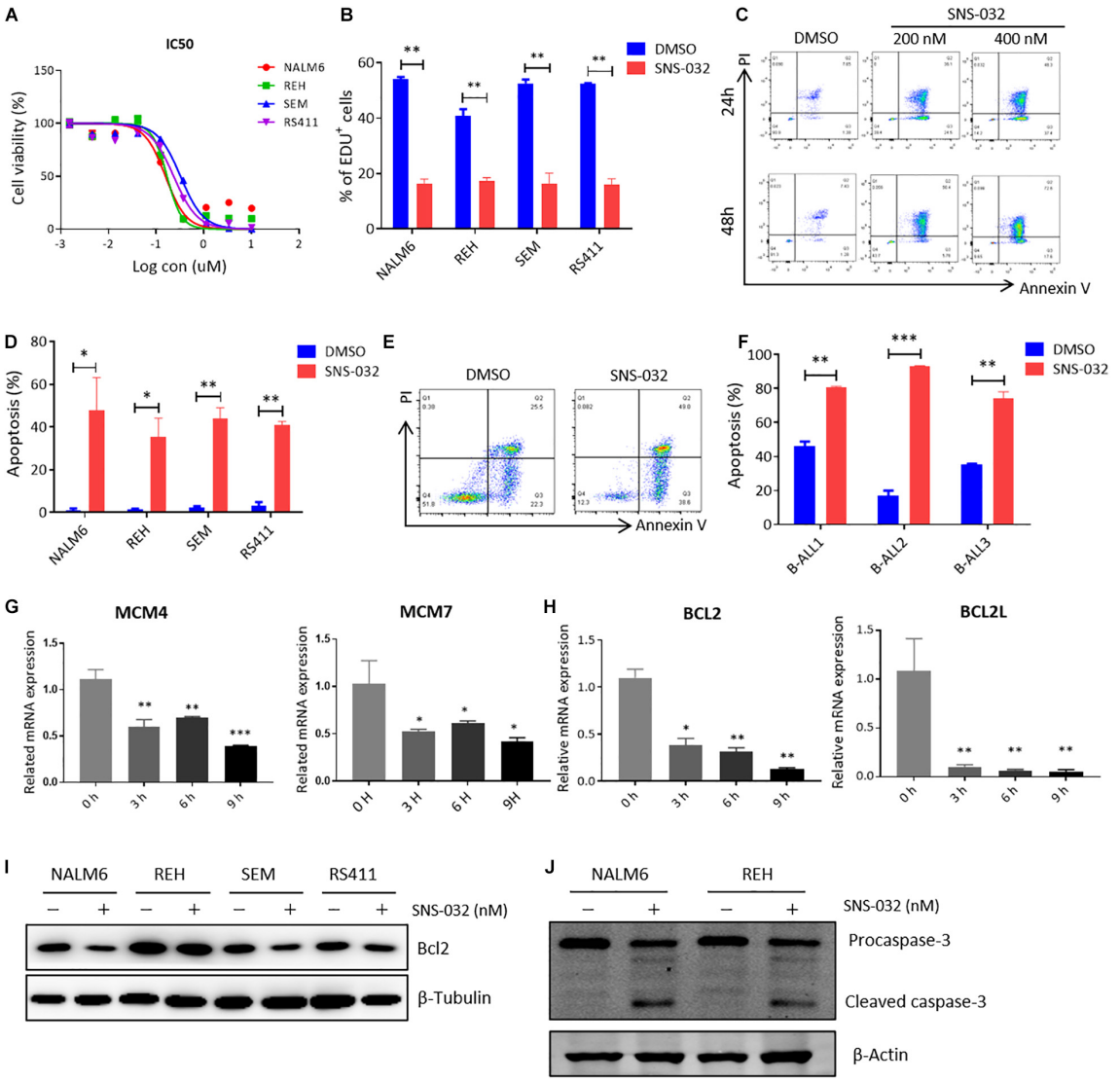

FIGURE 1 | SNS-032 treatment inhibits proliferation and induces apoptosis in B-ALL cells. **(A)** Drug sensitivity assay of NALM6, REH, SEM, and RS4;11 cell lines. B-ALL cells were treated with a gradient concentration of SNS-032 for 72 h. **(B)** EdU-labeled cell cycle of B-ALL cell lines was analyzed by flow cytometry. **(C)** Annexin V and PI labeled cell apoptosis of REH cells was analyzed by flow cytometry. **(D)** Statistical analysis of the cell apoptosis rates of B-ALL cell lines. **(E)** Annexin V- and PI-labeled cell apoptosis of B-ALL patient sample was analyzed by flow cytometry. **(F)** Statistical analysis of cell apoptosis rates in three patients. **(G,H)** The DNA replication and anti-apoptosis genes of B-ALL cells were measured by qRT-PCR. **(I,J)** The anti-apoptosis and apoptosis proteins of B-ALL cells were detected by Western blot analysis. B-ALL cells were treated with SNS-032 (200 nM for NALM6, 200 nM for REH, 350 nM for SEM, 250 nM for RS411, and 200 nM for primary B-ALL cells) for 24 h. Values were shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Moreover, the intermediates of glycolysis in SNS-032-treated B-ALL cells were measured by LC-MS/MS, and the data revealed that the levels of metabolic intermediates of glycolysis, such as glucose-6-phosphate, glyceraldehyde-3-phosphate, pyruvate, and lactate, considerably dropped in SNS-032-treated B-ALL cells (**Figure 3L**). Hence, SNS-032 restrains the glycolysis of B-ALL cells *in vitro*.

Metabolic shift occurs in the survival, invasion, and metastasis of cancer cells. Glycolysis, which is the main energy source of tumor cells, is inextricably coupled with cell proliferation and death (Buchakjian and Kornbluth, 2010; Kishton et al., 2016). To testify that SNS-032 results in the cell death of B-ALL cells by restraining glycolysis, cell apoptosis after co-treatment with a glycolysis inhibitor, 2-Deoxy-D-glucose (2-DG), was measured by flow cytometry. We uncloaked that the cell apoptosis induced by SNS-032 was markedly enhanced in 2-DG co-treated cells (**Figure 3M**). Additionally, the cell apoptosis induced by SNS-032 was significantly improved in GLUT1 inhibitor WZB117 co-treated cells (**Figure 3N**). Overall, these results indicated that SNS-032 leads to the apoptosis of B-ALL cells by partially inhibiting glycolysis.

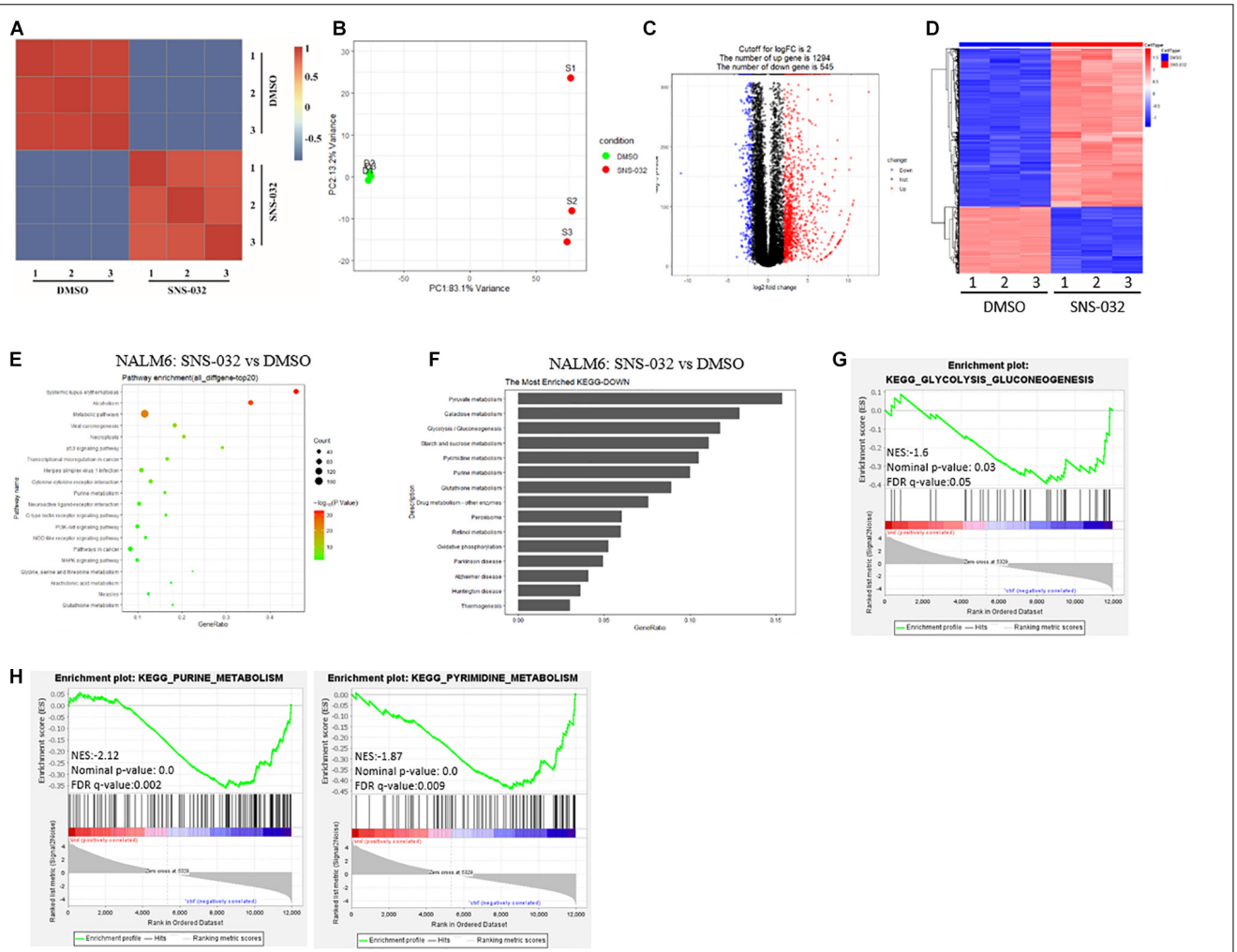

FIGURE 2 | Effect of SNS-032 treatment on the cellular metabolic pathways of B-ALL cells. **(A,B)** Correlation analysis and principal component analysis of the RNA-seq data from SNS-032-treated versus DMSO-treated REH cells. **(C,D)** Volcano plot and heatmap of differentially expressed genes by a log2-fold change ≥ 2.0 or ≤ -2.0 (padj < 0.001) in SNS-032-treated versus DMSO-treated REH cells. **(E)** Pathway analysis of up- and downregulated genes (padj < 0.001) in SNS-032-treated versus DMSO-treated REH cells. **(F)** Pathway analysis of downregulated metabolic genes (padj < 0.001) in SNS-032-treated versus DMSO-treated REH cells. **(G,H)** Gene set enrichment analysis of the genes related to purine, pyrimidine, and glycolysis.

CDK9 Inhibitor AZD4573 Facilitates the Apoptosis of B-ALL Cells by Inhibiting Glycolysis

To further confirm that CDK9i restrains the glycolytic metabolism of B-ALL cells *in vitro*, we used AZD4573, a highly selective CDK9 inhibitor, to evaluate the effects of CDK9i on the cell apoptosis of B-ALL cells. As shown in **Figure 4A**, the IC50 values of NALM6, REH, SEM, and RS411 are 5, 10, 10, and 1 nM, respectively. In addition, we found that AZD4573 induces the apoptosis of REH cells in a dose-dependent manner (**Figure 4B**). Meanwhile, AZD4573-treated REH cells exhibited lower glucose uptake activities compared with those of DMSO-treated cells (**Figures 4C,D**). Furthermore, AZD4573 treatment decreased levels of MMP, ROS and the ATP content in a dose-dependent manner (**Figures 4E–G**). Moreover, AZD4573 treatment reduced the ratios of SoNar-high cells in B-ALL cells (**Figures 4H,I**), indicating that AZD4573 restrains the glycolysis of B-ALL cells *in vitro*. More importantly, the cell apoptosis induced by AZD4573 was increased in cells co-treated with glycolysis inhibitors 2-DG and WZB117 (**Figures 4J,K**). We also confirmed that AZD4573 resulted in the glycolysis inhibition of B-ALL cells by degrading CDK9 and phosphorylating serine 2 and 5 in the CTD of RNA Pol II (**Supplementary Figure 2**). Hence, CDK9 inhibitors induce cell apoptosis by partially suppressing the glycolysis of B-ALL cells *in vitro*.

CDK9i Curbs the Glycolysis of B-ALL Cells by Downregulating the Expression of Metabolic Enzymes

As the initial step in glucose metabolism, glycolysis consists of several reactions that are involved in several key rate-limiting enzymes, such as hexokinase (HK), phosphofructokinase (PFK),

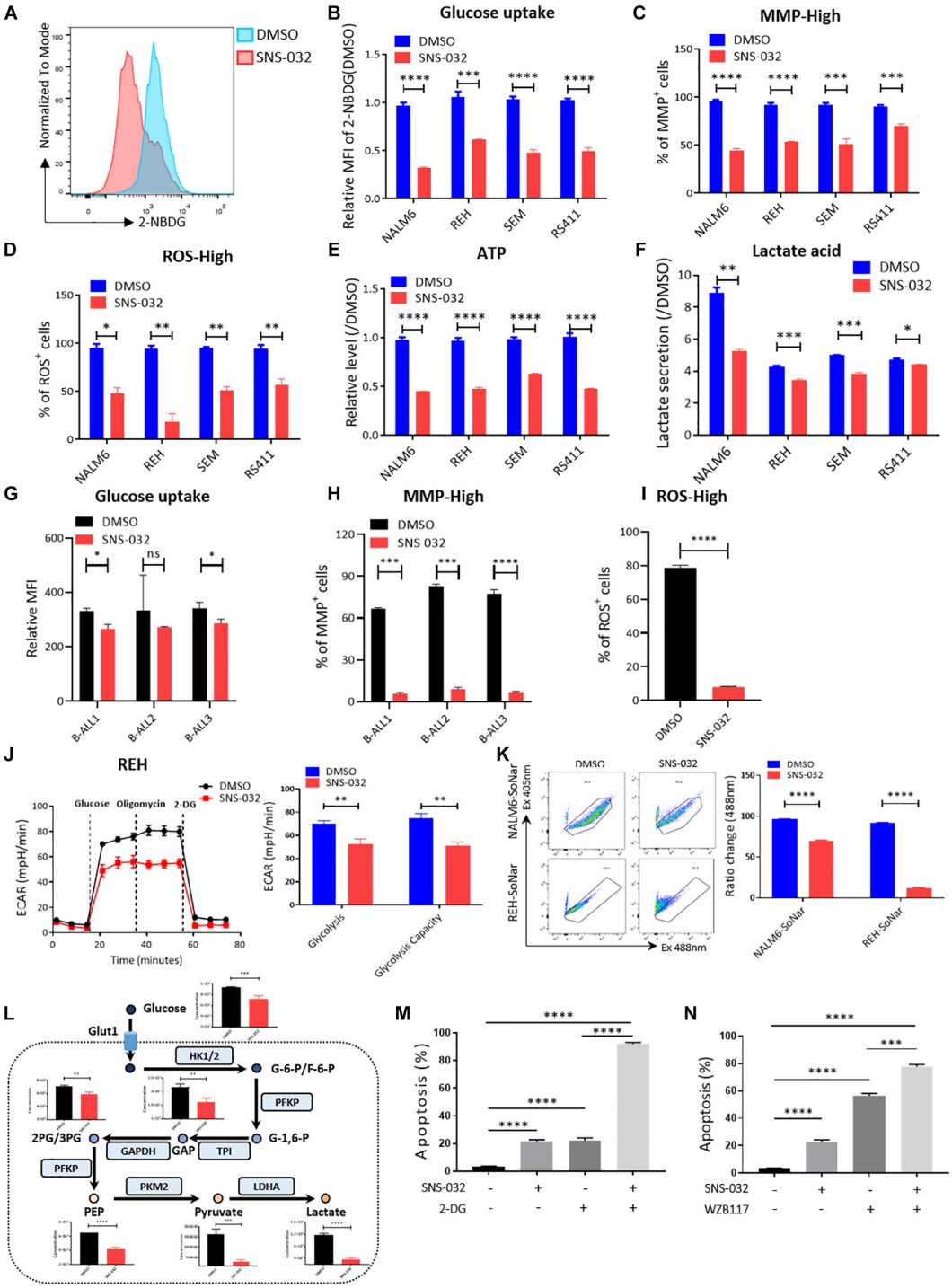

FIGURE 3 | SNS-032 treatment downregulates glucose metabolism in B-ALL cells. **(A)** The glucose uptake of NALM6, REH, SEM, and RS411 cells was detected by flow cytometry. **(B)** Statistical analysis of the glucose uptake of B-ALL cell lines. **(C,D)** The mitochondrial membrane potential (MMP) and total ROS of B-ALL cells were detected by flow cytometry. **(E)** The quantification of intracellular ATP in B-ALL cells was assessed using the ATP determination kit. **(F)** Quantification of intracellular lactate in B-ALL cell lines using the lactate assay kit. **(G)** The glucose uptake of primary B-ALL cells was detected by flow cytometry. **(H,I)** The MMP and total ROS of primary B-ALL cells were detected by flow cytometry. **(J)** Detection of ECAR in REH cell using Seahorse XF 96. **(K)** The SoNar-low or -high cells of B-ALL cells were measured by flow cytometry with 405 and 488 nm excitation. **(L)** Schematic map of glycolytic metabolism and the metabolic intermediates of glycolysis measured by LC-MS/MS. **(M,N)** Cell apoptosis of SNS-032-treated REH cells analyzed by flow cytometry after co-treatment with 2-DG (1 mM) and WZB117 (10 nM) for 24 h. Values were shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

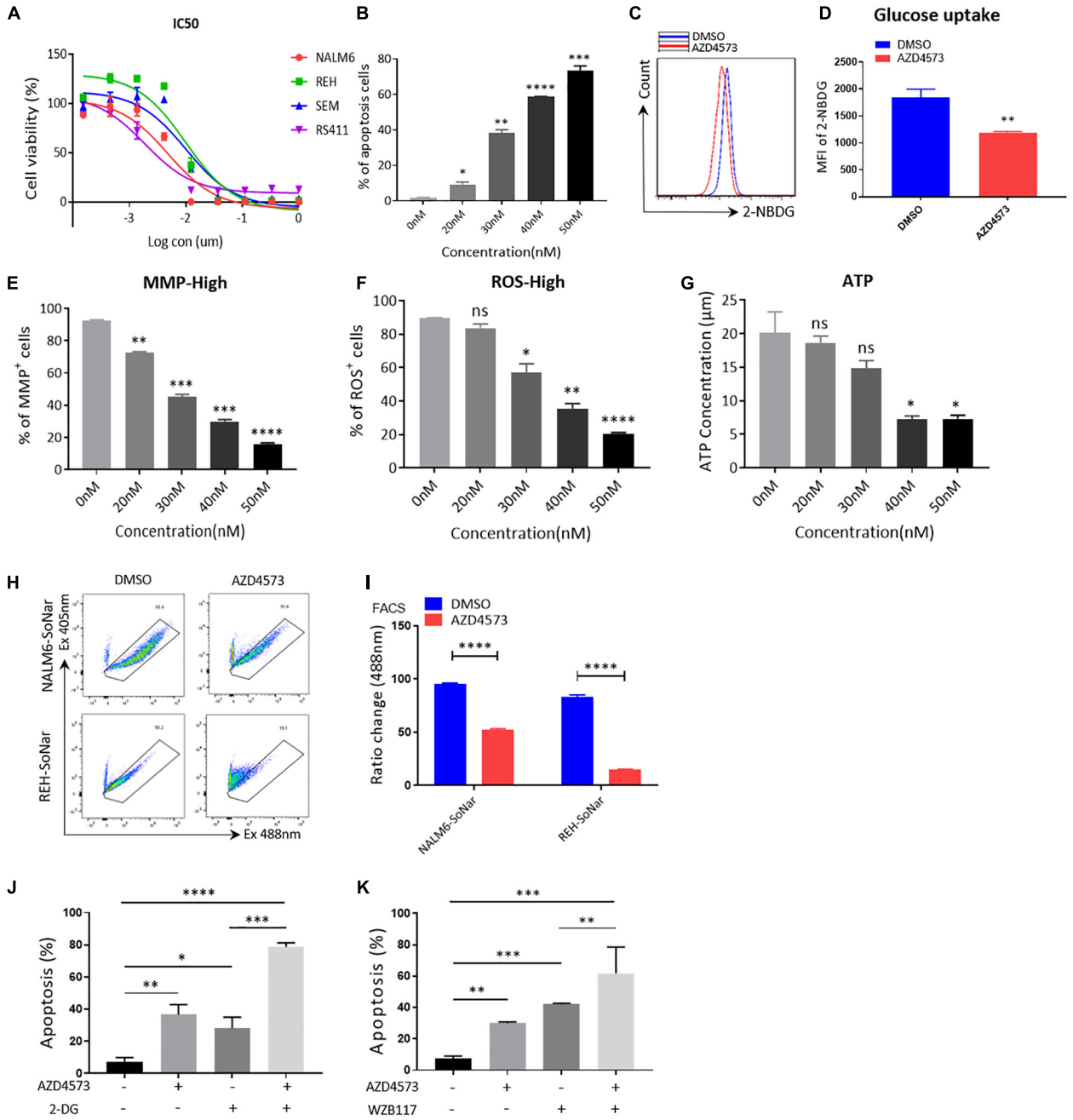

FIGURE 4 | AZD4573 treatment results in the cell death of B-ALL cell lines. **(A)** Drug sensitivity assay of NALM6, REH, SEM, and RS411 cell lines. B-ALL cells were treated with a gradient concentration of AZD4573 for 72 h. **(B)** Annexin V- and PI-labeled cell apoptosis of REH cells was analyzed by flow cytometry. **(C)** The glucose uptake of REH cells was detected by flow cytometry. **(D)** Statistical analysis of glucose uptake of REH cells. **(E,F)** MMP and total ROS of REH cells was analyzed by flow cytometry. **(G)** The quantification of intracellular ATP in B-ALL cells was assessed using the ATP determination kit. **(H)** The SoNar-low or -high cells of B-ALL cells were measured by flow cytometry with 405 and 488 nm excitation. **(I)** Statistical analysis of SoNar-high B-ALL cells. **(J,K)** Cell apoptosis of AZD4573-treated NALM6 cells analyzed by flow cytometry after co-treatment with 2-DG (1 mM) and WZB117 (10 nM) for 24 h. B-ALL cells were treated with AZD4573 for 24 h. Values were shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

and pyruvate kinase (PK) (Faubert et al., 2020). The RNA-seq data was re-analyzed to prove whether CDK9i suppressed the glycolysis of B-ALL cells by down-regulating the expression of metabolic enzymes. We discovered that SNS-032 remarkably down-regulated the key rate-limiting enzymes of glycolysis, such as GLUT1, HK2, and LDHA (**Figure 5A**). We performed qRT-PCR to validate the expression levels of glycolysis-related enzymes, and the results exhibited that SNS-032 dramatically

downregulated the expression of GLUT1, HK2, and LDHA (**Figures 5B–D**). We then detected the protein expression levels of the rate-limiting enzymes in the glycolytic pathway. The results exhibited that SNS-032 markedly downregulated the expression levels of GLUT1, HK2, and LDHA (**Figure 5E**). Moreover, AZD4573 downregulated the expression levels of GLUT1, HK2, and LDHA (**Figure 5F**). These findings indicated that CDK9i restrains the glycolysis of B-ALL cells by reducing the expression of metabolic enzymes.

CDK9i Engenders the Cell Apoptosis of B-ALL by Suppressing c-Myc-Mediated Glycolysis

CDK9 inhibition prevents productive transcription and downregulates the expression of many genes, such as c-Myc and Mcl-1 (Boffo et al., 2018). C-Myc stimulates the anabolism of cancer cells by directly modulates the expression of several glycolysis genes, such as GLUT1, PKM2, and LDHA (Liang et al., 2016; Fang et al., 2019). We deduced that CDK9i induces cell apoptosis by downregulating the expression of c-Myc-mediated glycolysis genes. To prove this hypothesis, we first confirmed that SNS-032 suppressed the mRNA and the protein expression of c-Myc in B-ALL cells (**Figures 6A,B**). To check whether the SNS-032-induced reduction of glycolysis in leukemia cells is mediated by c-Myc, we over-expressed c-Myc on REH cells by lentivirus infection. The overexpressed c-Myc protein in REH cells was verified by Western blot (**Figure 6C**). SNS-032 treatment did not affect the overexpression of c-Myc (**Figure 6D**). We also demonstrated that the glycolytic enzymes were reversed by overexpressing c-Myc upon treatment with SNS-032 (**Figure 6D**). Furthermore, the levels of glucose uptake, MMP, total ROS, and intracellular lactate were partially rescued in c-Myc-overexpressing B-ALL cells after treatment with SNS-032 (**Figures 6E–I**), implying that CDK9i blocked the glycolysis of B-ALL cells by reducing c-Myc expression. Additionally, EdU-positive proliferating cells were evidently restored in c-Myc-overexpressing B-ALL cells after intervention with SNS-032 (**Figures 6J,K**). More importantly, the cell apoptosis was abolished in c-Myc-overexpressing B-ALL cells after intervention with SNS-032 (**Figures 6L,M**). These data suggested that CDK9i induces the apoptosis of B-ALL cells by partly inhibiting c-Myc-mediated glycolytic gene expression.

DISCUSSION

Leukemic cells infiltrated and destructed the bone marrow, and then disrupted the normal hematopoiesis, leading to the death of patients. Through multi-modal combination chemotherapy or hematopoietic stem cell transplantation, the 5-year overall survival rate of patients with childhood B-ALL has reached over 80% (Malard and Mohty, 2020). However, a proportion of B-ALL patients is not sensitive to chemotherapy and still suffer from relapse, leading to treatment failure (Teachey and Pui, 2019; Malard and Mohty, 2020). Therefore, new drugs should be developed to improve the treatment rates and overcome drug resistance and B-ALL relapse. In preclinical studies, CDK9 inhibitors have demonstrated anti-tumor effects in many different types of tumor (Morales and Giordano, 2016). SNS-032, a selective and potent inhibitor of CDK 2, 7, and 9 and AZD4573, a highly selective inhibitor of CDK9, exhibited the inhibitory effects of hematological malignant cell lines *in vitro* and clinical therapeutic activity in patients with MM and CLL (Tong et al., 2010; Walsby et al., 2011). A study reported that CDK9 is overexpressed in B-ALL through hub analysis. They also observed that the RNA and protein expression levels of CDK9 were high in MOLT4 and REH leukemic cell lines in the Human Protein Atlas database. These data indicated that CDK9 could serve as potential biomarkers and predictors of leukemogenesis in B-ALL (Jayaraman et al., 2015). In the present study, we found that SNS-032 and AZD4573 induced cell apoptosis of both B-ALL cell lines and patients' samples in a dose- and time-dependent manner *in vitro*. Notably, the IC50 values of AZD4573 were lower than those of SNS-032, indicating that CDK9 is a highly selective inhibitor with high potential in the treatment of B-ALL. Moreover, our data indicated that triggering programmed cell death, resulting in B-ALL cell apoptosis, is the key to the treatment with CDK9 inhibitors. Thus, CDK9 could also serve as a novel target for B-ALL therapy.

Enhanced glycolysis is prerequisites for the rapid proliferation of tumor cells (Faubert et al., 2020). CDKs affect the catalytic activity of metabolic rate-enzymes and modulate the cell cycle arrest and apoptosis of tumor cells (Wang et al., 2017; Icard et al., 2019). However, the effect of CDK9 inhibitors on the cellular metabolism of B-ALL cells is unknown. In the study, we first observed that SNS-032 perturbs the cellular metabolic pathways of B-ALL cells, especially the glycolytic pathway. Therefore, we inferred that CDK9 inhibitors induced the cell apoptosis of B-ALL cells by suppressing glycolysis. By using Seahorse and LC-MS/MS to detect the metabolism of drug-treated cells, we uncovered that SNS-032 can significantly restrain the glycolysis of B-ALL cells by repressing glucose metabolism, thus reducing the metabolic intermediates, such as ATP and lactate, which are the energy sources and main materials for cellular anabolism (Ganapathy-Kanniappan, 2018; Abdel-Wahab et al., 2019). To further confirm our results, we used SoNar probe to dynamically detect the metabolic change and revealed that the ratios of SoNar-high cells significantly decreased upon treatment with SNS-032 and AZD4573, suggesting that CDK9 inhibitors suppressed the glycolysis of B-ALL cells. The results of RNA-seq indicated that SNS-032 restrained the glycolytic process by downregulating the expression of key enzymes, such as HK2, PFK, and LDHA. Moreover, the glycolysis inhibitors WZB117 and 2-DG enhanced the cell apoptosis of B-ALL cells induced by SNS-032 and AZD4573, suggesting that CDK9 inhibitors resulted in the apoptosis of B-ALL by partially inhibiting glycolysis. CDK9 inhibition promotes prostate cancer cells switch to fatty acid oxidation by inducing metabolic stress (Itkonen et al., 2019). In the present study, we did not observe the fatty acid metabolism on the top of SNS-032-inhibited pathway. Notably, SNS-032 not only affected glycolysis but also the purine/pyrimidine metabolism and oxidative phosphorylation of B-ALL cells, thus requiring further mechanism exploration.

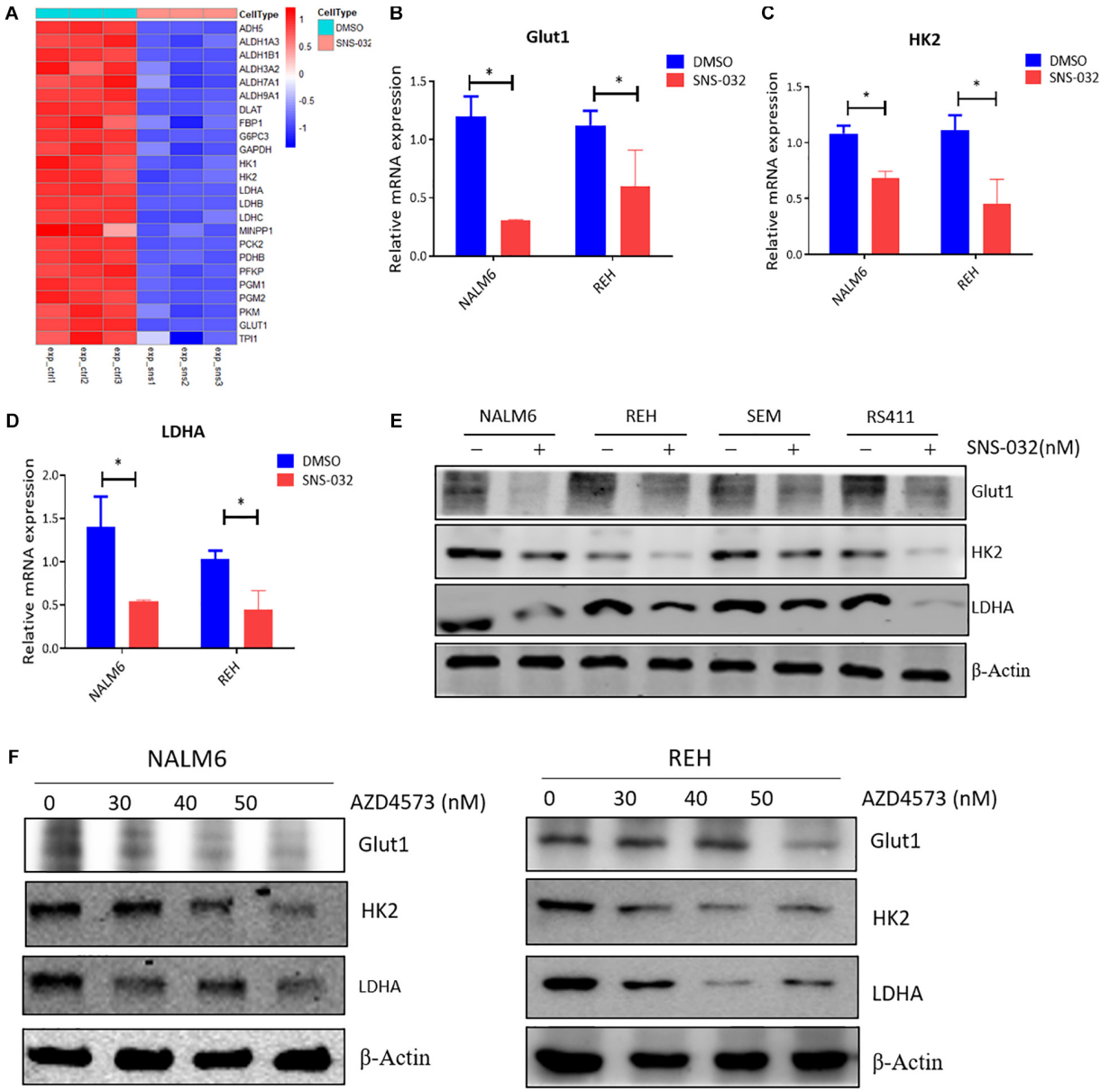

FIGURE 5 | CDK9i treatment alters the expression of metabolic enzymes in B-ALL cells. **(A)** Heatmap of glycolysis-related genes in REH cells analyzed by RNA-seq. **(B–D)** Relative mRNA expression levels of GLUT1, HK2, and LDHA measured by qRT-PCR in NALM6 and REH cells after treatment with SNS-032 for 24 h. **(E)** Protein expression levels of GLUT1, HK2, and LDHA in B-ALL cells detected by Western blot analysis after treatment with SNS-032 for 24 h. **(F)** Protein expression levels of GLUT1, HK2, and LDHA in NALM6 and REH cells detected by Western blot after treatment with AZD4573 for 24 h. Values were shown as mean ± SEM. $^*p < 0.05$.

As CDK9 inhibitors, SNS-032 and AZD4573 stop the gene transcription and results in the downregulated expression of a large proportion of genes, such as c-Myc and Mcl-1 (Boffo et al., 2018). Based on the results of RNA-seq, we found that SNS-032 dramatically reduced the expression of c-Myc. Moreover, the protein level of c-Myc decreased in B-ALL cells after SNS-032 and AZD4573 treatment. The metabolic reprogramming of tumor cells attributes to the regulation of target genes expression mediated by c-Myc (Dang et al., 2009; Hsieh et al., 2015). Thus, we infer that glycolysis is inhibited by CDK9 inhibitors because of the reduction of c-Myc level. The rescue experiment was performed to observed that the therapeutic effect of CDK9 inhibitors by overexpressing c-Myc in B-ALL cells. The cell apoptosis was abolished in c-Myc-overexpressing B-ALL cells after treatment with CDK9 inhibitors, accompanied by the relief of glycolysis, suggesting that the inhibitory effect in glycolysis of CDK9 inhibitors was mediated by downregulating c-Myc. This finding can be supported

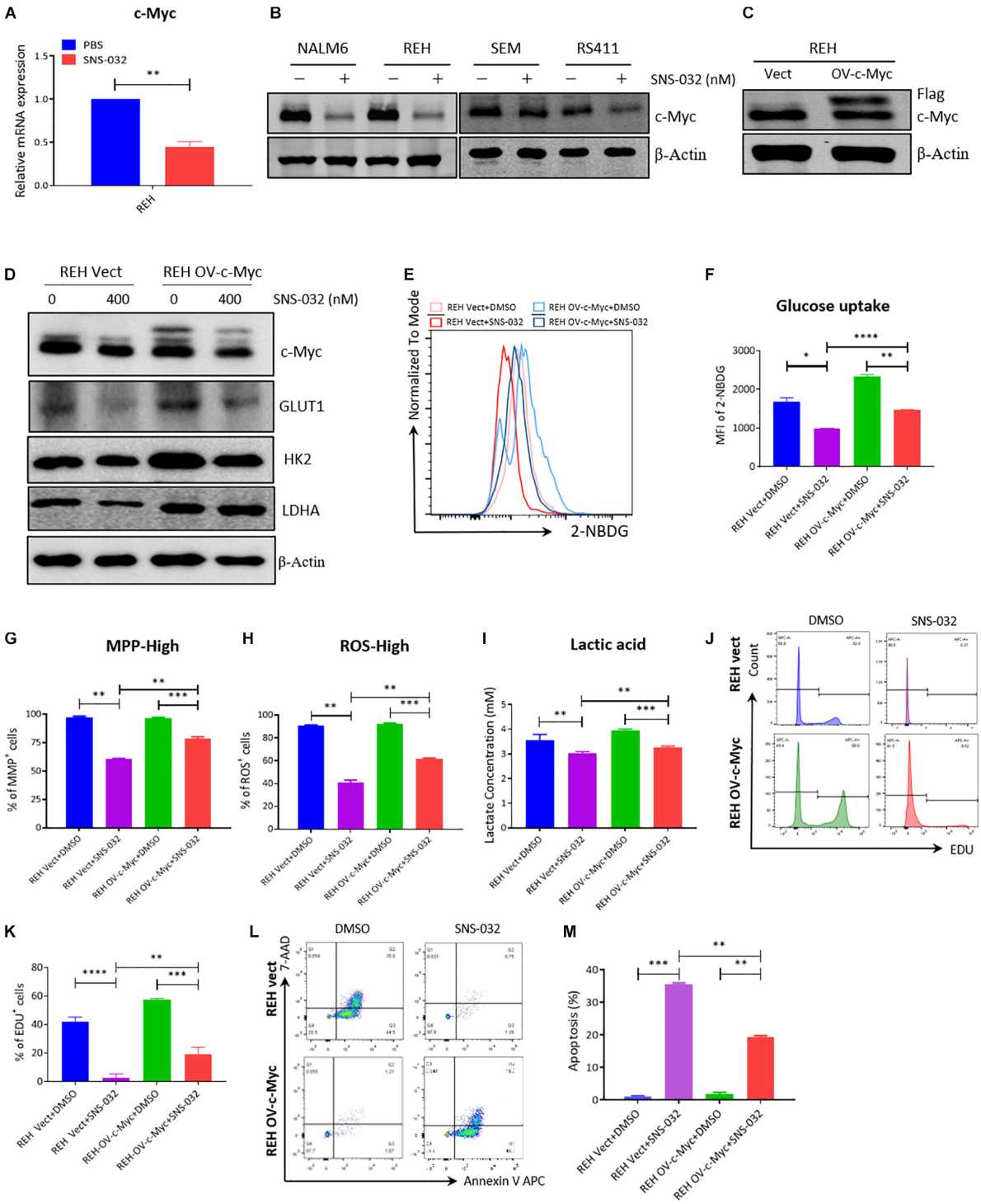

FIGURE 6 | Overexpressed c-Myc in B-ALL cells rescues CDK9i induced cell apoptosis. **(A)** Relative mRNA expression levels of c-Myc in REH cells measured by qRT-PCR. **(B)** Protein expression of c-Myc in B-ALL cells determined by Western blot analysis. **(C)** Overexpressed level of c-Myc in REH cells detected by Western blot analysis. **(D)** Levels of HK2 and LDHA in c-Myc-overexpressing REH cells tested by Western blot analysis after intervention with SNS-032 at 0 and 400 nM. **(E)** Glucose uptake of vector and c-Myc-overexpressing REH cells was analyzed by flow cytometry. **(F)** Statistical analysis of glucose uptake of REH cells. **(G,H)** MMP and total ROS of vector and c-Myc-overexpressing REH cells were analyzed by flow cytometry. **(I)** Quantification of intracellular lactate in vector and c-Myc-overexpressing REH cells using the lactate assay kit. **(J)** EdU-labeled cell cycle of c-Myc-overexpressing REH cells analyzed by flow cytometry. **(K)** Statistical analysis of EdU-positive REH cells. **(L)** Annexin V- and 7-AAD-labeled cell apoptosis of c-Myc-overexpressing REH cells was analyzed by flow cytometry. **(M)** Statistical analysis of cell apoptosis rates of B-ALL cells. B-ALL cells were treated with SNS-032 for 24 h. Values were shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

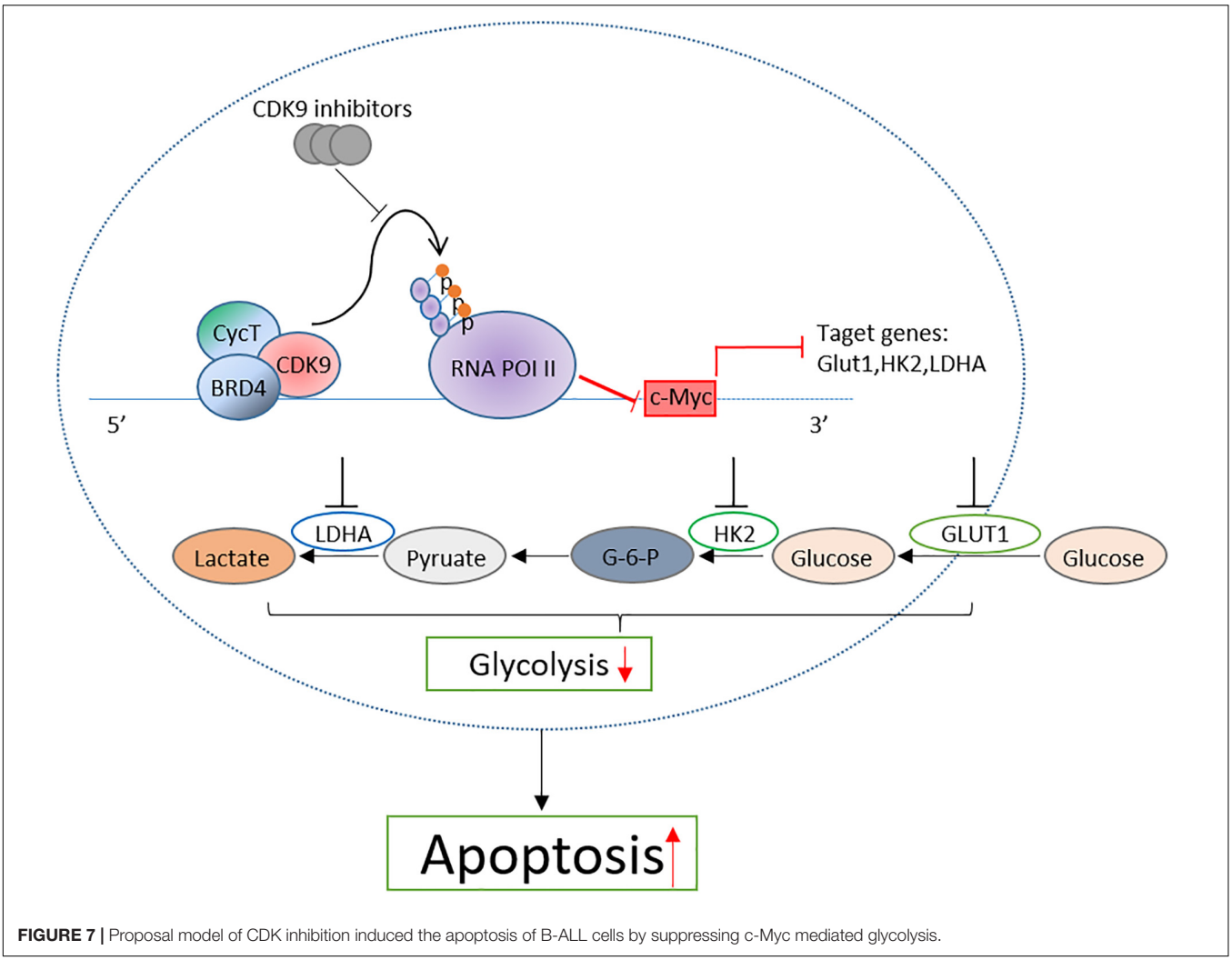

FIGURE 7 | Proposal model of CDK inhibition induced the apoptosis of B-ALL cells by suppressing c-Myc mediated glycolysis.

by flow cytometry with FITC Annexin V and PI staining that c-Myc overexpression could suppress SNS-032-induced apoptosis in REH cells. Meanwhile, the overexpression of c-Myc in REH cell enhanced glucose utilization, lactate production, and cell proliferation and inhibited apoptosis. Many glucose metabolism genes, such as GLUT1, HK2, PFKM, and LDHA, were documented to be directly regulated by c-Myc (Miller et al., 2012). To further explore the potential mechanism of c-Myc-mediated apoptosis upon SNS-032 treatment, we examined the protein expression of glycolysis-related gene. Our data demonstrated that the overexpression of c-Myc upregulated the mRNA and protein expression of GLUT1, HK2, and LDHA, thereby increasing glycolysis in REH cells. In addition, the expression of glycolysis-related genes was inversely correlated with the c-Myc expression level in the REH cell line treated with SNS-032, suggesting that c-Myc exerted an antagonistic effect on SNS-032-induced apoptosis by regulating glycolytic-related protein expression. In our study, whether c-Myc can reverse SNS-032 induced apoptosis by directly binding to the promoters of glycolytic genes requires further exploration.

CONCLUSION

Taken together, by detecting the therapeutic effect of CDK9 inhibitors on B-ALL cell lines, we confirmed that CDK9 inhibitors induced the cell apoptosis of leukemic cells by inhibiting the c-Myc-mediated glycolysis and revealed the mechanism of CDK9 inhibitors in the treatment of B-ALL (**Figure 7**). This study provides a new treatment strategy for B-ALL in clinical practice.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GEO repository, accession number GSE166339.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the SCMC Ethics Committee. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-WD, GY, and SG designed the study, analyzed and interpreted the data, and wrote the manuscript. W-LH and TA performed the experiments, and analyzed and interpreted the data. JX analyzed the RNA-seq data. HZ, W-WZ, NZ, R-YS, M-HL, J-MZ, and CJ performed the experiments. K-WL, KQ, and LC discussed the results and contributed to data interpretation. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.641271/full#supplementary-material

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REVIEW
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Activating Mutation of SHP2 Establishes a Tumorigenic Phonotype Through Cell-Autonomous and Non-Cell-Autonomous Mechanisms

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Gain-of-function mutation of SHP2 is a central regulator in tumorigenesis and cancer progression through cell-autonomous mechanisms. Activating mutation of SHP2 in microenvironment was identified to promote cancerous transformation of hematopoietic stem cell in non-autonomous mechanisms. It is interesting to see whether therapies directed against SHP2 in tumor or microenvironmental cells augment antitumor efficacy. In this review, we summarized different types of gain-of-function SHP2 mutations from a human disease. In general, gain-of-function mutations destroy the auto-inhibition state from wild-type SHP2, leading to consistency activation of SHP2. We illustrated how somatic or germline mutation of SHP2 plays an oncogenic role in tumorigenesis, stemness maintenance, invasion, etc. Moreover, the small-molecule SHP2 inhibitors are considered as a potential strategy for enhancing the efficacy of antitumor immunotherapy and chemotherapy. We also discussed the interconnection between phase separation and activating mutation of SHP2 in drug resistance of antitumor therapy.

Keywords: SHP2 mutation, tumor, cell-autonomous/non-cell autonomous mechanisms, SHP2 inhibition, tumor microenvironment

INTRODUCTION

Protein tyrosine phosphatases (PTPs) are widely expressed in most tissues. They play a regulatory role in various cell signaling events, such as mitogenic activation, metabolic control, transcription regulation, and cell migration. Src homology region 2 protein tyrosine phosphatase 2 (SHP2), encoded by *PTPN11*, is the first reported non-receptor protein oncogenic tyrosine phosphatase and required for the survival, proliferation, and differentiation of multiple cell types (Yang et al., 2013). Studies reported that germline mutations in the *PTPN11* gene contribute to Noonan syndrome (NS) (Niemeyer, 2018; Pierpont and Digilio, 2018; Bellio et al., 2019), which is a multisystem developmental disorder disease characterized by short stature, chest deformity, webbed neck, bleeding diatheses, cardiac defects, and mental retardation (Grossmann et al., 2010; Roberts et al., 2013; Liu et al., 2020). Patients with NS tend to develop juvenile myelomonocytic leukemia (JMML)-like myeloproliferative neoplasm (MPN) (Strullu et al., 2014). Hyperactive Ras signaling

is the main driving event caused by somatic mutations in *KRAS*, *NRAS*, or *PTPN11* in about 50% of JMML patients (Tartaglia et al., 2004; Lipka et al., 2017). Mutations in *NF1*, *NRAS*, *KRAS*, *CBL*, and *PTPN11* account for diagnosis in 85% of JMML patients (Stieglitz et al., 2015). Germline mutation of *PTPN11* is found in 50% of the patients with NS (Dong et al., 2016). Somatic *PTPN11* mutations are also associated with multiple types of human malignancies, such as leukemia and other solid tumors (Yang et al., 2013). According to previous reports, *PTPN11* mutations affect disease progression by unblocking PTP activity and enhancement of the catalytic activity via disrupting the auto-inhibition status or regulating the substrate binding ability of the catalytic pocket (Guo et al., 2017). SHP2 is proved to promote tumor proliferation, invasion, metastasis, and chemotherapeutic resistance (Zhang et al., 2015).

Gain-of-function (GOF) mutation SHP2 promotes tumor progression in cell-autonomous and non-autonomous mechanisms. SHP2 plays a central and indispensable role in hematopoiesis and leukemogenesis via its complex involvement with cellular signaling pathways (Pandey et al., 2017). Furthermore, activating mutations SHP2 in the bone marrow microenvironment, but not in the tumor cells, also promote childhood MPN development and progression through detrimental effects on hematopoietic stem cells (HSCs) in non-autonomous mechanism (Dong et al., 2016). Thus, a comprehensive understanding of how SHP2 contributes to oncogenesis will provide novel insights into pathogenesis.

It was of great interest to discover small-molecule SHP2 inhibitors as a potential cancer therapeutic target in recent years. The study in SHP2 inhibition did not make a breakthrough until the discovery of inhibitors that occupied allosteric sites of SHP2 (Chen et al., 2016; Shen et al., 2020). This novel discovery shed light on efficient SHP2 inhibitors (Garcia Fortanet et al., 2016). Targeting these non-conserved allosteric sites tends to improve drug selectivity. Consequently, several other allosteric drugs were continuously discovered with higher expectation for cell permeability, oral availability, etc. (Chen et al., 2016; Shen et al., 2020). Currently, a few clinical trials of SHP2 allosteric inhibitors showed remarkable antitumor benefits (Liu et al., 2020).

In this review, we summarized the structural change and functional regulation of oncogenic SHP2 mutations. We discussed how SHP2 affects tumor progressions in cell-autonomous and non-autonomous mechanisms. Since SHP2 is considered as a novel antitumor target, we also summarized currently used SHP2 inhibitors as well as their potentials in the application of cancer treatment.

THE STRUCTURAL CONFORMATION CHANGES AND FUNCTIONAL REGULATION OF ONCOGENIC SHP2

SHP2 consists of one PTP catalytic domain that locates at the C-terminal region, two tandem C-SH2 and N-SH2 domains, and a C-terminal tail with tyrosyl phosphorylation sites (Feng et al., 1993). Human SHP2 encodes 593 amino acids, among which the N-SH2 domain locates at 3–104, C-SH2 domain locates at 112–216, the PTP domain locates at 221–524, and the C-terminal locates at 525–593. The N-SH2 domain has two non-overlapping ligand binding sites to regulate its de-phosphorylated activity. The C-SH2 domain provides binding energy and specificity (Zhang et al., 2015). The PTP domain contains the catalytic structures, such as the P ring (Yu et al., 2013), to de-phosphorylate substrates.

SHP2 activity is regulated by conformational switch that N-SH2 binds to PTP to block or binds to phosphorylated proteins to unblock its phosphatase activity (Zhang et al., 2015). SHP2 mainly exists in a closed self-inhibitory conformation (Zhang et al., 2020). In the inactive state, the D-E ring of the N-SH2 domain is inserted into the PTP domain to block the phosphatase activity site (Rehman et al., 2019). Studies reported that the stimulation of growth factor receptor [e.g., epidermal growth factor receptor (EGFR)] or the interaction between the N-SH2 domain with phosphorylated tyrosine residues of scaffold proteins led to the dissociation of N-SH2 with the PTP domain; thus, the active region of PTP will be exposed and SHP2 is activated (Liu et al., 2016). The structure and function regulation of SHP2 is shown in **Figure 1**. In addition, SHP2 is activated via the phosphorylation on two tyrosine residues (Y542 and Y580) within the C-terminal region (Voena et al., 2007).

Gain-of-function mutations of SHP2 affect the interaction between N-SH2 and PTP (Tajan et al., 2015). GOF mutations mainly occur in the N-SH2 or PTP domains of cancer patients. These mutations lead to partial or complete dissociation of the binding domain of N-SH2 and PTP, and enhancement of phosphatase activity (Pannone et al., 2017). For example, the N-SH2 domain of $PTPN11^{E76K/+}$ mutant leads to consistently expose the catalytic site of the PTP domain (LaRochelle et al., 2018).

Protein tyrosine phosphatase is the catalytic domain that mediates a variety of cellular signaling processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. Several GOF mutations or loss-of-function (LOF) mutations of SHP2 are reported to be associated with cancer progression and other diseases (**Table 1**). Multiple researches reported that GOF mutations $SHP2^{D61G}$ and $SHP2^{E76K}$ are related to MPN (Xu et al., 2010), NS (De Rocca Serra-Nédélec et al., 2012), or JMML (Yang et al., 2008) through activation of Ras/Erk signaling *in vivo* and *in vitro*. $SHP2^{E76K}$ promotes tumorigenesis of colorectal cancer (CAC) and induces epithelial-to-mesenchymal transition (EMT) through the Wnt/β-catenin signaling pathway (Schneeberger et al., 2014). Studies reported that SHP2 is required for the growth of KRAS-mutant non-small-cell lung cancers (NSCLCs), and its inhibition leads to a potential antitumor therapy (Mainardi et al., 2018). More details are shown in **Table 1**.

A recent study reported that multivalent electrostatic interaction among the PTP domains leads to liquid–liquid phase separation (LLPS) of mutated SHP2. It recruits wild-type SHP2

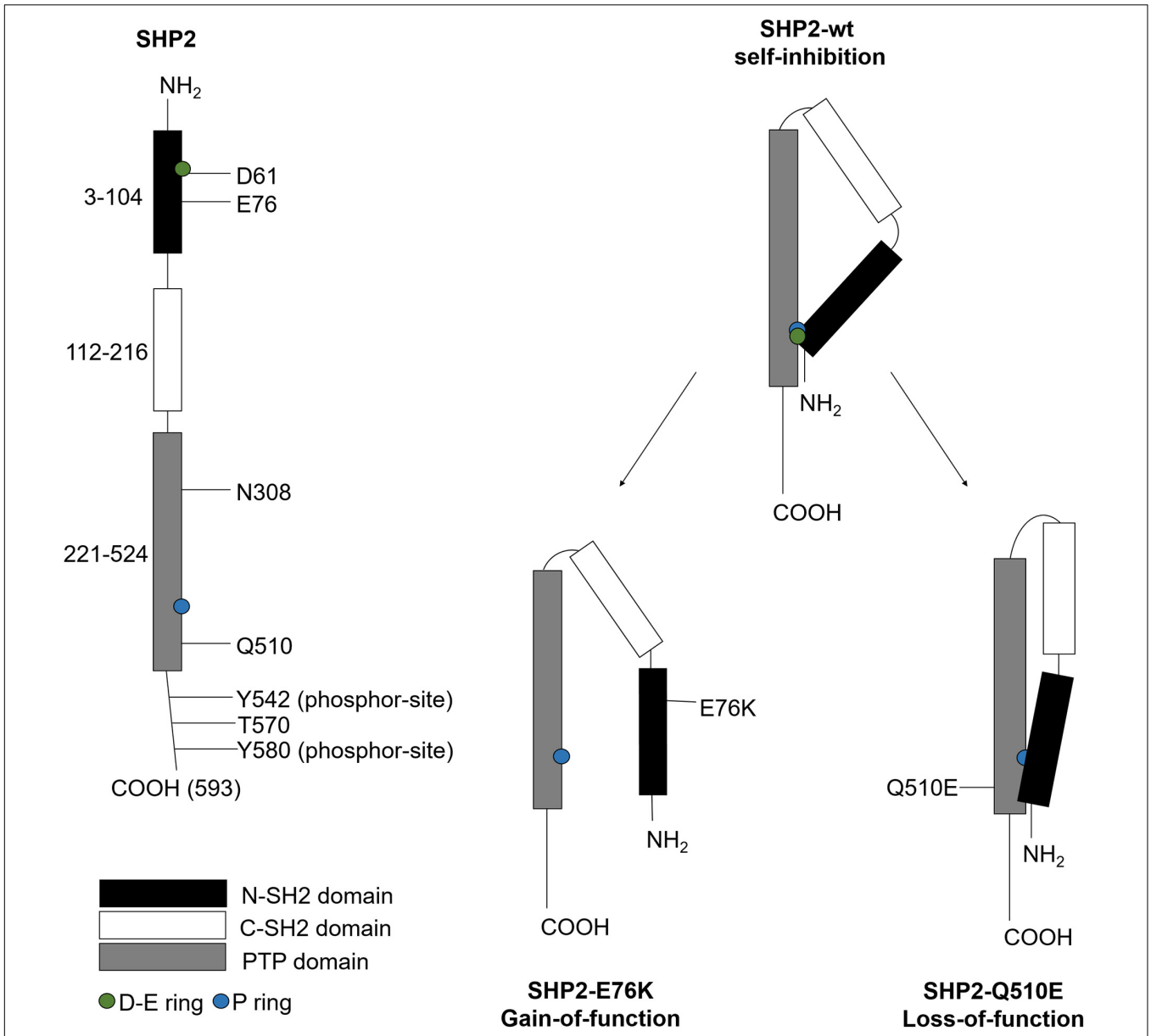

FIGURE 1 | The structure and function regulation of SHP2. SHP2 contains 593 amino acids, including various important active regulatory sites. When these sites are mutated, SHP2 gains or loses function. The D-E ring and P ring on the three-dimensional structure of SHP2 play an important role in regulating catalytic activity. When SHP2 is in self-inhibition state, the P ring on the PTP domain will cover the D-E ring on the N-SH2 domain. SHP2 is activated by gain-of-function mutations, such as E76K, or inactivated by loss-of-function mutations, such as Q510E.

and hyperactivates the phosphatase catalytic activity of SHP2, which further leads to hyperactivation of mitogen-activated protein kinase (MAPK) signaling pathway (Zhu et al., 2020). It also reported that SHP2 mutants in LEOPARD syndrome induce robust phase transition to liquid-like droplets in cells, which recruit and activate wild-type SHP2 to promote extracellular signal-regulated kinase1/2 (Erk1/2) activation (Zhu et al., 2020). It suggests that disease-associated SHP2 mutations promote GOF LLPS and consequently lead to overactivation of wild-type SHP2. The formation of LLPS is a mechanism of SHP2 activation and a potential contributor to developmental diseases and cancers.

GAIN-OF-FUNCTION SHP2 PROMOTES TUMOR PROGRESSION IN CELL-AUTONOMOUS OR NON-AUTONOMOUS MECHANISMS

The oncogenic SHP2 promotes cancer progression at a cellular level through two mechanisms. On the one hand, malignant proliferation results from tumor cell-autonomous oncogenic SHP2 mutations. Studies have reported that $SHP2^{D61Y}$ causes fatal myeloproliferative disorder via cell-autonomous effects on

TABLE 1 | Gain/loss-of-function mutations of SHP2.

Mutants	Disease	Influences and mechanisms	Function	References
D61G	/	$SHP2^{D61G}$ promotes U251 proliferation and inhibits apoptosis	GOF	Zhao et al., 2017
D61G	/	$SHP2^{D61G}$ enhances the production of ROS, leading to abnormal proliferation of bone marrow	GOF	Xu et al., 2013
D61G	JMML	JMML is a myeloproliferative neoplasm (MPN) of childhood with a poor prognosis. $SHP2^{D61G}$ promotes abnormal activation of hematopoietic stem cells and leads to JMML	GOF	Xu et al., 2010
D61G	NS	NS is a multisystem developmental disease. Patients with NS tend to develop JMML. $SHP2^{D61G}$ promotes hyperactivation of Ras/Erk1/2 to inhibit GH-induced IGF-1 release, leading to growth retardation and NS	GOF	De Rocca Serra-Nédélec et al., 2012
D61G	Breast cancer	$SHP2^{D61G}$ activates GAB1/Ras/Erk axis to promote breast cancer invasion and migration	GOF	Hu et al., 2016
D61Y	JMML	$SHP2^{D61Y}$ promotes the cell cycle development and survival of hematopoietic progenitor cells and further leads to JMML	GOF	Yang et al., 2008
E76K	JMML	$SHP2^{E76K}$ promotes the cell cycle development and survival of hematopoietic progenitor cells	GOF	Yang et al., 2008
E76K	GBM	GBM is the most aggressive and common form of brain malignancy in adults. $SHP2^{E76K}$ activates the Erk/CREB pathway to promote GBM cell proliferation, metastasis, and tumor growth	GOF	Yang et al., 2019
E76K	/	$SHP2^{E76K}$ leads to mitotic abnormalities	GOF	Liu et al., 2016
E76K	/	$SHP2^{E76K}$ promotes lung tumor development in transgenic mice	GOF	Schneeberger et al., 2014
E76K	/	$SHP2^{E76K}$ enhances the production of ROS, leading to abnormal proliferation of bone marrow	GOF	Xu et al., 2013
E76K	/	$SHP2^{E76K}$ has a non-pedigree-specific effect on hematopoietic malignant transformation and leads to acute leukemia in every stage of hematopoiesis	GOF	Xu et al., 2011
E76K	CRC	The mutation rate of SHP2 is the highest in CRC. $SHP2^{E76K}$ promotes tumorigenesis and induces EMT through the Wnt/β-catenin signaling pathway	GOF	Zhang et al., 2018
E76K	Hydrocephalus	$SHP2^{E76K}$ promotes the pathogenesis of hydrocephalus in mice by inhibition of STAT3 and enhancement of Erk/Akt activity. $SHP2^{C459S}$ suppresses this pathogenic effect	GOF	Zheng et al., 2018
E76Q	/	Phosphatase activity of $SHP2^{E76Q}$ was enhanced	GOF	Rehman et al., 2019
T507K	/	$SHP2^{T507K}$ dephosphorylates Sprouty1 to hyperactive Ras signaling pathway	GOF	Zhang et al., 2020
Q506P	/	Phosphatase activity of $SHP2^{Q506P}$ is reduced	LOF	Noda et al., 2016
Q510E	HCM	Dysregulation of mTOR signal pathway mediated by $SHP2^{Q510E}$ causes HCM, which is a common inherited cardiovascular disease	LOF	Schramm et al., 2012
Q510E	HCM	$SHP2^{Q510E}$ mutation reduces cardiac cell differentiation and promotes cardiac hypertrophy by disabling Akt/GSK-3/β-catenin signaling pathway	LOF	Ishida et al., 2011
T468M	/	Phosphatase activity of $SHP2^{T468M}$ is reduced	LOF	Noda et al., 2016
Y279C	/	Phosphatase activity of $SHP2^{Y279C}$ is reduced	LOF	Xu et al., 2010

NS, Noonan syndrome; JMML, juvenile myelomonocytic leukemia; CRC, colorectal cancer; GBM, glioblastoma multiforme; HCM, hypertrophic cardiomyopathy; LOF, loss of function; GOF, gain of function; EMT, epithelial-to-mesenchymal transition.

multiple stages of hematopoiesis (Chan et al., 2009). On the other hand, SHP2 in tumor microenvironment cells, such as mesenchymal stem cells (MSCs) and/or immunological cells, is responsible for tumor progression (Dong et al., 2016).

SHP2 Mutations Promote Tumor Progression in Cell-Autonomous Mechanism

It has been established that oncogenic alterations in the Ras/Raf/MEK/Erk pathway drive the neoplasia of multiple cancer types. SHP2 is expressed in multiple types of cells and regulates cell survival and proliferation through activation of the Ras/Erk signaling pathway (Chan et al., 2008; Bondeson, 2017). SHP2 negatively regulates the cytokine receptor-mediated JAK-STAT signaling pathway (Xu and Qu, 2008). Some studies also reported that $SHP2^{E76K}$ in the glioblastoma multiforme (GBM) cells promotes the malignant behavior of tumor cells through the Erk/cAMP responsive element binding protein (CREB) signaling pathway (Yang et al., 2019). These evidences indicated that activated SHP2 in tumor cells established oncogenic signaling pathways to promote tumor progression.

SHP2 established proliferative signaling pathways to promote tumorigenesis. Studies showed that $SHP2^{E76K}$

activates Erk and Src to promote the occurrence of lung tumors (Schneeberger et al., 2014). SHP2 dephosphorylates Ras to increase the association between Ras and Raf, thus activating the proliferation-promoting Ras/Erk/MAPK signaling pathway. Overexpression of SHP2 activates Erk/Akt signaling pathways and further leads to tumorigenesis of breast cancer (Hu et al., 2014). SHP2 activity is elevated by pathological analysis of astrocytes isolated from GBM. Patient-derived GBM specimens exhibit hyperactive Ras, while inhibition of SHP2 decelerates the progression of low-grade astrocytoma to GBM in a spontaneous transgenic glioma mouse model (Bunda et al., 2015). The observation that conditional knockout of SHP2 in the ErbB2 transgenic mice prevents tumorigenesis by blocking the expression of the ErbB2 indicates that SHP2 induces tumorigenesis through regulating the expression of oncogene (Zhao H. et al., 2019). Studies also revealed that SHP2 affects proliferation and tumorigenicity of glioblastoma stem cells (GSCs) through regulating the expression of transcription factor SOX2 (Roccograndi et al., 2017).

Oncogenic SHP2 promotes tumor progression. It has been demonstrated that targeting both Ras and its upstream or downstream proteins has no cancer-suppressing effect in Ras-mutant cancer (Bernards, 2012). However, studies revealed that SHP2 inhibition in KRAS-mutant NSCLCs *in vivo* under growth factor-limiting conditions triggers senescence response (Mainardi et al., 2018). Furthermore, genetic deletion of *PTPN11* or inhibition of SHP2 in KRAS-mutant-driven tumors delays tumor progression (Ruess et al., 2018). Another study pointed out that SHP2 small molecular allosteric inhibitor RMC-4550 decreases oncogenic-related Ras/Raf/MEK/Erk signaling to impair the growth of cancer-bearing Ras-GTP-dependent oncogenic BRAF mutation, NF1 loss, or nucleotide-cycling oncogenic Ras (Nichols et al., 2018). Wang et al. showed that $SHP2^{E76K}$ and $SHP2^{D61G}$ induce cytokine allergy of hematopoietic cells by enhancing the production of reactive oxygen species (ROS). They interact with a new substrate in the mitochondria to increase the aerobic metabolism of the mitochondria and drive the development of myeloproliferative diseases and malignant leukemia (Xu et al., 2013).

Additionally, SHP2 promotes tumor metastasis. SHP2 decreases the phosphorylation of PAR3 (partitioning-defective 3) to impair the formation of polarity-regulating protein complex, resulting in a disrupted cell polarity, dysregulated cell–cell junctions, and increased EMT, which is one of the essential steps for prostate cancer metastasis (Zhang et al., 2016). Other studies demonstrated that SHP2 overexpression enhances ovarian tumor invasion by activating the PI3K/Akt axis (Hu et al., 2017). SHP2 knockdown inhibits cell migration in the HeLa and SiHa cervical cancer cell lines, while SHP2 overexpression has the opposite effects. This study further pointed out that the tumor-promoting effect of SHP2 is partially related to Akt signaling (Cao et al., 2019). Other studies reported that $SHP2^{E76K}$ promotes GBM tumor metastasis via the activation of Erk/CREB axis (Yang et al., 2019).

Gain-of-function mutations of SHP2 in cancer stem cells (CSCs) promote cell expansion, proliferation, and stemness maintenance and are responsible for drug resistance. Studies reported that activated SHP2 in CSCs promotes liver CSC expansion by activating β-catenin signaling (Xiang et al., 2017). Treatment of NSCLC by tyrosine kinase inhibitor (TKI) failed because SHP2 induces the stemness of KRAS-mutant NSCLCs. The inhibition of SHP2 attenuates the enhanced stemness (Jiang et al., 2019), suggesting the important role of tumor cell-autonomous SHP2 in stemness maintenance of CSCs. Other studies revealed that SHP2 catalytic activity is required for proliferation and tumorigenic transformation of GSCs (Roccograndi et al., 2017). A recent study revealed that $PTPN11^{G226A}$ mutation is essential in hematopoietic differentiation of JMML-derived induced pluripotent stem cells (iPSCs), suggesting the significant role of SHP2 in regulating stem cell bioactivity (Shigemura et al., 2019). The oncogenic function of tumor cell-autonomous SHP2 is shown in **Figure 2**.

SHP2 in Tumor Microenvironment Affects Tumor Progression via Non-autonomous Mechanism

Cre/LoxP system is applied to elucidate the specific role of non-autonomous SHP2 mutations in cancer. Some studies examined the detailed pathogenesis of metachondromatosis by deleting *PTPN11* specifically in monocytes, macrophages, and osteoclasts (lysozyme M-Cre; LysM-Cre) or in cathepsin K (Ctsk)-expressing cells using $PTPN11^{flox/flox}$ and Cre recombinase transgenic mice (Yang et al., 2013). *PTPN11* deletion in $CD4^{+}$ cells driven by CD4 Cre recombinase demonstrated that although the ablation of SHP2 does not affect T cell development and functions, it causes cartilage tumors in a T cell-independent manner (Miah et al., 2017). More importantly, a previous study on the leukemogenic effect of SHP2 mutation in bone marrow microenvironment generated $PTPN11^{E76K/+}$/Nestin-Cre^{+} transgenic mice with a neo cassette and a stop codon inserted ahead of $PTPN11^{E76K}$, and thus, the $SHP2^{E76K}$ expressed with the deletion of neo cassette by Cre DNA recombinase (Dong et al., 2016). Ding et al. (2012) studied the cellular sources of Scf (stem cell factor) that affects HSC frequency and function by conditionally deleting Scf from hematopoietic cells, osteoblasts, nestin-cre- or nestin-creER-expressing cells, endothelial cells, or leptin receptor (Lepr)-expressing perivascular stromal cells. They found that HSCs were depleted when Scf was conditionally deleted in perivascular cells; thus, HSCs were proved to reside in a perivascular niche where they remained undifferentiated. Studying the oncogenic effects of SHP2 mutations in different cell populations in the tumor microenvironment could follow similar methods. In general, the use of transgenic mice combined with the Cre/LoxP system is a reliable approach for studying the role of SHP2 in diseases, especially in tumors.

SHP2 Mutations in Bone Marrow Microenvironment Promotes Leukemogenesis

SHP2 mutations promote the leukemogenesis of HSCs in non-cell-autonomous mechanisms. HSCs reside in distinct bone marrow niches defined by the surrounding stromal cells and the regulatory molecules they produce (Greenbaum et al., 2013), wherein the transduction signaling generated by surrounding

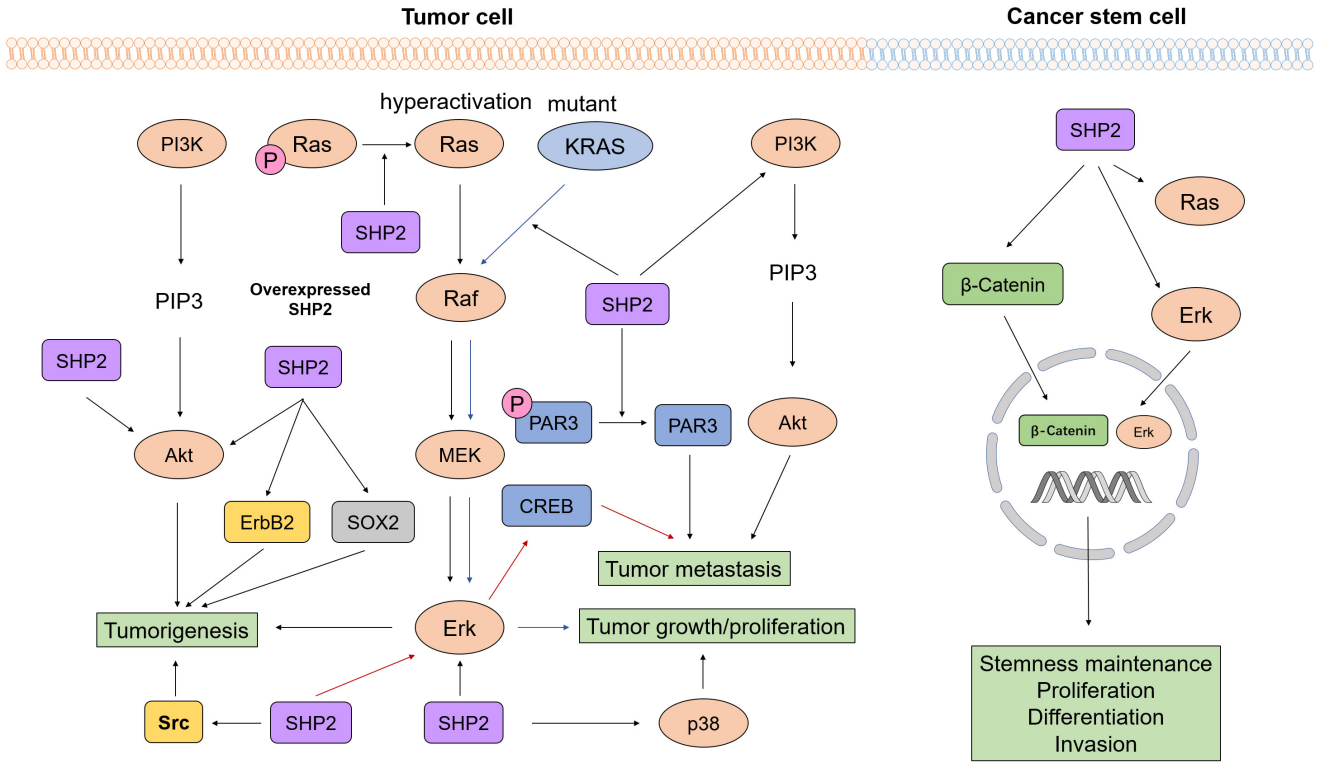

FIGURE 2 | The oncogenic function of tumor cell-autonomous SHP2 gain-of-function mutations. As a signaling hub, SHP2 affects various tumor developmental stages by regulating multiple signaling pathways, such as PI3K/Akt, Ras/Raf/MEK/Erk signaling. SHP2 promotes proliferation, differentiation, and invasion and maintains stemness of cancer stem cells via activation of β-catenin and Ras/Erk signaling.

microenvironment cells affects the self-renewal, proliferation of HSCs, and MPN formation. Studies have demonstrated that *PTPN11* mutation in the bone marrow microenvironment, such as MSCs, promotes the development and progression of childhood MPN through the profound negative effects on HSCs (Dong et al., 2016), which demonstrated that not only tumor cell-autonomous SHP2 but also mutations in microenvironment cells leads to tumorigenesis. This study clarified the mechanism of leukemia recurrence. Using specifically expressed cre, such as Prx1-cre (Greenbaum et al., 2013), nestin-cre (Méndez-Ferrer et al., 2010), Lepr-cre (Ding et al., 2012), and Osterix-cre (Tang et al., 2016), to induce *PTPN11* mutations in bone marrow mesenchymal cells leads to MPN, but SHP2 mutations in endothelial cells (VE-Cadherin-cre) and osteoblasts (Oc-cre) will not lead to MPN formation (Dong et al., 2016), which indicates that SHP2 mutations in the specific components of bone marrow microenvironment show leukemogenic effects (**Figure 3**).

SHP2 in Immune Microenvironment Promotes Immune Escape of Tumor Cells

Gain-of-function mutations of SHP2 in tumor microenvironment cells affect tumor progression by non-autonomous mechanisms. SHP2 regulates immune cell functions in the tumor immune microenvironment to affect tumor progressions (Liu et al., 2020; **Figure 4**). For example, SHP2 regulates the function of T cells by binding to programmed cell death 1 (PD-1) (Liu et al., 2020). PD-1, a key immune checkpoint target for cancer immunotherapy and negative costimulatory receptor, is important to inhibit T cell activation. PD-1 binds to ligand PD-L1 and clusters with T cell receptor (TCR), which is temporarily related to phosphatase SHP2. These negative costimulatory clusters induce dephosphorylation of TCR signal molecules and inhibit the activation of T cells to block TCR induced stop signal (Yokosuka et al., 2012). Dimeric PD-1 activates SHP2-mediated immunosuppression by binding to SH2 domains of SHP2 (N-SH2 and C-SH2) via the C-terminal tyrosine-based switch motif (ITSM) of immune receptor (Okazaki et al., 2001; Sheppard et al., 2004; Yokosuka et al., 2012), thus promoting the immune escape of tumor cells. Other studies reported that cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is also an immune checkpoint and a negative regulator of T cell immune function (Buchbinder and Desai, 2016; Rowshanravan et al., 2018). Phosphorylation of YYKM motif in CTLA-4 cytoplasmic tail recruits SHP2 to dephosphorylate and inactivate CD28 (Salmond and Alexander, 2006; Lorenz, 2009; Rudd et al., 2009) and to promote the tumor cell survival. Furthermore, SHP2 regulates another signaling to impair the antitumor immunotherapy. SHP2 in the cytoplasm dephosphorylates STAT1, which ultimately inhibits the proliferation of T lymphocytes, leads to a decline in antitumor immunity, and promotes the development of cancer (Liu et al., 2020). Li et al. (2015) analyzed tumor-infiltrating

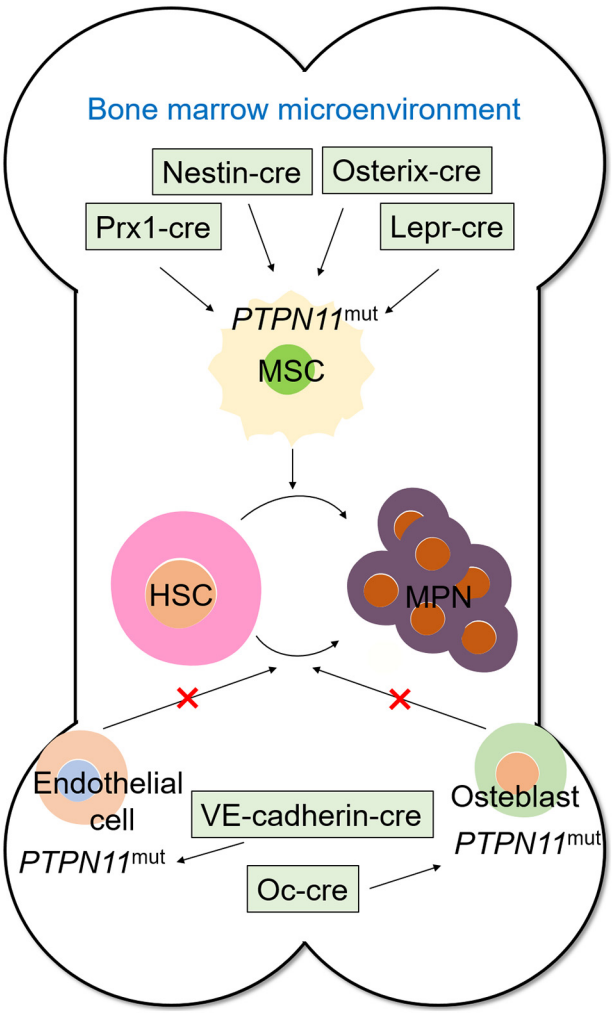

FIGURE 3 | SHP2 mutation in bone marrow microenvironment promotes myeloproliferative neoplasm. *PTPN11* mutation induced by Prx1-cre, Nestin-cre, Osterix-cre, and Lepr-cre in mesenchymal stem cells promotes tumorigenicity of hematopoietic stem cells, whereas *PTPN11* mutation induced by VE-Cadherin-cre in endothelial cells and Oc-cre in osteoblasts shows no myeloproliferative neoplasm formation-promoting effect.

and peripheral blood lymphocytes in head and neck squamous cell carcinoma patients and concluded on the inhibitory effect of SHP2-mediated PD-1 on tumor Th1 cell immunity and that the PD-1 or SHP2 blockade was sufficient to restore Th1 immune activity and to activate T cells, thus reversing immunosuppression in tumor microenvironment.

SHP2 is involved in multiple signaling pathways in tumor-associated macrophages. Stimulated by the colony-stimulating factor-1 (CSF-1), SHP2 binds to the CSF receptor (CSF-1R) complex on the inner membrane of tumor-associated macrophage (TAM), leading to the activation of the Ras/Erk signaling pathway in TAM and supporting the survival, proliferation, and migration of tumor cells (Achkova and Maher, 2016). Furthermore, SHP2 in the macrophages is associated with chronic inflammation-related cancers. Recently, Barkal et al. (2019) have shown that tumor-expressed CD24 binds macrophage sialic-acid-binding Ig-like lectin 10 (Siglec-10) to promote tumor avoidance in the tumor microenvironment by recruiting SHP2 to the cytoplasmic tail ITIM motif of Siglec-10. Besides, Xiao et al. (2019) found that SHP2 deficiency in macrophages disrupts the IL-10/STAT3 signaling pathway, worsening the colons of mice.

Gain-of-Function SHP2 Promotes Chemoresistance in Cell-Autonomous and/or Non-autonomous Mechanisms

SHP2 promotes chemoresistance in cell-autonomous and/or non-autonomous mechanisms. A previous study observed high expression of SHP2 in both chemoresistant hepatocellular carcinomas (HCCs) and recurrent HCCs derived from patients (Xiang et al., 2017), suggesting a relationship between aberrant SHP2 and chemoresistance. In fact, numerous studies reported that tumor cell-autonomous SHP2 participates in multiple signaling that promotes chemoresistance. For example, SHP2 mutations in tumor cells induce Erk inhibitor resistance through feedback activation of receptor tyrosine kinase (RTK) signaling and rebounding of Erk activity in Erk-driven tumors (Ahmed et al., 2019). SHP2 activates several different tyrosine kinases to drive anaplastic lymphoma kinase (ALK) inhibitor resistance during chemotherapy of ALK-rearranged NSCLCs (Dardaei et al., 2018). SHP2 mediates cisplatin resistance by inhibiting apoptosis and activating the Ras/PI3K/Akt/survivin pathway in lung cancer cells (Tang et al., 2018). Other studies demonstrated that SHP2 activation mutation confers resistance to imatinib in drug-tolerant chronic myeloid leukemia cells. The blockage of Raf/MEK/Erk and PI3K/Akt/mTOR pathways via SHP2 inhibition leads to apoptosis of drug-resistant cells (Li et al., 2018). In PTEN-null senescent tumors, there is a downregulation of SHP2 and activation of JAK/STAT3 pathway, which contributes to the establishment of an immunosuppressive tumor microenvironment that promotes chemoresistance (Toso et al., 2014). SHP2 also influences cancer resistance through other mechanisms. $SHP2^{E76K}$ activation mutation in bone marrow mesenchymal stromal cells (BMSCs) upregulates vascular cell adhesion molecule 1 (VCAM-1) expression by increasing the PI3K/Akt phosphorylation level and further induces BMSC-mediated chemoresistance in B-cell acute lymphoblastic leukemia (B-ALL) (Yu et al., 2020). This is a typical instance of SHP2 promoting drug resistance in non-autonomous mechanism. In general, these findings illuminate a pivotal oncogenic function of SHP2 in cancers; thus, pharmacological inhibition of SHP2 is a valid therapeutic approach for the treatment of cancers.

SHP2 INHIBITION IS A PROMISING ANTITUMOR STRATEGY

Small-Molecule Inhibitors of SHP2

SHP2 is a potential target for cancer therapy. At present, several SHP2 small-molecule inhibitors are available (**Table 2**).

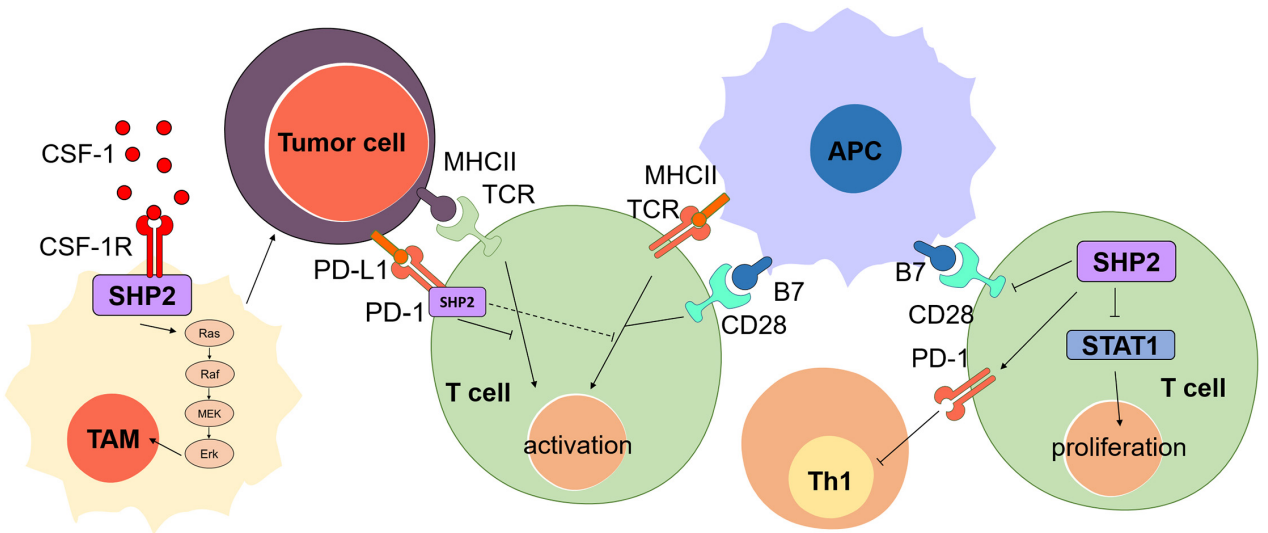

FIGURE 4 | The oncogenic role of SHP2 in tumor immune microenvironment. SHP2 binds to the T cell receptor (TCR) and prevents the activation of T cell induced by the signaling transduction among tumor cells, antigen-presenting cells (APCs), and T cells. SHP2 binds to the colony-stimulating factor-1 receptor (CSF-1R) under the stimulation of CSF-1 and then consequently activates Ras/Raf/MEK/Erk signaling and promotes the proliferation of tumor-associated macrophage (TAM), thus boosting the cancer cell survival. SHP2 in T cells inhibits the activation of T cell via blockage of the function of CD28 and STAT1.

TABLE 2 | SHP2 small-molecule inhibitors.

SHP2 inhibitors	Characteristics	References
SHP099	Allosteric small-molecule inhibitor, which binds to a tunnel-like pocket formed by the confluence of three domains of SHP2	Chen et al., 2016; Fodor et al., 2018
SHP244	Allosteric small-molecule inhibitor, which binds and stabilizes the inactive, closed conformation of SHP2	Fodor et al., 2018
SHP389	Allosteric small-molecule inhibitor, which binds to a tunnel-like pocket formed by the confluence of three domains of SHP2 and modulates MAPK signaling *in vivo*	Bagdanoff et al., 2019
SHP394	Allosteric small-molecule inhibitor, an orally efficacious inhibitor of SHP2, with high lipophilic efficiency, improved potency, and enhanced pharmacokinetic properties	Sarver et al., 2019
MRC-4550	Allosteric small-molecule inhibitor, which targets phosphatase activity of SHP2	Nichols et al., 2018
RMC-4630	Allotropic selective inhibitor, which is being evaluated in a multi-cohort phase I/II clinical program	Moore et al., 2020
PCC0208023	Allosteric small-molecule inhibitor, which shows higher affinity with key residues in the SHP2 allosteric pocket	Chen et al., 2020
NSC-87877	Binds to the catalytic cracking of SHP1/2 PTP and inhibits EGF-induced Erk1/2 activation *in vitro*	Song et al., 2009; Shi et al., 2015
PHPS1	Effective cell permeation inhibitor, which shows efficacy in blocking the downstream signal pathway dependent on SHP2	Chen et al., 2018; Salem et al., 2018
Cefsulodin	Blocks SHP2-mediated signal transduction and proliferation of several cancer cell *in vitro*	He et al., 2015

One study reported an allosteric small-molecule SHP2 inhibitor SHP099, which binds to a tunnel-like pocket formed by the confluence of three domains of SHP2 to stabilize its self-inhibiting conformation, while it has no significant activity against other PTP families (including SHP1) and kinases (Fodor et al., 2018). In addition, SHP099 inhibits the proliferation of RTK-driven human cancer cells by inhibiting Ras/Erk signaling, so drug inhibition of SHP2 is one of the effective strategies for cancer treatment (Chen et al., 2016). Meanwhile, Chen et al. identified a weak SHP2 inhibitor SHP244. X-ray crystallography shows that SHP244 binds to SHP2 and stabilizes the inactive closed conformation of SHP2 by forming cracks at the N-terminal interface between SH2 and PTP (Fodor et al., 2018). In addition, it is possible that the allosteric sites are occupied by SHP099 and SHP244 at the same time, and the combination of SHP099 and SHP244 enhances the pharmacological inhibition of cells (Fodor et al., 2018). A recent study demonstrated that mutated SHP2-mediated LLPS formation is inhibited by SHP2 allosteric inhibitors, which prevent SHP2 from releasing the self-inhibition status. Therefore, the application of SHP2 inhibitors is a promising therapeutic strategy to treat SHP2-involved developmental disorders and tumors (Zhu et al., 2020).

Recently, Bagdanoff et al. (2019) identified SHP2 inhibitor SHP389, which regulates MAPK signal *in vivo*. Another

study improved the basis of the allosteric inhibitors described previously and identified a new effective oral SHP2 inhibitor, SHP394, which shows high lipid efficiency, improved efficacy, and enhanced pharmacokinetics (Sarver et al., 2019). Nichols et al. (2018) showed that MRC-4550 affects human tumor models. MRC-4550 treatment reduces Ras/Raf/MEK/Erk signal transduction and cancer growth. RMC-4630 is an oral and effective allotropic selective inhibitor of SHP2 in the Ras signaling pathway. This inhibitor is currently in clinical trials and is being evaluated in a multi-cohort phase 1/2 clinical program (Moore et al., 2020). An effective SHP2 variable structure allosteric inhibitor PCC0208023 was synthesized recently. It non-competitively inhibits the activity of SHP2. In addition, PCC0208023 inhibits the proliferation of human CAC cells driven by KRAS mutation by inhibiting Ras/MAPK signaling pathway *in vitro*. It also shows an antitumor effect on KRAS-driven xenograft model (Chen et al., 2020). Other studies have identified NSC-87877, which binds to the catalytic cracking of SHP2 PTP (Chen et al., 2006). Additionally, PHPS1 is an effective cell permeation inhibitor, which inhibits SHP2-dependent cell processes, such as hepatocyte growth factor/dispersant factor (HGF/SF)-induced epithelial cell scattering and branching. PHPS1 also blocks the SHP2-associated downstream signaling pathway, such that it inhibits the $SHP2^{E76K}$-mediated activation of Erk1/2 to prevent the growth of a variety of human tumor cell lines (Hellmuth et al., 2008). Wang et al. found that cefsulodin blocks SHP2-mediated signaling transduction and proliferation of several cancer cell lines (He et al., 2015).

Small-Molecule Inhibition of Cell-Autonomous SHP2 to Prevent Chemoresistance

SHP2 is a potential therapeutic target for cancer treatment, as it plays a significant role in promoting chemoresistance. Chemotherapy is one of the most commonly used methods in the clinical diagnosis and treatment of malignant tumors. It is an effective means of systemic treatment for not only the tumors at the treatment site but also the clinical metastasis tumors at the potential lesions (Arbour and Riely, 2019). However, the resistance of tumor cells to chemotherapy drugs often impairs its efficacy and finally leads to failure, which has become a huge challenge for cancer treatment (Vasan et al., 2019). Increasing evidences have shown that mutated SHP2 plays an important role in chemoresistance (Ruess et al., 2018). At present, multiple allosteric inhibitors of SHP2 are discovered (Chen et al., 2016), and traditional chemotherapeutic drugs combined with SHP2 inhibition have become a potential approach to enhance efficacy of chemotherapy and immunotherapy.

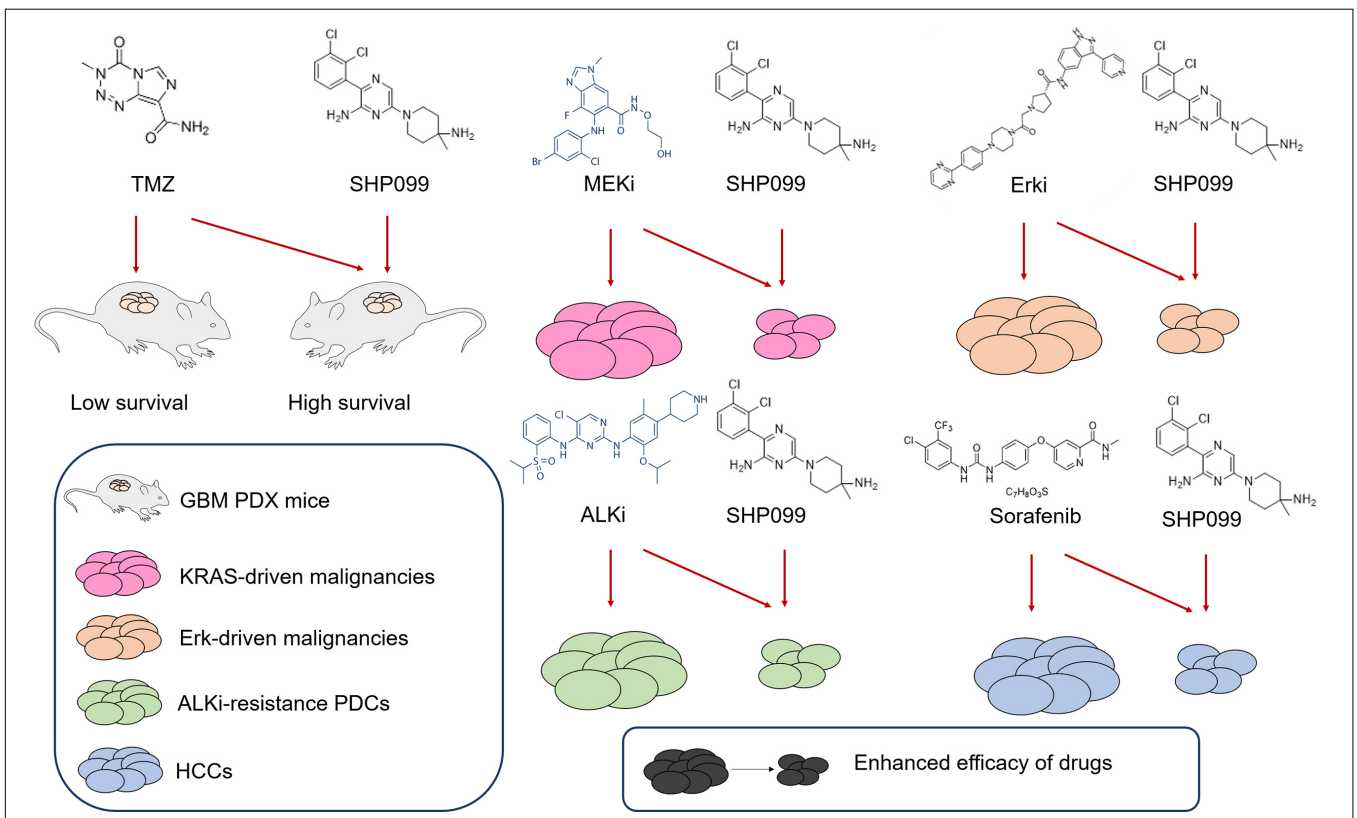

FIGURE 5 | Combination of SHP099 with other drugs enhances antitumor efficacy. In patient-derived xenograft (PDX) mouse model, temozolomide (TMZ) combines with SHP099 to increase the survival rate of tumor-carrying mice. The combination of SHP099 with MEK inhibitor, Erk inhibitor, ALK inhibitor, or sorafenib impairs the survival of malignancies, which indicates that the drug toxicity is enhanced through the drug combination.

Some studies reported that the combination of SHP2 inhibitors with other drugs shows promising application prospects (**Figure 5**). SHP099 exhibited antitumor activity either as a single agent or in combination with temozolomide (TMZ) and provided significant survival benefits for GBM tumor xenograft-bearing animals (Sang et al., 2019). MEK inhibitors show limited efficacy, because of the rapid development of adaptive resistance, whereas SHP2 inhibitor SHP099 combined with MEK inhibition prevents adaptive resistance in multiple KRAS-driven malignancies (Fedele et al., 2018). Pharmacologically targeting Erk signaling in Erk-dependent tumors is also limited by adaptive resistance, due to the feedback activation of RTK signaling, which is mediated by SHP2. Thus, targeting Erk signaling and SHP2 prevents such resistance in Erk-dependent tumors (Ahmed et al., 2019). Most ALK-rearranged NSCLCs initially respond to small-molecule ALK inhibitors, but drug resistance often develops. Researchers identified SHP2 as a common targetable resistance node in multiple ALK inhibitor-resistance patient-derived cells (PDCs), and treatment with SHP099 in combination with the ALK TKI ceritinib blocked the growth of resistant PDCs by preventing compensatory Ras and Erk reactivation (Dardaei et al., 2018). The survival benefit of sorafenib for patients with HCC is unsatisfactory due to the development of adaptive resistance, and SHP2 was observably upregulated in sorafenib-resistant HCC cell lines as well as patient-derived xenografts (Leung et al., 2019). SHP2 inhibition by SHP099 in combination with sorafenib attenuated the adaptive resistance to sorafenib by impeding RTK-induced reactivation of the MEK/Erk and Akt signaling pathways. Dioscin inhibits MEK/Erk and PI3K/Akt signaling pathways to abrogate TKI resistance through dysregulation of SHP2 expression in lung adenocarcinoma (Wang Y.C. et al., 2018). Thus, the combination of dioscin and TKI is potentially therapeutic for chemoresistant tumor treatment.

SHP2 Inhibition Is a Potential Strategy for Immunotherapy

SHP2 inhibition is a potential strategy for enhancing the efficacy of antitumor immunotherapy. Pharmacological inhibition of SHP2 through SHP099 combined with PD-1 antibody is a valid therapeutic approach for the treatment of cancers through enhancing the efficacy of antitumor immunity (Zhao M. et al., 2019). Some preclinical findings revealed that SHP2 promotes immune suppression in the tumor microenvironment; thus, the allosteric inhibition of SHP2 by RMC-4550 could induce antitumor immunity (Quintana et al., 2020). Other studies demonstrated that the co-inhibition of CSF1-R and SHP2 using nanoparticles loaded with inhibitors for tumor TAM activation and enhancement of phagocytosis is an effective strategy for macrophage-based antitumor immunotherapy (Ramesh et al., 2019). A recent study reported that allosteric inhibition of SHP2 leads to direct and selective depletion of pro-tumorigenic M2 macrophages and promotes antitumor immunity, suggesting a therapeutic approach for Ras-driven cancers (Quintana et al., 2020).

CONCLUSION AND DISCUSSION

SHP2 serves as a pivotal hub to connect multiple oncogenic signaling pathways, such as PI3K/Akt, Ras/Raf/MAPK, and PD-1/PD-L1 pathways. It promotes tumor progression via cell-autonomous and non-cell-autonomous mechanisms. That is, on the one hand, activation mutations of SHP2 in specific cells directly establish tumorigenic phenotype to promote the tumor progressions; on the other hand, SHP2 mutations in the tumor microenvironment promote tumor development. Oncogenic SHP2 is regarded as a potential cancer treatment target. Recently, multiple types of SHP2 inhibitors have been discovered to enhance cancer treatments.

At present, there are several techniques to study gene functions and explore new antitumor targets. Except for the Cre/LoxP system, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome-wide screening is widely applied to study the gene functions and to discover novel targets for treatment. CRISPR/Cas9 is a gene-editing tool for operating specific genes in the genome, which was first found as part of the adaptive immune system in bacteria. In recent years, CRISPR/Cas9 has been widely applied in altering genomes to activate or to repress the expression of genes; thus, its application accelerates the study of the mechanism of tumorigenesis and the development of cancer therapy (Lee et al., 2019). A recent study demonstrated that genome-scale CRISPR/Cas9 gene-knockout screening is applied in discovering potential therapeutic antitumor targets in the cancer cells' genomes and identified the important role of *PTPN11* in pediatric rhabdoid tumors (Oberlick et al., 2019). Genome-scale CRISPR/Cas9 gene-knockout screening can also be applied to study protein function and to explore new therapeutic targets. Recent studies applied the CRISPR/Cas9 system to gene therapy. AAV-CRISPR/Cas9-mediated gene editing corrects *Ldlr* mutation *in vivo* and effectively ameliorates atherosclerosis phenotypes, which is a potential therapeutic approach for patients with familial hypercholesterolemia (Zhao et al., 2020). Since transfusing the PD-1 knockout T-cells to patients with solid tumor induces immunological responses against tumor cells, the CRISPR/Cas9 system is regarded as therapeutic tool (Zhan et al., 2019). With the development of drug delivery systems (Chen et al., 2017; Wang P.et al., 2018), whether CRISPR/Cas9 could be applied to edit SHP2 mutation for enhancement of cancer therapy is worth exploring.

Other targeting SHP2 degradation techniques are also potential adjuvant approaches for enhancement of cancer therapeutic efficacy, including proteolysis-targeting chimera (PROTAC). PROTAC is designed to allosterically target specific proteins and recruit the E3 ligase Von Hippel-Lindau (VHL), resulting in ubiquitination and subsequent degradation of the target protein (Burslem et al., 2019). This technique is widely applied to drug development and research on mechanisms of chemoresistance. To date, about 50 proteins, including clinically validated drug targets, are targeted by PROTAC for degradation, and these PROTACs have been successfully developed in

clinical trials for cancer therapy (Li and Song, 2020). For example, PROTAC-induced bromodomain and extra-terminal (BET) protein degradation showed anti-prostate cancer efficacy (Raina et al., 2016). A recent study demonstrated that induced SHP2 degradation through PROTAC is an effective approach to inhibit the function of SHP2, and it further pointed out that optimization of these SHP2 degraders may lead to the development of a new class of therapies for cancers and other human diseases (Wang et al., 2020).

Recently, hyperactivation of SHP2 through the formation of LLPS has been elucidated (Zhu et al., 2020). Both GOF and LOF disease-associated SHP2 variants promote LLPS to increase the catalytic activity of mutant and wild-type SHP2, leading to MAPK hyperactivation. SHP2 is an important signal hub in normal conditions, and its hyperactivation will undoubtedly lead to the breakdown of cell signal balance. Therefore, in developmental diseases, especially tumors, LLPS possibly acts as an important driver for disease occurrence and chemotherapeutic resistance. As it has been found that allosteric inhibitors of SHP2 have an inhibitory effect on the formation of LLPS, it is very promising to develop new therapies based on SHP2 inhibition and LLPS blockage.

AUTHOR CONTRIBUTIONS

LD and DH drafted the manuscript and designed the structure. QX, MX, and XM proposed useful comments, suggestions, and revised the manuscript. CZ revised the language of the manuscript. All authors contributed to the article and approved the submitted version.

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The C-X-C Motif Chemokine Ligand 1 Sustains Breast Cancer Stem Cell Self-Renewal and Promotes Tumor Progression and Immune Escape Programs

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Breast cancer (BC) mortality is mainly due to metastatic disease, which is primarily driven by cancer stem cells (CSC). The chemokine C-X-C motif ligand-1 (CXCL1) is involved in BC metastasis, but the question of whether it regulates breast cancer stem cell (BCSC) behavior is yet to be explored. Here, we demonstrate that BCSCs express CXCR2 and produce CXCL1, which stimulates their proliferation and self-renewal, and that CXCL1 blockade inhibits both BCSC proliferation and mammosphere formation efficiency. CXCL1 amplifies its own production and remarkably induces both tumor-promoting and immunosuppressive factors, including *SPP1/OPN*, *ACKR3/CXCR7*, *TLR4*, *TNFSF10/TRAIL* and *CCL18* and, to a lesser extent, immunostimulatory cytokines, including *IL15*, while it downregulates *CCL2*, *CCL28*, and *CXCR4*. CXCL1 downregulates *TWIST2* and *SNAI2*, while it boosts *TWIST1* expression in association with the loss of E-Cadherin, ultimately promoting BCSC epithelial-mesenchymal transition. Bioinformatic analyses of transcriptional data obtained from BC samples of 1,084 patients, reveals that *CXCL1* expressing BCs mostly belong to the Triple-Negative (TN) subtype, and that BC expression of *CXCL1* strongly correlates with that of pro-angiogenic and cancer promoting genes, such as *CXCL2-3-5-6*, *FGFBP1*, *BCL11A*, *PI3*, *B3GNT5*, *BBOX1*, and *PTX3*, suggesting that the CXCL1 signaling cascade is part of a broader tumor-promoting signaling network. Our findings reveal that CXCL1 functions as an autocrine growth factor for BCSCs and elicits primarily tumor progression and immune escape programs. Targeting the CXCL1/CXCR2 axis could restrain the BCSC compartment and improve the treatment of aggressive BC.

Keywords: breast cancer stem cells, chemokines, CXCL1, tumor microenvironment, immunity genes, triple-negative breast cancer

INTRODUCTION

Breast cancer (BC) is the second most common cause of death from cancer in women (Sung et al., 2021). BC related deaths are mainly due to disease progression or recurrences, which are estimated to range between 20 and 30% of all BC cases (DeSantis et al., 2019). Moreover, approximately 6–10% of newly diagnosed BCs are initially stage IV or metastatic (DeSantis et al., 2019). Cancer stem cells (CSC), endowed with high plasticity and self-renewal properties, are the driving force of cancer progression and metastasis (Dittmer, 2018). Their quiescent, slow cycling, state accounts for chemo- and radiotherapy resistance (De Angelis et al., 2019), while their exit from dormancy and cell cycle re-activation, which precedes spreading and proliferation to distant organs, accounts for cancer relapse (De Angelis et al., 2019). The transition between these two CSC states is tightly regulated by cell-intrinsic mechanisms, systemic factors and interactions with the microenvironment, such as those mediated by immunoregulatory messengers (Prager et al., 2019).

Chemokines are soluble, small molecular weight (8–14 kDa) immunoregulatory proteins, which are essential for immune cell homing and play a key role in inflammation, host defense, angiogenesis, wound healing, but also in tumorigenesis and cancer immunoediting (Griffith et al., 2014; Nagarsheth et al., 2017).

The chemokine C-X-C motif ligand 1 (CXCL1), also named GROα, signals through the G protein-coupled receptor, C-X-C motif chemokine receptor 2 (CXCR2), to promote angiogenesis (Strieter et al., 2005) and to attract and activate neutrophils and basophils during inflammation (Baggiolini et al., 1994; Clark-Lewis et al., 1995).

Growing evidence supports a role for CXCL1 in cancer progression and recurrence. Originally identified as a melanoma growth stimulatory activity protein (Dhawan and Richmond, 2002), CXCL1 is constitutively highly expressed in melanoma cells and cooperates with oncogenic drivers, or loss of tumor suppressors, to promote tumor development (Luan et al., 1997).

In gastric cancer, overexpression of the CXCL1–CXCR2 axis is closely associated with the migration and invasiveness of malignant cells (Cheng et al., 2011), and CXCL1 release by the lymphatic endothelium promotes lymph node metastasis (Wang Z. et al., 2017).

In bladder cancer patients, urinary CXCL1 can serve as a molecular marker for tumor detection and as a predictor of local recurrence (Kawanishi et al., 2008; Nakashima et al., 2015).

In castration-resistant prostate cancer (Shamaladevi et al., 2009), overexpression of *CXCL1* promotes cancer cell epithelial-mesenchymal transition (EMT) and invasiveness, *via* AKT/NF-κB signaling pathway, thus favoring tumor progression (Kuo et al., 2012).

In colorectal cancer, a high level of *CXCL1* expression correlates with advanced tumor stage, shorter overall survival (OS) and disease-free survival (Wen et al., 2006; Zhuo et al., 2018).

In BC, expression of *CXCL1* is elicited by chemotherapy and promotes intratumoral recruitment of myeloid cells, which release chemokines that support BC cell survival and metastasis (Acharyya et al., 2012).

Although CXCL1 has been shown to be involved in BC progression and chemotherapy resistance (Minn et al., 2005; Acharyya et al., 2012), the question of whether it has a role in breast cancer stem cell (BCSC) behavior, which is the cornerstone of metastasis and resistance to chemotherapy, has never been addressed.

This study provides evidence that BCSCs produce and release CXCL1 and respond to the chemokine, which affects their viability and shapes their transcriptional profile. Bioinformatic analyses of microarray data, provided by the *TCGA PanCancer collection*, highlight the clinico-pathological relevance of our findings and reveal that expression of CXCL1 is prevalent in Triple-Negative (TN) BC, the molecular subtype with the highest CSC content and the worst clinical outcome (Honeth et al., 2008; Ma et al., 2014). Uncovering key pathways of BC progression is essential for the development of successful metastasis prevention and treatment strategies.

MATERIALS AND METHODS

Cell Cultures and MTT Cell Proliferation Assays

The human (h) BC cell lines, namely BCSC-105 and BCSC-608, were generated and provided by Prof. G. Stassi (University of Palermo, Italy), who characterized them as BC stem cells (Todaro et al., 2013). BCSCs were authenticated by surface staining for characteristic markers, as described (Todaro et al., 2013). The culture medium consisted of serum-free DMEM:F12 (1:1), enriched with GlutaMAX-I supplement (Thermo Fisher Scientific, Waltham, MA, United States), 50 ng/ml heparin (Sigma-Aldrich, St. Louis, MO, United States), 20 ng/ml EGF, 10 ng/ml βFGF (R&D Systems, Minneapolis, MN, United States), and free from proteins, lipids, or growth factors.

Cell proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, United States), according to manufacturer's instructions. Briefly, cells were seeded on a 96-well plate, at a density of 1×10^3 cells per well, and incubated, for 48 h, with recombinant (r) hCXCL1 (#300-11, Peprotech, Cranbury, NJ, United States), at concentration of 5, 10, 30, and 50 ng/ml, or with neutralizing anti-CXCL1 Ab (R&D Systems Cat# AF275, RRID:AB_355288), at concentration of 0.5, 1, 2, 5 μg/ml, for BCSC-105, and 0.030, 0.075, 0.150, 0.300 μg/ml, for BCSC-608.

Then, the plates were read at 490 nm, using the SpectraMax 190 microplate reader (RRID:SCR_018932; Molecular Devices San Jose, CA, United States). The proliferation was measured using untreated control cells as reference and the results were given as mean ± SD of three independent experiments carried out in triplicate.

Flow Cytometry

To assess phenotype markers, BCSCs were harvested and mechanically dissociated into a single cell suspension. Then, the cells were pelleted, resuspended in PBS and

incubated for 30 min, at 4°C, with the following antibodies (Abs): anti-CD24 (RRID:AB_10562033), anti-CD29 (RRID:AB_395836), anti-CD44 (RRID:AB_398683), and anti-CD117 (RRID:AB_398461) (all from BD Biosciences, Franklin Lakes, NJ, United States), anti-CD133 (RRID:AB_2726287) (Miltenyi Biotec, Bergisch Gladbach, Germany), and anti-CXCR2 (RRID:AB_2296102) (R&D Systems, Minneapolis, MN, United States), at a concentration of 0.25 μg/100μl. Acquisition was performed using a BD Scientific Canto II Flow Cytometer (RRID:SCR_018056) and the data were analyzed using FlowJo software (FlowJo, RRID:SCR_008520). Dead cells were excluded by 7AAD staining. All experiments were performed in triplicate.

ELISA

Assessment of CXCL1 protein in the supernatant derived from BCSCs was performed using the GRO alpha (CXCL1) Human ELISA Kit (BMS2122, Thermo Fisher Scientific, Waltham, MA, United States; detection sensitivity: 0.7–7.6 pg/ml), according to manufacturer's instructions, using the same culture conditions described above.

Sphere Formation Assay

The sphere-forming potential of BCSC-105 and BCSC-608 was assessed by using the Extreme Limiting Dilution Analysis (ELDA, RRID:SCR_018933) (Hu and Smyth, 2009).

Briefly, cells were seeded at concentrations of one cell, two cells, four cells, or eight cells per well, on 96-well ultra-low attachment plates, and incubated at 37°C, with 5% CO_2, in a humidified incubator for 8 days, with or without rhCXCL1 (Peprotech, London, United Kingdom; 10 ng/ml for BCSC-105 and 30 ng/ml for BCSC-608) or anti-CXCL1 Ab (R&D Systems, Minneapolis, MN, United States; 5 μg/ml for BCSC-105 and 0.15 μg/ml for BCSC-608).

Spheres containing $\geq$ 3 cells were counted under a Leica light microscope.

Histopathology and Immunohistochemistry

Histology and immunohistochemistry on formalin fixed paraffin embedded human TNBC samples, were performed as reported (Di Meo et al., 2014), by using anti-CXCL1 (R&D Systems Cat# AF275, RRID:AB_355288), anti-CD68 (Agilent Cat# M0876, RRID:AB_2074844), and anti-CD133 Abs (Cell Signaling Technology Cat# 3663, RRID:AB_2172866). Written informed consent was obtained from patients. The study was performed in accordance with the principles outlined in the Declaration of Helsinki and approved by the Ethical Committee of the "G. d'Annunzio" University and Local Health Authority of Chieti, Italy (Protocol ONCO-2017-1, 04/19/2018).

Immunofluorescence Staining and Confocal Microscopy

Immunofluorescent stainings of 4% PFA-fixed BCSCs were performed as follows: primary Abs goat anti-CXCL1 (R&D Systems Cat# AF275, RRID:AB_355288), rabbit anti-OPN (Abcam Cat# ab14175, RRID:AB_2194831), goat anti-TRAIL (Santa Cruz Biotechnology Cat# sc-6079, RRID:AB_2205918), and mouse anti-TWIST1 (Abcam Cat# ab50887, RRID:AB_883294) were incubated overnight at 4°C, followed by incubation with Alexa Fluor 633 anti-goat IgG (Thermo Fisher Scientific Cat# A-21082, RRID:AB_2535739), Alexa Fluor 488 anti-rabbit IgG (Thermo Fisher Scientific Cat# A32731, RRID:AB_2633280), or Alexa Fluor 594 anti-mouse IgG (Molecular Probes Cat# A-11005, RRID:AB_141372) for 2 h at room temperature.

For double immunofluorescent stainings, slides were incubated, for 3 h at room temperature, with mouse anti-TWIST1 Ab (Abcam Cat# ab50887, RRID:AB_883294), followed by incubation with Alexa Fluor 594 anti-mouse IgG (Molecular Probes Cat# A-11005, RRID:AB_141372), for 2 h at room temperature. Then, slides were incubated, overnight at 4°C, with anti-E-cadherin Ab (Agilent Cat# M3612, RRID:AB_2076672), followed by incubation with Oregon Green 488 anti-mouse IgG (Thermo Fisher Scientific Cat# O-11033, RRID:AB_2539797), for 2 h at room temperature.

Nuclei were counterstained with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (#D1306, Thermo Fisher Scientific, Waltham, MA, United States) for 2 min and slides were analyzed under an LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany; RRID:SCR_018062).

PCR Array and Real-Time RT-PCR

RNA was extracted by using the RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany), and reverse-transcribed with the RT2 First Strand Kit (#33040, Qiagen, Hilden, Germany). PCR array analyses were run on a Qiagen Rotor Gene Q (Qiagen Rotor-Gene Q, RRID:SCR_018976), using the RT^2 Profiler Human Cancer Inflammation and Immunity Crosstalk PCR Array (#PAHS-181Z, Qiagen, Hilden, Germany) and RT2 SYBR Green ROX Fast Master mix (#330623, Qiagen, Hilden, Germany). The results from each plate were normalized to the median value of a set of housekeeping genes. Changes in the gene expression were calculated using the ΔΔCt method. Results from experiments were performed in triplicate, pooled and analyzed with the manufacturer's software. A significant threshold of a twofold change in gene expression corresponded to a $p < 0.001$.

For single gene analyses, real-time RT-PCR was performed using the Quantifast SYBR Green PCR Kit (#204054, Qiagen, Hilden, Germany) and a MiniOpticon System (#CFB-3120, Bio-Rad, Hercules, CA, United States).

Primers for housekeeping gene *hypoxanthine phosphoribosyltransferase* (*HPRT*), *MMP2, MMP9, OCT4A*, *SNAI1, SNAI2, TWIST1, TWIST2, YAP1, ZEB1, ZEB2* were designed and synthesized by Sigma-Aldrich Corporation (St. Louis, MO, United States): *HPRT* Forward 5′- AGA CTTTGCTTTCCTTGGTCAGG-3′ and *HPRT* Reverse 5′-GTCTGGCTTA TATCCAACACTTCG-3′; *MMP2* Forward 5′-AGCGAGTGGATGCCGCCTTTAA-3′ and *MMP2* Reverse 5′-CATTCCAGGCATCTGCGATGAG-3′; *MMP9* Forward 5′-GCC ACTACTGTGCCTTTGAGTC-3′ and *MMP9* Reverse 5′-CCCTCAGAGAATCGCCAGTACT-3′; *OCT4A* Forward 5′-CCCCTGGTGCCGTGA-3′ and *OCT4A* Reverse 5′-GCAAATTGCTCGAGTTCTTTCTG-3′; *SNAI1* Forward

5′-CCTCTTCCTCTCCATACCT-3′ and *SNAI1* Reverse 5′-TTC ATCAAAGTCCTGTGGG-3′; *SNAI2* Forward 5′-TGT CATACCACAACCAGAGA-3′ and *SNAI2* Reverse 5′-CTTGGAGGAGGTGTCAGAT-3′; *SOX2* Forward 5′-AGA GAGAAAGAAAGGGAGAGA-3′ and *SOX2* Reverse 5′-AAT CAGGCGAAGAATAATTTGG-3′; *TWIST1* Forward 5′-CGG AGACCTAGATGTCATT-3′ and *TWIST1* Reverse 5′-CTGTCTCGCTTTCTCTTTT-3′; *TWIST2* Forward 5′-AACTGGACCAAGGCTCTC-3′ and *TWIST2* Reverse 5′-GCGGCGTGAAAGTAAGAAT-3′; *YAP1* Forward 5′-TTCCTCTCCAGCTTCTCTGC-3′ and *YAP1* Reverse 5′-GATGCTGAGCTGTGGGTGTA; *ZEB1* Forward 5′-CCAACAGACCAGACAGTG-3′ and *ZEB1* Reverse 5′-TGACTCGCATTCATCATCTT; *ZEB2* Forward 5′-CGGAGACTTCAAGGTATAATCTATC-3′ and *ZEB2* Reverse 5′-GTTACGCCTCTTCTAATGACAT-3′. Primers for *BMI1* (#QT00052654), *KLF4* (#QT00061033), *MET* (#QT00023408), *MMP14* (#QT00001533), *MYC* (#QT00035406), *NOTCH1* (#QT00231056), *SHH* (#QT01156799) and *WWTR1* (#QT01017996) were purchased from Qiagen, Hilden, Germany.

Melting curve analysis was done to assess the specificity of PCR products and the efficiency of reaction for each target was evaluated by amplifying serial dilutions of cDNA. Relative quantification of mRNA was done according to the comparative threshold cycle method with HPRT as calibrator, using the Bio-Rad CFX Manager software. The samples were processed in triplicate, and wells without added cDNA served as negative controls.

Pooled results ± SD are from two experiments performed in duplicate. A significant threshold of fourfold change in gene

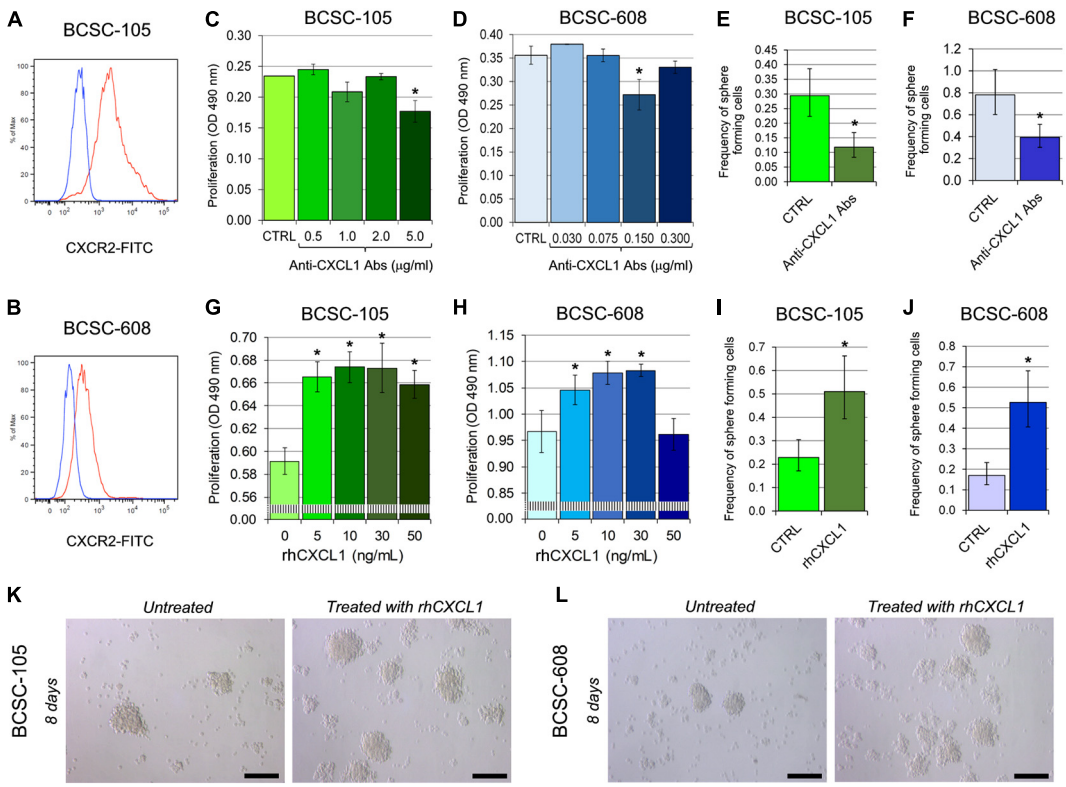

FIGURE 1 | CXCL1 regulates the proliferation and self-renewal ability of BCSCs. **(A,B)** Flow cytometry analysis of CXCR2 expression in **(A)** BCSC-105 and **(B)** BCSC-608. Red profiles illustrate the expression of CXCR2, while blue profiles represent isotype controls. Each panel is representative of three independent experiments. **(C)** MTT assay of BCSC-105 cells treated with different concentrations of anti-CXCL1 Abs. CTRL: untreated cells. ANOVA: $p = 0.0002$. $^*p < 0.05$, Tukey HSD Test compared with CTRL, 0.5, 1.0, 2.0, and 5.0 μg/ml. Experiments were performed in triplicate. **(D)** MTT assay of BCSC-608 cells treated with different concentrations of anti-CXCL1 Abs. CTRL: untreated cells. ANOVA: $p = 0.0022$. $^*p < 0.05$, Tukey HSD Test compared with CTRL, 0.030, 0.075, 0.150, and 0.300 μg/ml. Experiments were performed in triplicate. **(E)** Sphere forming capability of BCSC-105, evaluated by ELDA, after 8 days of treatment with 5.0 μg/ml of anti-CXCL1 Abs. $^*p = 0.0001$, Chi-squared test compared with untreated cells (CTRL). Experiments were performed in triplicate. **(F)** Sphere forming capability of BCSC-608, evaluated by ELDA, after 8 days of treatment with 0.15 μg/ml of anti-CXCL1 Abs. $^*p = 0.0002$, Chi-squared test compared with untreated cells (CTRL). Experiments were performed in triplicate. **(G)** MTT assay of BCSC-105 stimulated with different concentrations of rhCXCL1. ANOVA: $p < 0.001$. $^*p < 0.01$, Tukey HSD Test compared with 0 ng/ml. **(H)** MTT assay of BCSC-608 stimulated with different concentrations of rhCXCL1. ANOVA: $p < 0.001$. $^*p < 0.01$, Tukey HSD Test compared with 0 ng/ml. **(I)** Sphere forming capability of BCSC-105, evaluated by ELDA, after 8 days of treatment with 10 ng/ml of rhCXCL1. $^*p < 0.0001$, Chi-squared test compared with untreated cells (CTRL). Experiments were performed in triplicate. **(J)** Sphere forming capability of BCSC-608, evaluated by ELDA, after 8 days of treatment with 30 ng/ml of rhCXCL1. $^*p < 0.0001$, Chi-squared test compared with untreated cells (CTRL). Experiments were performed in triplicate. **(K)** BCSC-105 and **(L)** BCSC-608–derived spheres were dissociated, seeded at concentrations of 1 cell/well, and untreated or treated with 10 and 30 ng/ml of rhCXCL1, respectively. Magnification: X400. Scale bars: 100 μm.

expression corresponded to $p < 0.001$, and only genes above the threshold, in both cell lines, are represented.

Bioinformatic Analyses

For bioinformatic analyses (cBioPortal, RRID:SCR_014555), gene expression data from the "*Breast Invasive Carcinoma TCGA PanCancer collection*" dataset (Berger et al., 2018), which includes 1,084 BC cases, were downloaded from the cBioportal for Cancer Genomics database ([1] cBioPortal, RRID:SCR_014555). For each sample, the Z-scores of *CXCL1*mRNA levels were calculated, compared to the mean of all samples in the study, and all samples with a Z-score ≥ 2 were considered *CXCL1*-expressing. Subsequently, the association between *CXCL1*mRNA expression and BC subtypes was assessed using Fisher's exact test, whereas the correlation between *CXCL1*mRNA expression and the expression of other genes was assessed using the Spearman's correlation coefficient (ρ). All statistical tests were evaluated at an α level of 0.05.

Statistical Analyses

For *in vitro* studies, between-group differences were assessed by Student's *t*-test or ANOVA (followed by Tukey's HSD test). Between groups differences in sphere-forming potential were evaluated by ELDA (Hu and Smyth, 2009). All statistical tests were evaluated at an α level of 0.05, using Stata, version 13 (StataCorp, College Station, TX, United States; RRID:SCR_012763).

RESULTS

CXCL1 Autocrine Signaling Sustains BCSC Proliferation and Mammosphere Formation Efficiency

Human CSCs, BCSC-105 and BCSC-608, were isolated from distinct infiltrating ductal BCs with different genetic and molecular background, and showed a $CD133^{+}CD44^{+}CD24^{low}$ phenotype (Todaro et al., 2013; Sorrentino et al., 2021, submitted). BCSCs fulfilled the functional properties of CSCs, such as the ability to grow in tumor spheres and to reproduce the histological and immunophenotipical features of the tumor of origin, when implanted, at low cell numbers, in immunocompromised mice (Todaro et al., 2013).

To investigate whether BCSCs are responsive to CXCL1, we first analyzed, by flow cytometry, the expression of its cognate receptor CXCR2. Both BCSC-105 and BCSC-608 expressed CXCR2 (**Figures 1A,B**) and constitutively produced and released CXCL1, specifically, 155.07 and 3.33 pg/ml, respectively. The addition of anti-CXCL1 neutralizing Abs to the culture medium of BCSC-105 and BCSC-608 significantly inhibited their proliferation (BCSC-105: ANOVA, $p = 0.0002$; BCSC-608: ANOVA, $p = 0.0022$) (**Figures 1C,D**) and mammosphere-formation efficiency (BCSC-105: Chi-squared test, $p = 0.0001$; BCSC-608: Chi-squared test, $p = 0.0002$, **Figures 1E,F**). By contrast, treatment with rhCXCL1 (5–50 ng/ml for 48 h) increased the proliferation of BCSCs (ANOVA, $p < 0.001$; **Figures 1G,H**), and boosted their mammosphere formation ability (Chi-squared test, $p < 0.0001$; **Figures 1I–L**).

CXCL1 Shapes Immune Gene Expression Profile of BCSCs and Promotes Tumor Progression and Immune Evasion Programs

In both BCSC-105 and BCSC-608, treatment with rhCXCL1 (10 ng/ml) considerably amplified its own expression (267.19 times in BCSC-105; 115.53 times in BCSC-608; **Figures 2A,C**), and strongly promoted the expression of *ACKR3/CXCR7* (560.94 times in BCSC-105; 2,820.98 times in BCSC-608), and *SPP1/OPN* (2,212.88 times in BCSC-105; 2,094.11 times in BCSC-608; **Figures 2A,D**).

Treatment of hBCSCs with rhCXCL1 also increased the expression of cytokines, chemokines, chemokine receptors, growth factors and metalloproteinase, in particular, *IL1*β (5.25 times in BCSC-105; 7.53 times in BCSC-608), *IL15* (5.40 times in BCSC-105; 6.74 times in BCSC-608), *CCL18* (333.54 times in BCSC-105; 58.17 times in BCSC-608), *CXCL9* (192.03 times in BCSC-105; 46.60 times in BCSC-608), *CXCL10* (53.88 times in BCSC-105; 64.54 times in BCSC-608), *CXCL11* (71.10 times in BCSC-105; 187.89 times in BCSC-608), *CSF2* (7.63 times in BCSC-105; 9.53 times in BCSC-608), *IGF1* (4.09 times in BCSC-608; 4.80 times in BCSC-105), *HGF* (10.17 times in BCSC-105; 18.97 times in BCSC-608) and *MMP14* (9.95 times in BCSC-105; 27.34 times in BCSC-608) (**Figure 2A**).

The expression of specific surface molecules, which regulate immune evasion mechanisms (Koyama et al., 2002; Sato et al., 2009; Kuonen et al., 2012) was also significantly increased. In particular, the treatment with rhCXCL1 stimulated the expression of *TLR4* (605.39 and 512.75 times, in BCSC-105 and BCSC-608), of *KITLG* (4.10 and 4.86 times, in BCSC-105 and BCSC-608), and of *TNFS10/TRAIL* (94.47 and 47.25 times, in BCSC-105 and BCSC-608; **Figures 2A,E**).

By contrast, the treatment of BCSCs with rhCXCL1 inhibited their expression of *CCL2* (−53.59 and −90.00 times in BCSC-105 and BCSC-608), *CCL28* (−14.20 and −13.16 times in BCSC-605 and BCSC-608), *CXCR4* (−70.89 and −22.75 times, in BCSC-105 and BCSC -608) (**Figures 2B, 3A**), *TNF* (−8.99 and −6.58 times, in BCSC-105 and BCSC-608), *IDO1* (−12.89 and −13.07 times, in BCSC-105 and BCSC-608), *PTGS2* (−8.62 and −7.10 times, in BCSC-105 and BCSC-608) and *MICA/MHCI* (−6.14 and −4.40 times, in BCSC-105 and BCSC-608) (**Figure 2B**).

Analyses of genes that regulate stemness (*SHH, OCT4, SOX2, KLF4, NOTCH, MYC, YAP,* and *WWTR1*), and EMT, (*SNAI1, SNAI2, ZEB1, ZEB2, TWIST1, TWIST2,* and *MET*), showed that CXCL1 downregulated *TWIST2* and *SNAI2* (−9.69 and −9.10 times in BCSC-105, and −9.89 and −4.05 times in BCSC-608, respectively; **Figure 2B**), but strongly stimulated BCSC expression of *TWIST1* (73.38 times in BCSC-105; 108.57 times in BCSC-608) in association with a distinct loss of E-Cadherin expression (**Figures 2A, 3B**).

[1] https://www.cbioportal.org

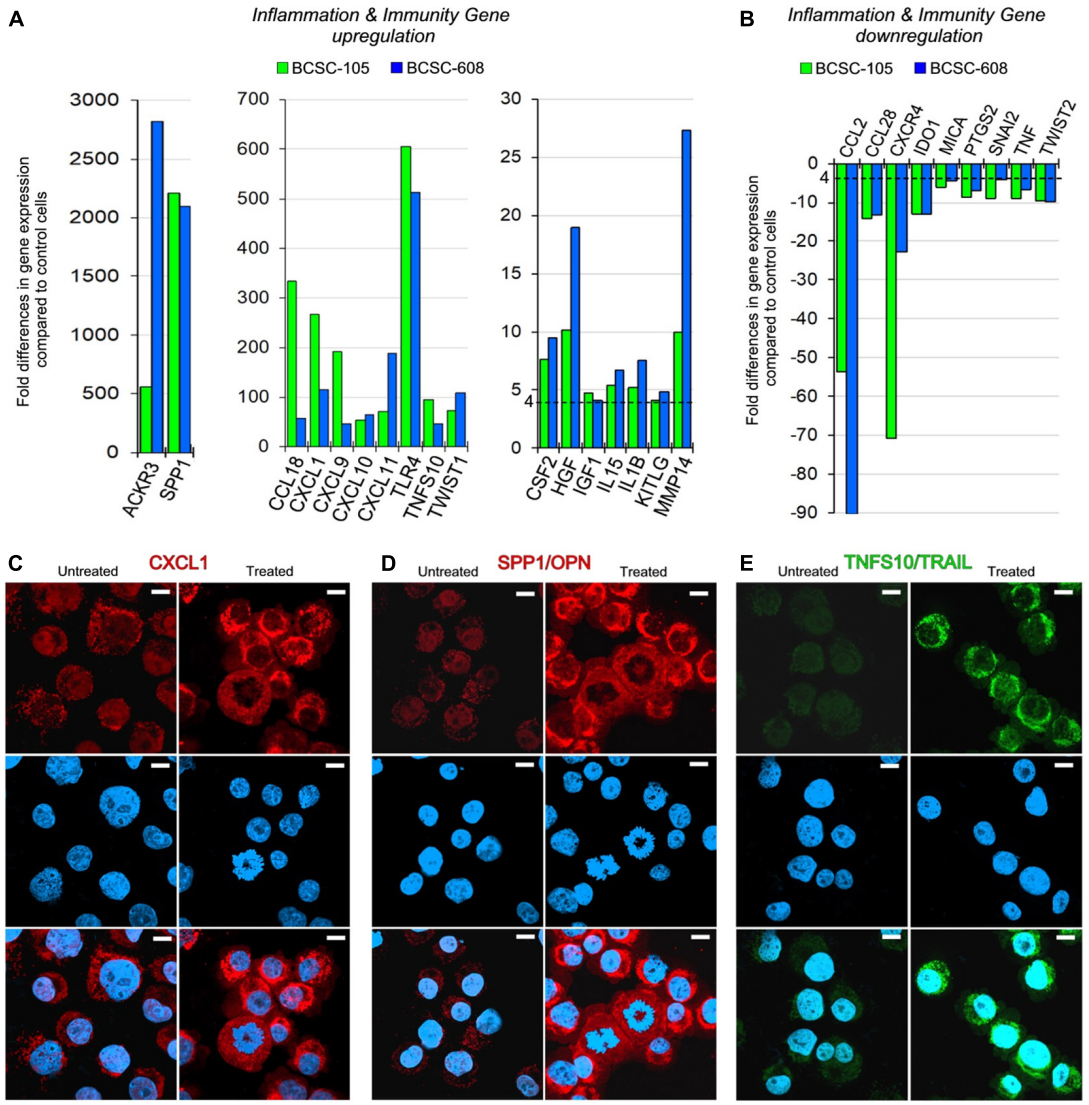

FIGURE 2 | CXCL1 upregulates its own expression and shapes the transcriptional profile of BCSCs. **(A,B)** Fold differences in mRNAs of inflammation and immunity genes expression [upregulated genes are represented in panel **(A)**, and downregulated genes are represented in panel **(B)**] between rhCXCL1 treated and untreated BCSC-105 (green bars) and BCSC-608 (blue bars). Pooled results ± SD are from two experiments performed in duplicate. A significant threshold of a fourfold change in gene expression corresponded to $p < 0.001$. **(C)** Confocal microscopy images of CXCL1 (red) in untreated and rhCXCL1 treated BCSC-105 cells. DAPI-DNA stained nuclei. Magnification: X630. Scale bars: 3 μm. **(D)** Confocal microscopy images of OPN (red) in untreated and rhCXCL1 treated BCSC-105 cells. DAPI-DNA stained nuclei. Magnification: X630. Scale bars: 3 μm. **(E)** Confocal microscopy images of TRAIL (green) in untreated and rhCXCL1 treated BCSC-105 cells. DAPI-DNA stained nuclei. Magnification: X630. Scale bars: 3 μm.

Expression of CXCL1 Is Prevalent in Triple-Negative BC and Positively Correlates with the Expression of Pro-Angiogenic Factors and Tumor Progression Genes

Previous studies have revealed that *CXCL1* expression is correlated with overall survival (OS) and relapse-free survival (RFS) in BC patients, and is predictive of a poor prognosis (Divella et al., 2013; Zou et al., 2014). The growth factor activity of CXCL1 in BCSCs led us to assess the extent of its expression in tumor tissues from BC patients in order to determine its clinic-pathological impact.

Bioinformatic analyses of gene expression data obtained from whole tumor samples of 1,084 BC patients included in the "*Breast Invasive Carcinoma TCGA PanCancer collection*" dataset (Berger et al., 2018), identified the expression of *CXCL1mRNA* in 3.67% of BCs, and its association with the TNBC subtype (Chi-squared test: $p < 0.0001$; **Figure 3C**). As represented in **Figure 3D**, 69% of $CXCL1^{+}$BCs, and only 15% of $CXCL1^{-}$BCs, were diagnosed as TNBC.

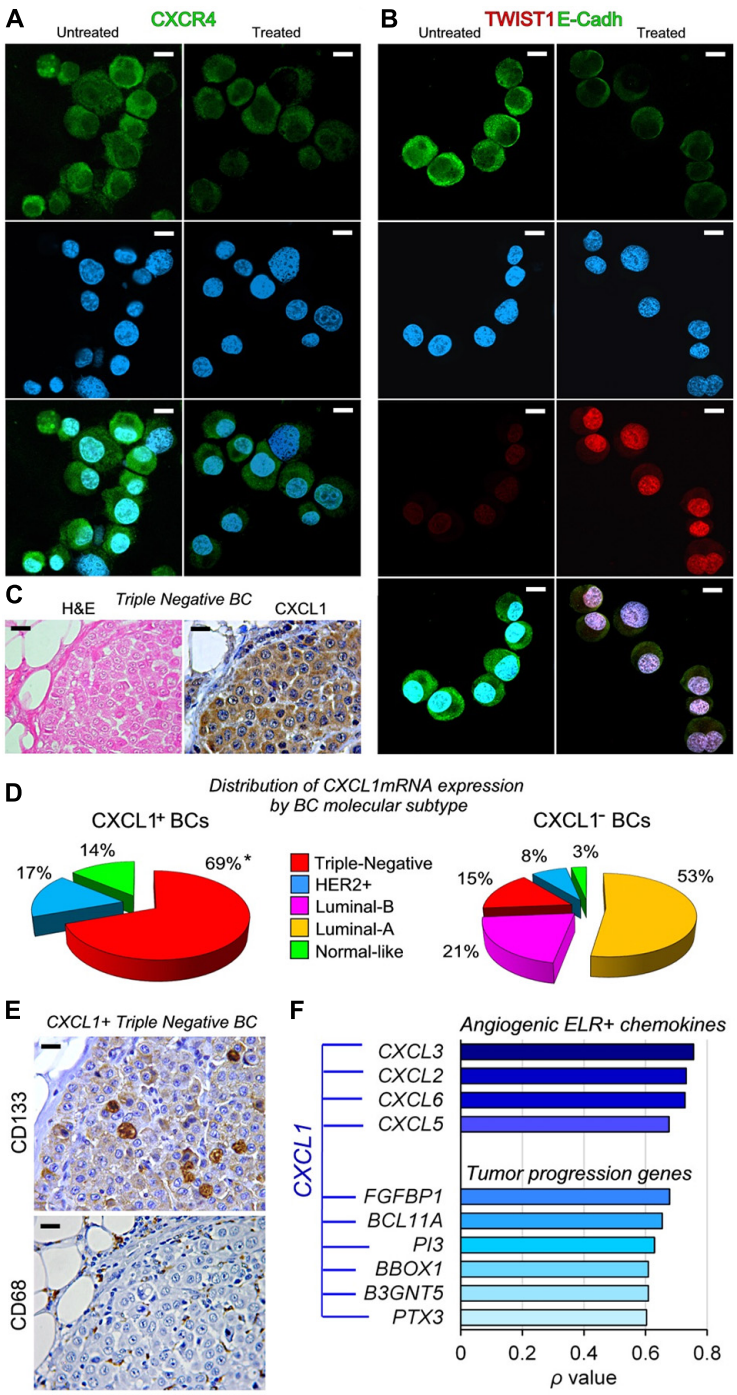

FIGURE 3 | CXCL1 has a dual role in regulating tumor progression genes in BCSCs and is primarily expressed in Triple-Negative BC molecular subtype. **(A)** Confocal microscopy images of CXCR4 (green) in untreated and rhCXCL1 treated BCSC-105 cells. DAPI-DNA stained nuclei. Magnification: X630. Scale bars: 3 μm. **(B)** Confocal microscopy images of TWIST1 (red) and E-Cadherin (green) in untreated and rhCXCL1 treated BCSC-105 cells. DAPI-DNA stained nuclei. Magnification: X630. Scale bars: 3 μm. **(C)** Hematoxylin and eosin (H&E) staining and immunostaining with anti-CXCL1 Abs of a representative Triple-Negative BC sample. Magnification: X400. Scale bars: 20 μm. **(D)** Distribution of $CXCL1^{+}$BCs and $CXCL1^{-}$BCs by molecular subtypes, represented as percentage of the total number of BC expressing or not *CXCL1*mRNA. $^{*}p < 0.0001$, Chi-squared test vs Triple-Negative $CXCL1^{-}$BCs. **(E)** Immunostaining with anti-CD133 stem cell marker and anti-CD68 Abs of a representative $CXCL1^{+}$Triple-Negative BC sample. Magnification: X400. Scale bars: 20 μm. **(F)** Correlation between the expression of *CXCL1mRNA* in human BC samples (from the *"Breast Invasive Carcinoma TCGA PanCancer collection")* and that of angiogenic ELR^{+} chemokines and tumor progression genes, measured by Spearman's rank correlation coefficient (ρ). Strength of the Correlation: $0.00 \leq \rho \leq 0.19$, very weak; $0.20 \leq \rho \leq 0.39$, weak; $0.40 \leq \rho \leq 0.59$, moderate; $0.60 \leq \rho \leq 0.79$, strong; $0.80 \leq \rho \leq 1.0$, very strong.

Immunohistochemistry revealed that, in CXCL1^{+}TNBCs, the cellular sources of CXCL1 included, in addition to CD133^{+}BCSCs (Park et al., 2010), tumor infiltrating immune cells, such as CD68^{+} macrophages (**Figure 3E**), and the vast majority of BC cells.

Irrespective of the molecular subtype of BC, analyses of microarray data also revealed, a strong positive correlation between the expression of *CXCL1mRNA* in BC samples, and that of angiogenic ELR^{+} chemokines, such as *CXCL3* (ρ = 0.756), *CXCL2* (ρ = 0.732), *CXCL6* (ρ = 0.728), *CXCL5* (ρ = 0.676), and tumor progression genes, specifically, *FGFBP1* (ρ = 0.678), *BCL11A* (ρ = 0.654), *PI3* (ρ = 0.629), *B3GNT5* (ρ = 0.609), *BBOX1* (ρ = 0.609), and *PTX3* (ρ = 0.603). This finding suggests that the CXCL1 signaling pathway is part of a broader BC progression program with important clinical implications (**Figure 3F**).

DISCUSSION

Overcoming the challenge of metastatic BC is a major public health issue, as it causes about 630,000 deaths worldwide each year (Sung et al., 2021). Understanding the molecular pathways regulating BCSCs, which are the driving force of metastasis, is critical to achieve this goal.

Here we demonstrate that CSCs, derived from BCs with different genetic and molecular background (Todaro et al., 2013), reveal different levels of production and responsiveness to CXCL1 that acts as an autocrine growth factor for BCSCs, regardless of the BC subtype from which they originate. BCSCs constitutively release and respond to the chemokine, which sustains their proliferation and self-renewal, and reshapes their transcriptional profile, ultimately promoting tumor invasion and immune evasion programs.

The ELR^{+} chemokine CXCL1 signals through CXCR2 to promote angiogenesis and regulates host immune response, by recruiting and activating neutrophils and basophils during inflammation (Baggiolini et al., 1994; Clark-Lewis et al., 1995; Strieter et al., 2005).

In the inflammatory tumor microenvironment, macrophages, myeloid derived suppressor cells (MDSC), endothelial cells and stromal fibroblasts secrete CXCL1, which activates the NF-kB pathway in CXCR2^{+}BC cells (Wang et al., 2018) and promotes tumor growth, while contributing to local immunosuppression. CXCL1, produced in the primary tumor, recruits MDSCs to form the pre-metastatic niche, sustaining homing, survival, and

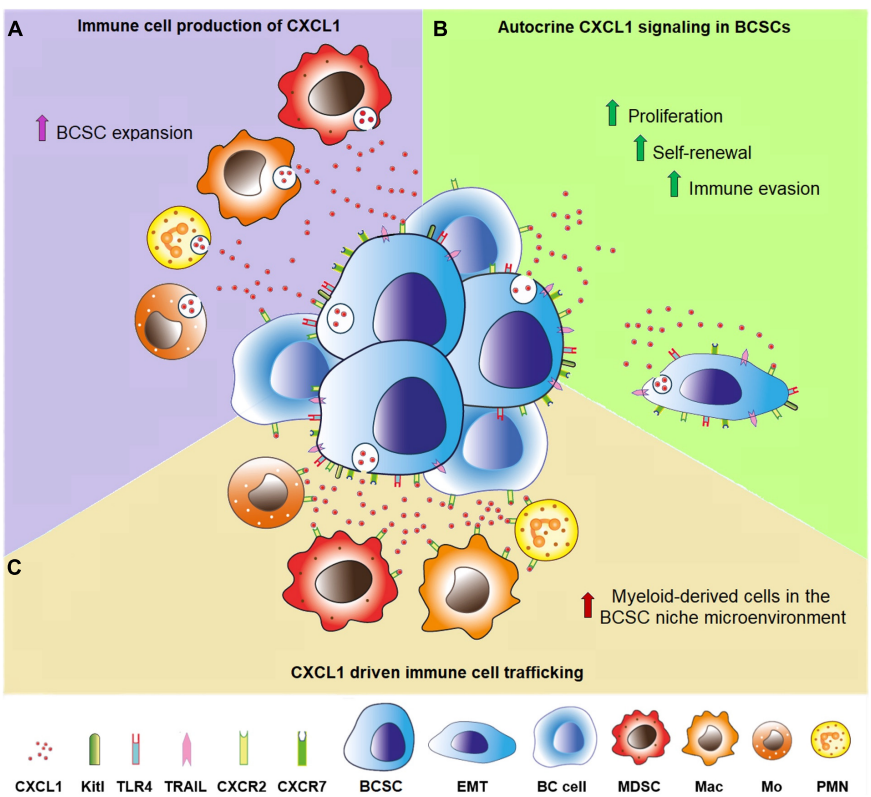

FIGURE 4 | CXCL1 production in the BCSC niche microenvironment. **(A)** Purple room. Polymorphonuclear neutrophils (PMN), myeloid-derived suppressor cells (MDSC), monocytes (Mo) and macrophages (Mac) produce and release CXCL1, which regulates the behavior of BCSCs and BC cells endowed with CXCR2. **(B)** Green room. CXCL1 secreted by BCSCs fosters, *via* autocrine and paracrine signaling, their proliferation and self-renewal, promotes their epithelial-mesenchimal transition (EMT) and immune evasion, by the induction of Kitl, TLR4, TRAIL, and regulates the behavior of the surrounding BC cells. **(C)** Brown room. CXCL1 secreted by BCSCs regulates immune cell trafficking, especially by recruiting polymorphonuclear neutrophils, monocytes, macrophages and myeloid-derived suppressor cells.

growth of circulating tumor cells in secondary organs leading to metastasis development (Wang D. et al., 2017).

Although bioinformatics reveal that only 3.67% of BCs, from different stages of disease, included in the PanCancer collection, express *CXCL1*, the high incidence of this tumor, estimated at 2,000,000 cases per year worldwide, means that ∼73,000 patients are expected to be diagnosed with CXCL1$^+$BC each year.

Our study adds a new piece to the puzzle of the BC microenvironment (**Figure 4**), by revealing that BCSCs can be a prominent source of CXCL1, particularly in TNBC, which is enriched in CSCs (Honeth et al., 2008; Ma et al., 2014) and more frequently expresses *CXCL1mRNA*, as evidenced by bioinformatic analyses of data obtained from the PanCancer database (Berger et al., 2018). Defined by the lack of ER, PR, and HER2 expression, TNBC represents about 15–20% of invasive BCs and is associated with high risk of metastasis and chemotherapy resistance (Dai et al., 2015). *CXCL1* expression likely contributes to this malignant phenotype, since 69% of patients bearing CXCL1$^+$BCs, were diagnosed as TNBCs.

In addition to the constitutive production by BCSCs, and possibly other BC cells and stromal mesenchymal stem cells (Wang Y. et al., 2017), CXCL1 can be dynamically induced, during tumor progression, in macrophages, MDSCs, granulocytes, endothelium and fibroblasts, by a variety of stimuli such as IL1β, TNFα (Wen et al., 1989; Acharyya et al., 2012), IL6 (Roy et al., 2012), PGE2 (Wang et al., 2006), adipokines (Wang et al., 2020), and TLR3/4 (Zhao et al., 2014).

In BCSCs, CXCL1 strengthens its own production and dramatically boosts *SPP1/OPN* and *ACKR3/CXCR7* expression. *Secreted Phosphoprotein 1 (SPP1)* gene encodes for *Osteopontin (OPN)*, a sialic acid rich, chemokine-like, matricellular phosphoglycoprotein, with well-defined roles in cell-matrix interaction, inflammatory responses, angiogenesis, and tumor metastasis (Shevde and Samant, 2014). OPN regulates the expression of genes leading to multiple signal transduction events associated with BC growth and progression (Cook et al., 2005). *ACKR3* gene encodes for *CXCR7,* the receptor for CXCL11 and CXCL12/SDF1, that promotes cell proliferation and invasive migration (Miao et al., 2007; Stacer et al., 2016), and has proven to be crucial for BCSC tumorigenicity and maintenance of stemness properties (Tang et al., 2016).

The considerable CXCL1-induced up-regulation of *TLR4*, *TNFSF10/TRAIL*, and *KITLG* expression along with *MICA/MHCI* down-regulation, enable tumor evasion from immune surveillance.

TLR4 is a pattern recognition receptor (PRR) family member that confers the ability to "sense" damage signals, and activates innate immunity, that can amplify the tumor-associated inflammation (Medzhitov, 2001). Activated TLR signals on cancer cells promote their migration and induce immunosuppressive cytokines and apoptosis resistance (Sato et al., 2009; Yang et al., 2010). Invasiveness and angiogenetic potential of BC cells are supported by TLR4-mediated signaling pathways (Ahmed et al., 2013; Yang et al., 2014), leading to pro-tumoral effects, which may be TP53 dependent (Haricharan and Brown, 2015).

TNFSF10, also known as Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that triggers apoptosis by binding to death receptors, DR4 and DR5 (Falschlehner et al., 2007). While TRAIL expression on activated NK and T cells increases their cytotoxicity, CXCL1 induction of TRAIL on BCSCs can turn them into apoptosis inducers, which suppress neighbor cancer cells (Griffith et al., 2000; Koyama et al., 2002; Papageorgiou et al., 2004), but also T cell activation and proliferation, favoring tumor immune evasion (Inoue et al., 2002).

Expression of KIT Ligand, encoded by the *KITLG* gene, on BCSCs can lead to intratumoral recruitment of immunosuppressive KIT$^+$CD11b$^+$cells. Blocking of the KIT Ligand/KIT axis has been demonstrated to slow-down BC progression and metastasis (Kuonen et al., 2012).

MHC class I chain related-protein A (MICA) is a natural killer group 2D (NKG2D) ligand that triggers NK and Vδ1 γδ T cells and co-stimulates CD8αβ$^+$T cells (Groh et al., 1998; Bauer et al., 1999). Reduced BCSC expression of MICA/MHCI is expected to weaken the cytolytic ability of effector cells and promote immune escape.

The tumor progression program triggered by CXCL1 in BCSCs also includes the expression of inflammatory mediators, proteases and growth factors, specifically, *IL1*β, *CCL18*, *CSF2, MMP14, HGF*, and *IGF1*. *IL1*β is involved in multiple aspects of tumor initiation and progression, and has shown to promote metastatic colonization of BCSCs to the bone (Mantovani et al., 2018; Eyre et al., 2019). CCL18 attracts naïve T cells, T regulatory cells, Th2 cells and immature dendritic cells (DC) (Adema et al., 1997; Chenivesse et al., 2012), and has been demonstrated to promote BC cell invasiveness and adherence to the extracellular matrix (Chen et al., 2011). CSF2/GM-CSF, produced by BC cells, activates plasmacytoid DCs leading to a regulatory Th2 response by naive CD4$^+$T cells, which is associated with aggressive BC subtypes (Ghirelli et al., 2015). MMP14 sustains cancer cell trafficking through the extracellular matrix ECM (Rowe and Weiss, 2009), and strengthens BCSC ability in anchorage-independent growth, tumor initiation, invasion, and migration under hypoxic nutrient-deprived conditions (Hillebrand et al., 2019). Both HGF and IGF1 activated signaling pathways lead to BC cell proliferation, migration and invasion, and are critically involved in the induction/maintenance of EMT and cell stemness, which are fundamental in metastatic spread and resistance to anti-cancer treatments (Malaguarnera and Belfiore, 2014; Christopoulos et al., 2015; Owusu et al., 2017). Therefore, the EMT program activated by CXCL1 in BCSCs, and revealed by the loss of E-Cadherin and gain of a strong nuclear expression of TWIST1, likely results from a complex network of signaling pathways triggered by secondary mediators, that ultimately overwhelm the effects expected by CXCL1-dependent TWIST2 and SNAI2 down-regulation.

Intriguingly, while CXCL1 induces, by hundreds and thousands of times, tumor-promoting and immunosuppressive factors, it also promotes, though to a lesser extent, the expression of *IL15, CXCL9, CXCL10, CXCL11*, which can lead to T and NK

cell recruitment and anti-tumor responses (Tagaya et al., 1996; Palacios-Arreola et al., 2014), and down-regulates *CCL2, CCL28, IDO, PTGS2, TNF* and *CXCR4*, that may inhibit inflammation and cancer cell migration (Müller et al., 2001; Ali and Lazennec, 2007; Howe, 2007). These findings reveal the unprecedented, apparently, dual role of CXCL1 in shaping the immunobiology of BCSCs, since it elicits a range of immunity genes with heterogeneous and opposing functions, including both pro- and anti-tumor mediators. Yet, the prominent expression of the former could explain why CXCL1 expression is associated with tumor progression (Divella et al., 2013; Zou et al., 2014) and with the highly malignant TNBC subtype.

The considerable CXCL1-dependent inhibition of the expression of CCL2 and CCL28, also endowed with an immunostimulating, but also pro-tumoral effect (Mohan et al., 2017; Yoshimura, 2018), emphasizes the critical role of the final equilibrium among the multiple microenvironmental signals in driving BCSC fate and tumor behavior.

Interestingly, independently of the molecular subtype, in BC the strong correlation between the expression of *CXCL1* and a range of pro-angiogenic and tumor promoting genes, including *CXCL3, CXCL2, CXCL6, CXCL5, FGFBP1* (Tassi et al., 2001; Strieter et al., 2005; Zheng et al., 2009), *BCL11A, PI3, B3GNT5, BBOX1* (Potapenko et al., 2010, 2015; Khaled et al., 2015; Pascual and Turner, 2019; Liao et al., 2020), and *PTX3* (Thomas et al., 2017; Zhang et al., 2020), suggests that CXCL1 regulated immunity genes are part of a wider signaling network that fuels BC progression.

Targeting CXCL1 signaling cascade, and closely associated pro-tumoral cues, could be a valuable strategy to restrain BCSC compartment and improve the efficacy of modern immunotherapeutic approaches to aggressive BCs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the "G. d'Annunzio" University and Local Health Authority of Chieti, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EDC conceived the study. SLC, LDA, CF, and PL performed the experiments, collected, and assembled the data. EDC, SLC, LDA, and CS performed data analyses. MT and GS provided cell lines for the study. EDC interpreted the data and wrote the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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Strategy for Leukemia Treatment Targeting SHP-1,2 and SHIP

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Protein tyrosine phosphatases (PTPs) are modulators of cellular functions such as differentiation, metabolism, migration, and survival. PTPs antagonize tyrosine kinases by removing phosphate moieties from molecular signaling residues, thus inhibiting signal transduction. Two PTPs, SHP-1 and SHP-2 (SH2 domain-containing phosphatases 1 and 2, respectively) and another inhibitory phosphatase, SH2 domain-containing inositol phosphatase (SHIP), are essential for cell function, which is reflected in the defective phenotype of mutant mice. Interestingly, SHP-1, SHP-2, and SHIP mutations are identified in many cases of human leukemia. However, the impact of these phosphatases and their mutations regarding the onset and progression of leukemia is controversial. The ambiguity of the role of these phosphatases imposes challenges on the development of targeting therapies for leukemia. This fundamental problem, confronted by the expanding investigational field of leukemia, will be addressed in this review, which will include a discussion of the molecular mechanisms of SHP-1, SHP-2, and SHIP in normal hematopoiesis and their role in leukemia. Clinical development of leukemic therapies achieved by targeting these phosphatases will be addressed as well.

Keywords: SHP-1, SHP-2, SHIP, leukemia, AML, PTP inhibitor, signaling pathway, thrapeutic target

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INTRODUCTION

SH2 domain-containing phosphatase 1 (SHP-1), encoded by the *PTPN6* gene, is expressed mainly in hemopoietic cellular systems. SHP-1, with a molecular weight of 68 kDa, is made up of three domains: the N-terminal Src homology-2 (SH2) domain, the C-terminal SH2 domain, and the C-terminal catalytic Protein tyrosine phosphatase (PTP) domain (**Figure 1A**; Lorenz, 2009). Two tyrosine residues at the C-terminus of SHP-1 (Y536 and Y564) are phosphorylated by various stimuli. The phosphorylation of tyrosine residues modifies the function and activity of SHP-1 depending on the property of the stimulus. SHP-1 exists in an auto-inhibited conformation (Yang et al., 2003). The N-SH2 domain binds tightly to the PTP domain and blocks substrate access to the catalytic domain, thus keeping the enzyme in its inactive conformation. On the contrary, when the phospho-peptide is bound to the C-SH2 domain, the N-terminal SH2 domain is released from the PTP domain, which relieves this autoinhibition and catalytically activates the enzyme (**Figure 1B**). The "moth-eaten" mice demonstrated a deficiency of SHP-1 and were affected by many hemopoietic disorders, including autoimmune hyperactivation of macrophages, which that suggested a defect in negative regulation (Shultz et al., 1997; Tsui et al., 2006). The SHP-1 gene has two promoters: the distal promoter, which is only present in epithelial cells, and the proximal promoter, which is active in both epithelial and hemopoietic cells. Therefore, under normal conditions, expression levels of SHP-1 vary between epithelial and hematopoietic cells. This

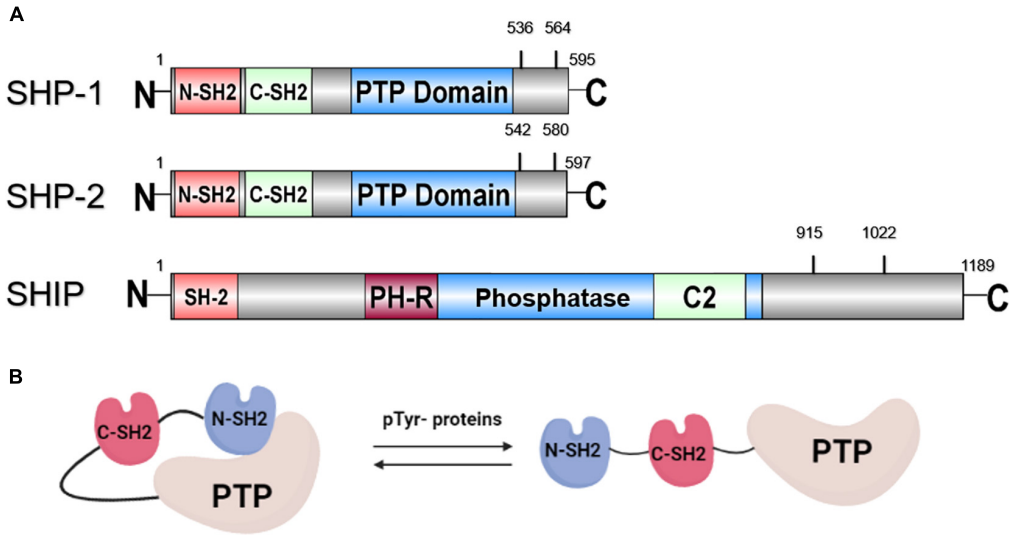

FIGURE 1 | (A) Schematic representation of the structure of SHP-1, SHP-2, and SHIP with tyrosine phosphorylation sites. **(B)** Structure of SHP protein in auto-inhibited and activated conformation.

concept is supported by an aberrant level of SHP-1 in cancers (Tsui et al., 2006). In hematopoietic cancers, such as myeloma, promoter methylation inhibited SHP-1 expression. Conversely, SHP-1 has been found to be overexpressed in epithelial cancer, such as breast cancer (Tsui et al., 2006).

Next, SH2 domain-containing phosphatase 2 (SHP-2), encoded by the *PTPN11* gene, with a molecular weight of 68 kDa, is another member of the PTP family. SHP-2 shares a structure similar to SHP-1: both are composed of two SH2 domains at the N-terminus followed by a PTP domain and two tyrosine residues for phosphorylation by various stimuli at the C-terminus (**Figure 1A**). Comparable to SHP-1, SHP-2 is also auto-inhibited by its N-SH2 domain (**Figure 1B**). Activating SHP-2 mutations have been detected in many cancers, such as melanoma, acute myeloid leukemia (AML), lung cancer, colorectal cancer, etc. (Bentires-Alj et al., 2004; Miyamoto et al., 2008). This result suggests that *PTPN11* may be a proto-oncogene. Despite its oncogenic potential, SHP-2 plays a role in tissue development. This is illustrated as SHP-2 expression was found to be present in neurons during brain development. A loss-of-function mutation of SHP-2 in mice led to inhibition of sympathetic neurite outgrowth (Chen et al., 2002). Furthermore, SHP-2 deficiency is related to autoimmune disorders with varying defectives in organ development, such as Noonan syndrome and Leopard syndrome. *PTPN11* mutations were detected in 50 and 80% cases, respectively, in patients with these two syndromes (**Table 1**) (Shen et al., 2020).

Lastly, SH2 domain-containing inositol phosphatase (SHIP) is a 145 kDa sized protein that is encoded by the *INPP5* (inositol polyphosphate-5-phosphatase) gene. SHIP interferes with the PI3K (phosphatidylinositol 3-kinase)/Akt pathway by dephosphorylating the P13K product PI(3,4,5)P3 to PI(3,4)P2 (Blunt and Ward, 2012). In the presence of SHIP, a controlled level of PI(3,4,5)P3 is produced, which activates downstream Akt and induces cell proliferation. Alternate transcriptional splice variants of SHIP encoding different isoforms of the protein have been characterized. One isoform of SHIP, SHIP-1, is expressed primarily in the hematopoietic system and suppresses the proliferation of hematopoietic progenitor cells (Fu et al., 2019). As shown in **Figure 1A**, the SH2 domain of SHIP is not involved in autoinhibition, but instead is responsible for membrane translocation and recruitment to upstream kinases. SHIP is activated by phosphorylation of Ser440 in the phosphatase domain by PKA (cyclic AMP-dependent protein kinase) (Zhang et al., 2009), or it is allosterically activated by binding of PI(3,4)P2 to the C2 domain (Ong et al., 2007). The dephosphorylation activity of SHIP to produce PI (3,4,5)P3 is carried out by both the Pleckstrin homology-related (PH-R) and phosphatase domain (Pauls and Marshall, 2017). In addition, the tyrosine residues Y915 and Y1022, located on the C-terminal region of SHIP, regulate the binding of the adapter protein and the activation of subsequent signaling (Lamkin et al., 1997). Furthermore, deficient SHIP led to inflammatory disorders *in vivo* since SHIP serves to suppress production of inflammatory cytokine by innate immune cells, such as neutrophils and basophils (Kerr, 2011). Severe inflammatory lung disease has been observed in SHIP knockout mice (Lo et al., 2019). Crohn's disease-like intestinal inflammation and fibrosis were revealed in SHIP knockout mice and irradiated wild-type mice reconstituted with splenocytes from SHIP knockout mice (Kerr, 2011; Maxwell et al., 2011; Lo et al., 2019) as well (**Table 1**).

In this review, we will interpret SHP-1, SHP-2, and SHIP in terms of their association with cell surface receptors, downstream signaling pathways, and roles in hematopoiesis. We will also focus on how SHP-1, SHP-2, and SHIP are involved in hematopoietic malignant diseases, particularly in AML, and the current development of leukemic therapy involving manipulation of their expression.

CELL SURFACE RECEPTORS AND DOWNSTREAM SIGNALING PATHWAYS

SH2 domain-containing phosphatase 1, SHP-2, and SHIP are recruited by and bound to multiple receptors on the cell surface through their SH2 domains. In myeloid cells, SHP-1 is associated with growth factor receptors, such as c-Kit (tyrosine protein kinase, CD117), and several immunoreceptor tyrosine-based inhibitory motif (ITIM) containing receptors, including paired immunoglobulin-like receptor B (PIR-B), leukocyte immunoglobulin-like receptor 1 (LIR-1), and leukocyte immunoglobulin-like receptor 2 (LIR-2; Zhang et al., 2000). In hematopoietic stem cells (HSCs), SHP-2 is activated by Kit, a mast/stem cell growth factor receptor (Kan et al., 2018). In addition, SHP-2 is expressed in stem cells of other tissues, whereas SHP-1 expression is mainly in hematopoietic cells. For example, in neural stem cells, SHP-2 is activated by fibroblast growth factor (FGF) receptor, Vascular endothelial growth factor (VEGF) receptor, and other receptor tyrosine kinases. SHP-2 is also present in mesenchymal stem/progenitor cells and satellite cells, where its activity is regulated by various tyrosine kinases on the cell membrane. SHIP, through its SH2 domain, binds to ITIM receptors such as FcγRIIB and killer cell immunoglobulin-like receptor (KIR) as well as immunoreceptor tyrosine-based activation motif (ITAM) receptors in hematopoietic cells (Dempke et al., 2018).

SH2 domain-containing phosphatase 1 negatively regulates hematopoietic cell proliferation through many intracellular signals. SHP-1 inhibits cytokine receptors, including Epo-R, IL3-R, and IL-2R, as well as growth factor receptors with intrinsic tyrosine kinase activity, such as CSF-1 and GM-CSF that reduce the proliferation of macrophages and granulocytes (Zhang et al., 2000). SHP-1 also suppresses the activation of the growth factor-induced signaling pathway of PI3K/Akt and nuclear factor-kappa B (NF-κB). SHP-1 regulates extracellular signal-related kinases (ERKs) and c-Jun-amino terminal kinases (JNKs) in a positive or negative manner. In addition, SHP-1 binds to erythropoietin (EPO) receptor and blocks subsequent activation of Janus kinase 2 (Jak2), resulting in downregulation of signal transducer and activator of transcription (STAT) (Chong and Maiese, 2007; Dempke et al., 2018). Most of the downstream factors of SHP-1 are also modulated by SHP-2, however, they are regulated in different ways. Upon induction by growth factor, SHP-2 promotes the PI3K/Akt pathway, ERK, and NF-κB through its association with signal regulatory protein α1 (SIRPα1) or Grb2-associated binder-1 (Gab1). Depending on the circumstance, SHP-2 can negatively or positively regulate STAT activation through Jak2 and JNK activation via Ras (Chong and Maiese, 2007; Dempke et al., 2018). Moreover, SHP-2 directly binds to and activates receptors for many growth factors and cytokines, such as IL-3 and GM-CSF (Tamir et al., 2000). Finally, SHIP dephosphorylates the product of its upstream kinase, P13K, and therefore has an inhibitory effect on downstream factors of P13K, including Akt, ERK, and NF-κB. SHIP also affects other kinases, including Burton tyrosine kinase (BTK) and phospholipase-C gamma (PLC-γ), and various transcription factors, such as nuclear factor of activated T cells (NFAT) (Blunt and Ward, 2012; Dempke et al., 2018).

TABLE 1 | Hematopoietic and non-hematopoietic abnormalities *in vivo* mediated by dysregulated SHP-1, SHP-2, and SHIP.

	Hematopoietic	Non-hematopoietic
SHP-1	Impaired stemness of HSCs Macrophage autoimmune hyperactivation	Fatal pneumonitis
SHP-2	BM aplasia and lethality Defective HSCs in homing, self-renewal, and survival	Compromised sympathetic neurite outgrowth Hepatic inflammation and hepatocyte mortality Noonan syndrome and Leopard syndrome
SHIP	Impaired stemness of HSCs Increased mobilization of BM cells	Intestinal and lung inflammation

FUNCTION IN NORMAL HEMATOPOIESIS

Several receptor tyrosine kinases (RTK) have been identified in hematopoietic cells and are critical mediators of cell signaling (Reilly, 2003). RTKs respond to chemokines, other cytokines, and numerous ligands. Ligand-induced phosphorylation of the RTK tyrosine residues invokes physiological actions, including cell growth, differentiation, metabolism, migration, and survival (Ruvolo, 2019). Phosphatases serve to regulate the actions of RTK through dephosphorylation of tyrosine residues and are required for maintenance of hematopoiesis in the hematopoietic microenvironment. Aberrant tyrosine phosphorylation induced by an imbalance between the activity of RTKs and phosphatases, such as SHP-1, SHP-2, and SHIP, can lead to abnormal cell signaling and hematopoietic defects (**Table 1**).

SHP-1 and its isoforms are widely expressed in all hematopoietic lineages and maturation stages. SHP-1 inhibits RTK pathways activated by various growth factors and cytokines (Neel et al., 2003; Lorenz, 2009; Abram and Lowell, 2017). Therefore, SHP-1 negatively affects hematopoietic differentiation of embryonic stem cells (Paling and Welham, 2005). The expression of dominant-negative SHP-1 in embryonic stem cells increased the formation of myeloid colonies during differentiation and was reduced by the expression of wild-type SHP-1. Mice lacking SHP-1 exhibited a plethora of perturbations in their hematopoietic and immune systems. Defective myelopoiesis has been found in mice with the SHP-1 inactivation mutation. SHP-1 mutant mice demonstrated an enlarged neutrophil and monocyte population in peripheral blood and increased macrophage proliferation, contributing to the development of fatal pneumonitis (**Table 1**). In lymphocytes, SHP-1 stimulated cell growth and suppressed their oncogenic capacity (Tibaldi et al., 2011). Mice with specific deletion of SHP-1 in B cells or dendritic cells exhibited increased differentiation and autoimmunity of B-1a and Th1 cells (Pao et al., 2007;

Kaneko et al., 2012). Loss of SHP-1 expression in tumor-specific T cells or natural killer cells promoted immune response and antitumor function in a mouse model of disseminated leukemia (Stromnes et al., 2012; Viant et al., 2014). Our previous *in vitro* study reported that HSCs from SHP-1 knockout mice have attenuated quiescence and impaired long-term self-renewal (Jiang et al., 2018). Therefore, we identified SHP-1 as a regulatory PTP to maintain the microenvironmental homeostasis of HSCs.

SH2 domain-containing phosphatase 2 is also required to maintain hematopoietic growth and homeostasis. SHP-2 contributes to cytokine-mediated signaling through several tyrosine kinases, and therefore loss of SHP-2 results in many dysfunctions in hematopoiesis (Nabinger and Chan, 2012; Rehman et al., 2019). The absence of the *PTPN11* gene in murine hematopoietic cells has been shown to result in BM (bone marrow) aplasia and lethality *in vivo* (Chan et al., 2011). Mice with *PTPN11* deletion also exhibited a rapid loss of functional HSCs and myeloid progenitors as well as reduced cellularity in the BM, spleen, and peripheral blood (Chan et al., 2011; Zhu et al., 2011). *PTPN11* knockout led to aberrant proliferation and promoted apoptosis of HSCs and progenitor cells *in vivo*, resulting in defects in homing, self-renewal, and survival (Zhu et al., 2011). Therefore, HSCs with deficient SHP-2 cannot reconstitute peripheral blood in lethally irradiated mouse recipients. SHP-2 is required not only for mouse hematopoiesis, but it is also necessary for human hematopoiesis. Upon SHP-2 knockout in human $CD34^+$ cord blood cells, cell proliferation and colony formation decreased (Li et al., 2011). HSCs of a patient with a point mutation in *PTPN11*, eliminating the phosphatase activity of SHP-2 in human $CD34^+$ cord blood cells, lost the ability to form colonies (Broxmeyer et al., 2013). These findings reveal a critical role for SHP-2 in the maintenance of functional HSCs and progenitors.

SH2 domain-containing inositol phosphatase is involved in the regulation of HSC proliferation and self-renewal. SHIP knockout induced an increase in the number of HSCs in the BM, spleen, and peripheral blood *in vivo* (Desponts et al., 2006). Mice with SHIP deletion demonstrated increased HSC cycling that was originally quiescent, which is justified by an increase in 5-FU sensitivity (Helgason et al., 2003). Although HSCs in SHIP knockout mice showed increased proliferation and reduced apoptosis, they also exhibited decreased homing ability to the BM after transplantation due to lower expression of CXCR4 and VCAM-1 receptors (Desponts et al., 2006). HSCs with SHIP knockout also revealed less self-renewal behavior *in vivo* as the expansion level of competitive repopulating cells (CRU) in the absence of SHIP was significantly lower than wild-type CRUs following transplantation (Helgason et al., 2003). Additionally, SHIP affects HSC adhesion in BM niches, and mobilization of primitive BM cells was expanded in SHIP knockout mice due to increased chemokine responsiveness (Helgason et al., 2003). These data demonstrate that SHIP plays a negative regulatory role in HSC proliferation and survival, and that SHIP is important in the maintenance of primitive hematopoietic cell homeostasis and regeneration.

RELEVANCE TO HEMATOPOIETIC DISEASE AND LEUKEMIA

Dysregulation of SHP-1, SHP-2, and SHIP is associated with uncontrolled cell growth and metabolism, which results in the activation of multiple pro-oncogenic cascades and eventually leads to leukemia. In fact, mutations of these phosphatases are identified in a fair percentage of patients with leukemia, and abnormal levels of SHP-1, SHP-2, and SHIP have been detected in mouse models of leukemia (Dempke et al., 2018). This suggests that they are associated with leukemia development. Therefore, agents that modify the levels or activity of these phosphatases are currently investigated for potential leukemic therapies.

SHP-1

Many studies have identified suppressive effects of SHP-1 on leukemia. For example, promotor methylation leading to silencing of SHP-1 has been reported in 10% of AML cases (Johan et al., 2005). Reexpression of SHP-1 by 5-Azacytidine, a DNA methyltransferase inhibitor, led to increased apoptosis of MV4-11 cells through down-regulation of STAT3 (Al-Jamal et al., 2015). In adult T cell leukemia/lymphoma, the SHP-1 protein has been shown to dephosphorylate and inactivate Sirtuin-1 (SIRT1) that repairs DNA of leukemia cells through homologous recombination (Yu et al., 2018). Upregulation of SHP-1 in Jurkat cells resulted in increased DNA damage, a higher incidence of apoptosis, and reduced colony formation *in vitro*. On the contrary, other studies indicate that the presence of SHP-1 is associated with the development of leukemia. *In vivo* myeloproliferative diseases induced by FMS like tyrosine kinase 3-internal tandem duplications (FLT3-ITD), a mutation present in approximately 30% of AML patients, were compromised by deletion of SHP-1 (Reich et al., 2020). Furthermore, our group has demonstrated that LAIR1-mediated SHP-1 activation recruited CAMK1 as an autonomous phosphatase signal adapter for downstream activation of CREB in AML cells, which contributes to the self-renewal of AML cells (Kang et al., 2015).

Likely due to the undetermined role of SHP-1 in the pathogenesis of AML, treatments that alter the expression level of SHP-1 do not currently exist for AML patient therapy. From 2009 to 2018, sodium stibogluconate (SS), a SHP inhibitor with a potent inhibitory effect on SHP-1, underwent several clinical trials and was proven to treat Leishmaniasis (**Table 2**). On the other hand, the antileukemia effect of SS was reported *in vitro* in early 2002 (Pathak et al., 2002). This study showed that NB4, a human AML cell line, exhibited higher differentiation, with cell growth arrest in S phase, and increased apoptosis following SS treatment. SS has also been shown to inhibit *in vitro* growth and induced differentiation of HL-60 and U937 cells. However, no study has confirmed a significant SS-mediated anti-AML effect *in vivo*, and this may be due to various bodily microenvironments weakening the efficacy of the drug. In addition to the lack of *in vivo* studies of the SHP-1 inhibitor, the clinical trial of AML treatments involving alterations of SHP-1 activity does not show a significant result. An AML preclinical trial consisting of a therapy that combines azacytidine and

TABLE 2 | SHP and SHIP inhibitors with clinical trial or with tumor suppression effect *in vivo*.

	Name	IC50 or inhibition concentration	*In vivo* effect	Clinical trial time	Trial number/ phase	Disease	Status
SHP-1 inhibitor	Sodium stibogluconate	Inhibits 99% of SHP-1 and SHP-2 activity at 10 and 100 μg/mL.	Growth inhibition of inoculated Renca tumors in BALB/c mice (Fan et al., 2005)	2007 2010 2018 2009	NCT00498979/ Phase 1 NCT01067443/ Phase 2 NCT03129646/ Phase 3 NCT01661296/ Phase 4	Stage IV Melanoma Primary Visceral Leishmaniasis Visceral Leishmaniasis Cutaneous Leishmaniasis	Completed in Jan 2012; negative response (Dempke et al., 2018) Completed in Jan 2012; positive response (Wasunna et al., 2016) Completed in Dec 2020; no result posted Completed in Dec 2011; no result posted
	TPI-1	40 nM	Growth inhibition of B16 melanoma tumors (Kundu et al., 2010)	No clinical trial			
SHP-2 inhibitor	TNO-155	0.011 μM	Growth inhibition of xenograft KYSE-520 tumor (LaMarche et al., 2020)	2021 2020 2020 2020	NCT04699188/ Phase 1| Phase2 NCT04292119/ Phase 1| Phase2 NCT04330664/ Phase 1| Phase2 NCT04294160/ Phase 1	Pulmonary and colorectal cancer Lung cancer and anaplastic Lymphoma Advanced cancer, metastatic cancer, malignant neoplastic disease Braf v600 colorectal cancer	Recruiting; estimated completion in Mar 2022 Recruiting; estimated completion in Mar 2023 Recruiting; estimated completion in Oct 2022 Recruiting; estimated completion in Aug 2023
	JAB-3068	N/A	N/A	2018	NCT03518554/ Phase 1	Non-small cell lung cancer, head and neck cancer, esophageal cancer, other metastatic solid tumors	Recruiting; estimated completion in Jul 2021
	RMC-4630	N/A	N/A	2018 2019	NCT03634982/ Phase 1 NCT03989115/ Phase 1| Phase 2	Relapsed/refractory solid tumor Relapsed/refractory solid tumor	Recruiting; estimated completion in Oct 2021 Recruiting; estimated completion in Apr 2022
	RLY-1971	N/A	N/A	2020	NCT04252339/ Phase 1	Advanced or metastatic solid tumors	Recruiting; estimated completion in Apr 2022
	IACS-13909	15.7 nM	Growth suppression of xenograft KYSE-520 tumor and suppression of MV4-11 induced FLT3-ITD AML (Sun et al., 2020)	No clinical trial			
	SHP 394	23 nM	Reduced tumor volume and raised tumor regression in mice carrying Detroit-562 pharyngeal carcinoma cells (Sarver et al., 2019)	No clinical trial			
	SHP099	70 nM	Reduced tumor volume of KYSE520 xenografts, reduced number of circulating leukemia cells and reduced splenomegaly in mice with patient-derived FLT3-ITD AML (Chen et al., 2016)	No clinical trial			
SHIP inhibitor	3α-Aminocholestane	2.5 μM	Reduced multiple myeloma (MM) growth, reduced number of circulating cancer cells, and enhanced survival rate (Fuhler et al., 2012)	No clinical trial			

gemtuzumab ozogamicin (GO) aimed to enhance the cytotoxicity of the CD33 antibody against AML blasts through epigenetic modifications of *PTPN6* (Medeiros et al., 2018). Although 24% of participating patients obtained complete remission, an association between SHP-1 expression and clinical response was not found. The contradicting effects of SHP-1 in AML *in vitro* and the lack of significant findings *in vivo* indicate a lack of understanding of SHP-1 signaling pathways and mechanisms involving AML pathogenesis. This blind spot in knowledge must be explored more explicitly in order to better understand the therapeutic potential of targeting SHP-1 in AML.

SH2 domain-containing phosphatase 1 has also demonstrated oncogenic properties in other leukemias. From the *in vitro* assay of chronic lymphocytic leukemia (CLL), SHP-1 underwent differential phosphorylation and, resultantly, exhibited differential functions and cellular localizations (Tibaldi et al., 2017). SHP-1 with phospho-S591 supported aberrant Lyn-dependent tyrosine phosphorylation of proteins in the cytosol of CLL cells and eventually formed a network of anti-apoptotic signaling. In B cell acute lymphoblastic leukemia (B-ALL), inducible ablation of SHP-1 reduced proliferation and stemness and increased cell cycle arrest in murine B-ALL cells. *In vivo* deletion of SHP-1 also extended the latency of leukemia and improved the survival rate of mice (Chen Z. et al., 2015). In patients with acute leukemia (AL), the differential expression of SHP-1 and the six-cytokine signaling suppressor (SOCS6) has been detected. SHP-1 and SOCS6 mRNA levels tended to be higher among patients in AL remission than in newly diagnosed patients. Therefore, the expression of SHP-1 and SOCS6 is associated with favorable outcomes, suggesting an anticancer property in AL and potential targets for gene therapy (Liu et al., 2017).

A SHP-1 inhibitor specific for leukemia treatment has not yet been developed. SS, a SHP-1 antagonist that showed effective suppression on inoculated tumors *in vivo,* underwent two Phase I clinical trials for malignant melanoma (**Table 2**). However, the outcome was poor, demonstrated by no objective response, life-threatening events in 68% of patients, and side effects such as pancreatitis, BM suppression, and nausea (Dempke et al., 2018). The pessimistic results of these clinical trials further imply an elusive role of SHP-1 in tumor development, and additional is needed to elucidate the potential of SHP-1 as a drug target for tumor treatment.

SHP-2

SH2 domain-containing phosphatase 2 has been determined to have an oncogenic effect on cell proliferation and growth. Mutations in the N-SH2 domain of SHP-2 cause constitutive activation of the SHP-2 protein in the hematopoietic stem and progenitor compartment, resulting in the development of clinical leukemia. A *PTPN11* mutation was common in diagnosed patients with secondary AML (Makishima et al., 2017), relapsed pediatric AML (Farrar et al., 2016), and acute lymphoblastic leukemia (ALL; Oshima et al., 2016). The activating mutation of SHP-2 has been identified in 10% of AML cases (Dempke et al., 2018). The prevalence of *PTPN11* mutations has been found to be higher in patients over 60 years of age and associated with a poor prognosis (Tsai et al., 2016). Secondary AML patients with *PTPN11* mutations tended to progress rapidly and have lower overall survival (Makishima et al., 2017). The *PTPN11* mutation exists not only in older patients with AML but also in children with *de novo* AML. A study identified the substitution of trinucleotides at position 211–213 in 24 pediatric patients as well as other *PTPN11* mutations in children with Juvenile myelomonocytic leukemia (JMML) and myelodysplastic syndrome (MDS; Tartaglia et al., 2003). Interestingly, almost all of the mutations identified in these leukemias are located in the N-SH2 domain, which confirms the essential role of the N-SH2 domain in regulating SHP-2 activity and thus the growth and proliferation of hematopoietic cells. The SHP-2 protein also plays an oncogenic role in the development of AML from a molecular perspective. SHP-2 inhibition negatively regulates the activation of downstream factors in FLT3-ITD pathways, which was illustrated *in vitro* as SHP-2 inhibition led to proliferation of blast cells with FLT3-ITD. Mutation of the FLT3-ITD residue that recruits SHP-2 also reduced FLT3-ITD-induced myeloproliferative disease *in vivo* (Richine et al., 2016). Furthermore, SHP-2 is involved in the signaling pathway of leukocyte immunoglobulin-like receptor B4 (LILRB4), an immunoreceptor tyrosine-based inhibition motif-containing receptor (Deng et al., 2018). LILRB4 inhibited T cell proliferation and improved the infiltration of AML cells into organs both *in vitro* and *in vivo*, and these effects were reversed by knockout of SHP-2 in human AML cell lines.

In addition, the mixed-lineage leukemia (MLL) translocation, *MLL-AF10*, is known to occur in patients with a *G503A* mutation in *PTPN11*. With the inclusion of both the *MLL-AF10* and *G503A* mutations in a mouse model, there was an accelerated rate of disease development compared to the control group (Fu et al., 2017). *PTPN11 E76K* mutation resulted in increased proliferation of mouse HSCs *in vitro*. Transplantation of HSCs co-expressing *PTPN11 E76K* and *MLL-AF9* fusion oncogenes induced a lower survival rate and more severe AML phenotype *in vivo*, such as splenomegaly (Chen L. et al., 2015). Given that SHP-2 imposes an oncogenic effect on hematopoietic cells and that its mutation has been identified in multiple malignancies, efforts have been devoted to discovering inhibitors of SHP-2 and examining their effects against cancers. For example, IACS-13909 (Sun et al., 2020) and SHP099 (Chen et al., 2016) inhibited the proliferation of human AML cell lines and reduced tumor burden derived from xenograft AML cells *in vivo* (**Table 2**). Importantly, there are several SHP-2 inhibitors, including TNO-155 (LaMarche et al., 2020; Liu et al., 2021), currently in clinical trials for multiple solid tumors and cancers that will be completed in spring 2022. Although results have yet to be announced, the potential of SHP-2 inhibitors as a treatment option for AML is expected given the strong oncogenicity of SHP-2 and its entanglement in AML oncogenic pathways.

SHIP

SH2 domain-containing inositol phosphatase 1, a SHIP isoform, has been proposed to play a regulatory role in the development of AML. This is because approximately 50–70% of patients

with AML demonstrated constitutive activation of the SHIP-1-regulated P13K/Akt pathway, and approximately 3% displayed a missense mutation in *INPP5D* (Täger et al., 2017). A higher level of SHIP-1 correlated with prolonged overall survival among 290 patients with AML as well. Furthermore, overexpression of SHIP-1 reduced the proliferation of $CD34^+$ cells in AML patients (Metzner et al., 2009). Studies based on AML cells and the AML mouse model also supported that SHIP-1 acts as an AML suppressor. SHIP-1 transfection to THP-1, a human AML cell line lacking endogenous expression of SHIP-1, resulted in a higher proportion of apoptotic cells. Moreover, SHIP-1 was significantly down-regulated in patients with late-stage MDS, and SHIP-1 expression reduced the number of colonies formed by primary patient myeloid leukemia blasts (Lee et al., 2012). Furthermore, SHIP-1 overexpression extended the life span of the NSG mice model transplanted with human AML cells (Täger et al., 2017).

Considering the role of SHIP-1 as an AML suppressor, factors that suppress SHIP-1 have been investigated. SHIP-1 has been demonstrated to be targeted and down-regulated by miR-155. Compared to normal $CD34^+$ cells, miR-155 expression was significantly higher in cells isolated from patients with late-stage MDS (Lee et al., 2012). Therefore, it is suggested that inhibition of miR-155 can restore suppression of AML by SHIP-1. One study identified Silvestrol as a miR-155 inhibitor and found it to down-regulate miR-155 levels in MV4-11 cells as well as inhibit the growth of MV4-11 and THP-1 cells (Brooks et al., 2010). Silvestrol treatment also improved the survival rate of AML mice *in vivo* and increased *in vitro* apoptosis of primary blasts from AML patients. Another study showed that MLN4924 decreased the miR-155 level in AML cells (Khalife et al., 2015). Treatment of MV4-11 cells with MLN4924 resulted in a reduced level of miR-155, upregulation of SHIP-1, suppression of the P13K/Akt pathway, and monocytic differentiation. MLN4924 also reduced the viability of MV4-11 cells and blasts from AML patients. Furthermore, the administration of MLN4924 to an AML mouse model prolonged the survival period, although all treated mice eventually died.

Based on these studies, SHIP-1 is recognized as an AML suppressor via attenuation of the P13K/Akt pathway and reduction of subsequent cell proliferation. However, some studies exhibit dual roles of SHIP-1. In some cases, SHIP-1 amplified survival or proliferative signals in neoplastic cells. The enzyme product of SHIP-1, PI(3,4) P2, has been shown to have higher affinity for Akt and led to more potent activation of the Akt pathway *in vitro* (Brooks et al., 2010). Treatment of the KG-1 AML cell line with 3α-aminocholestane (3-AC), a SHIP-1 inhibitor, resulted in reduced cell viability *in vitro*. Furthermore, SHIP-1 expression in patients with AML are largely variable and are not inversely associated with activated Akt level. Therefore, the role of SHIP-1 in the P13K/Akt pathway and AML cell proliferation needs to be clarified further. Considering that the antileukemic effect of 3-AC has not been tested in an AML mouse model, effects of SHIP-1 inhibition *in vivo* may provide more insight regarding the role and function of the phosphatase in AML.

CONCLUDING REMARKS AND PERSPECTIVES

The identification of SHP-1, SHP-2, and SHIP and their downstream signaling in hematopoietic cells provides new clues to the development and treatment of leukemia. The studies reviewed here demonstrate that an abnormal level of these phosphatases, instead of altered signaling pathways, is associated with aberrant proliferation of hematopoietic cells and leukemia development. Abnormal expression levels derive either from genetic mutations, such as a loss-of-function mutation of SHP-1 or a gain-of-function SHP-2 mutation, or dysregulated regulatory signals, such as upregulated miR-155 levels that inhibit transcription of the SHIP-1 gene in AML patients. Therefore, SHP-1, SHP-2, and SHIP can be potential targets for anti-leukemia therapy. However, scientists are still not in uniform agreement on the true role of these phosphatases in leukemia, which imposes challenges on the development of leukemia-treating reagents that manipulate expression levels of these proteins. Until now, most studies have supported the oncogenic property of SHP-2 and the tumor suppressor property of SHIP, but the effects of SHP-1 on leukemia are still controversial. Consequently, future studies must focus on confirming the effects of SHP-1 and its downstream pathways in different types of leukemia. In regard to SHP-2, it is necessary to refine its antagonists to suppress leukemia development *in vivo* for clinical trials. Furthermore, SHP-2 inhibitors, which have already undergone clinical trials to treat other tumors and cancers, need to be evaluated for their efficacy in the treatment of leukemia. Finally, miR-155 inhibitors, which upregulate SHIP-1, exhibit suppressive effects in AML *in vivo* and should also be refined for AML clinical trials.

AUTHOR CONTRIBUTIONS

FH and CW wrote the manuscript and prepared figures. CS provided critical comments and proofreading the manuscript. MC provided comments and joined the discussion. XK organized the structure of the manuscript, provided critical comments and proofreading the manuscript.

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REVIEW
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p130Cas/*BCAR1* and p140Cap/*SRCIN1* Adaptors: The Yin Yang in Breast Cancer?

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p130Cas/*BCAR1* is an adaptor protein devoid of any enzymatic or transcriptional activity, whose modular structure with various binding motifs, allows the formation of multi-protein signaling complexes. This results in the induction and/or maintenance of signaling pathways with pleiotropic effects on cell motility, cell adhesion, cytoskeleton remodeling, invasion, survival, and proliferation. Deregulation of p130Cas/*BCAR1* adaptor protein has been extensively demonstrated in a variety of human cancers in which overexpression of p130Cas/BCAR1 correlates with increased malignancy. p140Cap (p130Cas associated protein), encoded by the *SRCIN1* gene, has been discovered by affinity chromatography and mass spectrometry analysis of putative interactors of p130Cas. It came out that p140Cap associates with p130Cas not directly but through its interaction with the Src Kinase. p140Cap is highly expressed in neurons and to a lesser extent in epithelial tissues such as the mammary gland. Strikingly, *in vivo* and *in vitro* analysis identified its tumor suppressive role in breast cancer and in neuroblastoma, showing an inverse correlation between p140Cap expression in tumors and tumor progression. In this review, a synopsis of 15 years of research on the role of p130Cas/*BCAR1* and p140Cap/*SRCIN1* in breast cancer will be presented.

Keywords: breast cancer, cell signaling, mouse model, adaptor protein, epithelial – mesenchymal – transition, protein interactome

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P130CAS/*BCAR1* GENE AND PROTEIN

The *BCAR1* gene, the human ortholog of p130Cas, was independently identified as Breast Cancer Antiestrogen Resistance 1. *BCAR1* is localized on chromosome 16 on region q, on the negative strand and has seven exons, with multiple alternative first exons. p130Cas/BCAR1 protein (p130 Crk-associated substrate) is a member of the Cas (Crk-associated substrate) family that consists of five distinct adaptor proteins: p130Cas/BCAR1, Nedd9 (Neural precursor cell expressed, developmentally downregulated 9), Human enhancer of filamentation-1 (HEF-1 or CAs-L), EFS (Embryonal Fyn-associated substrate), and CASS4 (Cas scaffolding protein family member 4). All the family proteins are characterized by a similar modular structure with several interaction domains and multiple tyrosine and serine phosphorylation motifs (Defilippi et al., 2006; Cabodi et al., 2010; Tikhmyanova et al., 2010). The structural features of the p130Cas/BCAR1 protein includes an amino (N)-terminal Src-homology 3 (SH3) domain, an adjacent large substrate-binding domain containing 15 repetitions of the YxxP motif, a main site of tyrosine phosphorylation on

the p130Cas/*BCAR1* molecule that provides SH2-binding sites, a proline and serine rich region, a C-terminal part composed by binding sites for the SH2 and SH3 domains of Src (YDYVHL and RPLPSPP, respectively) and a highly conserved four-helix bundle [Focal Adhesion Targeting (FAT) domain] (Defilippi et al., 2006; Cabodi et al., 2010; Tikhmyanova et al., 2010).

p130Cas serves as an adaptor protein in multiprotein complexes, integrating signals from the extracellular matrix environment, soluble ligands, and mechanical stress. Tyrosine phosphorylation of the substrate-binding domain by Src-family kinases enables its interaction with Crk, Crk-L, CRKII, and Nck adaptors (Schlaepfer et al., 1997). The assembly of p130Cas-Crk-dedicator of cytokinesis 1 (DOCK1; also known as DOCK180) complex allows efficient recruitment and localization of small GTPase RAC1 at the membrane. This event induces actin cytoskeleton remodeling, pseudopodia extension and focal adhesion turnover resulting in increased cell migration (Cabodi et al., 2010). The extent of this phosphorylation is regulated by the mechanical forces acting on the cell that stretch the substrate-binding domain and expose hidden tyrosines to phosphorylation (Sawada et al., 2006). The SH3 domain mediates the interaction of p130Cas with polyproline motifs of FAK, PYK2/RAFTK, and FRNK kinases, PTP1B, PTP-PEST phosphatases, and other proteins (C3G, CMS, CIZ, and Vinculin) that regulate the recruitment and/or activation of Src-family kinases (Polte and Hanks, 1995; Liu et al., 1996; Garton et al., 1997; Li and Earp, 1997; Kirsch et al., 1998, 1999; Nakamoto et al., 2000; Janostiak et al., 2014). The C-terminal domain of p130Cas is crucial to allow the binding of Src-family kinases and the targeting of p130Cas to focal adhesions (Nakamoto et al., 1997). Moreover, previous studies demonstrated the contribution of the C-terminal domain to dimer formation and association with BCAR3, NEDD9, and p140Cap (Nakamoto et al., 1997; Law et al., 1999; Di Stefano et al., 2004; Branis et al., 2017).

p130Cas is ubiquitously expressed and plays a crucial role in early mouse development. Indeed, mouse embryos in which p130Cas was knocked-out die at 12.5 for massive cardiac and circulatory dysfunction (Honda et al., 1998). Although the expression of p130Cas has been implicated in the regulation of mammary, bone, brain, muscle, and liver homeostasis (Camacho Leal Mdel et al., 2015), the detailed mechanisms through which p130Cas regulates these physiological processes is yet to be understood.

In this review we will focus on the role of p130Cas in breast cancer by first discussing its importance during normal mammary development and then its relevance in breast cancer.

P130 CRK-ASSOCIATED SUBSTRATE EXPRESSION IN NORMAL MAMMARY GLAND DEVELOPMENT

Mammary gland development is a step process involving the activation of signaling pathways due to the coordinated stimulations of hormones, growth factors and extracellular matrix remodeling. It has been demonstrated that alteration either in the sequence of events or in the signaling pathways that governs mammary gland development can contribute to the onset and progression of breast cancer.

p130Cas/BCAR1 expression is detected in all the cell subtypes of the mouse mammary epithelium but it is highly enriched in the basal compartment. The mammary stroma shows very low levels of expression of p130Cas/BCAR1 (Tornillo et al., 2013). p130Cas/BCAR1 expression level is a crucial regulator of proper mammary development homeostasis. Indeed, transgenic mice overexpressing p130Cas/BCAR1 in the mammary gland show increased mammary branching morphogenesis *in vivo* during puberty, that results in extensive hyperplasia during pregnancy and lactation and delayed involution at the end of lactation. It has been shown that the morphological events occurring in presence of p130Cas overexpression are due to activation of proliferative signaling pathways involving Src, Erk1/2 MAPK, and Akt (Cabodi et al., 2006). The putative mechanism underlying Akt activation might be ascribed to the induction of the Src/PI3K axis, whereas Erk1/2 MAPKs are likely stimulated following Crk/C3G association with p130Cas and subsequent GTPases activation (Defilippi et al., 2006; **Figure 1**).

DYSREGULATION OF P130 CRK-ASSOCIATED SUBSTRATE IN BREAST CANCER

p130Cas/*BCAR1* and ER Positive Breast Cancer

As mentioned before, the gene encoding p130Cas was identified by screening estrogen receptor-positive human breast cancer cells as responsible for promoting tamoxifen resistance and for this reason named *BCAR1* (Breast Cancer Antiestrogen Resistance 1). In human breast tumors, high levels of p130Cas expression correlates with a poor response to tamoxifen

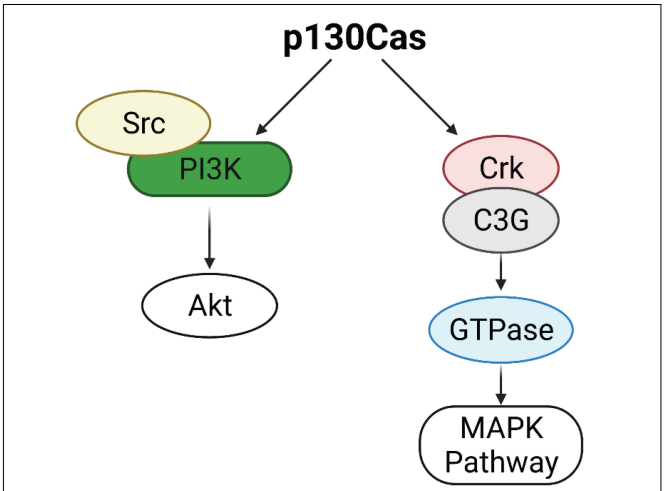

FIGURE 1 | Activation of Erk1/2 MAPK and Akt pathways by p130Cas. p130Cas may activate AKT through the Src/PI3K axis. p130Cas association with Crk and C3G induces GTPases activation, leading to the upregulation of the MAPK pathway.

TABLE 1 | Expression and prognostic value of p130Cas and p140Cas in breast cancer.

	Breast cancer subtype	Prognostic value	References
p130Cas	Invasive carcinomas	–	Cabodi et al., 2006
	ER and PR positive carcinomas	Poor relapse-free survival, poor overall survival, Tamoxifen resistance	van der Flier et al., 2000
	ERBB2-positive carcinoma	Poor overall survival, higher probability of developing a distant event	Cabodi et al., 2006; Tornillo et al., 2011
p140Cap	ERBB2-positive carcinoma	Lower probability of developing a distant event, increased survival	Grasso et al., 2017

therapy, quicker disease recurrence and decreased patient survival (Dorssers et al., 2001), suggesting that p130Cas/*BCAR1* expression might be a convenient prognostic marker for patients affected by primary or metastatic breast cancer (van der Flier et al., 2000; **Table 1**). However, the mechanism through which p130Cas/*BCAR1* can promote resistance to tamoxifen, increased recurrence and poor patient survival was unknown. By using human estrogen receptor positive breast carcinoma T47D cells we came out with a model of the mechanism by which p130Cas regulates ER activity. We demonstrated that upon estrogen treatment, p130Cas rapidly and transiently associates with estrogen receptor alpha in a multimolecular complex containing Src kinase and the p85 subunit of PI3K (**Figure 2A**). Transient overexpression of p130Cas increases and accelerates estrogen-dependent Src kinase, Erk1/2–MAPKs activity and Cyclin D1 expression. Accordingly, p130Cas RNAi inhibits estrogen-dependent Erk1/2–MAPKs and Cyclin D1 induction, demonstrating that p130Cas is directly involved in the non-transcriptional activity of the ER, regulating estrogen-dependent cytosolic signaling pathways (Cabodi et al., 2004).

p130Cas/*BCAR1* and *ErbB2* Positive Breast Cancer

The ErbB2 positive breast cancer subtype represents around 20% of human breast cancer. The first hint of the implication of p130Cas in this aggressive subtype came by crossing transgenic animals overexpressing p130Cas in the mammary gland (MMTV-p130Cas) and those expressing the oncogenic form of the rat *Neu* gene, the homolog of the *ERBB2* human gene, called MMTV-NeuT. The resulting double-transgenic mice were characterized by accelerated onset of mammary tumor formation. Accordingly, *in vivo* and *in silico* analyses of human breast cancer confirmed that the amplification of *ERBB2* in combination with the overexpression of p130Cas induces a higher proliferation rate and an increased number of distant metastases, as well as a correlation with poor prognosis (Cabodi et al., 2006; Tornillo et al., 2011). To unravel the molecular mechanisms responsible for the synergic effect of p130Cas and ErbB2 in tumorigenesis, 3D cultures of MCF10A.B2 mammary epithelial cells were used. MCF10A.B2 cells express a chimeric and activatable ErbB2 receptor that can form spheroid structures called acini when grown in Matrigel (Muthuswamy et al., 2001), mirroring the architecture of the ductal lobular unit in the human mammary gland and representing a faithful model with which to study mammary gland biology *in vitro*. In this model, the concomitant activation of ErbB2 and overexpression of the p130Cas protein give rise to invasive protrusions. The invasive behavior induced by the synergism of p130Cas and ErbB2 results in the stimulation of Akt/PI3K and Erk1/2 MAPKs signaling pathways that in turn, lead to the activation of Rac1 GTPase and the secretion of the metalloproteinase MMP9 (**Figure 2B**; Cabodi et al., 2010; Tornillo et al., 2011). The molecular mechanisms by which p130Cas drive the 3D invasive phenotype are still not understood. It's likely that its role as an adaptor protein is crucial in building a molecular hub very close to ErbB2. Indeed, in ErbB2-transformed cells, p130Cas is a crucial component of a functional molecular complex consisting of ErbB2, c-Src, and Fak. We demonstrated by using pharmacological inhibitors, that both MAPK and PI3K signaling cascades are required for the invasive behavior of p130Cas over-expressing and ErbB2 activated acini triggering invasion through distinct downstream effectors involving mTOR/p70S6K and Rac1 activation, respectively. It has also been demonstrated that Src activity seems to be dispensable, since treatment with the c-Src inhibitor SU6656 does not block cell invasion. The exact mechanisms through which p130Cas leads to activation of MAPK or PI3K are still not known. We can speculate that the activation of PI3K might be mediated by the interaction of p130Cas with the p85 subunit of PI3K, and the data with Src inhibitors suggest that at least Src is not involved in upstream regulation of PI3K.

In the p130Cas/ErbB2-mediated invasive process, the activation of MAPK and miR-23b downmodulation are instrumental to upregulate the transcriptional repressor Blimp1. Consistently, Blimp1 overexpression is detected in invasive breast cancer and correlates with metastatic status (Sciortino et al., 2017). Interestingly, the level of Blimp1 mRNA is upregulated in multi acinar structures of MCF10.B2 cells that result from p130Cas overexpression. In this experimental setting, Blimp1 expression is tightly controlled by the MAPK pathway, since treating the acini with MAPK inhibitor PD98059 dramatically reduces Blimp1 expression, leading to reduced formation of invasive protrusions. Cancer invasion represents a crucial event that allows tumor cells to disseminate in the stromal compartment by acquiring motile characteristics. Indeed, cancer cell invasiveness strictly depends on the possibility to undergo Epithelial-Mesenchymal Transition (EMT), a process during which cells lose adhesions to their neighbors and become more motile. It has been shown that p130Cas/*BCAR1* levels of expression impact on breast cancer cells EMT. As a matter of fact, in the highly invasive A17 mouse mammary tumor cells, p130Cas/*BCAR1* silencing induces loss of mesenchymal features and acquirement of epithelial-like traits, including the re-expression of the cell-cell adhesion molecule E-cadherin, thus affecting the EMT process involved in cancer progression. The mechanism through which p130Cas/BCAR1 expression induces

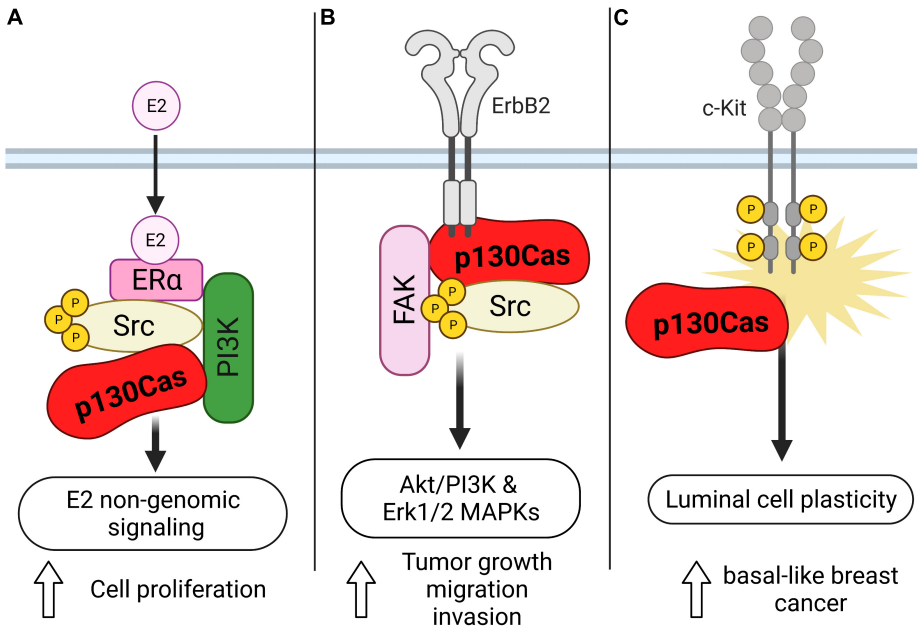

FIGURE 2 | Schematic representation of the role of p130Cas in the different types of breast cancer. **(A)** Upon estrogen treatment, p130Cas promotes cell proliferation mediated by the non-transcriptional activity of ER in T47D cells. In this context, p130Cas forms a complex with ERa, Src, and the p85 subunit of PI3K. **(B)** p130Cas/ERBB2 direct interaction stimulates the Akt/PI3K and Erk1/2 MAPKs pathways, facilitating tumor growth, cell migration, and invasion. **(C)** p130Cap expression promotes the hyperactivation of c-Kit in mouse mammary epithelial cells, inducing luminal cell plasticity.

the A17 cell invasive phenotype relies on its ability to increase the expression of cyclooxygenase-2 (Cox-2) (Bisaro et al., 2012). The p130Cas/Cox2-dependent EMT is effective both in the mouse and in the human setting. Indeed, while the role of Erk MAPK or PI3K/Akt has not been unraveled, c-Src and JNK kinases appear as sequential players in this axis and their pharmacological inhibition was sufficient to downregulate Cox-2 and to induce an epithelial phenotype. p130Cas is thus emerging as a critical player for onset and progression of many aggressive cancers, strengthening its relevance as an unfavorable prognostic marker and a putative therapeutic target, mostly in combination with high levels of ER, HER2, or Cox-2, respectively.

p130Cas/*BCAR1* and Triple Negative Breast Cancer

In addition, it has been described that defective differentiation of mammary luminal progenitors predisposes to basal-like breast cancer (Molyneux et al., 2010). Indeed, the punctual analysis of p130Cas/MMTV transgenic mice, revealed that p130Cas overexpression occurs mainly in the luminal progenitor cell compartment and results in the expansion of luminal cells. The detailed characterization of the luminal cells showed that they aberrantly display basal cell features and reduced differentiation in response to lactogenic stimuli. Consistently, experiments performed in mouse mammary epithelial cells (MMECs) overexpressing p130Cas demonstrate that p130Cas expression leads to hyperactivation of the tyrosine kinase receptor c-Kit, indicating that high levels of p130Cas, via abnormal c-Kit activation, promote mammary luminal cell plasticity, thus providing the conditions for the development of basal-like breast cancer (**Figure 2C**; Tornillo et al., 2013). Accordingly, p130Cas is overexpressed in human triple-negative breast cancer (Tornillo et al., 2013; **Table 1**), implying that the increased expression of p130Cas may be a priming event for the onset of basal-like breast cancer.

P130 CRK-ASSOCIATED SUBSTRATE: TRANSLATIONAL APPLICATIONS

The signaling implicated in p130Cas/ErbB2-dependent tumorigenesis accounts for the direct interaction of p130Cas and ErbB2 (Cabodi et al., 2010; Tornillo et al., 2011). It was demonstrated that the direct binding of p130Cas to ErbB2 stabilizes ErbB2 protecting it from autophagy-mediated degradation by interfering with its ubiquitination. The increased stability of the receptor in presence of high p130Cas expression can be also responsible for resistance to Trastuzumab (Bisaro et al., 2016).

These data suggest important therapeutic and translational value of p130Cas in ErbB2 breast cancer and supports the hypothesis that p130Cas/ErbB2 interaction can serve as a potential target for the discovery and development of new anticancer agents, that can be used in combination with standard therapy to manage and control Trastuzumab resistance.

Recently, two potential inhibitors of p130Cas/ErbB2 interaction were identified by structure-based virtual screening. Their experimental validation was performed *in vitro* and

in ErbB2-positive breast cancer cellular models. The results highlight that both compounds interfere with p130Cas/ErbB2 binding and significantly affect cell proliferation and sensitivity to Trastuzumab (Costamagna et al., 2019). This study supports p130Cas/ErbB2 complex as a potential breast cancer target and shows the druggability of this protein-protein interaction (PPI) that might benefit from a more advanced optimization effort for therapeutic applications.

Compared with other breast cancer subtypes, TNBC is highly invasive, has a high early recurrence rate and is exceptionally difficult to treat. The lack of ER, PR, and ErbB2 expression renders the tumor unresponsive to hormonal therapies or ErbB2-targeted therapies. Therefore, development of new TNBC treatment strategies has become an urgent clinical need (Yin et al., 2020). It has been demonstrated that TNBCs express high levels of p130Cas, that promote mammary luminal cell plasticity, thus providing the conditions for the development of basal-like breast cancer (Tornillo et al., 2013). It is possible to envision an application for protein-protein interaction inhibitors against p130Cas also in this context. Previous data suggest that p130Cas mediates its role in TNBC through increased c-Kit activation, therefore it would be reasonable to validate p130Cas/c-Kit direct interaction and then start a drug discovery screening to identify potential p130Cas/c-Kit interaction inhibitors.

IDENTIFICATION OF P140CAP (P130CAS-ASSOCIATED PROTEIN) AND ITS MAIN CHARACTERISTICS

As previously described, p130Cas is able to interact with different molecules, involved in several signaling processes, in particular cell adhesion, motility, and transformation (O'Neill et al., 2000; Bouton et al., 2001). In order to identify and characterize new p130Cas interactors, an affinity chromatography experiment was performed in human cells. Among others, a 140 kDa protein was detected, named p140Cap for p130Cas-associated protein, also known as SNIP, as Snap25 Interacting Protein in rat brain (Chin et al., 2000). p140Cap is an adaptor protein that indirectly associates with p130Cas and it has been shown to be involved in integrin- and Epidermal Growth Factor (EGF)-dependent signaling (Di Stefano et al., 2004). It is encoded by the *SRCIN1* gene, located on chromosome 17q12. One of its main functions is the binding and activation of C-terminal Src kinase (CSK) leading to Src inhibition, as well the downstream signaling with the related tumor properties (Di Stefano et al., 2007). In particular, 140Cap stabilizes adherens junctions and inhibits EGF Receptor (EGFR) and Ras signaling through the dual control of both Src and Ras activities, thus affecting crucial tumor features such as growth and invasion (Damiano et al., 2010).

While p130Cs is ubiquitously expressed, p140Cap expression is highly tissue-specific. Indeed, analyzing several tissues and cellular lines (Di Stefano et al., 2004), it was apparent that the p140Cap protein is physiologically expressed in neural and epithelial tissues and in a significant subset of cancers including breast cancer and neuroblastoma (Salemme et al., 2021). Interestingly, in both these types of cancers p140Cap has been demonstrated to act as tumor suppressor protein, impairing cancer properties and correlating with a better prognosis (Di Stefano et al., 2007; Grasso et al., 2017, 2020). In addition to Src inhibition, p140Cap has been shown to affect Rac1 GTPase activity and to decrease Tiam1 activation in breast cancer cells (Grasso et al., 2017; Chapelle et al., 2020).

P140CAP EXPRESSION IN MAMMARY GLAND AND ITS ROLE IN BREAST CANCER

In physiological conditions, the p140Cap protein is detectable in the human mammary gland, where it is specifically expressed in alveolar luminal cells. A first immunohistochemical analysis with specific monoclonal antibodies on a small cohort of human breast cancers revealed that p140Cap is lost in the most aggressive and highly proliferative cancers, suggesting an inverse correlation between its expression and the state of malignancy (Damiano et al., 2010). However, in order to assess a prognostic relevance for p140Cap expression, a second study was performed. In particular, a cohort of 622 breast cancer patients in tissue microarray was analyzed. The results revealed that a p140Cap positive status was associated with negative lymph node status, ER and progesterone receptor (PgR)-positive status, small tumor size, low grade, low Ki67 status, and correlates with a better prognosis in ERBB2-positive patients.

Interestingly, it emerged that the p140Cap coding gene *SRCIN1* could be included in the *ERBB2* amplicon, being the *SRCIN1* gene located on human chromosome 17q12, one million base pairs centromeric to the *ERBB2* gene. In fact, the analysis of 200 *ERBB2*-amplified tumors showed that the *SRCIN1* gene is often (55–60% of *ERBB2*-amplified breast cancer patients), but not necessarily co-amplified with *ERBB2*. The prognostic effect of p140Cap was finally underlined in this subgroup of *ERBB2*-amplified breast cancer patients, where the Kaplan–Meier analysis of these tumors evidenced that *SRCIN1* amplification is associated with a significant improved survival and an high expression of p140Cap predict a lower probability of developing metastasis.

Taken together these data suggest a relevant role for p140Cap as prognostic marker in *ERBB2*-amplified breast cancer patients, highlighting its tumor suppressor functions in this breast cancer subtype (Grasso et al., 2017).

P140CAP AND SIGNALING

Src and Csk Phosphorylation

As reported above, upon integrin-mediated adhesion, Src kinase regulates cell growth, spreading and migration through increased phosphorylation of Fak as well as of other key adaptor molecules, like p130Cas (Mitra et al., 2005; Defilippi et al., 2006). Interestingly, by loss- and gain-of-function approaches in *in vitro* models of breast cancer, it was found that p140Cap affects breast cancer cell motility and invasion. Indeed, high levels of p140Cap result in inhibition of integrin- and EGFR-dependent

Src activation, through its ability to directly associate with it (Di Stefano et al., 2007; Damiano et al., 2010). Mechanistically, it was found that p140Cap behaves as a novel binding partner in the cell machinery recruiting Csk and Src, through the binding of Csk on p140Cap with the tyrosines inserted in EGLYA/EPLYA motif and of Src with the proline rich domains, respectively, regulating in this way their activity and downstream signaling in breast cancer cells (**Figure 3A**; Di Stefano et al., 2007; Repetto et al., 2013). Effectively, the direct mutagenesis of the tyrosine present in the EPLYA/EGLYA peptides, is sufficient to disrupt the ability of p140Cap to bind Csk and thus to inhibit Src activity.

p140Cap and Epithelial-Mesenchymal Transition

As shown before, in the *ERBB2*-amplified subgroup of breast cancer patients, p140Cap expression predicts a significantly lower probability of developing distant events (Grasso et al., 2017). EMT is the first step in the metastatic process, increasing the migratory ability of cancer cells. Effectively, in primary epithelial cancer cells derived from NeuT and p140-NeuT tumors in the BALB/c background, the presence of p140Cap correlates with the up-regulation of both E-cadherin mRNA and cell surface protein levels (Grasso et al., 2017). Furthermore, p140Cap exerts an overall inhibitory effect on counteracting the EMT invasive program of ERBB2 tumors, as shown by a marked down-regulation of the EMT transcription factors Snail, Slug, and Zeb1 as well as by the reduction of the mesenchymal cell–cell adhesion protein N-cadherin. Noteworthy, p140Cap is also able to interact with E-Cadherin, both with classical biochemical approaches (Damiano et al., 2010) or by a comprehensive analysis of the p140Cap interactome in breast cancer cells (Chapelle et al., 2019). Overall, these data indicate its possible contribution in strengthening the adherence junction stability through the relocalization and immobilization of E-cadherin at the plasma membrane (**Figure 3B**). Future studies will determine how p140Cap participates in these molecular interactions in a spatial and temporal manner and how it affects E-cadherin junction stability, which is a key step in counteracting the EMT process and more in general the tumor progression.

p140Cap Affects Tiam1-Dependent Rac1 Migration

The clinical evidence that p140Cap correlates with a favorable outcome in ERBB2 breast cancer patients suggest that p140Cap is able to curb the intrinsic biological aggressiveness of ERBB2 tumor (Grasso et al., 2017). Indeed, p140Cap confers to ERBB2 transformed cells both limited *in vivo* tumor growth ability and impaired spontaneous lung metastasis formation. This less aggressive phenotype is likely linked to reduced cell proliferation, assessed by a decreased staining of the proliferative marker PCNA in tumors, increased sensitivity to apoptosis, and strong inhibition in the EMT program observed in p140Cap expressing tumor cells. As reported above, p140Cap limits the integrin and the EGFR signaling pathways by activating Csk and inhibiting Src activity, thus leading to impaired cell spreading, motility, and invasion (Di Stefano et al., 2007). In the same context, p140Cap limiting integrin or growth factor signalings, is able to counteract tumor features such as cell migration and invasion. On the same line, Grasso et al. by using SKBR3 breast cancer cells as a model of ERBB2 amplification, reported that in a transwell assay, migration was significantly decreased in p140Cap-overexpressing cells as well as increased in MDA-MB-453 p140Cap-silenced cells, an additional model of *ERBB2* gene amplification.

Unexpectedly, both in NeuT and in SKBR3 cells, p140Cap expression did not affect the activation of the Src kinase and the

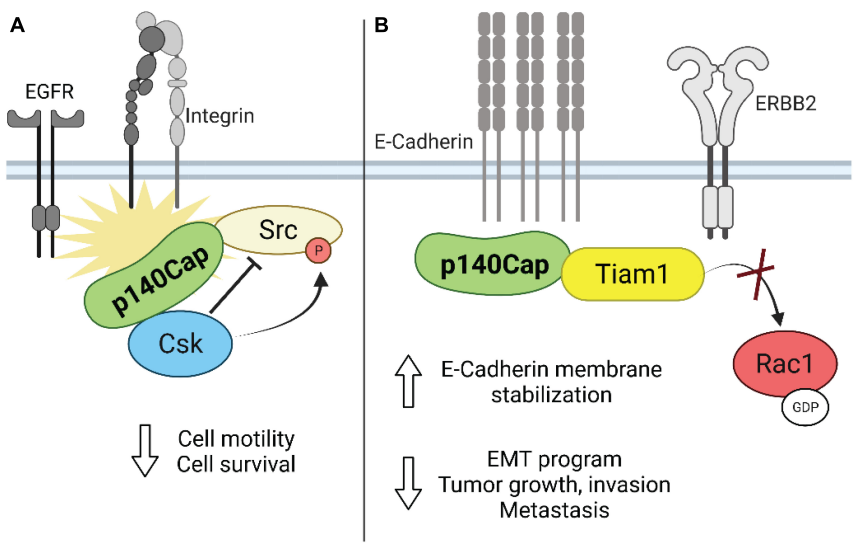

FIGURE 3 | Schematic representation of the role of p140Cap in breast cancer. **(A)** Upon integrin and growth factor-mediated signaling, p140Cap inhibits Src activity by binding to the Src inhibitor Csk. **(B)** p140Cap counteracts the EMT process by increasing E-cadherin stability at the plasma membrane in luminal A cell line MCF7. Moreover, p140Cap downregulates Rac1 activation by interfering with the Rac1-GEF Tiam in ERBB2 transformed cells.

phosphorylation of its effectors such as p130Cas and paxillin, suggesting that in ERBB2 transformed cells p140Cap controls cell migration in an additional way. In presence of p140Cap the Rac1 GTPase activity was significantly decreased as reported by the *in vitro* pull-down of active Rac1. Concomitantly, the decreased Rac1 activity in p140Cap expressing cells was dependent on decreased activity of a specific Rac1-GEF, namely Tiam1, as found in a GST-RacG15A *in vitro* pull-down assay. The same results were also obtained in p140Cap-silenced MDA-MB-453 cells. Moreover, p140Cap and Tiam1 co-immunoprecipitated, indicating that p140Cap associates in a molecular complex with Tiam1, and suggesting that this interaction may be responsible for reducing Tiam1 activity (Grasso et al., 2017). As a consequence of impaired motility, p140Cap expression in ERBB2 preclinical models is also able to reduce the number of metastases in *in vivo* assays of spontaneous and experimental metastasization (**Figure 3B**).

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL CONTROL OF P130 CRK-ASSOCIATED SUBSTRATE AND P140CAP EXPRESSION

The mechanisms underlying the transcriptional regulation of p130Cas and p140Cap in physiology and pathology are still poorly investigated. The transcriptional control of p130Cas/*BCAR1* in human breast cancer cells is partly due to the transcription factor EGR1 and its coregulator NAB2 (Kumbrink and Kirsch, 2012). Interestingly, p130Cas signaling mediates the induction of both EGR1 and NAB2, which, in turn, up-regulates p130Cas expression in a positive feedback loop.

To date, we are not aware of any transcription factors involved in the regulation of *SRCIN1* expression. The identified mechanisms responsible for its deregulation in cancer are mainly related to chromosome rearrangements and microRNA control. Besides the co-amplification with *ERBB2* in breast cancer, the *SRCIN1* gene might be lost or disrupted in some cases of aggressive neuroblastoma due to the 17q12 chromosomal rearrangement (Grasso et al., 2020). Although the clinical relevance of *SRCIN1* expression in neuroblastoma has been demonstrated, since it is an independent risk factor inversely correlated to disease aggressiveness, the importance of these genetic aberrations should be better investigated in a larger cohort of patients. Recently, Damez-Werno et al. (2016) discovered that the *SRCIN1* gene is transcriptionally activated in the nucleus accumbens (NAc) of mice brains following cocaine exposure thanks to a particular chromatin modification. In this specific brain area, *SRCIN1* exhibited reduced asymmetric dimethylation of R2 on the histone H3 (H3R2me2a), leading to consequent *SRCIN1* induction in the NAc, decreased Src signaling, and reduction of the rewarding effects of cocaine, including self-administration of the drug. It is crucial to identify the transcriptional and epigenetic mechanisms determining p140Cap and p130Cas deregulation in cancer to develop new therapeutic strategies.

Several microRNAs (miRNAs) affect the expression of these adaptor proteins. The post-transcriptional control of p140Cap by miRNAs occurs in different types of cancer and involves many miRNAs, as extensively discussed elsewhere (Salemme et al., 2021). The only miRNA that acts as a negative

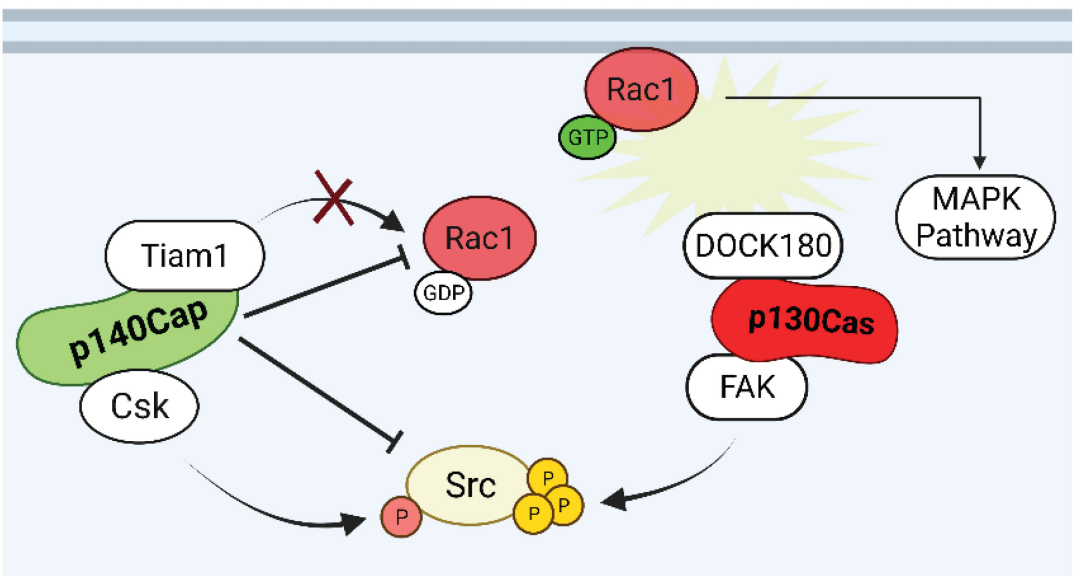

FIGURE 4 | Putative p130Cas and p140Cap points of crosstalk in breast cancer. Rac1 and Src are the possible points of crosstalk in which p130Cas and p140Cap may exert their antagonistic functions in the regulation of tumorigenic signaling. While Rac1 GTPase is kept inactive by p140Cap through the inhibition of the GEF Tiam1, the assembly of p130Cas/DOCK180 complex induces the recruitment of active Rac1 to the cell membrane. The p140Cap/CSK and p130Cas/FAK interactions contribute to the inhibition and activation of Src activity, respectively.

BOX 1 | Critical unanswered questions on p130Cas and p140Cap in breast cancer.
Key Unanswered Questions

- How is BCAR1/p130Cas transcriptionally regulated and which are the mechanisms responsible for its overexpression In breast cancer?
- Despite the already identified p130Cas interactors, which is the comprehensive p130Cas interactome in breast cancer cells?
- Which is the prognostic value of p140Cap in the other subtypes of breast cancer, except for the ErbB2-amplified tumors?
- Are there any point of cross-talk between p130Cas and p140Cap in breast cancer?
- Are the multi-protein complexes associated with p130Cas and p140Cap critical to uncover new biologically relevant and potentially targetable pathways in the context of breast cancer?

regulator of p140Cap in breast cancer is miR-150, which promotes cell migration, invasion, and expression of EMT markers in breast cancer cells. Three miRNAs, namely miR-24-3p, miR-362-3p, and miR-329, are known to direct target p130Cas and inhibit its protein levels, leading to impaired breast cancer cell migration and invasion (Kang et al., 2016, 2017).

TARGETABLE PATHWAYS IN BREAST CANCER FROM P140CAP AND P130CAS ASSOCIATED PROTEIN INTERACTOME STUDIES

Given the presence of multiple protein-binding modules, adaptor proteins can facilitate the assembly of signaling complexes regulating cellular signals both spatially and temporally (Flynn, 2001). Understanding the nature and the functional role of the multi-protein complexes associated with p140Cap and p130Cas adaptor proteins might be critical to discover new biologically relevant and potentially targetable pathways in the context of breast cancer.

Recently, we generated a p140Cap interactome from the ERBB2-positive breast cancer TuBo cell, a clonal line established *in vitro* from a BALB-NeuT mouse mammary carcinoma (Rovero et al., 2000; Chapelle et al., 2019). The identification and functional characterization of p140Cap co-immunoprecipitated proteins, performed by Mass Spectrometry and subsequent bioinformatic analysis, revealed 374 putative interacting partners. Among these proteins are present crucial components of signaling pathways that regulate specific cellular functions such as cell-substrate junction, focal adhesion organization, cell-cell adhesions, cell cycle and apoptosis, and protein homeostasis (Chapelle et al., 2019).

One putative interactor is E-cadherin, which was already demonstrated to co-immunoprecipitate with p140Cap in the luminal A MCF-7 cells, where E-Cadherin is immobilized by p140Cap at the cell surface, strengthening adhesion between cells and curbing breast cancer cell migration (Damiano et al., 2010). Members of the catenin family, such as α-, β-, and δ catenin, were also identified as interactors, emphasizing the role of p140Cap in the context of the cadherin/catenin-based adhesion system in breast cancer cells. Besides the well-characterized roles in cell adhesion, β-catenin acts as a transcriptional co-activator in the canonical Wnt signaling (Clevers and Nusse, 2012), raising the hypothesis that p140Cap might participate in the regulation of the Wnt signaling pathway in breast cancer.

Analysis of the functional relationships between proteins within the interactome highlighted the presence of defined protein clusters characterized by specific functions. One protein cluster was enriched with kinase domain-containing proteins, such as Src, ERBB2, and ERBB2IP (ERBIN), reinforcing the idea that p140Cap can associate and regulate kinases implicated in breast cancer transformation and progression. The interactome also contains proteins involved in actin cytoskeleton remodeling, including Actinin B and Actin Remodeling Protein (FLII), several F-actin capping proteins, the motor protein Myosin 6 (MYO6) and Flotillin, which localizes to the caveolae and plays a role in vesicle trafficking. To better understand the function of these interactors in the tumor-suppressing roles of p140Cap, it's mandatory to address their biological role in an experimental breast cancer context and if this role is somewhat compromised by the interaction with p140Cap.

Although less is known on the global p130Cap interactome in breast cancer models, Evans et al. (2017) identified the interactome of p130Cas in human endothelial cells in response to VEGF treatment. They reported that VEGF stimulation induces an enrichment in p130Cas interactors implicated in cell motility, actin cytoskeletal dynamics, and angiogenesis, such as IQGAP, Profilin-1, FLII, MYO6, and MRCKβ. It is reasonable to hypothesize that some of these interactors might be directly or indirectly associated with p130Cas independent from the cellular context. Interestingly, FLII, MYO6, and IQGAP are interactors shared by both adaptor proteins, indicating that p140Cap and p130Cas might regulate common signaling pathways involved in cytoskeletal remodeling by interacting with similar molecular complexes. However, it would be of great interest to investigate the p130Cas interactome in the breast cancer TUBO cells, which will allow a better understanding of the opposing contributions of p140Cap and p130Cas adaptor proteins in controlling cancer cell signaling pathways (Cabodi et al., 2006).

CONCLUSION

The above findings provide evidence for a role of p130Cas as a positive regulator of both proliferation and survival in normal and transformed mammary epithelial cells. In contrast, based on the collected data, p140Cap, in the same breast cancer cell models, behaves as an oncosuppressor which opposes and interferes with cancer features. Several molecular pathways have already been depicted through which p130Cas and p140Cap may exert their opposite properties in breast cancer (**Figure 4**). The fact that the two proteins have common interacting partners, such as the Src kinase, with opposite effects, further indicate that the two proteins share a common network and that a temporal analysis of their reciprocal localization and kinetics of interactions with specific interactors would be of great interest

to shed light on their functions. The comprehensive analysis of p140Cap interactome in BC cells has recently provided data on its involvement in several additional biological networks relevant for cancer progression. The analysis of the p130Cas interactome in the same model would provide additional knowledge to the comprehension of this complex network. Moreover, we have recently generated genetically modified mice in which either p130Cas or p140Cap can be specifically ablated in a specific tissue (Del Pilar Camacho Leal et al., 2018; Russo et al., 2019). These models could be exploited in the mammary gland to further address the impact of each protein in physiological development or in tumor progression, focusing on the functional balance of the common network of interacting proteins.

Finally, further analysis is needed of both *BCAR1* and *SRCIN1* gene status and regulation in specific breast cancer subtypes, in order to address their real contribution to the biological heterogeneity of human tumors in terms of patient stratification. On the other hand, since many tumors express p130Cas, but do not express relevant levels of p140Cap, the existing knowledge on miRNAs acting on p130Cas and p140Cap expression in human tumors, will provide the testable hypothesis on the use of specific anti-miRNAs to enhance an appropriate tumor response in pre-clinical models. Overall, the dissection of p130Cas and p140Cap biological features and their regulated pathways in breast cancer highlight the potential clinical impact of their reciprocal expression on patient stratification, as being relevant key players for patient outcome.

From the ancient Chinese philosophy of the yin and yang (bright-black or positive-negative) concept of dualism, the detailed study of p130Cas and p140Cap proteins suggests that in normal tissues they can be not only opposite or contrary forces, but actually be complementary, interconnected, and interdependent in the natural world. According to this philosophy, everything has both yin and yang aspects (for instance, shadow cannot exist without light). Additional studies are needed to better address if a similar concept can be applied to the p130Cas/p140Cap network (**Box 1**).

AUTHOR CONTRIBUTIONS

GC, VS, and PD conceived and wrote the part relating to p140Cap/SRCIN1. DN, AC, and SC conceived and wrote the part relating to p130Cas/BCAR1. SC and PD revised the manuscript and the figures. All authors contributed to the article and approved the submitted version.

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6-Phosphogluconolactonase Promotes Hepatocellular Carcinogenesis by Activating Pentose Phosphate Pathway

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Hepatocellular carcinoma (HCC) has a poor prognosis due to the rapid disease progression and early metastasis. The metabolism program determines the proliferation and metastasis of HCC; however, the metabolic approach to treat HCC remains uncovered. Here, by analyzing the liver cell single-cell sequencing data from HCC patients and healthy individuals, we found that 6-phosphogluconolactonase (PGLS), a cytosolic enzyme in the oxidative phase of the pentose phosphate pathway (PPP), expressing cells are associated with undifferentiated HCC subtypes. The Cancer Genome Atlas database showed that high PGLS expression was correlated with the poor prognosis in HCC patients. Knockdown or pharmaceutical inhibition of PGLS impaired the proliferation, migration, and invasion capacities of HCC cell lines, Hep3b and Huh7. Mechanistically, PGLS inhibition repressed the PPP, resulting in increased reactive oxygen species level that decreased proliferation and metastasis and increased apoptosis in HCC cells. Overall, our study showed that PGLS is a potential therapeutic target for HCC treatment through impacting the metabolic program in HCC cells.

Keywords: hepatocellular carcinoma, 6-phosphogluconolactonase, metabolic reprogramming, pentose phosphate pathway, ROS

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide (Jiang et al., 2000; Imamura et al., 2003; Ye et al., 2016; Yang et al., 2019). HCC usually occurs in patients with chronic liver diseases related to viral infection (chronic hepatitis B and C viruses), alcoholism (alcohol and aflatoxin), and liver metabolic disorders (diabetes and non-alcoholic steatohepatitis) (Ganne-Carrie and Nahon, 2019; Kanwal and Singal, 2019). Hepatectomy and liver transplantation are the two main treatments for HCC currently, but the HCC recurrence rate is high because of the easy metastasis of liver cancer cells (Clavien et al., 2012; Sapisochin and Bruix, 2017;

Yoshida et al., 2019). Hence, limited cognition hinders clinical treatment of HCC. In order to seek effective clinical treatment for HCC, more knowledge about HCC is necessary.

HCC requires metabolic reprogramming for continuous growth and rapid proliferation (Cancer Genome Atlas Research Network, 2017; Tian et al., 2019). Hepatocytes mainly produce ATP through oxidative phosphorylation (OXPHOS), whereas HCC cells produce ATP through anaerobic glycolysis, instead of OXPHOS (Feng et al., 2020). The pentose phosphate pathway (PPP) is a metabolic pathway parallel to glycolysis (Jiang et al., 2014; Patra and Hay, 2014; Wu et al., 2018). The PPP pathway consumes intermediate glucose 6-phosphate (G6P) through the oxidized and non-oxidized branches to produce fructose 6-phosphate and glyceraldehyde 3-phosphate (Ma et al., 2020). The PPP pathway metabolites, ribose 5-phosphate (R5P) and NADPH, are mainly produced by rate-limiting enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconolactonase (PGLS), which are essential for the survival of HCC cells and the synthesis of fatty acids (Gao et al., 2019; Jing et al., 2019; Li M. et al., 2019; Ghergurovich et al., 2020). Emerging evidence has demonstrated that G6PD is involved in the occurrence of HCC, but the role of PGLS in HCC remains unclear. PGLS, a hydrolase, specifically catalyzes the hydrolysis of 6-phosphogluconolactone to 6-phosphogluconic acid (Beutler et al., 1985).

In our study, by analyzing the liver single-cell RNA sequencing data from HCC patients or healthy individuals, we found that PGLS was highly expressed in undifferentiated HCC cells, and down-regulation of PGLS *in vitro* could inhibit the proliferation, migration, and invasion of HCC cells. In addition, PGLS has a new tumor-promoting effect in HCC by activating the PPP pathway.

MATERIALS AND METHODS

Patients and Tissue Samples

A total of six pathologically diagnosed HCC tissues and matched tumor-adjacent tissues were obtained from patients at the Third Affiliated Hospital of Sun Yat-sen University. The use of clinical samples was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-sen University, and written informed consents were obtained from all enrolled patients. All patients did not receive preoperative therapies.

Cell Culture and Transfection

HCC cell lines (Hep3b, Huh7) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were maintained in a six-well plate in RPMI-1640 medium (Corning, 10-040) supplemented with 10% fetal bovine serum (FBS) (Hyclone, SH30084), 100 μg/mL streptomycin/penicillin (Hyclone, SV30010) in a humidified 37°C incubator with 5% CO_2. The PGLS siRNA (siPGLS) was designed and obtained from Gene Pharma (Guangzhou, China). The transfection assay was carried out using Lipofectamine 2000 (Invitrogen, 11668030) following the protocols. The culture media was supplemented with 6-aminonicotinamide (6-ANA) (10 μM, Target-mol, T7545) 48 h.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from HCC cell lines or clinical samples using a TRIZOL reagent (Magen, R4801-02) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR kit protocol (Bio-Rad, 1725150). PCR primer sequences are listed in **Supplementary Table 1**.

Western Blot Analysis

The same number of cells from each population to be analyzed was sorted into phosphate-buffered saline (PBS) with 2% FBS. The cells were washed with PBS and lysed by RIPA. Equal amounts of protein extracts were fractionated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (IPVH00010, Merck Millipore). After blocking with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST, pH 7.6) for 1 h at room temperature, the membranes were incubated with primary antibodies including anti-PGLS (rabbit, 1:1,000, GTX120327, Genetex), anti-CK18 (rabbit, 1:1,000, 10830-1-AP, Proteintech), and anti–β-actin (rabbit, 1:1,000, 4970s, Cell Signaling Technology) overnight at 4°C and then incubated with secondary antibodies (rabbit, 1:10,000, W401B, Promega) for 1 h at room temperature, which was detected by digital imaging with a charge-coupled device camera system (Odyssey Fc). The images shown are representative of images from at least three experiments.

Flow Cytometry

For apoptosis, the cells were fixed, permeabilized, and stained by Tunel Detection kit (C1086, Beyotime) according to manufacturer's instructions. For reactive oxygen species (ROS) activity analysis, the cells were stained by 5 μM DCFDA (D6883, Sigma). Cell sorting and analysis were performed using an Attune NxT analyzer (Thermo Fisher Scientific) or InFlux Cell Sorter (BD Biosciences). Data analysis was performed using FlowJo software.

Metabolic State Analysis

$PGLS^{high}$ and $PGLS^{low}$ cells were sorted and then lysed; intracellular $NADP^+$/NADPH ratio was measured using the $NADP^+$/NADPH Assay Kit (KA1663, Abnova) according to the manufacturer's instructions.

Cell Proliferation Assay

HCC *in vitro* proliferation was measured by calcein-AM/PI kit (C2015S, Beyotime) according to the manufacturer's instructions.

Transwell Assay

Hep3b and Huh7 cells were added into the upper chambers of Matrigel-uncoated (cell migration) or coated (cell invasion) Transwells (ET BIOFIL, Guangzhou, TCS004024). The lower

chambers were added medium with 10% FBS, and the upper chambers were serum-free medium. After 24 h culture, the migrated or invaded cells (on the bottom of the filters) were fixed using 4% paraformaldehyde (Mei Lun, China, MA0192) and stained with 0.5% crystal violet for 1 h. The number of migrated or invaded HCCs was counted under a light microscope by randomly selecting five fields.

Colony Formation Assay

A total of 5×10^4 Hep3b and Huh7 cells were plated into six-well plates. Colonies were fixed with 4% paraformaldehyde and stained with crystal violet (Beyotime, Shanghai, C0121-100ML for 30 min at room temperature. The visible colonies were counted manually.

Wound-Healing Assay

A total of 5×10^4 Hep3b and Huh7 cells were seeded into six-well plates and grown to 80% cell abundance. Then, a single layer wound was created using a pipette tip, and we took images (Olympus, BX51). Imaging was repeated at the same location and further analyzed by ImageJ software. All assays were conducted three times.

scRNA-Seq Data Processing

Raw genomic data have been deposited in the Gene Expression Omnibus database with accession number GSE149614. The scRNA-seq data are available from the corresponding author upon reasonable request; 28,687 non-tumor liver cells and 34,414 primary tumor cells from 10 HCC patients were included. Normalization, dimensionality reduction, and clustering were performed with the Seurat 3.2.3 R package (Butler et al., 2018) on RStudio. Cells were filtered to have > 500 and $< 5{,}000$ detected genes and $< 5\%$ of total UMIs mapping to the mitochondrial genome. Data set normalization was performed by dividing the UMI counts per genes by the total UMI counts in the corresponding cells and log-transforming, and following the results, scaling and centering. Cells underwent dimensionality reduction with the uniform manifold approximation and projection method (UMAP). HCC-like clusters were selected by HCC markers including GPC3, CD24, and MDK (Tsuchiya et al., 2015; Lu et al., 2018; Yu et al., 2018). Feature plots were generated by the Seurat function feature plot. Pseudotime trajectory was analyzed by monocle2 on basis of the Seurat clustering (Subramanian et al., 2005; Qiu et al., 2017). Signature genes of each cluster were obtained using the Seurat function FindMarkers with "wilcox" test. Venn plots were generated by Venn Diagram R packages. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and plots were performed using cluster Profiler and ggplot2 R package. Gene lists were preranked by the fold change values of the differential expression analysis using Seurat. Gene sets were obtained from Gene Ontology database as indicated. Heatmap was generated by the pheatmap R package.

Statistical Analyses

Data are expressed as means ± standard deviation (SD). All experiments were analyzed by Student *t*-test, and differences were considered statistically significant if $p < 0.05$. Differences were considered statistically significant if $p < 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

6-Phosphogluconolactonase Was Specifically Highly Expressed in Human Hepatocellular Carcinoma Samples

From the Gene Expression Omnibus database, we downloaded scRNA-seq data of non-tumor and HCC patient liver cells. In total, 28 clusters were shown after UMAP dimensionality reduction (**Figures 1A,B**). We found that clusters 8, 13, 16, and 17 were specifically presented in patient samples, labeled by HCC markers (GPC3, CD24, and MDK) (**Figures 1C,D**). Then we extracted these four clusters for pseudotime trajectory analysis, which showed that cluster 0 was the most primitive (**Figures 1E,F**). Venn plot presented the overlapped marker genes among the new four clusters (**Figure 1G**). The specifically high expression genes, in cluster 0, were used for KEGG enrichment analysis. It showed that several top pathways were associated with carbon metabolism (**Figure 1H**). Next, we found the expression of PGLS in carbon metabolism was the highest (**Figure 1I**). Consistent with this, the UMAP plot and violin plot showed specific high expression level of PGLS in new cluster 0 (**Figures 1J,K**). Similar rising level could be observed in human HCC samples from the results of IHC staining and The Cancer Genome Atlas (TCGA) (**Figures 1L–N**). Compared with the survival probability of the high PGLS expression group, the low PGLS expression group showed a longer survival period (**Figure 1O**). PGLS transcripts were profoundly higher in human HCC samples by quantitative PCR. Similar rising levels could be observed in the HCC markers GPC3, CD24, and MDK (**Figure 1P**).

6-Phosphogluconolactonase Pathway Led to Significant Activation of Pentose Phosphate Pathway in Hepatocellular Carcinoma

HCC cells from human liver cancer tissues were sorted by the expression level of PGLS for further exploring the difference between these two groups. PGLS was also highly expressed in $PGLS^{high}$ cells (**Figure 2A**), with a higher expression level of CK18, which was used for HCC diagnosis in clinic (**Figures 2B,C**). $PGLS^{high}$ cells presented a higher transcription level of GPC3, CD24, and MDK (**Figure 2D**). It also showed a lower $NADP^{+}$/NADPH ratio, which was associated with the reduction of ROS production and apoptosis (**Figures 2E–I**), we next used scRNA-seq data to investigate the role of PGLS in HCC; we found that the expression of apoptosis-related genes was negative correlated with the expression of PGLS (**Figure 2J**). Furthermore, this kind of apoptosis occurs because of the activation of ROS relative signaling pathways (metabolism of xenobiotics by cytochrome P450, chemical carcinogenesis–ROS) (**Figure 2K**).

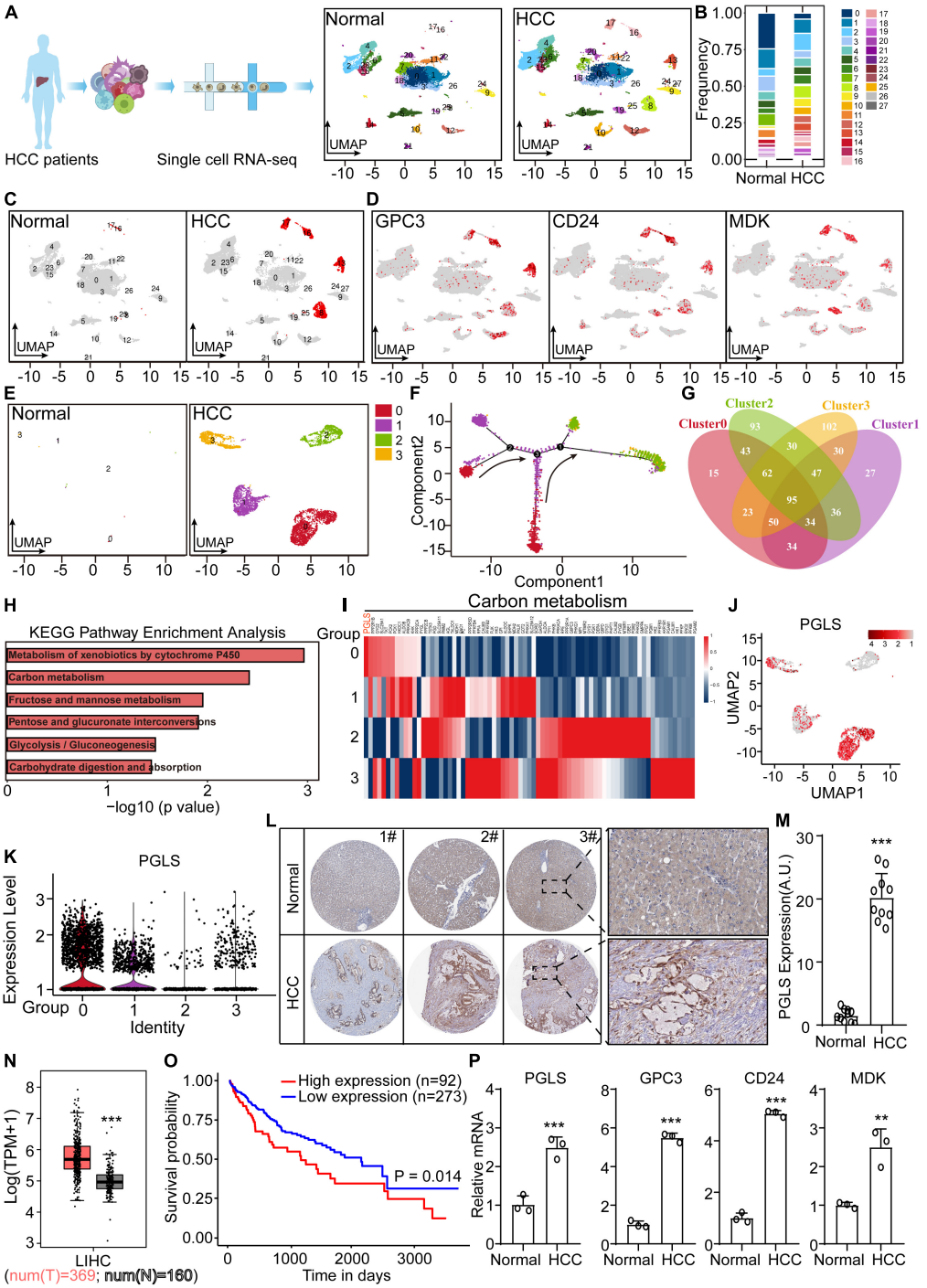

FIGURE 1 | PGLS was specifically highly expressed in human HCC samples. **(A)** The UMAP dimensionality reduction results of scRNA-seq data from non-tumor and HCC patients. **(B)** The frequency of cells in each cluster. **(C)** Four more clusters presented in HCC samples. **(D)** These four cell clusters were labeled by HCC markers (GPC3, CD24, and MDK). **(E)** Four clusters specifically presented in HCC samples. **(F)** The pseudotime trajectory analysis was done for the above four clusters, which showed that cluster 0 was the most primitive. **(G)** The overlapped marker genes among the new four clusters. **(H)** KEGG enrichment using the specifically high expression genes in new cluster 0. **(I)** High expression genes in new cluster 0, in carbon metabolism. **(J)** The expression of PGLS in the UMAP plot. **(K)** The expression of PGLS in the violin plot. **(L)** The expressions of PGLS in normal and HCC samples were detected by IHC staining. **(M)** The quantitative results of IHC staining in normal and HCC samples. **(N)** PGLS mRNA expressions between HCC tissue (n = 369) and non-tumor liver tissue (n = 160) of TCGA and GTE database. **(O)** The survival curve for the HCC patients. **(P)** The differential expressed marker genes between non-tumor and HCC samples. Data show individual values and mean ± SD. m, n, and p, unpaired two-tailed Student t-tests, assessed statistical significance, $^{**}p < 0.01$, $^{***}p < 0.001$.

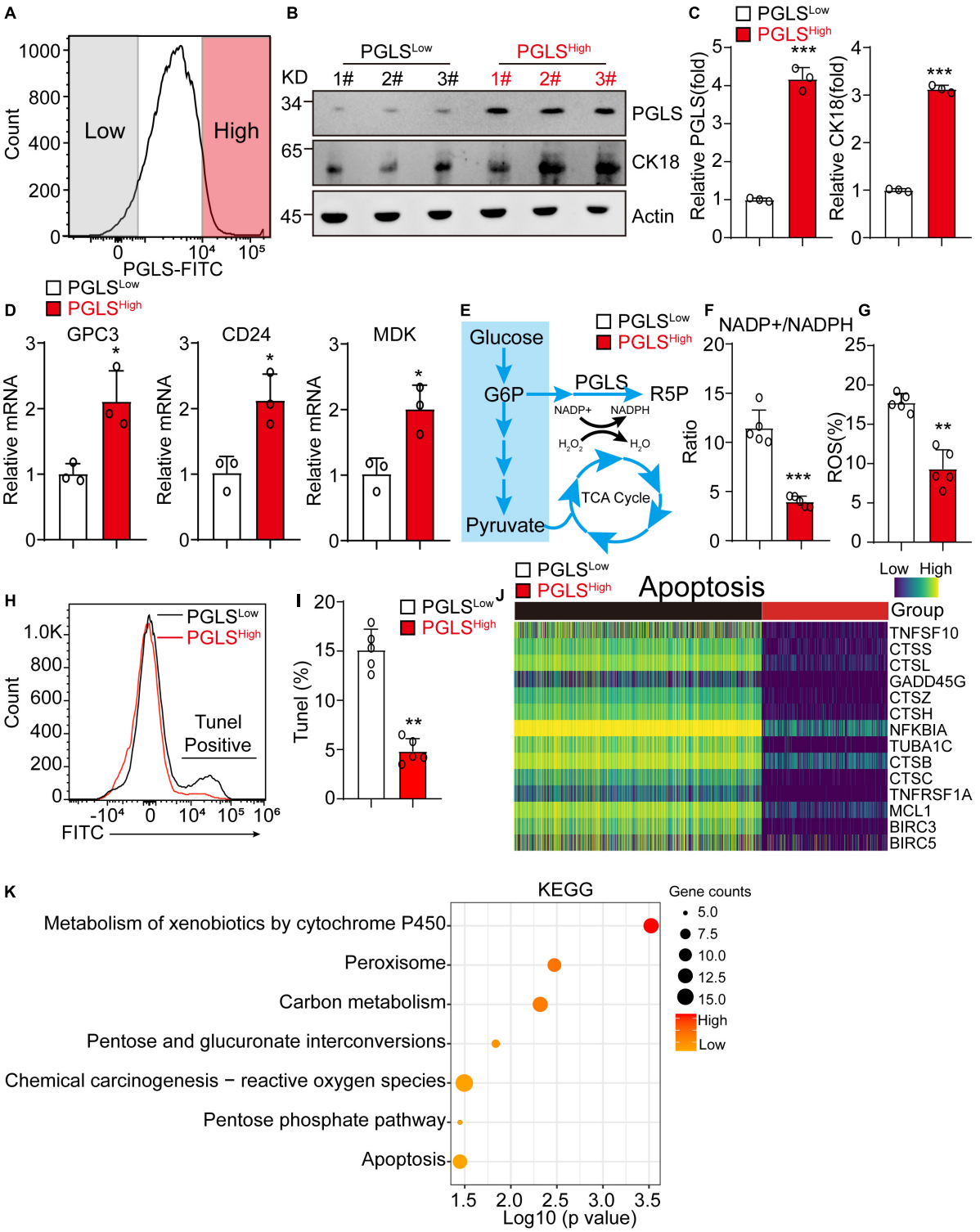

FIGURE 2 | PGLS pathway led to significant activation of PPP in HCC. **(A)** The representative FACS plot of PGLS^{high} and PGLS^{low} HCC cells. **(B,C)** Western blot of the PGLS and CK18 in PGLS^{high} and PGLS^{low} cells from human HCC patients. β-Actin is a loading control. **(D)** The relative expression of HCC marker genes in PGLS^{high} and PGLS^{low} cells (n = 3 replicates). **(E)** Glycometabolism diagram. **(F)** The NADP^{+}/NADPH ratio in PGLS^{high} and PGLS^{low} cells (n = 5 replicates). **(G)** The percentage of ROS in PGLS^{high} and PGLS^{low} cells (n = 5 replicates). **(H)** The representative FACS plot of PGLS^{high} and PGLS^{low} cells for apoptosis analysis. **(I)** The apoptosis rate of PGLS^{high} and PGLS^{low} cells. **(J)** The expression profile of genes associated with apoptosis pathway between PGLS^{high} and PGLS^{low} cells. **(K)** KEGG analysis of PGLS^{high} and PGLS^{low} cells. Data show individual values and mean ± SD. **(C,D,F,G,I)** Unpaired two-tailed Student t-tests, assessed statistical significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

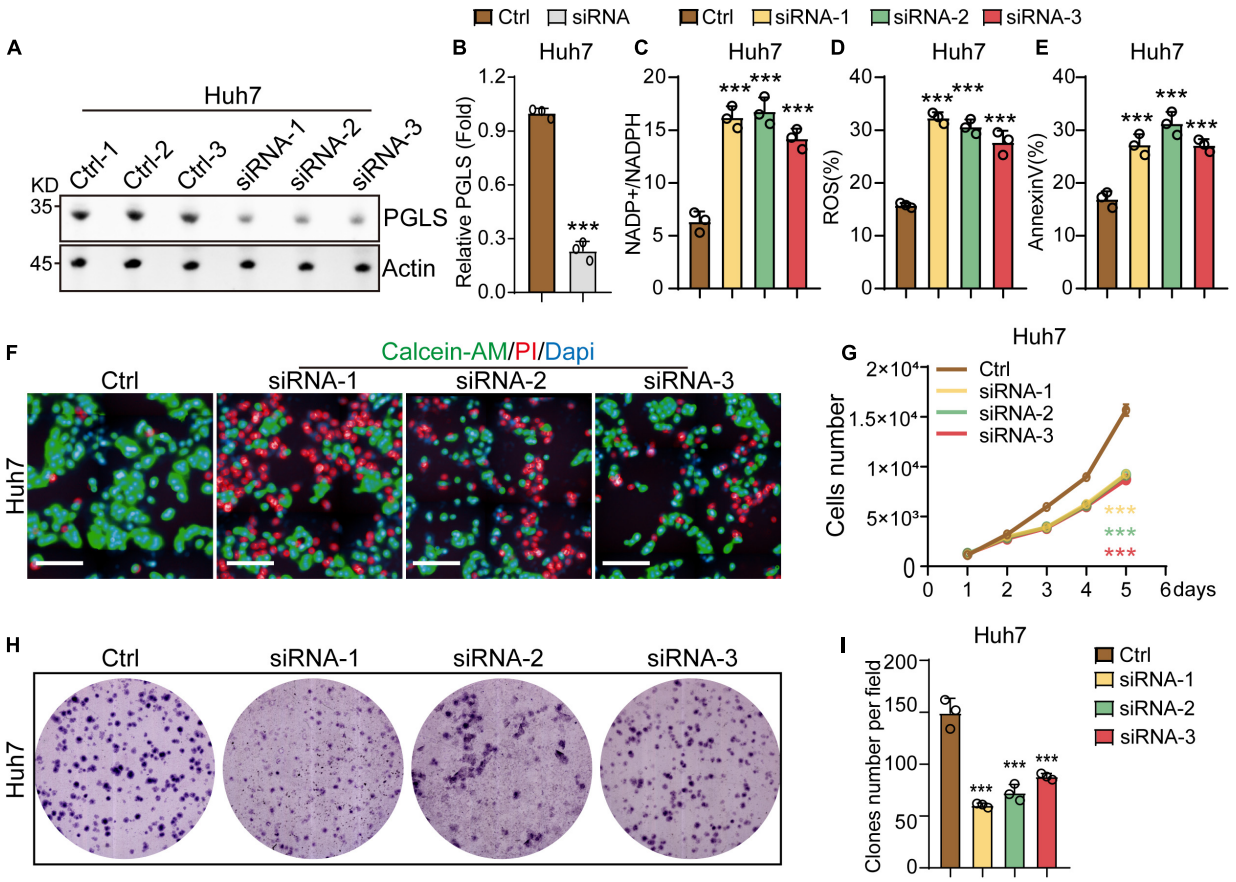

FIGURE 3 | PGLS regulated the proliferation and apoptosis of HCC. **(A)** Transfection with the three siRNAs could effectively knock down the expression of PGLS on Huh7. **(B)** Quantitative analysis of Western blot in **(A)**. **(C)** The $NADP^+$/NADPH ratio on Huh7. **(D)** The percentage of ROS on Huh7. **(E)** The apoptosis rate on Huh7. **(F)** Fluorescence micrographs of Huh7 after staining with calcein-AM, PI, and DAPI. **(G)** Proliferation curve of Huh7 transfected by siRNA-1, siRNA-2, and siRNA-3. **(H)** Representative field of CFUs, formed by Huh7 treated with siRNA-1, siRNA-2, and siRNA-3. **(I)** CFU number per field on Huh7. Data show individual values and mean ± SD. **(B–E,G,I)** Unpaired two-tailed Student *t*-tests, assessed statistical significance, $^{***}p < 0.001$.

6-Phosphogluconolactonase Regulated the Proliferation and Apoptosis of Hepatocellular Carcinoma

To investigate the effects of the rate-limiting enzymes of PGLS in HCC cell lines, Huh7 and Hep3b cells were transfected with siRNA-1, siRNA-2, or siRNA-3. We found that all three siRNAs could reduce the expression level of PGLS in Huh7 cell lines as compared to control groups (**Figures 3A,B**). Similar results could be observed in Hep3b cell lines (**Supplementary Figures 1A,B**). In addition, we found that the treatment of siRNA-1, siRNA-2, and siRNA-3 increased the $NADP^+$/NADPH ratio in HCC cell lines (**Figure 3C** and **Supplementary Figure 1C**). Meanwhile, ROS and apoptosis rate in the three knockdown (KD) groups were also significantly higher as compared to the control group (**Figures 3D,E** and **Supplementary Figures 1D,E**). After transfection by siRNA-1, siRNA-2, and siRNA-3, both HCC cell lines presented a slower proliferation rate at all six time points (**Figures 3F,G** and **Supplementary Figures 1F,G**). Consistent with this, Huh7 and Hep3b cells showed a significantly smaller size and fewer numbers of CFUs (colony-forming units) as compared to the control group (**Figures 3H,I** and **Supplementary Figures 1H,I**).

6-Phosphogluconolactonase Regulated the Migration and Invasion of Hepatocellular Carcinoma

In the migration assay, the two cell lines presented significantly fewer clones per field in the three KD groups as compared to the control group (**Figures 4A,B** and **Supplementary Figures 2A,B**). To analyze the effects of siRNA-1, siRNA-2, and siRNA-3 on HCC cell invasion, chamber invasion assay was performed on Hep3b and Huh7 cells. Significantly fewer cell clones were shown in the three siRNA groups, in both Hep3b and Huh7 cells (**Figures 4C,D** and **Supplementary Figures 2C,D**).

To further confirm that PGLS could regulate the migration of HCC. Wound-healing assay was performed, and the area covered by treated Huh7 cells was calculated by ImageJ. Nearly the same area was occupied at 0 h among four groups, and less migration length was shown in all three KD groups at 24 h as compared to the control group

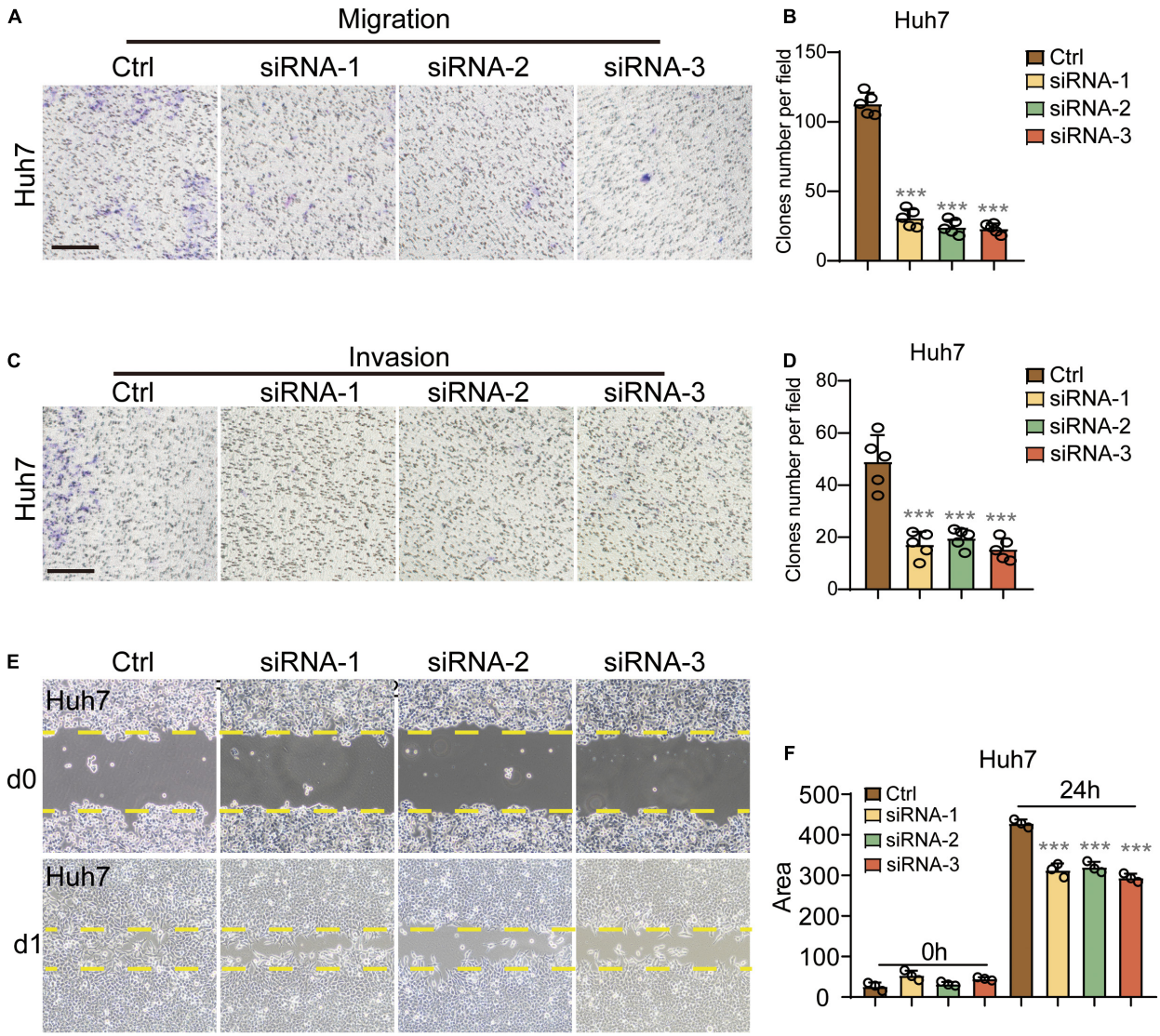

FIGURE 4 | PGLS regulated the migration and invasion of HCC. **(A)** Representative images of CFUs in migration assay. **(B)** CFU clone number per field on Huh7 in migration assay. Invaded cells from five representative fields were counted. **(C,D)** The number of invasive cells significantly decreased after the treatment of these three siRNAs, respectively ($n = 5$). **(E)** Wound healing in Huh7 cells treated with siRNA-1,siRNA-2, and siRNA-3. The lines indicated the edge of wound at 0 and 24 h. **(F)** Migration rate was analyzed and expressed as the area of cells migrating from the original wounds. Data show individual values and mean ± SD. **(B,D,F)** Unpaired two-tailed Student *t*-tests, assessed statistical significance, $^{***}p < 0.001$.

at the same time point (**Figures 4E,F**). Similar inhibitory effects were also observed in Hep3b cells (**Supplementary Figures 2E,F**).

Pentose Phosphate Pathway Inhibitor 6-Aminonicotinamide Functionally Attenuated Hepatocellular Carcinoma Migration and Invasion

6-ANA is a PPP inhibitor (Street et al., 1997; Arbe et al., 2020; Cheng et al., 2020). When Hep3b and Huh7 were cultured with a 10 μM 6-ANA concentration, we found that the $NADP^+$/NADPH ratios increased significantly (**Figure 5A** and **Supplementary Figure 3A**), and the level of ROS and apoptosis rate were also increased in both HCC cell lines as compared to the control group (**Figures 5B,C** and **Supplementary Figures 3B,C**). Then we analyzed the effect of 6-ANA on Hep3b and Huh7 cell on proliferation. Similar to previous results, 6-ANA significantly suppressed cell proliferation in 10 μM concentration (**Figure 5D** and **Supplementary Figure 3D**). Subsequently the CFU results showed 6-ANA could also effectively reduce the size and number of CFUs in the treated group (**Figures 5E,F** and **Supplementary Figures 3E,F**). To test the effects of 6-ANA on HCC cell line migration, we did both chamber assay and wound-healing assay. The results showed that 6-ANA could reduce the clone number and migration area of Hep3b and Huh7

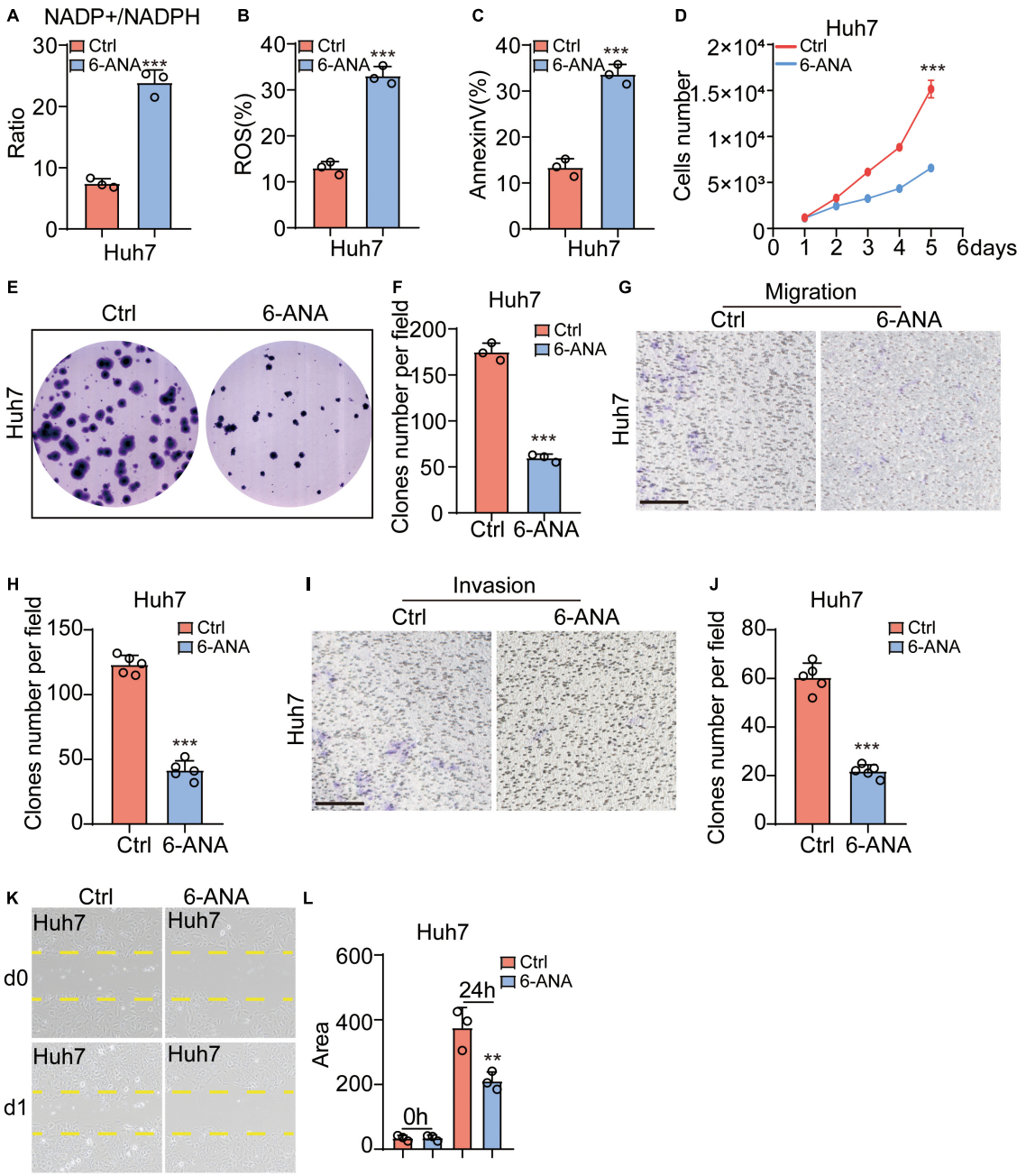

FIGURE 5 | PPP pathway inhibitor 6-ANA functionally attenuated HCC migration and invasion. **(A)** The $NADP^+$/NADPH ratio increased in the 6-ANA treated groups ($n = 3$). **(B,C)** The percentage of ROS and annexin V–positive cells increased as compared to the control group ($n = 3$). **(D)** The extent of cell proliferation was significantly reduced in the 6-ANA–treated group. **(E,F)** 6-ANA effectively suppressed the size and number of CFUs ($n = 3$). **(G,H)** Representative photographs showing the HCC cell lines that had passed through the well bottom to the lower surface of the membrane. The cells from five representative fields were counted. **(I,J)** Representative photographs showed the invasive cells that had passed through Matrigel to the lower surface of the membrane. Invaded cells from five representative fields were counted. **(K,L)** Migration rate was analyzed and expressed as the area of cells migrating from the original wounds. Data show individual values and mean ± SD. **(A–D,F,H,J,L)** Unpaired two-tailed Student *t*-tests, assessed statistical significance, $^{**}p < 0.01$, $^{***}p < 0.001$.

cells (**Figures 5G,H,K,L** and **Supplementary Figures 3G,H,K,L**). The number of invasive cells was also markedly decreased after the treatment of 6-ANA (**Figures 5I,J** and **Supplementary Figures 3I,J**). Overall, our data showed that PGLS was essential for the development of HCC.

DISCUSSION

In this study, by analyzing the liver single-cell sequencing data of HCC patients and healthy people, we were surprised to find an undifferentiated HCC population with high PGLS-specific

expression. In the selected cells with high PGLS expression in human liver cancer tissues, we found the PPP pathway activated, and ROS production and HCC apoptosis reduced. PGLS inhibition inhibited the metabolic reprogramming of HCC cell lines. For the purpose of clinical transformation, we used 6-ANA to inhibit the PPP and inhibit the proliferation, clonal formation, migration, and invasion of HCC cell lines.

More and more evidences show that metabolism-related genes (HK2, FBP1, and PKM2) are very important for the occurrence and the development of HCC, and these genes promote the growth of HCCs by promoting the transcription of oncogenes (Chen et al., 2017; DeWaal et al., 2018; Li Q. et al., 2019; Hou et al., 2020). Our project analyzed the single-cell sequencing technology data of HCC patients and found that carbon metabolism played an important role in the differentiation of HCCs, and PGLS was the most obvious change among the genes that differ in carbon metabolism. Previous reports have shown that PGLS has a significant correlation with the occurrence of breast cancer, but its relationship with HCC has not been reported (Sivaraksa and Lowe, 2008), whereas Huh7 and Hep3b cell lines that knock down PGLS have decreased proliferation and metastasis and increased apoptosis in HCC cells. Thus, inhibiting PGLS in HCCs could be a novel strategy to inhibit HCC proliferation.

Metabolic reprogramming has been recognized as a hallmark of HCC (Kowalik et al., 2017; Jin and Zhou, 2019). Although metabolism-related drugs are currently approved as molecular targeting agents for HCC, their effect on life expectancy is generally limited (Rudalska et al., 2014). In this study, for the first time, we found that the specific high expression of PGLS in HCC activates the PPP pathway and reduces cell apoptosis induced by oxidative stress injury. In hepatocytes, low levels of PGLS lead to low activity of the PPP pathway. At this point, cells mainly rely on oxidative phosphorylation and glycolysis for energy. However, the rapid proliferation of HCCs requires activation of the PPP pathway to generate large amounts of R5P and NADPH, which are vital for the survival and proliferation of HCCs. R5P is the cornerstone for nucleic acid synthesis (Andriotis and Smith, 2019). NADPH is essential for anabolic reactions and redox equilibrium. This shift in metabolic patterns is critical for HCC growth.

CONCLUSION

Finally, HCC patients with high PGLS expression have a poor prognosis. Interestingly, ROS levels and $NADP^+$/NADPH levels were significantly reduced when we knocked down PGLS in Huh7 and Hep3b cell lines. Therefore, inhibition of PGLS can promote the recovery of the PPP metabolic profile of HCC, which may be a new way to regulate the metabolic reprogramming of HCC. Taken together, our data suggest that inhibition of PGLS may provide a novel strategy to achieve effective inhibition of HCC cells.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149614.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Third Affiliated Hospital of Sun Yat-sen University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CL, JC, and YLi designed, performed most of the experiments, analyzed the data, and generated figures. YLi and BW contributed to single cell seq. ZY, XT, and YW contributed to bioinformatic analysis. ZH contributed to seahorse analysis. YP, HZ, and KY joined this project as rotation students for technique support. ZF and JX contributed for scientific discussion and manuscript preparation. YLu supervised the project and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Targeting Phosphatases and Kinases: How to Checkmate Cancer

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Metastatic disease represents the major cause of death in oncologic patients worldwide. Accumulating evidence have highlighted the relevance of a small population of cancer cells, named cancer stem cells (CSCs), in the resistance to therapies, as well as cancer recurrence and metastasis. Standard anti-cancer treatments are not always conclusively curative, posing an urgent need to discover new targets for an effective therapy. Kinases and phosphatases are implicated in many cellular processes, such as proliferation, differentiation and oncogenic transformation. These proteins are crucial regulators of intracellular signaling pathways mediating multiple cellular activities. Therefore, alterations in kinases and phosphatases functionality is a hallmark of cancer. Notwithstanding the role of kinases and phosphatases in cancer has been widely investigated, their aberrant activation in the compartment of CSCs is nowadays being explored as new potential Achille's heel to strike. Here, we provide a comprehensive overview of the major protein kinases and phosphatases pathways by which CSCs can evade normal physiological constraints on survival, growth, and invasion. Moreover, we discuss the potential of inhibitors of these proteins in counteracting CSCs expansion during cancer development and progression.

Keywords: phosphatase, kinase, cancer stem cell, phosphatase and kinase inhibitors, targeted therapies

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INTRODUCTION

The main role of kinases and phosphatases is to regulate post-translational modifications of proteins, which are essential to govern cellular signaling networks (Sacco et al., 2012). Mutations in kinases (Irby et al., 1999; Zhang et al., 2009) or phosphatases (Stebbing et al., 2014; Zhao et al., 2015) that lead to either a loss-of-function or gain-of-function are likely to cause cancer. Moreover, oncogenes can influence the balance between kinases and phosphatases activity, causing cell malignant transformation (Qi et al., 2018). The uncontrolled activation of kinases and the suppression of phosphatases has been frequently observed in cancer with consequent induction of cell proliferation, migration and survival to anti-cancer therapies. Failure in the balance between kinases and phosphatases activity has been shown in several types of solid cancer, such as colorectal,

gastric, liver and breast cancer (BC) (Martellucci et al., 2020). Thus, it is plausible that a better understanding of how kinases and phosphatase enzymes function and how they are regulated can aid the development of new anticancer agents.

Cancer recurrence and relapse are attributed to a small subpopulation of cancer cells, named cancer stem cells (CSCs) (Turdo et al., 2020). CSCs are also able to self-renewal and multilineage differentiation, as well as an ability to initiate and support tumorigenesis and metastasis formation (Veschi et al., 2020b).

This review focuses on dysregulation of kinase and phosphatase activity, since they represent the major players that sustain CSCs persistence in a variety of cancers. Moreover, herein we will discuss novel therapeutic compounds that inhibits kinase and phosphatase proteins involved in carcinogenesis.

THE ROLE OF KINASES AND PHOSPHATASES IN CANCER

Kinases and phosphatases carry out essential roles in a plethora of biological functions and regulatory network of cells (Ostman et al., 2006; Ventura and Nebreda, 2006; Malumbres and Barbacid, 2009; Bononi et al., 2011; Otto and Sicinski, 2017). Kinases catalyze the transfer of phosphate groups, released by ATP, to molecules while phosphatases remove phosphate groups from their substrate proteins.

Kinase phosphorylation can modify the function of a protein by increasing or decreasing its activity, enhancing its stabilization, marking it for destruction, localizing it within a specific cellular compartment, and initiating or disrupting its interaction with other proteins. Protein kinases often act on multiple substrates and different proteins can serve as substrates for more than one specific kinase. Kinases mediate most of the signal transduction of the cell, and consequently, control many cellular processes, including transcription, proliferation, apoptosis, metabolism, interplay with the immune systems, migration, cytoskeletal rearrangement and differentiation (Rubin et al., 2000; Lander et al., 2001; Venter et al., 2001). The largest group of kinases is composed by protein kinases, which phosphorylate proteins at serine/threonine, tyrosine or all three residues (dual-specificity kinases) (Krupa et al., 2004).

The family of protein tyrosine kinases consists of the receptor tyrosine kinases (RTKs) proteins and the non-receptor tyrosine kinases (nRTKs). Besides regulating several cellular processes in normal cells, RTKs are implicated in the development and progression of cancer. Indeed, mutations in RTKs lead to the constitutive activation of the receptor and uncontrolled activation of multiple signal transduction pathways (Schmidt-Arras and Bohmer, 2020). Almost 20 different RTKs classes have been described, which include EGFR, insulin R, PDGFR, VEGFR, FGFR, HGFR and RET (Blume-Jensen and Hunter, 2001). Differently from RTKs, the nRTKs are cytosolic enzymes, in some cases anchored to the cell membrane. Janus kinase (JAK) and Src families are the most important nRTK families involved in cancer. The JAK proteins transduce signals that are mediated by cytokines in the JAK-STAT pathway while Src, when activated, is known to phosphorylate PI3K, RAS and STAT to promote proliferation, survival and invasion of cancer cells (Kisseleva et al., 2002; Ishizawar and Parsons, 2004).

Among the protein serine/threonine kinases, PKA, MAPKs, RAF, PKB (also known as Akt), GSK-3, mTOR and cyclin-dependent kinases (CDKs) are among the most frequently occurring drivers of human cancer.

The major function of PKA in the cell include regulation of carbohydrate and lipid metabolism (Turnham and Scott, 2016). The MAPK is a complex series of signal transduction pathways connecting extracellular signals to intracellular responses, whose function is the regulation of important processes such as cell proliferation, differentiation, and death (Raman et al., 2007). During the last decades, the insight of each main MAPK signaling modules and their role in tumorigenesis has grown remarkably (Wagner and Nebreda, 2009). The four main MAPK signaling modules are (i) ERK1/2, (ii) JNK, (iii) p38, and (iv) ERK5 pathways, which are induced by specific extracellular signaling. RAF kinase is activated by growth factors, forms part of the RTKs/RAS/RAF/MEK/ERK pathway and its leading function is to stimulate cell division and growth (Chang and Karin, 2001). Moreover, the majority of solid tumors are explicitly characterized by their overall mutations along all the RTKs/RAS/RAF/MEK genes of the signaling pathway (Stern, 2018). Interestingly, MEK kinase is a component of the MAPK pathway which acts on both serine/threonine and tyrosine kinase residues.

GSK-3 is considered to be at the crossroads of various cancer pathways and a major component of the RTKs/RAS/PI3K/PTEN/Akt/GSK-3/mTORC1 axis. Upon Akt-mediated phosphorylation, GSK-3 is inactivated and targeted for proteasome degradation (Cross et al., 1995). As Akt is often active in human cancer, GSK-3 is consequently often inactivated. GSK-3 can also regulate NF-κB and WNT/β-catenin pathway activity (Gotschel et al., 2008).

Cyclin-dependent kinases are intracellular serine/threonine protein kinases whose central activity is the regulation of the cell cycle. Therefore, they are responsible for the progress of the cell through its various checkpoints (Harper and Adams, 2001). Dysregulation of CDKs, such as CDK1, CDK2, CDK3, CDK4 and CDK6, is a well-known hallmark of cancer. Several studies have focused on trying to establish a strategy to inhibit specific CDKs proteins involved in cell cycle progression, leading to uncontrolled cell proliferation in many solid cancer types (Asghar et al., 2015).

Lipid kinases are a smaller group of kinases that add phosphate groups to lipids causing a change in the reactivity and localization of the lipid with a consequent modulation of signal transmission (Whitman et al., 1988). Phosphoinositide 3 kinase (PI3K) is the main player of the PI3K/Akt pathway, which is the most common activated signaling in human's cancer (Lawrence et al., 2014). This signaling network is activated downstream of RTKs and regulates cell survival, growth, transcription and protein synthesis (Fruman et al., 2017). Phosphatases are known to exert dephosphorylation on RTKs, whose activity

can be modified in both a positive and negative manner (Volinsky and Kholodenko, 2013). Consequently, phosphatase dysregulation can hamper RTK regulation, emphasizing their critical implication in the onset and progression of cancer (Yao and Stagljar, 2017).

A total of 211 phosphatase domains have been characterized and then assigned to six different families, defined by catalytic domain sequence similarity (Sacco et al., 2012). The major categories include the phosphoprotein phosphatase (PPP) and the protein phosphatase Mg^{2+}- or Mn^{2+}- dependent (PPM) families that dephosphorylate phosphoserine and phosphothreonine residues, the haloacid dehalogenase (HADS), and the most dominant group of Cys-dependent protein tyrosine phosphatase (PTP) family that dephosphorylate phosphotyrosine amino acids. Notably, a subfamily of the PTPs, the dual-specificity phosphatises (DUSPs), dephosphorylate all three phosphoamino acids (Alonso et al., 2016).

The PPM family component PPM1D, also termed WIP1, is nowadays considered as an oncoprotein because of its negative regulation of target anti-cancer proteins such as p53, ATM, H2AX and p16. Amplifications and mutations of PPM1D has been frequently observed in cancer, and linked to the progression of the disease and therapy resistance phenomena (Deng et al., 2020b).

The phosphatase PP2A, belonging to the PPP family, is the most expressed serine/threonine phosphatase in mammalian cells and plays a fundamental role in the control of normal kinases activity (Westermarck and Hahn, 2008). PP2A has been primarily described as a tumor suppressor involved in diverse signaling networks regulating cancer progression (Mumby, 2007). The main PP2A function is to inhibit the RAF-MEK-ERK pathway (by reducing activity of both ERK and RAF), and to dephosphorylate and inhibit Akt, c-Myc, and RalA (Zhang and Claret, 2012).

Among the classical PTPs, the receptor-type T PTP (PTPRT) and the receptor-type D PTP (PTPRD) have been reported to be involved in the negative regulation of the JAK/STAT pathway and in particular of STAT3 (Veeriah et al., 2009). Both PTPRT and PTPRD have been described as tumor suppressors in a variety of cancers (Wang et al., 2004; Funato et al., 2011). Moreover, it has been reported that PTPRH and PTPRB directly exert dephosphorylation on EGFR, and thence, suppress its downstream signaling in PI3K/Akt/mTOR and MEK/MAPK pathways (Yao et al., 2017).

The classical PTPs non-receptor type comprise the PTP1B, whose activity has been associated with poor prognosis in breast, gastric, colorectal, hepatocellular and lung cancer by regulating variable tumor-specific mechanisms (Bollu et al., 2017). Also, it has been shown that SHP2 (also known as PTPN11), is a main functional regulator of RTK. Indeed, somatic *PTPN11* mutations has been linked to human malignancies, including juvenile leukemia and juvenile myelomonocytic leukemia (Grossmann et al., 2010). Finally, it has been suggested that protein *PTPN13* is a tumor-suppressor gene, for instance in non-small cell lung cancer, likely due to the control of phosphorylation of both RTK type receptors EGFR and HER2 (Scrima et al., 2012).

It has been shown that numerous DUSP are critical regulators of the MAPK family, which includes ERK and JNK (Liu et al., 1995; Muda et al., 1996; Nunes-Xavier et al., 2013). Besides, PTEN, which is a crucial member of the DUSP family (Myers et al., 1997), negatively regulates intracellular levels of PIP3 and functions as a tumor suppressor by exerting a negative regulation on the PI3K/Akt signaling pathway (Lee et al., 2018). Loss of PTEN, which results in hyperactivation of PI3K pathway, and thus an increase in cell proliferation, has been identified as a decisive genetic event triggering the onset of a wide variety of neoplasm. PTEN activity includes the control of apoptosis, migration, metabolism and anti-cancer therapy response of cancer cells (Lee et al., 2018).

PTEN has been frequently found to be downregulated by PRL-3 in colorectal cancer (CRC). Overexpression of phosphatase PRL-3 is associated with activation of the PI3K/Akt pathway, which can promote epithelial to mesenchymal (EMT) and tumor progression (Wang et al., 2007).

The CDK-associated protein phosphatase (KAP) is overexpressed in cancer cells. It participates to the G1/S transition of the cell cycle and forms a complex with CDK2. Indeed, KAP promotes growth of cancer cells and determines resistance to anti-tumor necrosis factor-α-induced apoptosis by preventing the activation of caspase-3 (Lai et al., 2012). Additionally, cells overexpressing KAP show a higher ability of cell invasion and tumorigenicity (Stebbing et al., 2014).

A class of DUSP proteins involved in the regulation of MAPK pathway, thus also referred to as MAP kinase phosphatases (MPK), dephosphorylate ERK, JNK and p38 at tyrosine and serine/threonine residues. In physiological conditions, DUSP expression levels are positively regulated at transcriptional level by ERK, generating a negative feedback loop to restrain RAS signaling. DUSPs are classified, in more than ten members, according to the specificity of the substrate and cell localization (Caunt and Keyse, 2013). DUSPs are generally defined as tumor suppressor even though several evidence reported a pro-tumorigenic role (Furukawa et al., 2003). For instance, DUSP1 and DUSP6 inhibition suppresses tumor growth in leukemia and BC. Indeed, in these cases DUSP activity seems to favor cancer cell adaptation to high proliferative stimuli (Kaltenmeier et al., 2017; Kesarwani et al., 2017).

Cyclin-dependent kinases dysregulation, which is often determined by altered phosphatases, is undoubtedly a notorious hallmark of cancer. As a result, it is important to understand the abnormal role of specific proteins, including phosphatases, which can facilitate cell cycle progression, leading to uncontrolled cell proliferation and malignancy (Asghar et al., 2015). Cell division cycle 25 (CDC25) families are DUSPs and determine the activation of CDKs, which in turn regulate cell-cycle progression. Three isoforms have been characterized CDC25A, CDC25B and CDC25C, which showed a correlation with poor survival and multi-drug resistance (Galaktionov et al., 1995; Karagoz et al., 2010; Albert et al., 2012).

Given the indispensable role of kinase and phosphatase enzymes in cancer onset and progression (an extensive review literature can be found in the following references Ostman et al., 2006; Ventura and Nebreda, 2006;

Malumbres and Barbacid, 2009; Bononi et al., 2011; Otto and Sicinski, 2017), several research studies, as reported below, are nowadays aimed at uncovering the contribution of these enzymes in cancer stemness.

KINASES AND PHOSPHATASES ARE CRUCIAL PLAYERS IN CANCER STEM CELLS

Cancer is a heterogeneous disease at phenotypic and genetic level. Many studies have investigated the role of CSCs in cancer progression and resistance to therapy. Kinases and phosphatases play important roles in maintaining CSC phenotypes, including self-renewal capacity, invasiveness, and tumorigenicity (**Figure 1**).

The PI3K/Akt pathway is among the best investigated in human biology, and its pathological activation is considered as a "driver" in numerous cancers. Growing evidence suggests an important role for PI3K signaling in the regulation of stemness, and the underlying mechanisms are still under investigation (Todaro et al., 2014; Di Franco et al., 2016; Mangiapane et al., 2021). Previously Dubrovska et al. (2009) showed that PTEN/PI3K/Akt pathway is critical for prostate CSCs maintenance. Prostate cancer cells treated with the PI3K inhibitors showed a reduced capacity to form spheres. Conversely, genetic silencing of *PTEN*, caused a significant increase of cancer progenitors and stem-like cells in prostate cancer (Dubrovska et al., 2009) and glioblastoma (Duan et al., 2015). Other activities in CSCs are mediated by the activation of PI3K/Akt/mTOR pathway. In particular, mTOR pathway has a central role in the maintenance of CSCs. The PI3K and mTOR pathway sustained the expansion of side population cells, expressing a $CD44^+/CD24^-$ phenotype, in MCF7 BC cell lines, and fostered tumorigenic potential *in vivo* (Li et al., 2018). More recently, it has been shown that the activation of PI3K/Akt pathway characterized breast CSCs (BCSCs) expressing high levels of multidrug resistance (MDR) (Hu et al., 2015). The PI3K/PTEN/Akt/mTOR signaling regulated CSC activity in gefitinib-resistant A549 cells, which contained a high proportion of $CXCR4^+$ cells endowed with an enhanced potential of self-renewal activity *in vitro* and tumor growth *in vivo* (Jung et al., 2013).

Chang et al. (2015) found that prostate cancer radioresistance is sustained by the activation of a stemness and EMT phenotype dictated by the activation of the PI3K/Akt/mTOR signaling pathway. PI3K/mTOR also positively regulated aldehyde dehydrogenase 1, member A1 (ALDH1A1) expression and ALDH activity, through SOX9 transcriptional activation, in head and neck squamous cancer cell (Keysar et al., 2017). PI3K/Akt pathway likely contributes to survival and resistance of brain CSCs. Specifically, among the PI3K isoforms, the p110α was decisive to sustain sphere formation and clonogenic capability of medulloblastoma cells. Notably, the PI3Kα catalytic isoform acted in synergism with MAPK-interacting kinase (MNK) to enhance medulloblastoma stem-like properties (Eckerdt et al., 2019).

Also MAPK signaling is involved stem cell biology. Blaj et al. (2017) showed that MAPK pathway is significantly related to intratumoral heterogeneity of CRC. The oncogenic effect of MAPK activity appeared consistently restricted to tumor cells placed at the leading edge, whereas more differentiated tumor cell placed at the central core of the tumor had lower MAPK activity. Moreover CRC cells, displaying high MAPK activity, had a distinct phenotype characterized by decreased epithelial markers expression, such as E-cadherin, and increased expression of the LAMC2 mesenchymal marker, which is a target of ZEB1 (Blaj et al., 2017). Recently it has been demonstrated in CRC that MAPK and FAK signaling pathways are able to maintain the $ALDH^+$ cell population, which has been described in various cancer, such as colon, breast, lung, head and neck squamous cancer, to possess tumor-initiating capabilities (Tomita et al., 2016).

JAK-STAT3 pathway regulated CSCs properties of thyroid anaplastic carcinoma. Anaplastic thyroid carcinoma is one of the most aggressive carcinoma refractory to current therapies. CSCs, responsible for his high malignancy, have been previously identified and characterized (Todaro et al., 2010). *In vitro* experiments have showed that JAK1 inhibitor suppressed specifically the stem cell compartment of THJ16T cells, suggesting a role of JAK/STAT pathway in thyroid CSCs growth (Shiraiwa et al., 2019). Zhou et al. (2007) demonstrated that the JAK/STAT3 pathway, in synergism with mTOR, was fundamental for breast cancer stem-like cell survival *in vitro* and in a preclinical model of nude mice. The screening of a large scale library of shRNAs revealed that the JAK2/STAT3 signaling was selectively activated in $CD44^+/CD24^-$ BCSCs as compared with the differentiated compartment (Marotta et al., 2011). Similarly, IL-6 secreted by CRC-derived mesenchymal stem cells promoted the onset of a stemness phenotype in CRC cells through the activation of JAK2/STAT3 pathway (Zhang et al., 2018). Tumor micro environmental cues are indeed the major upstream activators of the JAK/STAT pathway. Yang et al. (2019) demonstrated that IL-10 derived by tumor associated macrophages dictates the stem cell fate of non-small cell lung cancer (NSCLC) cells *via* the activation of JAK1/STAT1 signaling. Under the hypoxic tumor microenvironment, hypoxia-inducible factor 1 α (HIF-1α) activated the JAK1-2/STAT3 pathway in glioma stem-like cells causing an enhancement in self-renewal and a delay in *in vivo* tumor growth (Almiron Bonnin et al., 2018).

Recently, by using an unbiased proteomic profiling combined to *in vivo* transplantation studies, Coles et al. (2020) showed that PKA is a key kinase responsible for initiation and progression of small cell lung cancer (SCLC). Activation of PKA activity in SCLC cells increased significantly the stem cell frequency, the expression of stem cell markers, such as NCAM1, DLL3, MYCL and CD24 and tumor growth of xenografts. Beyond the role of PKA in SCLC pathogenesis, these data provided essential insights regarding PKA signaling networks, comprising the G-protein α subunit and the serine/threonine protein phosphatase PP2A as positive and negative regulators respectively (Coles et al., 2020).

Phosphatases have been also linked to stem cell biology. SHP2, currently under investigation for therapeutic proposal, has a

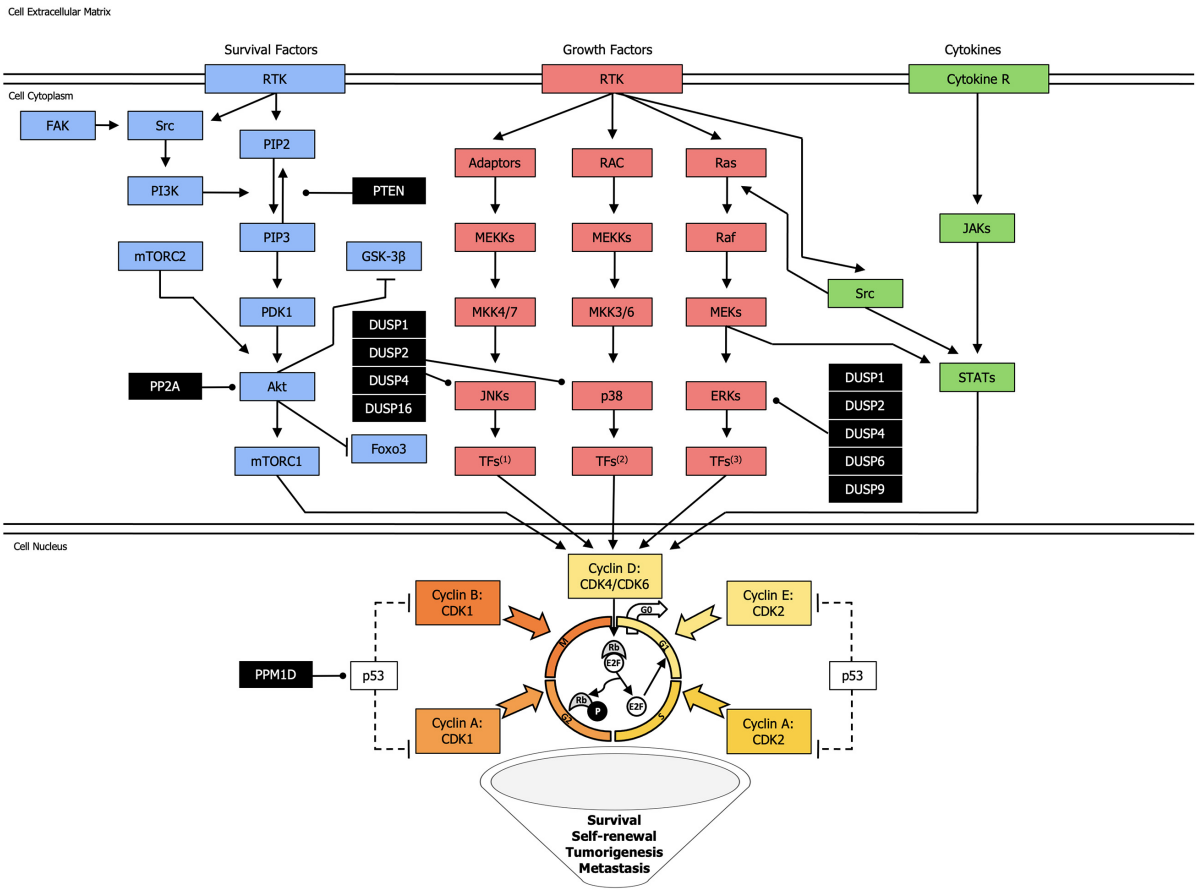

FIGURE 1 | Landscape of the most common kinases and phosphatases network in cancer stem cells. Kinase activating phosphorylation is shown with arrowed lines, inhibitory phosphorylation or non-phosphorylation is indicated with blocked lines, and dephosphorylation, exerted by phosphatases (black squares), is displayed with round-ended lines. Dotted lines represent indirect effect. TFs^1: Transcription Factors (Jun, ATF2, RNPK, p53, NFAT4, Shc); TFs^2: Transcription Factors (CHOP, ATF2, MNK, MSK, MEF2, Elk-1); TFs^3: Transcription Factors (Elk-1, Ets-2, RSK, MNK, MSK, cPLA2).

specific role in regulating CSCs biology. The function of SHP2 within CSCs has been characterized in several tumors, such as leukemia, BC, glioma and liver carcinoma. Aceto et al. (2012) showed that SHP2 was able to influence the self-renewal of tumor cells. In fact, *SHP2* knockdown reduced the self-renewal ability of breast cells in both HER2- positive and triple-negative tumors. Overexpression of both *HER2* and *HER3 in vitro* increased the number of $CD44^+/CD24^-$ cells. However, knockdown of *SHP2* decreased the $CD44^+/CD24^-$ and $ALDH^{high}$ cell population. Finally the depletion of *SHP2* in xenografts reduced tumor growth and abolished sphere formation suggesting that SHP2 contributes to the CSC phenotype (Aceto et al., 2012). Furthermore, *PTPN11* mutations have been frequently identified in glioblastomas, occurring in 7.5% of cases. SHP2 expression correlated with SOX2 expression in glioma stem cells and was decreased in the differentiated counterpart. The induction of differentiation of glioma stem cells resulted in decreased SHP2 expression (Roccograndi et al., 2017). In hepatocellular carcinoma (HCC), SHP2 promoted cell dedifferentiation and CSC expansion through the activation of β-catenin signaling (Xiang et al., 2017).

Among phosphatases, an important role in CSCs regulation has been attributed to DUSPs. Family members of DUSPs play pleiotropic and controversial roles in stemness maintenance, making their pro- or anti-cancer function particularly context-dependent. The treatment of HCC1806 BC cells with specific pharmacologic inhibitor or shRNA of DUSP9, significantly reduced DUSP9 levels and caused simultaneously reduction of the stem cells markers OCT4 and ALDH1. DUSP9 shRNA treated cells had reduced ability to form mammospheres when compared to controls. When transferred in xenograft, these cells showed reduced tumor growth compared to controls (Jimenez et al., 2020). DUSP1, DUSP4 and DUSP6 have been reported to associate with resistance to anti-cancer therapies and activation of an EMT program (Small et al., 2007; Liu et al., 2013; Boulding et al., 2016; Wu Q. N. et al., 2018). A recent study showed that DUSP6 supports a CSC phenotype in endometrial carcinoma. DUSP6 albeit inhibiting MAPK–ERK1/2 signaling, led to the activation of PI3K/Akt, with consequent increased expression of CSC-related genes in endometrial cancer cells (Kato et al., 2020). Conversely, Boulding et al. (2016) demonstrated that, although DUSP1 sustains CSC expansion, DUSP4 and DUSP6 negatively

influences BCSCs maintenance. DUSP2 also showed inhibitory effects on stemness. In particular, knockdown of *DUSP2* caused an expansion of the CSC population in CRC. This cell subset showed increased expression of OCT4, NANOG, and SOX2 and tumor-sphere formation ability. In addition, DUSP2 expression levels were significantly decreased in $CD133^+$ CRC cells as compared with the $CD133^-$ cell counterpart (Hou et al., 2017).

RECENT CLINICAL DEVELOPMENTS IN KINASE AND PHOSPHATASE INHIBITORS

As the knowledge obtained from different studies has shed light on the fundamental influence of kinase and phosphatase proteins in cancer biological processes, several protein tyrosine kinases and phosphatases inhibitors have granted the FDA approval or are under clinical evaluation for the treatment of various cancer types. One of the fundamental aspects for the development of molecules that inhibit protein kinases is the maximization of drug affinity for a specific target and the limited interaction with non-target enzymes. Some important physicochemical properties have to be taken into consideration for orally effective therapies. It is necessary to estimate the solubility, membrane permeability and efficacy in the drug setting. The assessment of those parameters follows the Lipinski's "rule of five" (RO5) (Lipinski et al., 2001). Additional characteristics considered are lipophilic efficiency and ligand efficiency (Roskoski, 2021a,c).

Since the first approval in 2001 of the imatinib, a small molecule inhibitor of ABL kinase, more than 60 protein tyrosine kinase inhibitors have received FDA authorization for cancer treatment (**Figure 2** and **Table 1**). According to the mechanisms of action, small molecule protein kinase inhibitors have been categorized in six different classes. Type I inhibitors compete for ATP binding in the ATP-binding sites of active conformations. Type II inhibitors bind to an inactive enzyme forms and in particular to an ATP-binding adjacent site. The allosteric inhibitors, type III, interact with an allosteric site, and possess the peculiarity of being a non-competitive ATP inhibitors since they leave the ATP site free (Dar and Shokat, 2011). Type III inhibitors have been further divided into different classes: type III antagonists, which bind inside the cleft between small and large lobes close to the ATP-binding site, and type IV antagonists, which bind sites outside the ATP-binding pocket (Gavrin and Saiah, 2013). Some antagonists, classified as type V inhibitors, are defined as bivalent since they bind to separate parts of the protein kinase domain (Lamba and Ghosh, 2012). Although owing numerous advantages, including efficacy at low doses, prolonged inhibition and capability to bind targets with shallow binding sites, the scientific community has been skeptical about covalent inhibitors due to their toxicity and safety concerns. In 2013, the BTK inhibitor ibrutinib was approved for clinical use after demonstrating efficacy in lymphoma and chronic leukemia, thus leading to a new area for irreversible drugs. Subsequently, the FDA approved other six irreversible kinase inhibitors drugs (acalabrutinib, zanubrutinib, afatinib, dacomitinib, osimertinib, and neratinib) (Roskoski, 2021b).

Moreover, over the past decades antibody-based therapies have significantly changed the probability of survival for oncological disorders. Some examples are further discussed below.

Besides PTK have been successfully targeted with almost a hundred of FDA-approved drugs, PTP druggability is still a major challenge due to difficulties in the design of safe and efficacious treatments (Mullard, 2018). Protein tyrosine phosphatase loss of function is due to genetic alterations including point mutations, deletion and epigenetic modification. For this reason, PTP inhibitors could comprise, in the near future, agents with different characteristics and mechanisms of action. As for now, the majority of drugs currently in clinic are mainly directed against SHP2 and PTP1B, which are central nodes for several signaling pathways. In this context, a list of phosphatase inhibitors under clinical evaluation is reported in **Table 2**.

KINASES AND PHOSPHATASES AS THERAPEUTIC TARGETS IN CANCER STEM CELLS

The fight against cancer has always been a challenge for the scientific community. Conventional anti-cancer therapies, as for example chemotherapy, have been pivotal treatments for cancer since the 1940s (DeVita and Chu, 2008). Over the past years, several studies have been conducted with the purpose to narrow the various chemotherapy drugs developed in order to select the best chemical compounds. Nevertheless cancer patients have received important benefits, the high mortality rate is due to chemoresistance phenomena that allow the persistence of therapy resistant cancer cell clones responsible for tumor outgrowth and metastasis formation (Turdo et al., 2019, 2021). Metastatic disease continues indeed to be a threat for cancer patients, raising the important point of the necessity of new efficacious and tailored therapies able to counteract the expansion and dissemination of the CSCs compartment. Despite the standard chemo-radiotherapy and specific kinase/phosphatase inhibitors having improved the life quality of cancer patients, metastatic disease, relapse and chemoresistance remain an outstanding issue. Hence, the discovery of kinase/phosphatase inhibitors constitute an attractive therapeutic option for the near future for the purpose to improve the efficiency of standard cancer therapy striking the refractory CSCs (Yang et al., 2020; Momeny et al., 2021).

PI3K is the major pathway considered deregulated in cancer (Fruman et al., 2017). In different types of cancer and in particular in HCC, alteration of the PI3K pathway was considered a master player for the support of CSCs. The striking increase in one of catalytic subunit type 3 of PI3K (PIK3C3) was detected in HCC tissues and liver CSCs. The inhibition of PIK3C3 hampered CSCs stability *via* the activation of AMPK. *In vitro* and *in vivo* models have shown that the combined use of PIK3C3 and PI3K inhibitor reduced spheroid formation and counteracted the tumors growth in mice models (Liu et al., 2020). In line with these observations, our group demonstrated that a subpopulation of colorectal CSCs (CR-CSC), endowed with metastatic potential, is characterized

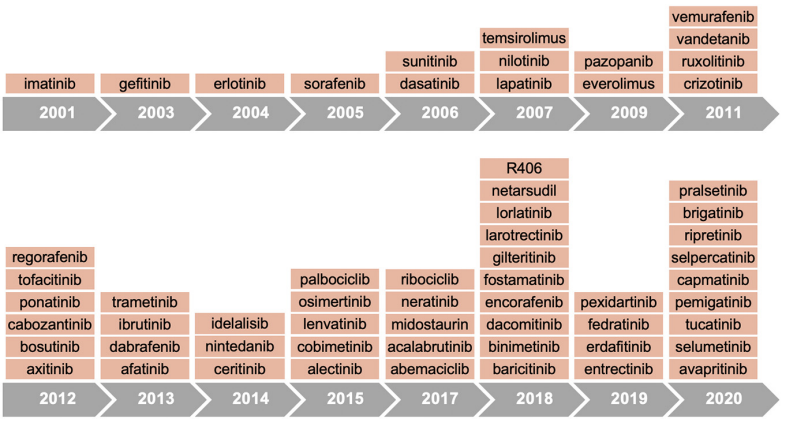

FIGURE 2 | Timeline of FDA approvals for kinase inhibitors.

TABLE 1 | Protein kinase inhibitors and their related targets.

Target	Drug									
ALK	Alectinib	Brigatinib	Ceritinib	Crizotinib	Gliteritinib	Lorlatinib				
BCR/ABL	Bosutinib	Imatinib	Nilotinib	Ponatinib						
BTK	Acalabrutinib	Ibrutinib								
CDK	Abemaciclib	Midostaurin	Palbociclib	Ribociclib						
C-RET	Lenvatinib	Pralsetinib	Selpercatinib							
CSF1R	Pexidartinib									
EGFR	Afatinib	Dacomitinib	Erlotinib	Gefitinib	Lapatinib	Neratinib	Osimertinib	Regorafenib	Tucatinib	Vandetanib
FGFR	Erdafitinib	Pemigatinib	Nintedanib	Lenvatinib						
FGR	Midostaurin									
FLT3	Gliteritinib	Midostaurin								
JAK	Baricitinib	Fedratinib	Ruxolitinib	Tofacitinib						
KIT	Avapritinib	Midostaurin	Pazopanib	Ripretinib						
MEK	Binimetinib	Cobimetinib	Encorafenib	Selumetinib	Trametinib					
MET	Cabozantinib	Capmatinib	Crizotinib							
m-TOR	Everolimus	Temsirolimus								
PDGFR	Avapritinib	Lenvatinib	Midostaurin	Nintedanib	Pazopanib	Ripretinib	Sorafenib			
PI3K	Idelasib									
RAF	Dabrafenib	Sorafenib	Vemurafenib							
RHO-K	Netarsudil									
ROS1	Crizotinib	Lorlatinib								
SRC	Dasatinib									
SYK	Fostamatinib	Midostaurin	R406							
TRK	Entrectinib	Larotrectinib								
VEGFR	Axitinib	Cabozantinib	Lenvatinib	Midostaurin	Nintedanib	Pazopanib	Regorafenib	Sunitinib	Vandetanib	Sorafenib

by the expression of CD44v6 and consequent activation of PI3K/Akt pathway. Targeting PI3K with the BKM120 selectively killed CD44v6^{+} CR-CSCs and reduced the growth of metastasis (Todaro et al., 2014; Veschi et al., 2020a). B591 is a novel inhibitor that targets class I PI3K isoforms, blocking the PI3K/mTOR pathway. This inhibitor proved to be effective in reducing the CSC compartment of different types of cancer. In particular, Zhou et al. (2019) observed a reduction in EMT and stemness markers, self-renewal and tumor initiating capabilities of CSCs. The combined treatment of cisplatin and the PI3K inhibitor, BEZ235, induced apoptosis of chemoresistant ovarian cancer cells. The authors also observed a reduction in colony formation capabilities, EMT and CSC markers expression (Deng et al., 2019). Akt isoforms play crucial roles in the maintenance of the CSC-like phenotype. The knock-down of the Akt1 isoform, and to a lesser extent of Akt2, affected the survival of BCSC and in particular of those displaying mesenchymal characteristics (Rivas et al., 2018). These results suggest that Akt1 is required not only for the self-renewal of CSC but also for their migratory capacity. On the other hand, glioblastoma and BCSCs deficient of Akt2 showed decreased expression of WIP and the stemness markers YAP and TAZ (Escoll et al., 2017).

TABLE 2 | Different phosphatase inhibitors under clinical investigation.

Drug	Target	Properties	Indication	NCT identifier	Status
AKB-9778	VE-PTP	Catalytic inhibitor	Diabetic retinopathy	NCT03197870	Phase IIb
Benznidazole	PTP1B	Allosteric inhibitor	Chagas disease	NCT03378661	Phase II
GSK2983559	RIP2	Allosteric inhibitor	Inflammatory Bowel Diseases	NCT03358407	Phase I
IFB-088	PPP1R15A	Allosteric inhibitor	Charcot-Marie-Tooth	NCT03610334	Phase I
LB-100	PP2A	Catalytic inhibitor	Solid tumors	NCT01837667	Phase I
MSI-1436C	PTP1B	Non-competitive allosteric inhibitor	Breast	NCT 02524951	Phase I
			Type II diabetes	NCT00606112	Phase I
PRL3-zumab	A*STAR	Monoclonal antibody	Solid tumors	NCT04118114	Phase II
RMC-4630	SHP2	Allosteric inhibitor	Solid tumors	NCT03989115	Phase I
			Leishnaniasis	NCT00662012	Phase I
SSG	SHP1, SHP2	Allosteric inhibitor	Melanoma	NCT00498979	Phase IV
			Solid tumors	NCT00629200	Phase I
TNQ155	SHP2	Allosteric inhibitor	Solid tumors	NCT04000529	Phase Ib

Indeed, it is now clear that the PI3K/Akt pathway activation is required for the maintenance of CSCs from a variety of cancers including colorectal, ovarian, breast and hepatocellular cancer. It should not also be underestimated that this aggressive cell subpopulation could activate mechanisms of resistance to PI3K/Akt inhibitors. Of note, high HER2 expression levels have been associated with the activity of the PI3K/Akt pathway, whose targeting was not sufficient to hamper the viability of CR-CSCs. Besides targeting PI3K, with the BKM120 or taselisib, together with the HER2, inhibitor Trastuzumab and MEK inhibitors, cobimetinib or trametinib, induced the regression of tumors in CRC xenografts (Mangiapane et al., 2021).

A better understanding of the signaling pathways driven by CDKs activity is an urgent need in order to develop new specific CDKs inhibitors to block cancer cell proliferation. For instance, the role of unusual CDK, such as CDK5 was studied in glioblastoma. It was reported that CDK5 was involved in stemness of brain tumor cells in mouse models. Therefore, CDK5 enhanced asymmetric cells division and promoted glioma stem cell self-renewal through bind and phosphorylation of CREB1. The hypothesis to inhibit this kinase, with a specific inhibitor, such as CP681301, could be a change to inhibit the brain CSCs and reduce risk of recurrence (Mukherjee et al., 2018). The CDK4 positively regulates cancer stemness in triple-negative BC, where it behaved as negative prognostic marker and therapeutic target. The pharmacological inhibition of CDK4 interfered with BCSC self-renewal, promoted the transition to an epithelial phenotype and eliminated chemotherapy-resistant cancer cells (Dai et al., 2016).

An interesting example that empathizes the clinical relevance of PTP and PTK double targeting has been demonstrated by Lai et al. (2018) in the context of BCR/ABL positive leukemia patients who experienced resistance to tyrosine kinase inhibitors (TKIs). The LB100 and LB102 inhibitors of PP2A, in combination with the most effective BRC-ABL inhibitors imatinib or dasatinib (Roskoski, 2018a), reduced the viability of leukemic stem cells both *in vitro* and *in vivo*. The PP2A is implicated in human cell transformation and in cell cycle progression and its inhibition eradicated the cancer subpopulation of leukemic stem cells responsible for TKI resistance and minimal residual disease (Lai et al., 2018). These findings corroborated previous observations regarding the additive anti-cancer effect, on chronic myeloid leukemia stem cells, of the inhibition of TKs and JAK2, which is a component of the PP2A/β-catenin/BCR-ABL complex (Lin et al., 2014). However, starting from the notion that PP2A activity is inhibited by BCR-ABL, Perrotti et al. (2019) raised some concerns regarding the synergistic effect of TKIs and the PP2A inhibiting drug LB100. Several experimental flaws regarding Lai et al. (2018) study have been brought to light, including the choice to use cell lines and not primary normal and leukemia stem cells resistant to TKIs, the evaluation of cell viability rather than apoptosis and the lack of data regarding the translatability of PP2A inhibitors into clinical settings due to expected serious adverse events. Thus, the therapeutic relevance of PP2A inhibition combined with TKIs, in BCR-ABL leukemia, seems still speculative (Perrotti et al., 2019).

Triple negative BC is known as a subtype of BC more aggressive with a remarkable likelihood of relapse and metastasis. The standard treatment for this molecular subtype of BC is chemotherapy. Lu et al. (2018) have recently highlighted that the HIF-1, induced by chemotherapy, positively regulated DUSP9, while inhibiting DUSP16, in turn leading to the dephosphorylation and nuclear translocation of FoxO3 and activation of the p38 MAPK, respectively. This in turn triggers a stemness program with the enrichment of $ALDH^+$ BCSCs and therapy resistance. *In vitro* treatment with the p38MAPK inhibitor SB203580 sensitized BCSCs to chemotherapy. Moreover, *in vivo* administration of another p38 MAPK inhibitor, the LY2228820, decreased tumor formation in immunocompromised mice. Interestingly, the small molecule LY2228820, has already been tested in early phases clinical trials showing tolerable side effects in advanced cancers (Patnaik et al., 2016; Lu et al., 2018).

Accordingly our group and others demonstrated that IL4, secreted by tumor and microenvironment cells interacts with

IL4R expressed by cancer cells and promotes metastatic spreading by the activation of the JAK/STAT6, PI3K/Akt and MAPK pathways (Gaggianesi et al., 2017). Targeting the autocrine and paracrine IL4 signaling by using an IL4Rα antagonist (IL4DM), attenuated MAPK pathway and reduced the tumorigenic and metastatic potential of $CD44^+/CD24^-$ BCSCs. Interestingly, IL4DM treatment also potentiate the response of the immune system against the aggressive subtype of CSCs responsible for cancer progression and resistance to standard therapeutic regimens. Additionally, the inhibition of the IL4-induced NF-κB, with withaferin or 5-aminosalicylic acid (5-ASA), restored DUSP4 expression levels and consequent ERK inactivation and inhibition of stemness, invasion and proliferation of BC cells (Gaggianesi et al., 2017). Interestingly, a decrease in IL6 levels abrogated sphere-formation and *in vivo* growth of primary tumors generated by paclitaxel-resistant cervical cancer cells. In particular, the treatment with the EGFR inhibitor, erlotinib, inhibited IL6 at transcriptional and translational levels through the MUC1-EGFR-CREB/GRβ axis, causing a depletion in CSC both *in vitro* and *in vivo* (Lv et al., 2019).

The protein phosphatase WIP1 (PPM1D) inhibitors are currently under clinical evaluation for their capability to subtract p53 from proteolytic degradation or inactivation. Deng et al. (2020a) described a novel function of the WIP1 inhibitor GSK283071, which suppresses stemness features in NSCLC through the activation of p38 MAPK. Specifically, the inhibition of WIP1 induced p38 MAPK phosphorylation and consequent activation of the downstream targets MK2 and HSP27 and reduction of the SOX2, OCT4, NANOG and ALDH1A1 stemness markers, sphere forming capability and tumor-initiating potential (Deng et al., 2020a).

The small molecule BBI608 is an inhibitor of STAT-3-driven transcription, which showed cytotoxic effects against several cancer types. The peculiarity of this compounds is that it showed superiority in counteracting CSCs propagation *in vitro* as compared to other PTK inhibitors and chemotherapy. Moreover, the BBI608 depleted stem-like cells *in vivo* and prevented metastasis formation (Li et al., 2015).

The Hippo kinases Mst1/2 and Lats1/2 are responsible for the phosphorylation and inactivation of the Yes-associated protein (YAP) and the transcriptional activator with PDZ-binding domain (TAZ). The oncogenic role of YAP and TAZ has always been debated (Hong et al., 2016). Indeed, recently it has been demonstrated that YAP overactivation following the loss of LATS1/2 caused a reduction in the $Lgr5^+$ CR-CSC compartment and acted as tumor suppressor role in preclinical models (Cheung et al., 2020).

The development of drugs targeting the most important oncogenic signaling, consisting in the RTKs/RAS/RAF/MEK/ERK pathway, has been the major challenge in the past 20 years (Roskoski, 2018b). One recent report aimed at demonstrating the impact of more than two thousands compounds on the activation of the Wnt pathway in colorectal cancer. While small molecules targeting BRAF and EGFR did not alter the stemness pathway, MEK1/2 inhibitors, such as trametinib, selumetinib, U0126 and PD318088, fostered an increase in Wnt signaling (Zhan et al., 2019). Accordingly, MEK inhibition induced the expression of pluripotency markers in thyroid cancer and melanoma cells (Dorris et al., 2016). On the other hand, targeting BRAF signaling pathway depleted the CD133 positive compartment in thyroid tumor (Bozorg-Ghalati et al., 2019) and synergized with cetuxiamb, an EGRF inhibitor, in reducing the CSC pool in colorectal cancer (Wu Z. et al., 2018).

DISCUSSION

In the present review, we propose an overview of the major kinases and phosphatases involved in many biological processes and in particular their roles in promoting tumor growth. Notwithstanding standard anti-cancer therapies have represented and actually represents the primary arm to counteract tumor bulk, the presence of a small subpopulation in the malignant cell pool, named CSCs, does not allow the total eradication of the tumor.

Therefore, CSCs are responsible for cancer relapse, higher invasiveness as well as chemoresistance.

Recent research has shown that the imbalance between kinases and phosphatases in tumor microenvironment are key elements to lead tumorigenesis, tumor growth and dissemination. In addition, gene deletions, mutations or epigenetic modifications have been shown to be crucial factors in aberrant activation of signaling pathways.

We herewith describe the latest findings regarding the contribution of kinases and phosphatases in cancer progression and dissemination. Recent advances discussed in this review regard drugs that target several kinases and phosphatases proteins deregulated in cancer. Several inhibitors have been used with the aim of counteracting advanced disease, in particular hampering CSCs.

Unfortunately, many types of advanced cancers are still appropriately untreated, in fact the challenge for the scientific community is to drive the discovery of novel therapeutic targets. Thus, there is a need to find new drugs that inhibit kinases and phosphatases proteins and, at the same time, sensitize CSCs compartments to chemotherapy.

Hereby, the aim is to enhance the efficacy of standard therapeutic approaches also using new compounds. Based on accumulated knowledge in recent years, new compounds have been used in experimental phases in patients affected by different types of cancer with promising results.

These data provide a new perspective in order to eradicate the tumor and offer a better life expectancy for the oncologic patients.

AUTHOR CONTRIBUTIONS

AT, CD'A, and AG contributed to design and drafting the content, figure and table in the review article. AT contributed to refine the manuscript. GPo, CC, LC, MM, NF, CM, GPi, and MB contributed to draft the manuscript. MT and GS revised the review article. All authors contributed to the article and approved the submitted version.

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Single-Cell RNA Sequencing Reveals the Role of Phosphorylation-Related Genes in Hepatocellular Carcinoma Stem Cells

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Abnormal activation of protein kinases and phosphatases is implicated in various tumorigenesis, including hepatocellular carcinoma (HCC). Advanced HCC patients are treated with systemic therapy, including tyrosine kinase inhibitors, which extend overall survival. Investigation of the underlying mechanism of protein kinase signaling will help to improve the efficacy of HCC therapy. Combining single-cell RNA sequencing data and TCGA RNA-seq data, we profiled the protein kinases, phosphatases, and other phosphorylation-related genes (PRGs) of HCC patients in this study. We found nine protein kinases and PRGs with high expression levels that were mainly detected in HCC cancer stem cells, including *POLR2G*, *PPP2R1A*, *POLR2L*, *PRC1*, *ITBG1BP1*, *MARCKSL1*, *EZH2*, *DTYMK,* and *AURKA*. Survival analysis with the TCGA dataset showed that these genes were associated with poor prognosis of HCC patients. Further correlation analysis showed that these genes were involved in cell cycle-related pathways that may contribute to the development of HCC. Among them, *AURKA* and *EZH2* were identified as two hub genes by Ingenuity Pathway Analysis. Treatment with an AURKA inhibitor (alisertib) and an EZH2 inhibitor (gambogenic) inhibited HCC cell proliferation, migration, and invasion. We also found that both *AURKA* and *EZH2* were highly expressed in *TP53*-mutant HCC samples. Our comprehensive analysis of PRGs contributes to illustrating the mechanisms underlying HCC progression and identifying potential therapeutic targets for future clinical trials.

Keywords: AURKA, EZH2, tyrosine kinase inhibitors, TKI, protein kinases, cell cycle, single-cell RNA sequencing, hepatocellular carcinoma

INTRODUCTION

The most frequent type of primary liver cancer is hepatocellular carcinoma (HCC) (Budny et al., 2017; Khemlina et al., 2017). It contains approximately 85% of cirrhosis cases and is currently the sixth-leading cause of cancer worldwide (Asafo-Agyei and Samant, 2021) According to GLOBOCAN 2020 statistics, the estimated liver cancer cases summed up to 905,677 and deaths to 830,180 in 2020 worldwide (Sung et al., 2021). HCC commonly occurs in patients with chronic liver diseases such as hepatitis B and C infection (El-Serag, 2012). Treatments such as excision or transplantation may be successful in the early stages of HCC, but only a few therapeutic options are available when advanced HCC develops (Lencioni et al., 2014).

The dysregulation of phosphorylation-dependent signaling pathways is a hallmark of oncogenesis (Bhatia et al., 2010; Berndt et al., 2013; Liu et al., 2018). Early this century, the first tyrosine kinase inhibitor (TKI) was approved as a potential precision therapy for HCC (da Fonseca et al., 2020). It was more efficient and safer than traditional chemotherapies because it was more specific to target and had a lower impact on normal cells (Forner et al., 2018). TKIs are used for patients with advanced unresectable HCC and have proven clinically efficacious (Wei et al., 2019; Lee et al., 2020). However, TKI resistance has become a great concern in recent years (Dal Bo et al., 2020; Huang et al., 2020). An example would be the treatment failure of sorafenib due to the high-level inter-tumoral and intra-tumoral heterogeneity (Cabral et al., 2020). Interrogating the underlying molecular basis of abnormal expression of protein kinases, phosphatases, and other phosphorylation-related genes (PRGs) will help to improve the efficacy of HCC therapy.

Cancer stem cells (CSCs) were identified and considered as one of the most important reasons for heterogeneity in HCC, generating diverse cell populations and leading to the selection of the tumor microenvironment (Yin et al., 2010; Eun et al., 2017; Fujii and Sato, 2017; Zhu et al., 2018). The resistance caused by CSCs to TKIs is an obstacle to the total eradication of carcinoma (Graham et al., 2002). Previous studies reported that abnormal phosphorylation was linked to the capacity of CSCs to proliferate and differentiate (Cianflone et al., 2020). However, the expression characteristics of all PRGs in CSCs of HCC have yet to be revealed. Recent high-throughput approaches of single-cell RNA sequencing (scRNA-seq) allow for a better understanding of tumor heterogeneity and transcriptional plasticity in HCC, which may provide additional insight to improve the efficacy of HCC therapy (Kim et al., 2018; Zheng et al., 2018; Aizarani et al., 2019; Zhang et al., 2019a; Kang et al., 2019; Ma et al., 2019). ScRNA-seq provides a viable strategy for the elucidation of abnormal phosphorylation-dependent signaling pathways in multiple cell types of tumors, especially CSCs, which could help to identify new effective drugs in the therapy of HCC.

In this study, we identified 18 PRGs involved in HCC initiation and progression based on The Cancer Genome Atlas (TCGA) RNA-Seq data. In addition, scRNA-seq data of HCC patients provides further evidence that nine of these PRGs were expressed in CSCs and correlated with tumor progression. We performed cell cycle inference analysis of PRGs in CSCs of HCC and found that they played crucial roles in cell cycle transition. Among these genes, Aurora kinase A (*AURKA*) and Enhancer of Zeste 2 (*EZH2*, Polycomb Repressive Complex 2 Subunit) were correlated with multiple cell cycle genes and may take part in cell cycle transition. HCC cell lines treated with an *AURKA* inhibitor (alisertib) or *EZH2* inhibitor (gambogenic acid) exhibited impeded cell proliferation and decreased clone formation, migration, and invasion capacities. Taken together, our results provide a framework for deeper investigation into PRGs in HCC patients. Alisertib and gambogenic acid, which act as inhibitors of *AURKA* and *EZH2*, hold promise as novel therapeutic drugs for HCC.

MATERIALS AND METHODS

Data Collection and Preprocessing

The gene expression data of 369 HCC patients and 50 adjacent cancer samples and clinical data of matched patients were obtained from The Cancer Genome Atlas (TCGA) data portal (https://TCGAData.nci.nih.gov/TCGA/). Fragments per kilobase million (FPKM) RNA-seq data were used for the following analysis. The scRNA-seq dataset of HCC was downloaded from the GEO dataset (GSE149614). The gene expression matrix was used for further analysis. A total of 3,012 phosphorylation-related genes (PRGs) were extracted from the Gene Ontology database (GO) (**Supplementary Table S1**).

Differentially Expressed Gene (DEG) Analysis and GO Enrichment Analysis

DEGs were analyzed by analysis of variance (ANOVA) with the cutoff of FDR<0.01 and fold change>2. GO enrichment analysis was performed by DAVID (https://david.ncifcrf.gov/). The results were visualized with the ggplot2 R package ($p < 0.01$) (Law et al., 2014).

Prognosis-Related Genes Analysis

Univariate Cox regression analysis was used to identify differentially expressed genes associated with overall survival in HCC patients from the TCGA dataset. A risk score was calculated for each patient, a median value was identified for all patients, and HCC patients were then divided into low-risk (score below the median) and high-risk (score above the median) groups. The high- and low-risk groups were stratified and visualized using Kaplan–Meier (K-M) survival curves and analyzed for statistical significance using the log-rank test. Cox regression analysis and Kaplan–Meier curves with the log-rank test were conducted by the glmnet and survival packages.

Gene Expression, Survival, Tumor Grade, and Nodal Metastasis Status Analysis of HCC

The boxplot of gene candidates was performed by GEPIA2 (http://gepia2.cancer-pku.cn/), which includes the gene expression data of TCGA and GTEX. The survival plot was generated by the survival module of GEPIA. Tumor grade, nodal metastasis status, and *TP53*-mutant status analyses were performed using the UALCAN database (http://ualcan.path.uab.edu/). The protein expression of AURKA and EZH2 in HCC was explored based on immunohistochemistry (IHC) data from the Human Protein Atlas (HPA) database (http://www.proteinatl.as.org/).

ScRNA-Seq Analysis

Basic filtering, classification, and visualization of the single-cell dataset of HCC were analyzed with the Seurat package (v.3.0) in R (v.3.4.0.5). We trimmed cells expressing fewer than 200 unique genes, more than 4,500 unique genes or over 20% mitochondrial reads. The top 2000 variable genes were used for further clustering. The function FindMarkers was used based on *t*-test. Fifteen principal components (PCs) remained for t-SNE analysis. Cell types were identified by the markers compared with information in the CellMarker database (http://biocc.hrbmu.edu.cn/CellMarker/index.jsp) (Satija et al., 2015; Zhang et al., 2019b). Single-cell RNA-seq analysis of ovarian carcinoma was performed by the CancerSCEM database (Cancer Single-cell Expression Map database) (Zeng et al., 2021). We applied the E-MTAB-8559 dataset for further analysis (Nelson et al., 2020).

Gene Expression Correlation Analysis and Cell Cycle Status Inference Analysis

Gene expression correlation analysis was performed by the Pearson method in R (4.0.5) (Pearson coefficient >0.2, p value <0.001). Cell cycle status inference was based on the cyclone method in the scran package in R (4.0.5) (Haghverdi et al., 2018).

QIAGEN Ingenuity Pathway Analysis

IPA was used to analyze the potential regulatory genes and pathways of the phosphorylation-related genes. The related diseases and biofunctions were analyzed by the disease and function module of IPA.

Cell Culture and Drugs

Human HCC cell lines (Hep3B and Huh7) were purchased from Beijing Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, HyClone, United States, SH30243-01) with 10% fetal bovine serum (FBS, HyClone, United States, SH30084) and 1% penicillin/streptomycin (HyClone, United States, SV30010) at 37°C in 5% CO2. The EZH2 inhibitor gambogenic acid was purchased from MCE (Shanghai, China). The AURKA inhibitor alisertib was purchased from SELLECK (Shanghai, China). The solvent for gambogenic acid and alisertib is DMSO (Sigma Aldrich, United States).

Cell Viability Assays

Cell viability was assessed using Calcein-AM/PI staining assays (Beyotime, Shanghai, C2015M). DAPI (Invitrogen, Carlsbad, CA) was used to stain the cell nuclei. A total of 1×10^6 Hep3B or Huh7 cells were plated into 96-well plates for 24 h to allow for cell attachment before being incubated for an additional 48 h with various concentrations of the tested compounds. The concentrations of gambogenic acid or DMSO (solvent of gambogenic acid) were 0 μM (Ctrl), 0.1, 2, 10, 50, and 100 μM. The concentrations of alisertib or DMSO (solvent of alisertib) were 0 μM (Ctrl), 0.1, 1, 10, 50, and 100 μM. For slope analysis, a total of 1×10^6 Hep3B or Huh7 cells were plated into 96-well plates for 24 h to allow for cell attachment before being incubated for additional time (0, 12, 24, 36, 48, and 60 h) with gambogenic acid (2 μM) and alisertib (10 μM), respectively. The cells were then cultured with Calcein-AM, PI and DAPI at 37°C for 30 min according to the manufacturer's protocol. Subsequently, images were captured using a PerkinElmer Operetta CLS High Content Screening System. All assays were conducted at least three times.

Colony Formation Assay

Hep3B and Huh7 cells (a total of 5×10^6) were plated into 6-well plates, respectively. After treatment with an EZH2 inhibitor (2 μM gambogenic) or an AURKA inhibitor (10 μM alisertib) for 72 h, colonies were fixed with 4% paraformaldehyde (Mei Lun, China, MA0192) and stained with crystal violet (Beyotime, Shanghai, C0121-100 ML) for 30 min at room temperature. The visible colonies were counted manually. All assays were conducted at least three times.

Wound-Healing Assay

A total of 5×10^6 Hep3B and Huh7 cells were seeded into 6-well plates and grown to 80% cell abundance. Then, a single layer wound was created using a pipette tip, and we took images (Olympus, BX51). After treatment with an EZH2 inhibitor (2 μM gambogenic) or AURKA inhibitor (10 μM alisertib) for 24 h, imaging was repeated at the same location and further analyzed by ImageJ software. All assays were conducted at least three times.

Transwell Assay

The capacity of cell migration and invasion was evaluated via Transwell (JET BIOFIL, Guangzhou, TCS004024) assays. The upper chamber was precoated with Matrigel (Corning, United States, 356234) for the invasion assay, whereas the migration assay was not precoated with Matrigel. A total of 5×10^6 Hep3B and Huh7 cells were resuspended in serum-free DMEM and placed in the upper chamber of the Transwell system. After culturing overnight, 10% FBS was used as a chemoattractant and placed in the lower chamber. The control (1XPBS), EZH2 inhibitor (2 μM gambogenic), or AURKA inhibitor (10 μM alisertib) were added to the top chamber. After culturing for 24 h, the chambers were fixed with 4% paraformaldehyde and stained with crystal violet (Beyotime, Shanghai, C0121-100 ml) for 30 min at room

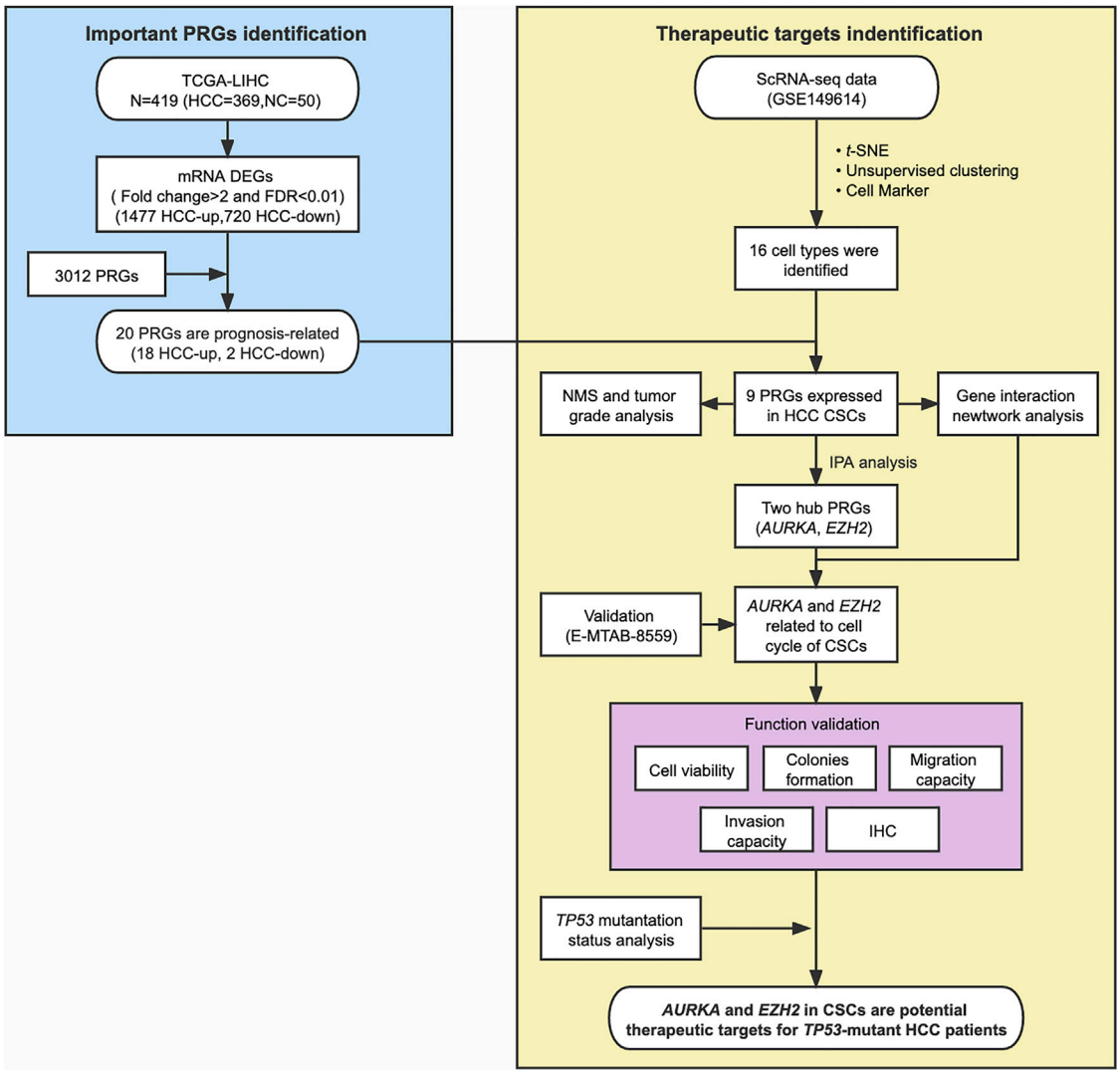

FIGURE 1 | Flowchart for bioinformatics analysis and validation of phosphorylation-related genes (PRGs) based on the TCGA dataset and scRNA-seq data of HCC. NMS, Nodal Metastasis Status; DEGs, differentially expressed genes.

temperature. After removing the noninvasive cells, five fields in the chamber were photographed using an optical microscope (Olympus, BX51), and the numbers of cells were counted. All Transwell assays were conducted at least three times.

Statistical Analyses

Data were expressed as means ± s.d. For all experiments were analyzed by Student's t-test. Differences were considered statistically significant if $p < 0.05$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The statistical analyses were conducted in R 3.4.0.5. The dendrogram was computed and visualized using the R package ggplot2.

RESULTS

Aberrant Phosphorylation Processes Were Related to Hepatocellular Carcinoma Progression

We showed the workflow chart of this study in **Figure 1**. To identify the key genes involved in the occurrence and progression of hepatocellular carcinoma (HCC) as well as prognosis, we conducted an in-depth analysis of public RNA-Seq data from the TCGA database of 369 HCC and 50 adjacent normal tissues. According to the results of differentially expressed gene (DEG) analysis, we identified 1,477 upregulated and 720 downregulated

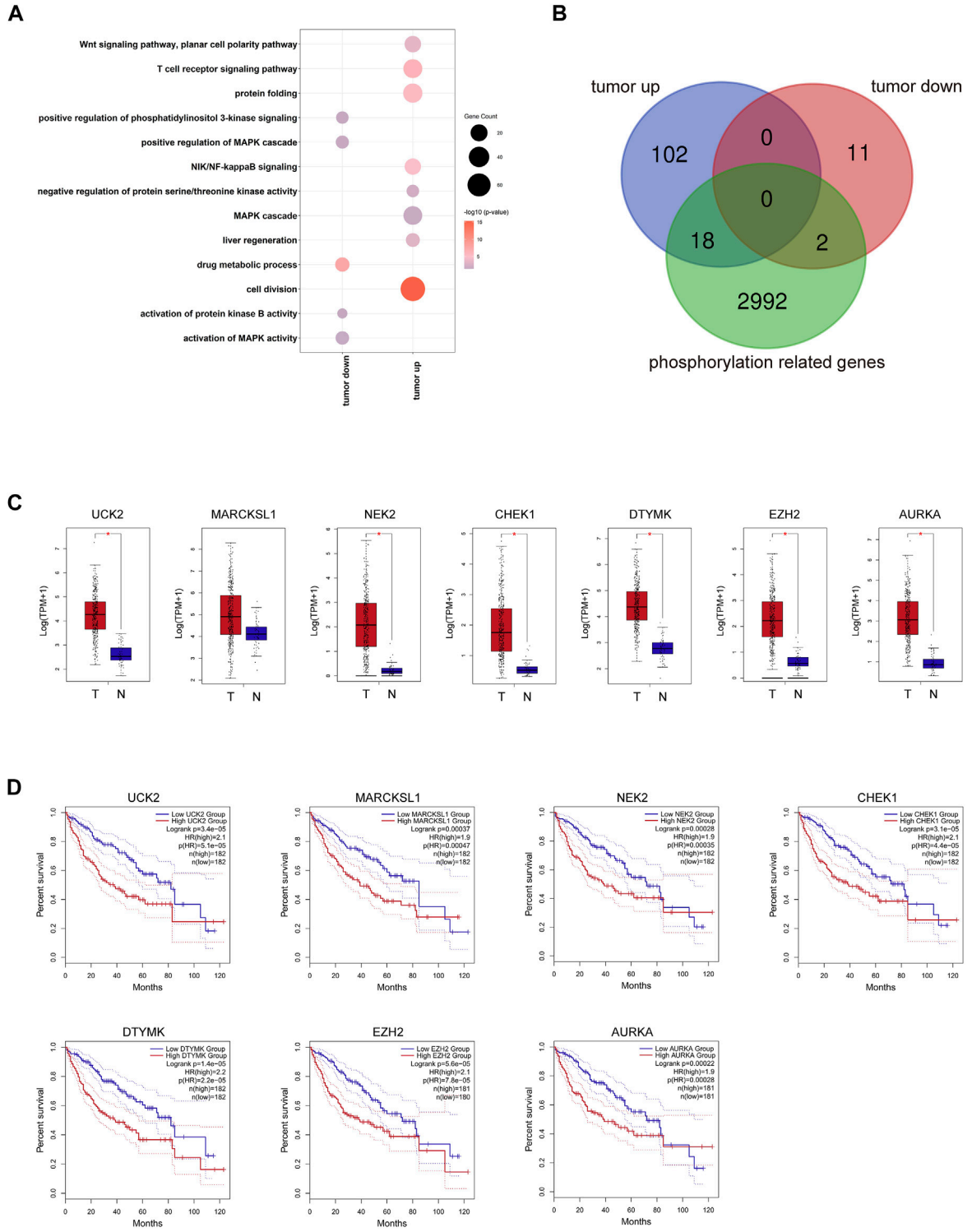

FIGURE 2 | Transcriptome profiling of PRGs in the TCGA LIHC dataset. **(A)** Representative GO terms of HCC related DEGs. Dot size represents the gene counts, and dot color represents the significance. **(B)** The overlay of 120 prognosis-related and HCC upregulated DEGs, 13 prognosis-related and HCC downregulated DEGs, and 3,012 kinase-related genes. **(C)** Expression of seven representative PRGs. **(D)** Kaplan–Meier overall survival curves of TCGA LIHC patients grouped by seven representative PRGs.

genes in HCC tissues compared with adjacent normal tissues (fold change >2, FDR <0.01) (**Supplementary Table S2**). Interestingly, after gene ontology (GO) enrichment by DAVID, we found that the upregulated genes were not only enriched in cell division, the T cell receptor signaling pathway, and other particular pathways that have been verified during tumorigenesis but also enriched in some phosphorylation-related GO terms, including negative regulation of protein serine/threonine kinase activity, regulation of cyclin-dependent protein serine/threonine kinase activity and MAPK cascade pathways (**Figure 2A**, **Supplementary Table S3**). The downregulated genes were highly associated with activation of protein kinase B activity and positive regulation of phosphatidylinositol 3-kinase signaling pathways (**Figure 2A**, **Supplementary Table S3**). In HCC, those DEGs enriched in protein phosphorylation pathways indicated that protein phosphorylation might play an important role during HCC tumorigenesis. Then, the top 500 most prognosis-related genes were selected by Cox regression analysis, among which 120 and 13 genes were significantly up- and downregulated in cancerous tissues (with the cutoff of fold change >2 and FDR<0.01), respectively (**Figure 2B**, **Supplementary Table S4**). Furthermore, filtered by the Gene Ontology database, we identified 20 DEGs that were associated with prognosis and were closely related to the activity and function of protein kinases (**Figure 2B**, **Supplementary Table S5**). Among them, the expression levels of the above 18 phosphorylation-related genes (PRGs) were significantly upregulated in HCC samples (**Figure 2C**, **Supplementary Figure S1A**), while the 2 phosphorylation-related genes were significantly downregulated in HCC samples (**Supplementary Figure S1A**). The survival curves illustrated that the patients with the higher expression levels of HCC-upregulated and PRGs showed significantly poor prognosis (**Figures 2D**, **Supplementary Figure S1B**). Among them, *UCK2*, *MARCKSL1*, *NEK2*, *CHEK2*, *AURKA*, and *DTYMK* are protein kinases, and others, such as *EZH2* and *PRC1*, are PRGs. Additionally, the two HCC-downregulated genes (*SLC11A1* and *ADRA2B*) showed significantly downregulated in HCC samples. The high expression of *SLC11A1* and *ADRA2B* was associated with poor and good prognosis, respectively, due to the diverse functions of phosphorylation (**Supplementary Figure S1B**). The abnormal expression of these genes might induce the dysregulation of the phosphorylation of HCC progression-related genes and enhance tumorigenesis and tumor development. We suspected that these important genes may participate in protein phosphorylation, affecting downstream phosphorylation-related pathways and then promoting the occurrence and progression of HCC.

ScRNA-Seq Dataset Indicates the High Expression of PRGs in Cancer Stem Cells

Traditional RNA-Seq can only detect transcriptome information of the entire tumor tissue with the limitations that it cannot distinguish different cell types, such as CSCs, malignant cells, and immune cells. To confirm which exact cell type the above-mentioned genes were affected, we downloaded the scRNA-seq data of HCC, containing the primary tumor, portal vein tumor thrombus (PVTT), metastatic lymph node, and nontumor liver controls of 10 patients from the GEO database (GSE149614). After quality filtering using the Seurat package, approximately 70,000 single cells were included in further analysis (**Supplementary Figure S2A**). The top 2000 variable genes were used for further clustering (**Supplementary Figure S2B**). Fifteen principal components (PCs) remained for t-SNE analysis (**Supplementary Figure S2C**). We identified 12 major cell types, including CSCs, tumor propagating cells, myeloid cells, $CD4^+$ T cells, $CD8^+$ T cells, Treg cells, HSCs, B cells, NK cells, plasma cells, malignant cells, and endothelial cells, which were labeled with canonical markers (**Figures 3A,B**, **Supplementary Figure S3**). These cells were mainly tumor and immune cells, some of which served as tumor-specific cell subgroups (**Figure 3C**). Since all of the 18 HCC-upregulated and PRGs were consistently related to poor prognosis, to investigate their potential locations in HCC, we used the scRNA-seq data and found that 18 HCC-upregulated and PRGs were mostly expressed in tumor-related cells but not in immune cells. Of all the tumor related cells, such as malignant cells and bud hepatic cells, we detected the expression of some of the PRGs to some extent. However, CSCs caught our attention due to its high expression levels of the PRGs and crucial functions in tumor progression. Considering the sample heterogeneity, we identified a total of nine (*DTYMK*, *EZH2*, *ITGBP1*, *POLR2G*, *POLR2L*, *PPP2R1A*, *PRC1*, *AURKA*, and *MARCKSL1*) genes that were significantly highly expressed in CSCs (**Figure 3D**). Seven of the remaining genes (*UCK2*, *CCNB1*, *CDC25C*, *CDK1*, *CDKN2C, CHEK1, and NEK2*) were expressed at low levels in CSCs, and two of the remaining genes were not expressed in CSCs (*BAMBI*, *STK39*) (**Supplementary Figure S4A**). We further examined their expression in HCC CSCs. Interestingly, of all 2795 *EPCAM* + liver CSCs, most expressed at least one of the nine genes, while only 106 cells expressed *EPCAM* only, indicating the crucial roles of the nine PRGs in liver CSCs (**Figure 3E**, **Supplementary Table S6**). Among them, *POLR2L*, *MARCKSL1*, *PPP2R1A*, *POLR2G*, and *ITGB1BP1* showed the highest expression levels in HCC CSCs, indicating that these five genes may participate in phosphorylation-related pathways in HCC CSCs (**Supplementary Figure S4B**). Furthermore, as the overlapping relationship of the five genes and CSC marker *EPCAM* showed in the Venn diagram, most cells expressed two or more PRGs, while cells expressing both *POLR2L* and *MARCKSL1* accounted for the highest proportion (**Supplementary Figure S4B**). To further investigate the potential biological mechanisms and functions of the key genes we identified, QIAGEN Ingenuity Pathway Analysis (IPA) was used to analyze the potential regulatory genes and pathways of the nine PRGs. The results showed that they might be involved in regulating the estrogen receptor 1 (ESR1), vascular endothelial growth factor A (VEGA), and MAP kinase (ERK1/2) pathways (**Figure 3F**). The hub genes in this IPA network were *AURKA* and *EZH2*, which might exercise a core influence on phosphorylation-dependent pathways in HCC. In addition, the most related disease of those genes was presumed to

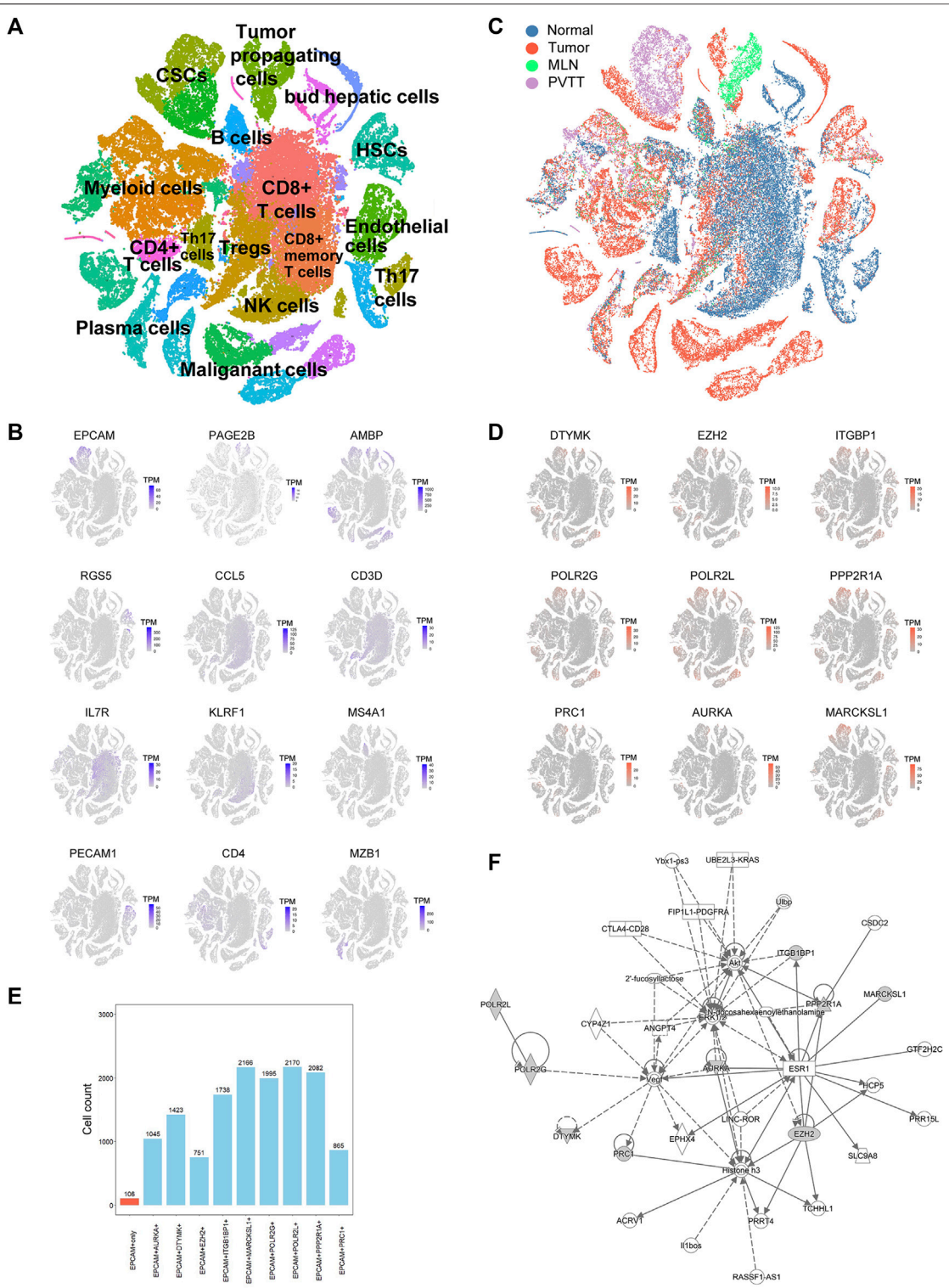

FIGURE 3 | Single-cell RNA sequencing (ScRNA-seq) analysis of HCC patients. **(A)** t-SNE clustering of scRNA-seq colored by significant cell types. **(B)** t-SNE plot of expression for genes specifically upregulated in each of the clusters. **(C)** t-SNE clustering of scRNA-seq as in **(A)** but colored by patient type. Normal: nontumor liver, Tumor: primary tumor, MLN: metastatic lymph node, PVTT: portal vein tumor thrombus. **(D)** t-SNE plot of expression for nine PRGs that were significantly highly expressed in tumor-related cell clusters. **(E)** Statistics of the cell counts of the nine PRGs coexpressed with EPCAM in 2,795 cancer stem cells (CSCs). **(F)** Regulatory network analysis of the nine PRGs using Ingenuity Pathway Analysis (IPA).

TABLE 1 | Clinical characteristics of TCGA LIHC patients included in this study.

Characteristics	Number of patients
Grage	
Normal	50
Grade1	54
Grade2	173
Grade3	118
Grade4	12
Nodal metastasis status	
Normal	50
N0	252
N1	4
TP53 mutation	
Normal	50
TP53-Mutant	105
TP53-NonMutant	255

be cancer-related (**Supplementary Figure S4C**, **Supplementary Table S7**). The above results illustrated the possible important functions and potential regulatory mechanisms of PRGs in CSCs.

Expression of PRGs Is Related to Tumor Grade and Nodal Metastasis Status

Important PRGs may regulate the occurrence and progression of tumors by affecting the activity of CSCs. Tumor progression can be defined by tumor grade and nodal metastasis status (NMS). Tumor grade was defined as normal, G1, G2, G3, and G4. The lower the tumor grade, the better the tumor differentiation. Nodal metastasis statuses N0 and N1 indicate no regional lymph node metastasis and 1 to 3 axillary lymph nodes, respectively. The clinical information of the TCGA LIHC patients included in this study was shown in **Table 1**. Among the highly expressed important PRGs, the increased expression levels of *POLR2G*, *PPP2R1A*, *POLR2L*, *PRC1*, *ITGBP1*, *MARCKSL1*, *EZH2*, *DTYMK*, and *AURKA* were accompanied by increased tumor grade (**Figure 4A**, **Supplementary Table S8**), which indicated that the RNA abundance of these genes was positively correlated with higher tumor grade and lower tumor differentiation level. Furthermore, the higher the nodal metastasis status, the higher the expression levels of *POLR2G*, *PPP2R1A*, *POLR2L*, *PRC1*, *ITGB1BP1*, *MARCKSL1*, *EZH2*, *DTYMK*, and *AURKA* (**Figure 4B**, **Supplementary Table S8**), which illustrated the potential relationship of these genes and nodal metastasis. The results demonstrated a positive correlation between PRGs and tumor grade as well as nodal metastasis status, which were associated with tumor progression.

PRGs Participate in the Cell Cycle Transition of HCC CSCs

To explore the possible mechanism of how phosphorylation-related genes affect HCC progression, we predicted the co-expressed genes of each important PRGs, identified the genes with a Pearson correlation coefficient >0.2 ($p < 0.001$), and further constructed a possible gene expression regulatory network for HCC CSCs (**Figure 5A**, **Supplementary Table S9**). We found that different PRGs shared many related genes in the network, and these genes were enriched in cell division, mitotic nuclear division, and DNA replication (**Figure 5B**), indicating that PRGs might mediate multiple biological processes of tumor stem cells by regulating the expression of cell cycle-related genes such as *TOP2A*, *UBE2C*, and *CENPN* (**Figure 5A**). Additionally, there were strong correlations between the expression levels of *UBE2C*, *TOP2A*, and *AURKA* and between the expression levels of *TUBB*, *UBE2T*, and *MARCKSL1,* suggesting that *UBE2C* and *TOP2A* and *TUBB* and *UBE2T* may serve as potential targets for *AURKA* and in regulating the cell cycle (**Figure 5C**, **Supplementary Table S9**). The above results showed that the PRGs we identified, such as *AURKA*, *MARCKSL1*, and *EZH2,* might mediate the occurrence and progression of HCC by regulating cell cycle-related pathways in liver cancer stem cells. To investigate the effect of PRGs on the cell cycle, we used the cyclone function of the R package scran to infer the cell cycle status of CSCs based on the expression levels of different cell cycle genes. Interestingly, after correlation analysis, which was performed between the expression level of phosphorylation-related genes and the cell cycle inference score of each cell in the G1, S, and G2/M phases, we found that the correlation coefficient with S phase and G2/M phase was significantly higher than that of G1 phase, indicating the potential roles of PRGs in the activation of cell cycle checkpoints (**Supplementary Figure S5A**). The expression levels of *PRC1*, *AURKA*, *DTYMK*, and *EZH2* had the most significant correlation with the S phase and G2/M phase scores (**Supplementary Figure S5A**).

Among all these PRGs, *AURKA* and *EZH2* drew our attention because these two genes were hub genes in the network sourced from IPA (**Figure 3F**) and were previously considered functional mostly in tumor progression. To further confirm the roles of *AURKA* and *EZH2* in the cell cycle pathway of cancer stem cells, we analyzed single-cell RNA-seq datasets of ovarian carcinoma in the CancerSCEM database (Cancer Single-cell Expression Map database) based on the E-MTAB-8559 dataset (Wu et al., 2016; Davient et al., 2018; Doan et al., 2019). We found that *AURKA* and *EZH2* were mostly expressed in cells expressing *EPCAM*, which is consistent with the results of GSE149614 (**Figure 3**, **Supplementary Figure S5B**). The cell cycle scores of in G2/M and S phases of $EPCAM^+$ cells were positively correlated with the expression of *AURKA* and *EZH2* (**Supplementary Figure S5C**). These results indicated that PRGs such as *AURKA* and *EZH2* might participate in cell cycle-related pathways and then mediate the proliferation of tumor cells, especially CSCs.

Gambogenic Acid (EZH2 Inhibitor) and Alisertib (AURKA Inhibitor) Inhibit HCC Cell Proliferation, Migration, and Invasion

To verify the protein levels of AURKA and EZH2 in HCC between HCC tissues and normal tissues, we applied the IHC data in the HPA database and found that the expression of

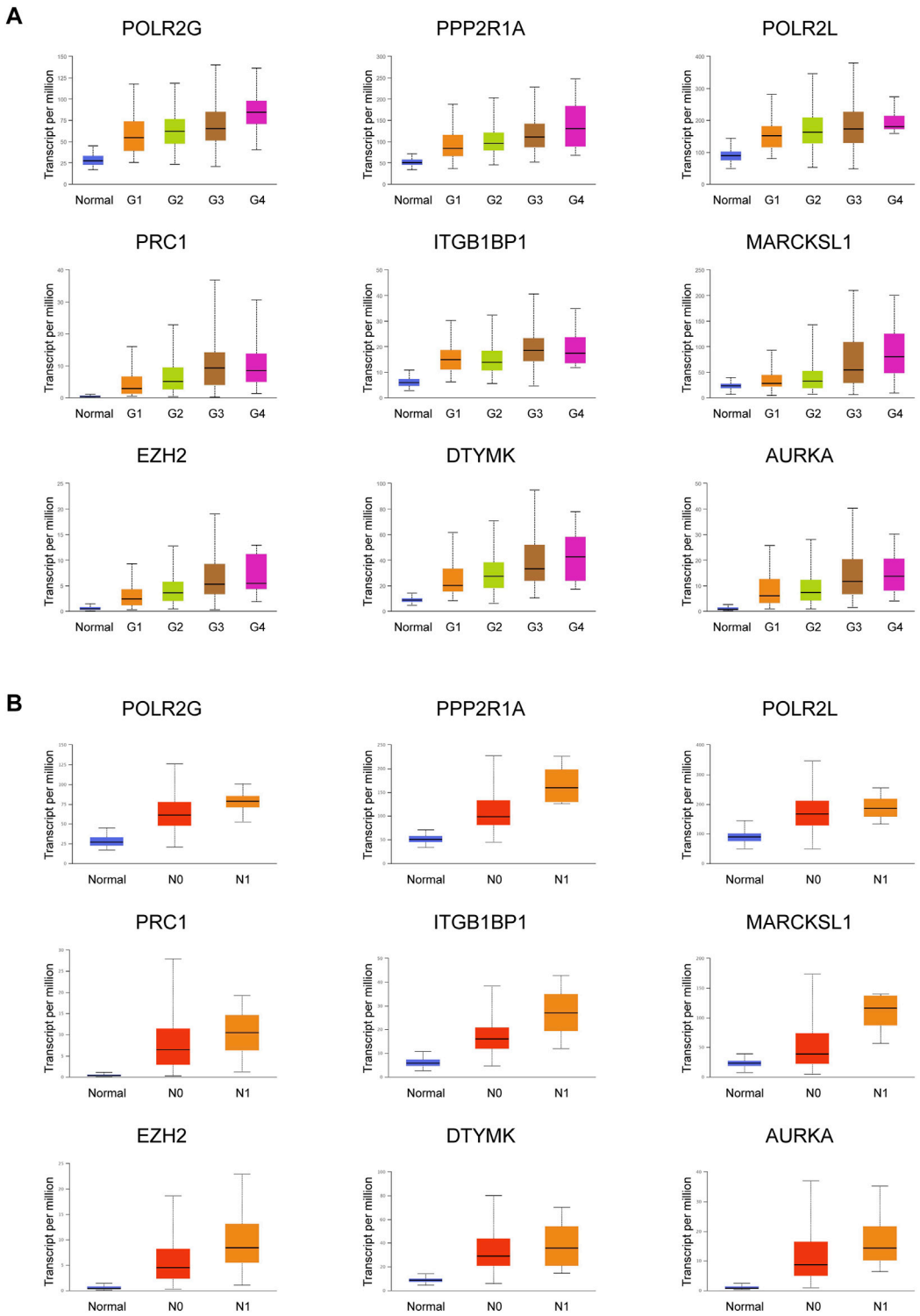

FIGURE 4 | Expression of nine important PRGs is related to tumor grade and nodal metastasis status. **(A)** Expression of the nine PRGs at different tumor grades in HCC patients (n_Normal = 50, n_Grade 1 = 54, n_Grade 2 = 173, n_Grade 3 = 118, and n_Grade 4 = 12). **(B)** Expression of the nine PRGs at different nodal metastasis statuses in HCC patients (n_Normal = 50, n_N0 = 252, n_N1 = 4). The statistical significance of the relationship between genes and tumor grade/nodal metastasis status was shown in **Supplementary Table S8**.

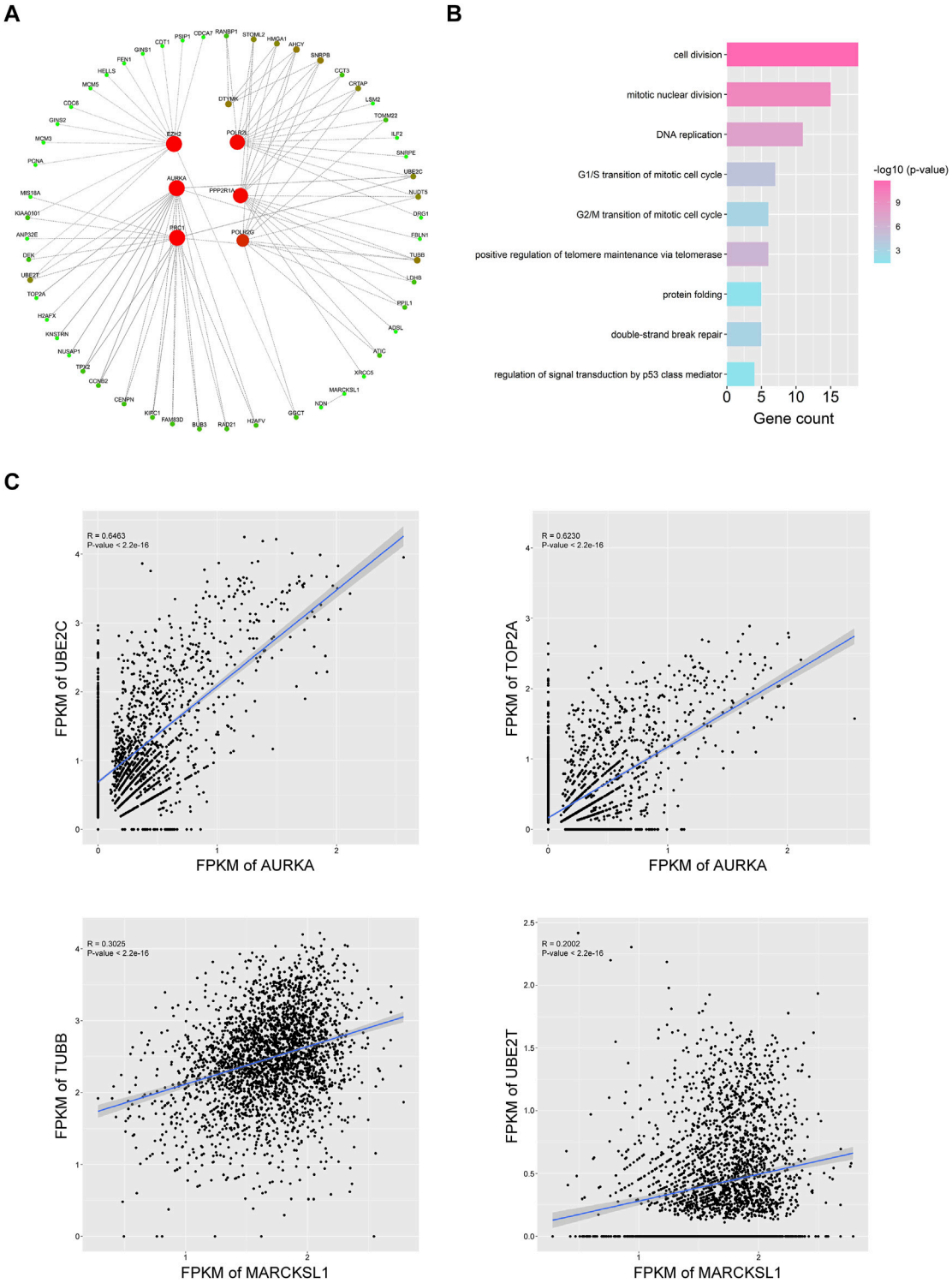

FIGURE 5 | Nine important PRGs participate in the cell cycle transition of HCC CSCs. **(A)** Coexpression network of the nine PRGs and their representative correlated genes in HCC CSCs. Interactions with coefficient >0.3 and *p* value < 0.001 were selected for plotting. **(B)** Representative GO terms of correlated genes in **(A)**. **(C)** Typical examples of coexpression of PRGs and cell cycle-related genes in HCC CSCs.

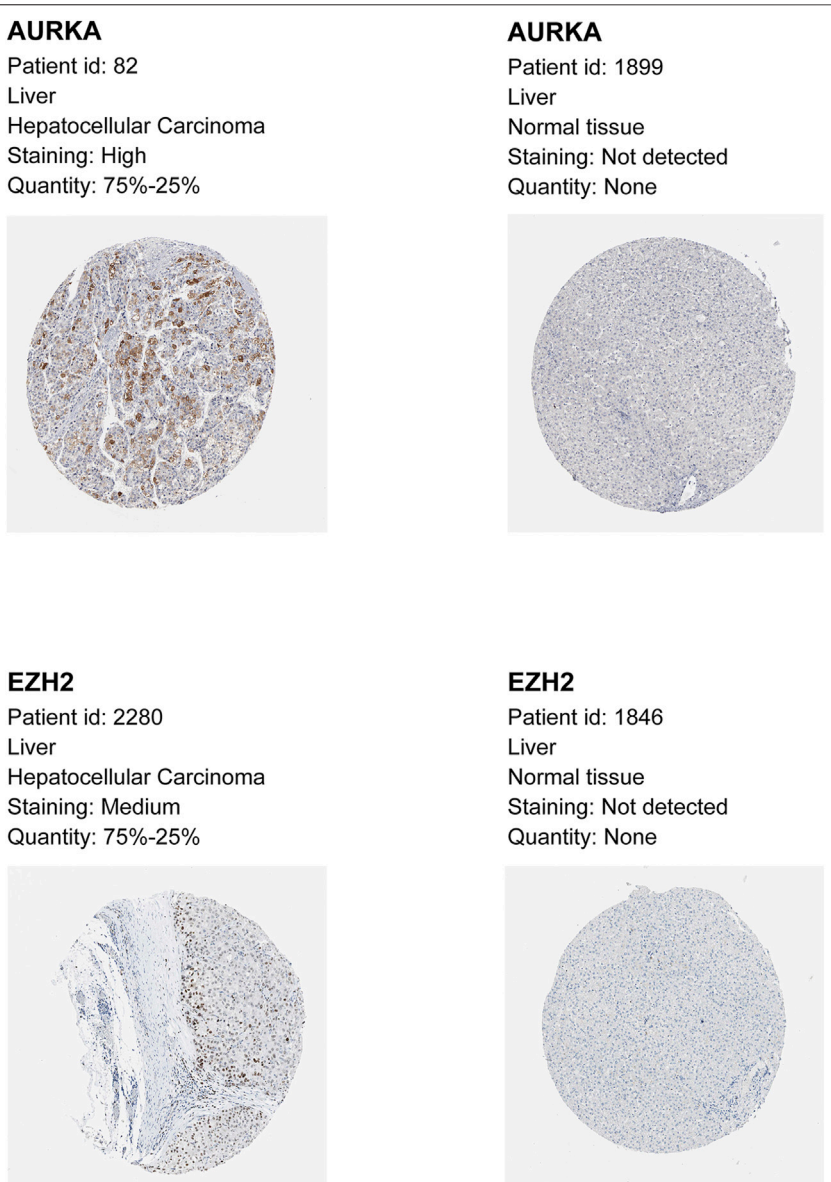

FIGURE 6 | Immunohistochemistry (IHC) data of AURKA and EZH2 from the Human Protein Atlas. Higher expression of AURKA and EZH2 by immunohistochemistry in HCC compared with normal tissue.

AURKA and EZH2 was significantly higher in HCC tissue than in normal tissues (**Figure 6**). To study the functions of *EZH2* and *AURKA* in HCC, we treated HCC cell lines, including Hep3B and Huh7 cells, with an EZH2 inhibitor (gambogenic acid) and an AURKA inhibitor (alisertib) *in vitro*. The Calcein-AM/PI staining results showed that the EZH2 inhibitor (2 μM gambogenic acid) and AURKA inhibitor (10 μM alisertib) significantly impeded the proliferation of Hep3B and Huh7 cells after 48 h treatment (**Figure 7A**). Since inhibitors' concentration was increased gradually, then the corresponding solvent (DMSO) treatments were used as controls. The results proved that the observed effect of inhibitor was independent of the solvent (**Figure 7B**). Because of a high dose of inhibitors and or solvent can induce apoptosis, cell proliferation rate (slope analysis) was examined to prove that inhibitors impede cell proliferation at 48 h treatment (**Figure 7C**). These results were consistent with previous reports in breast cancer, nasopharyngeal carcinoma, and lung cancer (Korobeynikov et al., 2019; Yan et al., 2011; Yu et al., 2012). In addition, the number of colonies formed (**Figure 7D**), migration (**Figures 7E,F**), and invasion capacities (**Figure 7G**) were also significantly decreased in Hep3B and Huh7 cells treated with EZH2 and AURKA inhibitors. Notably, *TP53*-mutated or *TP53*-deleted human HCCs were more hypersensitive to the treatment of AURKA inhibitors in previous studies (Dauch et al., 2016; Caruso et al., 2019). We found that both *AURKA* and *EZH2* were highly expressed in *TP53*-mutant HCC samples

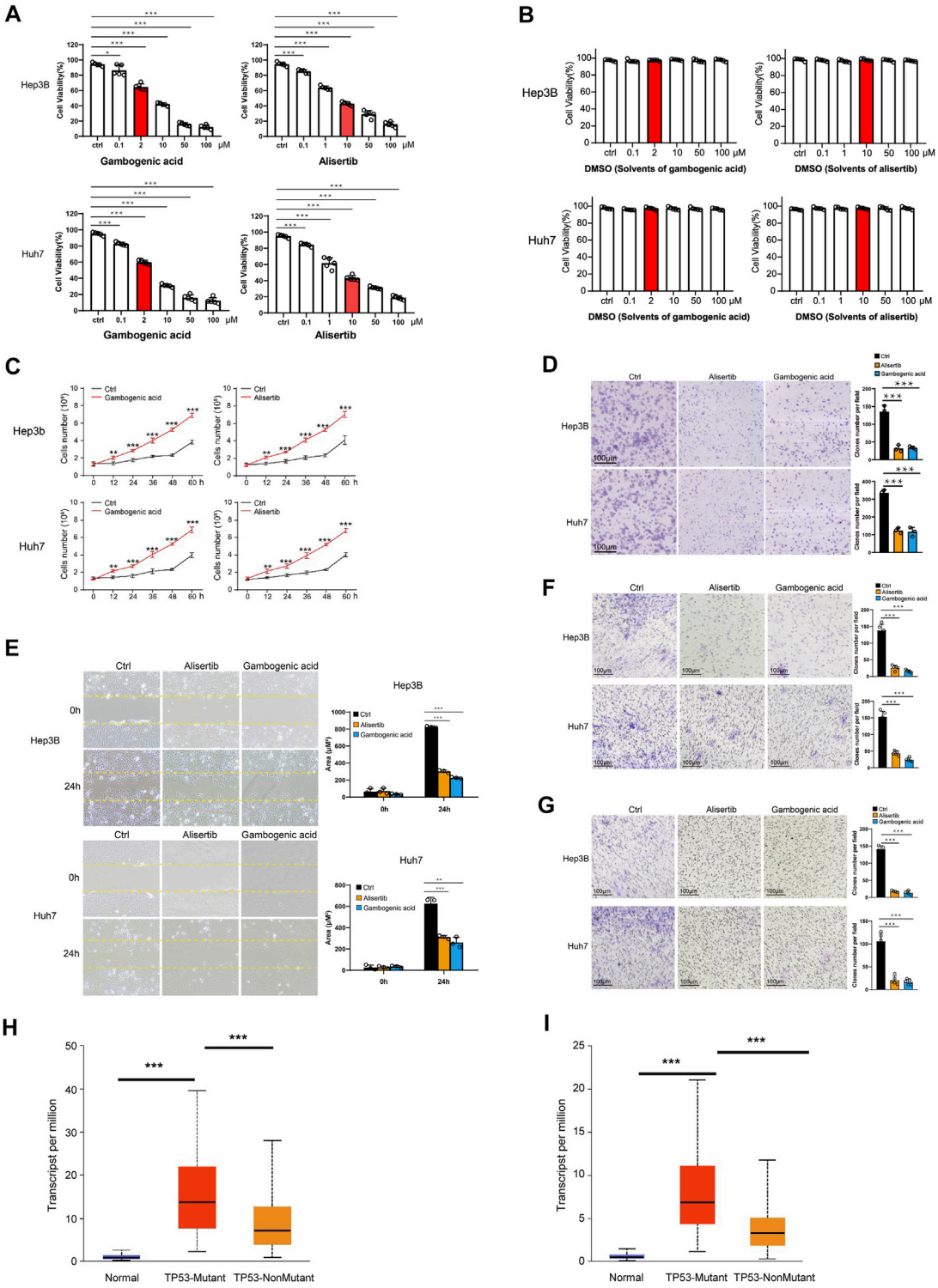

FIGURE 7 | Gambogenic acid (EZH2 inhibitor) and alisertib (AURKA inhibitor) inhibit HCC cell proliferation, migration, and invasion. **(A)** Hep3B and Huh7 cells were treated with gambogenic acid (EZH2 inhibitor) or alisertib (AURKA inhibitor) at different concentrations (0–100 μM) for 48 h, and cell viability was determined by Calcein-AM/PI staining assays. **(B)** Hep3B and Huh7 cells were treated with DMSO (solvents of gambogenic acid and alisertib) at different concentrations (0–100 μM) for 48 h,

(*Continued*)

FIGURE 7 | and cell viability was determined by Calcein-AM/PI staining assays. **(C)** Hep3B and Huh7 cells were treated with gambogenic acid (EZH2 inhibitor, 2 μM) or alisertib (AURKA inhibitor, 10 μM) for 0, 12, 24, 32, 48, and 60 h, and cell viability was determined by Calcein-AM/PI staining assays. **(D)** Colony formation assays were conducted to analyze Hep3B and Huh7 cell proliferation with gambogenic acid (2 μM) or alisertib (10 μM) treatment. (**E-F**) Wound healing assays **(E)** and Transwell assays **(F)** were performed to detect the cell migratory abilities of Hep3B and Huh7 cells treated with gambogenic acid (2 μM) or alisertib (10 μM). **(G)** Transwell assays were performed to detect the cell invasion abilities of Hep3B and Huh7 cells treated with gambogenic acid (2 μM) or alisertib (10 μM). Data are expressed as the means ± s.d. Differences were considered statistically significant if $p < 0.05$. ns, no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(H, I)** Expression of *AURKA* **(H)** and *EZH2* **(I)** in TCGA-LIHC based on *TP3* mutation status.

(**Figures 7H,I**), suggesting the relationship of *AURKA* and *EZH2* with *TP53* mutation. These results showed that *EZH2* and *AURKA* play an important role in promoting the proliferation, migration, and invasion of HCC, especially in *TP53*-mutant HCC, which indicates that they are potential targets for clinical therapy.

DISCUSSION

In this study, we provided a comprehensive understanding of protein kinases, phosphatases, and other phosphorylation-related genes (PRGs) at both the bulk RNA and single-cell RNA levels in HCC. We investigated their expression levels using TCGA datasets and scRNA-seq data from GEO datasets. Notably, we found that PRGs play crucial roles in liver cancer stem cells. We further validated two essential inhibitors of PRGs (*AURKA* and *EZH2*) in HCC cell lines, which suppressed cell proliferation, clone formation, migration, and invasion capacities.

To our knowledge, the PRGs in CSCs of HCC remain largely unknown. The application of scRNA-seq methods has enabled us to build precise profiling of multiple cell types in HCC, such as CSCs, cancer-associated structural cells, vascular cells, fibroblasts, and immune cells, and illustrate the subset of cell types that promote tumor progression and metastasis. Previous studies have already shown the heterogeneity of immune cells in HCC by scRNA-seq technology (Zheng et al., 2017; Zhang et al., 2019a). In this study, we focused on the PRGs of CSCs based on scRNA-seq data of 10 HCC patients from the GEO database (GSE149614). The results showed that PRGs tend to be expressed abnormally in CSCs instead of immune cells, indicating their potential roles in CSC proliferation, differentiation, and migration.

Due to the heterogeneity of individual HCC patients, only nine genes were detected in the scRNA-seq dataset among the 18 important PRGs identified in the TCGA LIHC dataset. This result illustrated the conservation of the nine genes in HCC patients. Among the nine PRGs, four of them (*EZH2*, *POLR2G*, *POLR2L*, and *PRC1*) also regulate gene expression through other pathways, such as histone methyltransferase and transcription regulation (Acker et al., 1996; Maeta et al., 2020; Xue et al., 2021). In our study, we found that the increased expression of those genes also correlated with higher progressive tumor grades and advanced metastatic stages. Among them, four genes (*PRC1*, *AURKA*, *DTYMK*, and *EZH2*) were calculated by cell cycle inference to be more correlated with the cell cycle. Since the hub genes in the IPA network were *AURKA* and *EZH2*, we further validated the role of *AURKA* and *EZH2* in tumor cell proliferation and migration in HCC cell lines. Our results indicate that an AURKA inhibitor (alisertib) and an EZH2 inhibitor (gambogenic) can obviously inhibit the proliferation, migration, and invasion of HCC cells, which are potential targets for clinical application.

AURKA plays an important role in mitosis, including centrosome function and maturation, spindle assembly, chromosome alignment, and mitotic entry (Bolanos-Garcia, 2005; Nikonova et al., 2013). Overexpression of *AURKA* correlates with tumor progression and poor prognosis in various carcinomas, including pancreatic carcinoma and breast carcinoma (Gomes-Filho et al., 2020). Furthermore, blockade of *AURKA* in preclinical models of ovarian carcinoma leads to decreased proliferation and increased apoptosis (Lin et al., 2008). Inhibition of AURKA affect the vasculogenic mimicry formation of CSCs in triple negative breast cancer (Sun et al., 2016). In HCC, the overexpression of *AURKA* has been reported to be related to aggressive tumor characteristics, chemotherapy resistance, and poor prognosis (Jeng et al., 2004; Lin et al., 2010; Zhang et al., 2014). Alisertib is a novel oral adenosine triphosphate-competitive AURKA inhibitor. A phase II study of alisertib in advanced sarcoma showed promising results for liposarcoma (PFS at 12 weeks of 73%), leiomyosarcoma (44%), and malignant peripheral nerve sheath tumors (60%), although each cohort only had a small number of patients (Dickson et al., 2016). Other studies have also shown the partial responses of alisertib in breast cancer, small-cell lung cancer, non-small-cell lung cancer, head and neck squamous cell carcinoma, and gastroesophageal adenocarcinoma (Melichar et al., 2015). *EZH2* is a catalytic subunit of polycomb repressive complex 2 (*PRC2*) (He et al., 2012). Based on H3K27me3-mediated gene expression silencing, *EZH2* often functions as a transcriptional repressor to downregulate tumor suppressors such as *ADRB2* and *DAB2IP* (Cao et al., 2002; Chen et al., 2005; Yu et al., 2007). Notably, the overexpression of EZH2 is reported to activate the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway, which is related to phosphorylation pathways (Gonzalez et al., 2011). Currently, *EZH2* is reported to be highly expressed in lymphomas, glioblastoma multiforme, ovarian, breast, and metastatic prostate cancers and is related to tumor progression, invasive growth, and poor prognosis in these tumors (Visser et al., 2001; Varambally et al., 2002; Bracken et al., 2003; Kleer et al., 2003; Yu et al., 2007; Hu et al., 2010; Orzan et al., 2011). Previous study has also revealed the activated EZH2 in glioma stem cells promoted cellular survival under stress and was potential to serve as tumor therapeutic targets (Jin et al., 2017). In addition, *EZH2* is reported to be related to proliferation and invasion in HCC cells (Liu et al., 2015; Zhang et al., 2017; Gao et al., 2020). Gambogenic acid (an *EZH2* inhibitor) is a natural compound derived from gamboge and is reported to be used as an antitumor drug in nasopharyngeal and lung cancer (Yan et al.,

2011; Yu et al., 2012). Previous studies revealed that *AURKA* modulates the PI3K/Akt/mTOR pathway in Hep3B cells, and NICD1 and JAG1 were regulated by EZH2 (Zhu et al., 2017; Wang et al., 2020). In addition, *EZH2* could also affect tumor progression via histone methyltransferase-related functions. For example, *EZH2* is related to epigenetic silencing of miR-200c and induces BMI1-mediated hepatocarcinogenesis (Xu et al., 2020). *In vivo* experimental studies are needed to validate the functions of these two inhibitors in the progression of HCC in the future.

In this study, we found that *AURKA* and *EZH2* might mediate the occurrence and progression of HCC by regulating the transition of the cell cycle to S phase and G2/M phase in CSCs. We also found that both *AURKA* and *EZH2* were highly expressed in *TP53*-mutant HCC samples, suggesting the relationship of *AURKA* and *EZH2* with *TP53* mutation, corroborating a recent study in HCC cell lines and mice (Dauch et al., 2016; Caruso et al., 2019). These studies also showed that HCC cells with inactivating mutations in *TP53* were sensitive to alisertib. Additionally, other studies also reported the roles of *AURKA* and *EZH2* inhibitors.

The EZH2 inhibitor gambogenic acid can inhibit the growth of Hep3B and Huh7 cells through apoptotic pathways, and the inhibition of AURKA by alisertib leads to the inhibition of cell proliferation and induces cell cycle arrest and autophagy in Hep3B cells (Lee and Ho, 2013; Zhu et al., 2017). Thus, the AURKA inhibitor (alisertib) and the EZH2 inhibitor (gambogenic acid) may offer a potential therapeutic opportunity for HCC patients with *TP53* mutations, as *TP53* is the most frequently mutated tumor suppressor gene in HCC.

Taken together, the results of this study provide insights into the expression characteristics and potential functions of PRGs in HCC. The limitation of this study is that AURKA and EZH2 as key molecules in HCC cell proliferation were widely acknowledged. However, For the first time, we found AURKA and EZH2 were highly expressed in HCC CSCs that may contribute to the development of HCC. We found that they might play roles in the cell cycle transition of CSCs and validated that inhibitors of AURKA and EZH2 could suppress HCC proliferation and migration. Hence, alisertib and gambogenic acid have the potential to hold promise as novel anticancer agents of HCC, especially for *TP53*-mutant HCC. The therapeutic efficacy and mechanism of action of these compounds combined with other anticancer drugs are worth further clinical investigation as alternative combination therapies, which show promising potential in oncotherapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

FY, YZ, and CL initiated the study, performed the analysis, prepared the figures, and wrote the manuscript. YL, JC, ZW, QL, YS, BC, JC, and KT proposed useful comments and suggestions and revised the manuscript. JD discussed and optimized the pictures in this manuscript. ZP, YN, and LM designed the structure and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.734287/full#supplementary-material

Supplementary Figure S1 | HCC prognosis-associated phosphorylation-related genes (PRGs). **(A)** Expression of 13 representative PRGs. **(B)** The Kaplan–Meier overall survival curves of TCGA LIHC patients grouped by the expression of 13 representative PRGs.

Supplementary Figure S2 | Overview of single-cell RNA-seq (scRNA-seq) of HCC patients. **(A)** Quality control of scRNA-seq data. We filtered out cells with fewer than 200 unique genes, more than 4,500 unique genes, or over 20% mitochondrial reads. **(B)** The top 2,000 variable genes are shown by a characteristic variance diagram. **(C)** Data standard deviations under different selections of PCs.

Supplementary Figure S3 | Top markers of different cell types of the scRNA-seq dataset (GSE149614). Top markers for CSCs are *EPCAM*, *MEG3*, *GLB1*, *VCX3A*, *VCX2*.

Supplementary Figure S4 | (A) t-SNE plot of the expression of PRGs that were expressed at lower levels in CSCs. **(B)** Overlay of the CSCs expressing *EPCAM*, *POLR2L*, *MARCKSL1*, *POLR2G*, and *ITB1BP1*. **(C)** Treemap of diseases related to the nine PRGs.

Supplementary Figure S5 | Gene expression and cell cycle scores of AURKA and EZH2 in scRNA-seq datasets. **(A)** Correlation between cell cycle score and expression of the nine PRGs in the GSE149614 dataset. **(B)** Gene expression of EPCAM, AURKA, and EZH2 in the single cells of the E-MTAB-8559 dataset. **(C)** Correlation between cell cycle score and expression of AURKA and EZH2 in the E-MTAB-8559 dataset.

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GLOSSARY

PRGs phosphorylation-related genes
FPKM Fragments per kilobase million
GEO Gene Expression Omnibus
GO Gene ontology
ANOVA analysis of variance
PC prinicipal components
HCC hepatocellular carcinoma
TKI tyrosine kinase inhibitor
TCGA the cancer genome atlas
CSC cancer stem cell
AURKA aurora kinase A
EZH2 enhancer of zeste homolog 2
DAVID database for annotation, visualization and integrated discovery
IPA Ingenuity Pathway Analysis
scRNA-seq single-cell RNA sequencing
DMEM Dulbecco's modified eagle medium
FBS fetal bovine serum
SLC11A1 Solute Carrier Family 11 Member 1
ADRA2B adrenoceptor alpha 2BAdrenoceptor Alpha 2B
UCK2 uridine-cytidine kinase 2
MARCKSL1 MARCKS like 1
NEK2 NIMA related kinase 2
CHEK2 checkpoint kinase 2
DTYMK deoxythymidylate kinase
PRC1 protein regulator of cytokinesis 1
EPCAM epithelial cell adhesion molecule
POLR2L RNA polymerase II, I and III subunit L
PPP2R1A protein phosphatase 2 scaffold subunit alpha
POLR2G RNA polymerase II subunit G
ITGB1BP1 integrin subunit beta 1 binding protein
ESR1 estrogen receptor 1
VEGA vascular endothelial growth factor A
NMS nodal metastasis status
TOP2A DNA topoisomerase II alpha
UBE2C ubiquitin conjugating enzyme E2 C
CENPN centromere protein N
TUBB tubulin beta class I
UBE2T ubiquitin conjugating enzyme E2 T
PRC2 protein regulator of cytokinesis 2
ADRB2 adrenoceptor beta 2
DAB2IP DAB2 interacting protein
CancerSCEM Cancer Single-cell Expression Map.

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REVIEW
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The Role of the Tumor Suppressor Gene Protein Tyrosine Phosphatase Gamma in Cancer

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Members of the Protein Tyrosine Phosphatase (PTPs) family are associated with growth regulation and cancer development. Acting as natural counterpart of tyrosine kinases (TKs), mainly involved in crucial signaling pathways such as regulation of cell cycle, proliferation, invasion and angiogenesis, they represent key parts of complex physiological homeostatic mechanisms. Protein tyrosine phosphatase gamma (PTPRG) is classified as a R5 of the receptor type (RPTPs) subfamily and is broadly expressed in various isoforms in different tissues. *PTPRG* is considered a tumor-suppressor gene (TSG) mapped on chromosome 3p14-21, a region frequently subject to loss of heterozygosity in various tumors. However, reported mechanisms of *PTPRG* downregulation include missense mutations, ncRNA gene regulation and epigenetic silencing by hypermethylation of CpG sites on promoter region causing loss of function of the gene product. Inactive forms or total loss of PTPRG protein have been described in sporadic and Lynch syndrome colorectal cancer, nasopharyngeal carcinoma, ovarian, breast, and lung cancers, gastric cancer or diseases affecting the hematopoietic compartment as Lymphoma and Leukemia. Noteworthy, in Central Nervous System (CNS) PTPRZ/PTPRG appears to be crucial in maintaining glioblastoma cell-related neuronal stemness, carving out a pathological functional role also in this tissue. In this review, we will summarize the current knowledge on the role of PTPRG in various human cancers.

Keywords: phophatase, PTPRG, cancer biology, tumor suppressor, pathway aberrant activation

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INTRODUCTION

Phosphorylation represents one of the best-characterized post-translational modifications, a form of structural change that can modify interactions and stability of the protein structure and modulate enzymatic activity. Since the discovery of proteins tyrosine kinase (PTKs) in the late 1970s, multiple evidences of the key pathogenetic role in cancer progression has emerged that led to countless investigations and discoveries on the regulatory mechanisms underlying signaling pathways governed by these critical enzyme (Hunter, 2009). On the other hand, the protein tyrosine phosphatase (PTPs) field developed with at least a 10 years delay and, being responsible for the removal of phosphate groups on tyrosine residues, they were viewed as a natural counter actors for oncogenic PTKs, becoming of great study interest as potential therapeutics target (Julien et al., 2011). A classic subdivision of the PTPs gene family is formed by receptors (RPTPs), particularly R1–R8 subgroups localized on cell membrane, and non-receptor (NRPTPs) including NR1–NR9 subgroups, localized predominantly in cellular interspaces such as cytoplasm (Alonso et al., 2004; Stoker, 2005). Other members of this large superfamily are represented by DSPs (Dual specificity phosphatases)

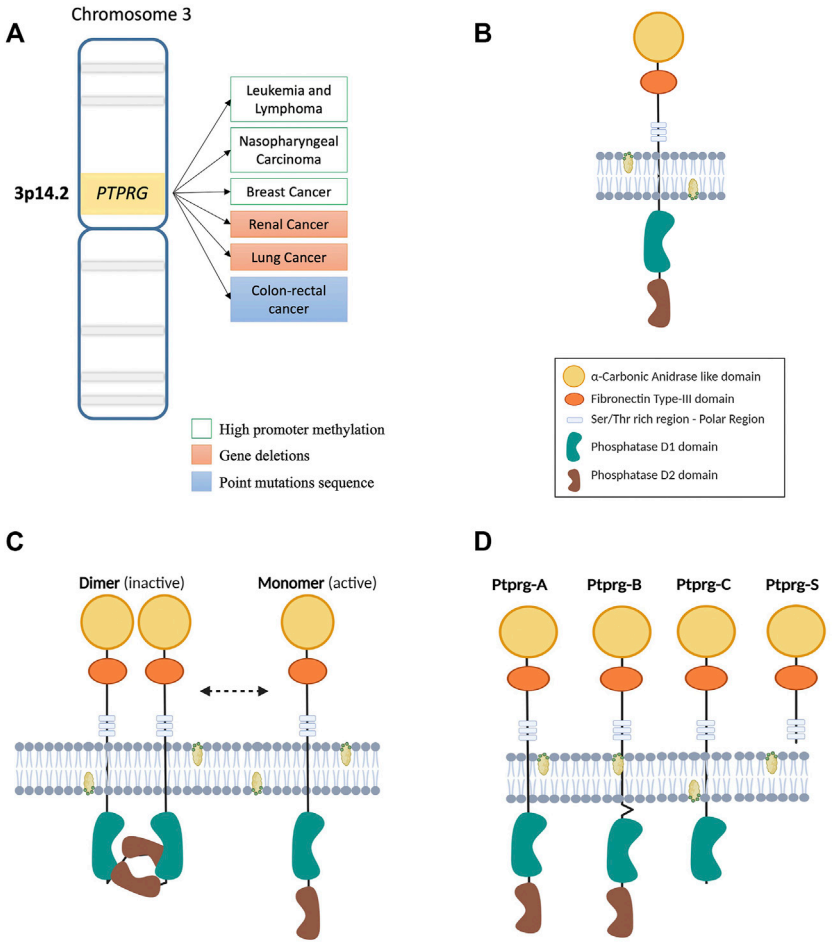

FIGURE 1 | (A) Map of the region of human chromosome 3 where the PTPRG gene is located. On the side, the main genetic and epigenetic alterations involving the expression of PTPRG in various malignancies. **(B)** Illustration of the transmembrane structure of the PTPRG phosphatase. The different intra- and extra-membrane domains that form the protein are indicated in the legend on the side. **(C)** Schematic representation of the "head to toe" dimerization model of PTPRG phosphatase proposed by Barr et al., 2009. The inactive D2 domain of the first monomer of PTPRG interacts with the D1 domain of the second monomer making it blocked. The transition from inactive (dimer) to active (monomer) state can be performed using the technology of Trojan peptides (P1-WD) proposed by Montresor et al., 2021. Meanwhile the inhibition of phosphatase activity was performed using a specific inhibitor 3- (3,4-dichlorobenzylthio) thiophene-2-carboxylic acid in the work of Tomasello et al., 2020. **(D)** Illustration of the four different isoforms proposed for PTPRG.

and LMPs (Low molecular weight phosphatases). Despite a rather low specificity in *in vitro* assays, these enzymes possess a high substrate specificity in cells, mainly derived from the specific tissue distribution, restricted subcellular localization and from other post-translational modifications (e.g., phosphorylation) that regulate its functions (Tiganis and Bennett, 2007). Receptor-phosphatase usually are composed of a variable extracellular region combined with intracellular segment including phosphatase domains commonly shared in this superfamily. This union makes them suitable for coordinating both extracellular activities (e.g., cell-cell or cell-matrix adhesion) and intracellular signaling. Protein tyrosine phosphatase receptor gamma (PTPRG) belongs to the class of receptor PTPs similar to PTPRB/Z (Krueger and Saito, 1992), characterized by the presence of the homologous α-carbonic anhydrase like domain (CAH) and a Fibronectin type III domain in the N-terminal region (protein structure is represented in **Figure 1B**) (Barnea et al., 1993; Sorio et al., 1997). Beyond the membrane-spanning region, two highly conserved phosphatase domains (tandem domains) extend into the intracellular side. The catalytically active phosphatase domain D1 is proximal to the membrane, while the proximal C-terminal domain D2, lack the enzyme activity and is defined as pseudophosphatase domain. This inactive domain might be involved in stability, substrate specificity and binding of docking proteins (Barnea et al., 1993). Of note, the mutated form D1028A lacks phosphatase activity, rendering the PTPRG completely inactive (Zhang et al., 2012). Domains organization of the PTPRG molecule is fundamental for all tasks performed. Particularly the intracellular structure (ICD) plays a critical role in the regulation of phosphatase activity. Despite the receptor phosphatases dimerization was already known as a mechanism of inhibition (Jiang et al., 2000; Sonnenburg et al., 2003; Groen et al., 2008), also PTPRG on the cell membrane seems to

self-associate forming a homodimer in a "head to toe" dimerization model. While further confirmations will be essential, the flexibility of the transmembrane domain of PTPRG would allow the inhibitory interaction, which is abolished with the mutation of several residues on the interface between both the D1 and D2 domains. Indeed, under normal dimerization conditions the PTPRG mutants have a higher catalytic activity than the wild type (Barr et al., 2009). Representation of the "head to toe" model of PTPRG is depicted in **Figure 1C**.

PTPRG FUNCTIONS IN A NON-NEOPLASTIC CONTEXT

Numerous cellular functions have been attributed to PTPRG due to its wide expression in different tissues (Vezzalini et al., 2007). The first specific functional data obtained from murine embryonic stem cells described a role in the differentiation of the hematopoietic compartment (Sorio et al., 1997), despite further analysis on another *Ptprg*-null mice model exhibited mild behavioral abnormalities without showing an obvious phenotype (Lamprianou et al., 2006; Zhang et al., 2012). A role in the regulation of blood flow/pressure and in vascular relaxation through intracellular Ca^{2+} response was described using the same murine model: under conditions of altered acid/base balance, such as low concentrations of [HCO^{3-}], PTPRG-induced vasorelaxant effects, that were reduced in *Ptprg*-null mice (Boedtkjer et al., 2016; Hansen et al., 2020). Moreover, a similar process was described the proximal renal tubules, where the presence of PTPRG increased the reabsorption of HCO^{3-} (Zhou Y. et al., 2016). Both represent useful information to understand vascular conditions related to alteration of acid/base balance, such as stroke or heart attack, but also cancer cells and their microenvironment. Studies of PTPRG has focused on the central nervous system (CNS), which is characterized for the expression of various phosphatases (Lamprianou and Harroch, 2006). PTPRG is involved in the development of the spinal cord in chick (Hashemi et al., 2011), indeed its expression was detected in neuronal cells, more specifically in sensory neurons (e.g., pyramidal neurons in cortical layers II and V) and astrocyte (with higher expression in neuro-inflammation) while glial cells were slightly positive (Lamprianou et al., 2006; Lorenzetto et al., 2014). The lack of knowledge regarding the ligand for the PTPRG protein prompted Bouyain et al. to analyze some molecules belonging to the contactin family (CNTN), which are involved in cell adhesion and already known to be a PTPRZ/B ligand. CNTN1 binds to PTPRZ-expressing glial cells, increasing neurite growth and playing a role in CNS development (Peles et al., 1995). Similarly, biochemical and structural approaches have indicated an interaction between PTPRG and several members of this family (CNTN3 4, 5 and 6) (Bouyain and Watkins, 2010b). Instead, on cell surface, both *cis* and *trans* interactions between CNTN3 and PTPRG have been highlighted in neurons, hypothesizing a possible role in neuronal development, as already demonstrated for PTPRA (Bouyain and Watkins, 2010a; Nikolaienko et al., 2016). Increased PTPRG levels were reported in neurological and liver inflammation processes, suggesting a higher complexity level in the post-translational regulation of the PTPRG (Lorenzetto et al., 2014; Moratti et al., 2015). Particularly, Ptprg expression in liver has been indicated as a specific indicator of inflammation and a possible cause of insulin resistance (Brenachot et al., 2017). So far, pro-inflammatory factors such as LPS or IL-1β and TNFα were reported to upregulate *PTPRG* in astrocytoma cell line or astrocyte culture (Schumann et al., 1998; Lorenzetto et al., 2014) and also appear to associate to specific myeloid lineages, such as the differentiation of monocytes to dendritic cells (Lissandrini et al., 2006). Despite these examples (surely further roles in physiological context are expected to be uncovered) of an important role in normal cell physiology, the main data on PTPRG function derive from studies on its tumor suppressor role, since genetic alterations were found in numerous malignancies. For this reason, we mainly describe the available data on the mechanisms of expression and regulation of PTPRG activity in the context of neoplasia and potential clinical applications.

MECHANISMS PROMOTING PTPRG SILENCING IN CANCER

Genomic and Epigenetic Processes

The first suggestion of an oncosuppressor role played by *PTPRG* is related to the non-random deletion in its chromosomal region in different types of carcinomas (**Figure 1A**) (LaForgia et al., 1991). Further analysis revealed loss of heterozygosity of the *PTPRG* locus in clear cell Renal Carcinomas (RCC), with no evidence of mutations in the 30 exons of the protein (Druck et al., 1995). Of note, this region was lately found to harbor the TS gene *FHIT* (Panagopoulos et al., 1996). On the other hand, observations on 12 microsatellites mapped on chromosome 3p12.2–21.1, specifically on marker D3S1239, showed a non-random loss both in NPC (nasopharyngeal carcinoma) cell lines and in three of seven (43%) of primary NPC samples (Cheung et al., 2008).

Studies on the genetic alteration of the phosphatases in human cancers evaluated the coding exons of 87 members of the PTPs genes superfamily. Examination of 3.3 Mb of sequences recognized somatic mutations affecting six PTP genes, including *PTPRG*. Specifically, the study of 157 colorectal cancers revealed eight cases (5%) harboring somatic mutations on the *PTPRG* gene (Wang et al., 2004). Detection of cancer alteration has shown that many mutations occurred in genes able to affect the DNA methylation status or controlling the chromatin structure. These analyses, performed with high-throughput technology, show that the epigenetic status of cancer can easily be a cause of the numerous mutations that occur in tumor cells (Shen and Laird, 2013; Klutstein et al., 2016). Exploring normal and cancerous colon mucosa using a CpG island microarray, specifically on a CpG-rich region in *PTPRG* intron 1, revealed that 17/18 colorectal carcinoma samples exhibited a fully methylated state. Furthermore, comparable data have also been identified in earlier carcinoma stages (e.g.,

serrated adenomas), and in Lynch syndrome-associated with colorectal cancers. Regardless of the methylation status and the tumor stages, no variation in *PTPRG* mRNA expression was detected. Nevertheless, the methylation of *PTPRG* intron 1, about 3 Kbp downstream of the transcription-starting site, reduced the binding of CTCF protein to the intron 1 sequence. (van Roon et al., 2011). The nuclear protein having 11 zinc-finger domains CTCF is highly conserved across species, from *drosophila* through mammalian radiation, and it enables vertebrates to regulate negatively and positively their transcription (Dunn and Davie, 2003; Kim et al., 2007). The reduced binding of CTCF with the intron 1 of *PTPRG* sequence could weaken the formation of chromatin regulatory structures essential for the expression of distant genes (van Roon et al., 2011). Additionally, high levels of methylation have been found in chronic myeloid leukemia (CML) patients (Ismail et al., 2020). Instead, *PTPRG* promoter methylation was regularly associated with decreased protein expression and has been reported in several malignancies. Shu et al. described a reduction of *PTPRG* mRNA in breast cancer cell lines MCF-7 and SK-Br-3, compared to a non-cancerous cell line MCF-10A. Small *PTPRG* mRNA values were identified in cancer cell lines that matched with the methylation pattern evaluated using TaqI restriction enzyme by COBRA assay (Shu et al., 2010). Similarly, hypermethylated status of the *PTPRG* promoter was characterized in NPC cell lines by methylation-specific PCR. Expanding the analysis to seven human NPC biopsies, paired with the counterpart of normal tissue showed *PTPRG* methylated alleles only in tumor tissues (Cheung et al., 2008). Similar alterations were reported in hematological malignancies, such as in acute lymphoblastic leukemia (ALL) and in cutaneous T-cell Lymphoma patients (Chatterton et al., 2014; Stevenson et al., 2014). A genome-wide investigation of promoter CpG islands identified several membrane-bound tyrosine phosphatases frequently methylated, including *PTPRG* (van Doorn et al., 2005; Kuang et al., 2008). This initial screening prompted Stevenson et al. to investigate the methylation status of 22 leukemic cell lines, demonstrating a strong promoter methylation of *PTPRO* phosphatase, while *PTPRG* and others showed variable patterns between myeloid and lymphoid cell lines. Significantly, higher methylation levels were also identified in 57 ALL patient samples, with a *PTPRG* promoter methylation rate of 63% (Stevenson et al., 2014). Concurrently, direct correlation was established between *PTPRG* methylation (both CpG in the promoter and in gene body) and *RAS*-mutated phenotype in ALL childhood patients (Chatterton et al., 2014; Xiao et al., 2014). Indeed, the KRAS-induced transcription factor RREB1 was shown to be able to bind a *RAS-Responsive Element* (R.R.E.) on the *PTPRG* promoter region. This feature emphasizes a KRAS-induced modulation of the phosphatase expression, especially after treatment with a demethylating agent, emphasizing the relevance of epigenetic regulation (Xiao et al., 2014).

The analysis of *PTPRG* expression led to its characterization also in the myeloid lineage (Lissandrini et al., 2006) and in myeloproliferative diseases (Della Peruta et al., 2010). Recently our laboratory demonstrated an intense correlation between *DNMT-1* and *3b* expression, two DNA methyl-transferases cooperating in tumor suppressor genes silencing (Rhee et al., 2002), and the reduction of *PTPRG* expression in CML. These two methyl-transferases were found highly expressed in CML cell and chromatin immunoprecipitation revealed the engagement of DNMT-1 to the PTPRG promoter sequence (Tomasello et al., 2020). Furthermore, recent results have highlighted a high frequency of CpG island methylation in CML patients compared with control group. Interestingly, hypermethylation of CpG islands in *PTPRG* intron 1 was identified in a group of patients that failed the tyrosine kinase inhibitors (TKIs) response compared to a newly diagnosed one (Ismail et al., 2020). In almost all studies performed on the methylation of *PTPRG* promoter, treatment with demethylating agents (including 5-azacytidine) restored the expression of *PTPRG* (van Doorn et al., 2005; Cheung et al., 2008; Della Peruta et al., 2010; Shu et al., 2010; Stevenson et al., 2014; Xiao et al., 2014; Tomasello et al., 2020). This results support how epigenetic silencing represent a general mechanism to modulate *PTPRG* expression, especially in leukemia (**Figure 1A**).

Post-Transcriptional and Post-translational Regulation

PTPRG protein is known to undergo some specific processing: four different isoforms have been described as alternative splicing in rat brain cells (**Figure 1D**) (Shintani et al., 1997). Besides the classic whole structure of the phosphatase, the truncated form of the extracellular domain seems to be of particular relevance. Indeed, increasingly evidence emphasizes a role in several tissues under inflammatory state (Lorenzetto et al., 2014; Moratti et al., 2015; Jiang et al., 2020). As previously discussed, PTPRG is regulated also in the context of myeloid cell differentiation (Lissandrini et al., 2006). In addition, the entire active form of PTPRG phosphatase protects breast cancer cell lines from the increase in cell growth and proliferation induced by estradiol-17β and zeranol, both of which may induce an estrogenic response (Liu et al., 2004). Considering this, Wang et al. found a lower amount of *PTPRG* mRNA in breast cancer tissues compared to cells from healthy tissues. Subsequently, they investigated the role of conjugated linoleic acids, natural compounds protecting breast cancer cells from estrogenic proliferative effects. The t10, c12-CLA and t9, c11-CLA were shown to enhance *PTPRG* expression in breast cancer cell lines but also in human cancerous tissues. This condition occurs mainly in epithelial cells, with no effects on stromal cells, indicating selective *PTPRG* regulation of these compounds and their antitumor role in breast cancer (Wang et al., 2006).

Forefront genomic techniques afforded the study of non-coding RNA forms, a massive component in the human genome that participates in the transcriptome regulation. Alteration of the levels of these critical ncRNAs have been shown to promote tumorigenesis (Goodall and Wickramasinghe, 2021). *PTPRG* expression is regulated by different ncRNAs, classified according to structurally different molecules and their biological roles exerted (**Table 1**). Several microRNAs (miRNAs) composed by 18–25 nucleotides might

TABLE 1 | ncRNA targeting *PTPRG* in disease processes.

ncRNA	Tissues source	Expression	References
mir-19b	Breast	Upregulation	Liu et al. (2016)
mir-567	Lung	Upregulation	Yu et al. (2019)
cMras	Lung	Downregulation	Yu et al. (2019)
mir-141	Kidney	Upregulation	Newbury et al. (2021)
lncRNA-AS1	Brest, Bone, others	Alteration	(Zhao et al., 2014; Iranpour et al., 2016; Ge et al., 2021)

induces mRNA modulation by guiding gene expression through the binding to the 3' UTR region of the mRNA (Ha and Kim, 2014). Altered expression of several miRNAs, such as those belonging to the polycistronic miR-17–92 cluster, has been associated with tumorigenesis (Hong et al., 2010). Liu et al. analyzed the role of *PTPRG* in human breast cancer, confirming the dramatic reduction of PTPRG protein compared to healthy human tissue. Post-transcriptional regulation of *PTPRG* has been indicated as a consequence of increased levels of miR-19b only in cancer tissues. Indeed, treatment with an anti-miR-19b subsequently restored PTPRG protein expression levels. Finally, a *PTPRG*-specific siRNA simulates the phosphatase protein reduction increasing the tumorigenic capability of cancer cell lines, confirming the tumor suppressor role played by PTPRG in human breast cancer (Liu et al., 2016). Similarly, miR-141 belonging to the miR-200 family affects the expression of *PTPRG* in renal tissue. Specifically, the effect demonstrated by Newbury et al. reveals the increased value of miR-141 in acute kidney injury (AKI). By inducing miR-141 and causing a similar cell oxidative stress (H_2O_2) the reduction of PTPRG expression was achieved. MiR-141 increased cell death and decreased viability in PTEC cells, obtaining the same results by siRNA transfection against *PTPRG* (Newbury et al., 2021).

CircRNAs are a novel class of the untranslated RNA, usually used as initiation/progression diseases markers, characterized by a special circular structure and a higher forbearance to exonucleases (Meng et al., 2017). Recent evidence in lung cancer indicating a crucial role for reduced levels of has_circ_100,395, a circRNA that operate as a sponge for miR-1228 that was involved in cancer development (Chen et al., 2018). In addition, the study involving another circRNA hsa_circ_0067512 (cMras) indicated its downregulation in human lung adenocarcinoma (LUAD) tissues and LUAD cell lines. Since circRNAs are known to control mRNA functions, Yu et al. identified a potential regulatory process enabling cMras/miR-567 to modulate *PTPRG* expression. Particularly, the reduction of cMras in LUAD left the miR-567 free to bind the 3' UTR of *PTPRG* mRNA, reducing its expression. The effects obtained by this cancer mechanism reflect the increase of proliferation/migration in lung cancer cells. Finally, a worse prognosis was revealed in LUAD patients with low levels of PTPRG expression suggesting a protective role by phosphatase in this cancer (Yu et al., 2019). The ncRNAs governing PTPRG-expression in disease processes are shown in **Table 1**.

Although we know only a little fraction of functional lncRNAs to date, these transcribed ncRNA molecules longer than 200 nt have been shown to modulate each level of gene expression. Post-transcriptional gene silencing can occur through the category of lncRNA antisense, such as PTPRG-AS1 (Faghihi and Wahlestedt, 2009; Wang and Chang, 2011). PTPRG-AS1 expression has been considered as an oncogenic factor in several cancers. Additionally, *PTPRG* gene expression may be affected by high manifestation of PTPRG-AS1 affecting its TSG functions. These evidences were associated with increased survival of breast cancer patients who had a specific pattern of three lncRNAs, including reduced levels of PTPRG-AS1 (Zhao et al., 2014; Iranpour et al., 2016) (**Table 1**).

PTPRG INTERACTS WITH CHARACTERISTIC ONCOGENES IN SPECIFIC CANCER TYPES

Leukemia and Lymphomas

Various evidence reflects the critical role of *PTPRG* in bone marrow and peripheral blood malignancies. Investigations carried out in the various leukemia and lymphoma subgroups report a significant involvement of *PTPRG* underlined by multiple data obtained on the epigenetic mechanisms affecting this gene (van Doorn et al., 2005; Chatterton et al., 2014; Stevenson et al., 2014; Xiao et al., 2014). In this context, PTPRG has been shown to negatively modify the ERK1/2 kinase phosphorylation in cell line model expressing mutant *KRAS* able to alter several signal cascades including AKT, ERK1/2, IκB-α, JNK and p38 MAPK (Xiao et al., 2014). Chronic lymphocytic leukemia (CLL) was defined as B lymphocytes accumulation, both in primary and secondary lymphoid organs, which are characterized by extended cell life (Pangalis et al., 2002). Laudanna group reported that PTPRG was found involved in the regulation of the BTK/JAK2 axis in the CXCR4-and BCR-triggered integrin activation (Mirenda et al., 2015; Montresor et al., 2018). Trojan peptide-mediated activation of PTPRG (P1-WD) demonstrated the ability to reduce both JAK2 and BTK phosphorylation by producing a strong reduction in the integrin-mediated adhesion capability of healthy and leukemic B-lymphocytes (**Figure 2**). Moreover, activated PTPRG was able to induce the apoptotic process as intensely as the BTK inhibitor Ibrutinib, specifically in CLL and not in healthy B-lymphocytes. These results were also confirmed by the use of the whole D1 catalytic domain engineered in a membrane permeable form (TAT-ICD) (Montresor et al., 2021).

The first hematological malignancy where a key role of *PTPRG* was discovered is the Ph^+ chronic myeloid leukemia (CML). Della

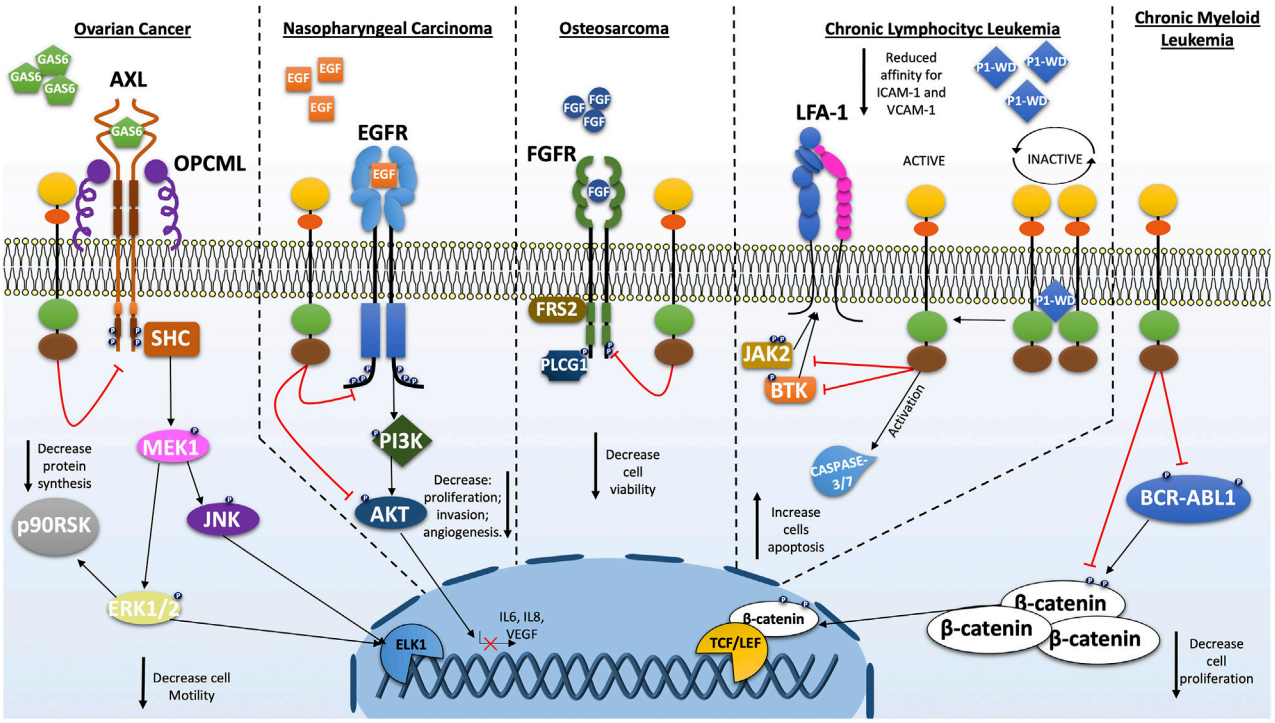

FIGURE 2 | Schematic representations of the cellular pathways most investigated on the role of PTPRG in various types of cancer. As a tumor suppressor, PTPRG regulates several oncogenes such as BCR-ABL1, EGFR, FGFR, and others. The alteration of PTPRG emphasizes some cancer-associated cellular characteristics such as proliferation, motility and invasion.

TABLE 2 | Cancerous RTKs interacting with PTPRG.

Proteins	Disease	PTPRG role	References
JAK2	CLL	Decrease integrin-mediated adhesion and increase apoptosis	(Mirenda et al., 2015; Montresor et al., 2021)
BTK	CLL	Decrease integrin-mediated adhesion and increase apoptosis	(Mirenda et al., 2015; Montresor et al., 2021)
BCR-ABL1	CML	Decrease cell proliferation	(Della Peruta et al., 2010; Vezzalini et al., 2017; Drube et al., 2018; Tomasello et al., 2020)
AXL	Ovarian Cancer	Decrease cell motility and cancer phenotype	(Antony et al., 2018; Zurzolo, 2018)
EGFR	NPC	Decrease cell proliferation, invasion and the angiogenesis processes	(Cheung et al., 2008; Cheung et al., 2015)
FGFR	Osteosarcoma	Decrease cell viability	Kostas et al. (2018)

Peruta et al. reported how the tyrosine phosphorylation of BCR-ABL1, CRKL and STAT5 were decreased in *PTPRG*-transfected K562 and reported downregulation of PTPRG mRNA and protein in CML patients (Della Peruta et al., 2010). This type of result was independently replicated confirming the inhibitory activity of PTPRG on BCR-ABL1 protein and on substrates influenced by itself such as ERK1/2 kinase (Drube et al., 2018). Additionally, BCR-ABL1 dephosphorylation on Y245, a key residue for the kinase activity, together with the impairment of tyrosine phosphorylated residues stabilizing β-catenin, constitutes a fundamental issue for the disease progression (Della Peruta et al., 2010; Tomasello et al., 2020). Treatment with TKI and subsequent MMR (major molecular response) achievement was associated with a recovery of *PTPRG* expression, while *PTPRG* was still absent in patients who failed to achieve the MMR (Della Peruta et al., 2010; Vezzalini et al., 2017) suggesting that recovery of non-neoplastic hematopoiesis is associated to restoration of *PTPRG* expression. Recent flow cytometry analysis has confirmed this association between MMR and *PTPRG*-restored expression in CML patients after TKI-treatment (**Figure 2**; **Table 2**; **Table 3**) (Drube et al., 2018; Ismail et al., 2021).

Carcinoma

Numerous reports indicate reduced *PTPRG* expression in epithelial-derived cancers. Data are available for several tissues

TABLE 3 | Hypothetical clinical role of PTPRG in cancers.

Disease	Predictive role	Prognostic effects	References
Chronic Myeloid Leukemia	CML patients with great response to therapy express high levels of PTPRG compared with levels at diagnosis	Increased levels of PTPRG mRNA have been found in patients who achieve the highest molecular response (MMR) after therapy compared to non-responders.	(Della Peruta et al., 2010; Vezzalini et al., 2017; Ismail et al., 2020; Ismail et al., 2021)
Lung Adenocarcinoma		Specific germline polymorphisms such as SNPs on PTPRG gene may influence the survival of patients with lung adenocarcinoma	Galvan et al. (2015)

and, more specifically, for lung (van Niekerk and Poels, 1999; Galvan et al., 2015), breast (Liu et al., 2016), gastric or esophageal squamous cell carcinoma (Wu et al., 2006; Lo et al., 2007), ovarian (van Niekerk and Poels, 1999), colorectal cancer (Wang et al., 2004; van Roon et al., 2011) and nasopharyngeal carcinoma (NPC) (Cheung et al., 2008; Cheung et al., 2015). A few studies addressed the role of PTPRG in nasopharyngeal carcinoma, where the extracellular matrix (ECM) seems to play a key role in cell-cycle progression. *PTPRG*-transfected NPC cells showed a significant cell-cycle arrest compared to the control particularly when these cell lines formed spheroids in 3D cultures, underlining the suppression induced by PTPRG through the interaction with ECM. Under these conditions, PTPRG reduces the phosphorylated form of Rb (active) producing the cell cycle G1-arrest through the regulation of cyclin D1 (Cheung et al., 2008). Subsequent investigations revealed an additional regulatory pathway involving PTPRG/EGFR/AKT in NPC (Yip et al., 2008). EGFR is regulated on Y1068 and Y1086 by PTPRG inducing downregulation of the PI3K/AKT pathway, also confirmed by the reduction of phosphorylation of several downstream substrates of AKT such as JNK, c-JUN and CREB (**Figure 2** summarizes all PTPRG interactors). Remarkably, PTPRG suppresses invasive capacity on NPC cells while also limiting angiogenesis (Cheung et al., 2015). In epithelial ovarian cancer (EOC), the suppression of *OPCML*, a TSG recognized in several cancers, together with the overexpression of RPTK *AXL*, confer a worse overall survival (Sellar et al., 2003). In normal ovarian cells, Anthony J. et al. unearthed the chaperone interaction between OPCML protein and the active form of AXL kinase in the cholesterol-enriched lipid domains on cell membrane, in which PTPRG also resides. The proximity PTPRG mediated by OPCML to the AXL kinase produces an inhibitory effect on the AXL pathway and other RPTKs network, reducing the expression of transcription factors related to epithelial-mesenchymal transition (EMT) such as ZEB1 and related to cell motility such as Slug. Furthermore, the *PTPRG*-expressing cells were more sensitive to the AXL inhibitors, improving the therapeutic effect. In EOC, OPCML can be downregulated thereby preventing the tumor suppressor effect of PTPRG (Antony et al., 2018; Zurzolo, 2018). In addition to EOC, the lncRNA PTPRG-AS1 was found to be highly expressed in primary samples and cell lines compared to the normal counterparts. Indeed, it seems that PTPRG-AS1 may function as a sponge for miR-545-3p, which binds the 3' UTR of the *HDAC4* gene causing both mRNA and protein repression. Interestingly, by interfering with PTPRG-AS1 expression, the tumorigenic capabilities of these cells have been considerably reduced both *in vitro* than in tumor xenograft model (Shi et al., 2020). The separation between PTPRG and AXL from one side (Antony et al., 2018) and the high expression of antisense lncRNA PTPRG-AS1 on the other (Shi et al., 2020), although with different pathways implication, suggest a fine and intricate regulation of PTPRG in the EOC. Similarly, in colorectal cancer the identification of somatic mutations suggests a complex scenario where *PTPRG* can be modified quantitatively (reduced expression driven by methylation/non-coding RNAs) and qualitatively (somatic mutations), a feature shared with the alteration of other classic TSGs in cancer development. (Capellini et al., 2021; Li et al., 2021). The case of Merkel Cell carcinoma is noteworthy, where integration of Merkel cell polyomavirus (MCV or MCPyV) to form different length fusions with intron 1 of the human *PTPRG* gene. Occurs virus incorporation was found associated in 80% of the cases and only 8% of controls (Feng et al., 2008), adding new scenario in the list of alterations occurring in the context of *PTPRG* gene and possibly contributing to the pathogenesis of this disease.

Sarcoma

The observation that PTPRG and Fibroblast Growth Factor Receptors (FGFR1) interact and co-localize at the plasma membrane exhibiting a further model of regulation of PTPRG. Interestingly in U2OS sarcoma cells, PTPRG directly dephosphorylates the active FGFR1, connecting for the first time PTPRG to the development of sarcomas (Kostas et al., 2018). Indeed, FGFR overexpression and activating mutations were shown to play an important role in several types of sarcomas such as: osteosarcoma, rhabdomyosarcoma and soft tissue sarcoma (Taylor et al., 2009; Guagnano et al., 2012; Weekes et al., 2016; Zhou W.-Y. et al., 2016; Chudasama et al., 2017). Meanwhile FGFR-specific downstream signaling adaptor, FGFR substrate 2 (FRS2), is overexpressed in liposarcoma and renders these cells sensitive to FGFR inhibitors (Zhang et al., 2013; Hanes et al., 2016). In osteosarcoma the loss of PTPRG represent and advantage for cancer cells (a representative diagram is shown in **Figure 2**). Precisely, PTPRG regulates FGFR1 and it further appeared to impinge the efficiency of the TK-inhibitor on the FGFR kinase. This would represent a possible drug-resistance mechanism of cancer cells and the presence of PTPRG could reduce the effective concentration of the drug. Moreover, PTPRG could also modulate the activity of FGFR4 in rhabdomyosarcoma, indeed using siRNA against PTPRG in FGF-treated RH30 cell line increases phosphorylation of the receptor FGFR4 and downstream molecule such as PLCG1 (Phospholipase C-gamma 1) compared to the scramble control (Kostas et al., 2018). Another mechanism was recently found to occur in patients with osteosarcoma involving the long non-coding RNA PTPRG-AS1. Overexpression of PTPRG-AS1 may predict the poor prognosis of patients and may have a

promoting effect on osteosarcoma cell metastasis being associated to increased migratory abilities of Saos-2 cells (Ge et al., 2021).

Cancers of the Central Nervous System

Astrocytoma cell line U373-MG and primary astrocytes express *PTPRG* whose expression was found to be regulated by IL-1 or TNFα (Schumann et al., 1998; Lorenzetto et al., 2014). Analysis of formalin-fixed paraffin-embedded human tissues showed overexpression of PTPRG in astrocytoma cases with no or limited expression in their healthy counterparts (Vezzalini et al., 2007; Lorenzetto et al., 2014). In these tumor types, although *PTPRG* overexpression may be the manifestation of a putative oncogenic role, it might also be associated with the undifferentiated state of the neoplastic cells, as suggested by previous studies showing a role of this phosphatase in hematopoietic differentiation of murine embryonic cells and in neurite outgrowth (Shintani et al., 1997; Sorio et al., 1997). Notable, PTPRG has been involved in Wnt/β-catenin pathway involved in differentiation, cell migration and proliferation during embryogenesis and in adult tissues where a number of small molecules that can modulate it may have opposing effects depending on cell-type. Al-Harthi et al. reported that small molecules acting on colon epithelial cells do not have the same effect in astrocytes suggesting that different pathways involving β-catenin are active in CNS cells (Al-Harthi, 2012). However, the lack of knowledge regarding the function of *PTPRG* in this type of tumor does not allow a precise classification of its role.

A little bit features are available about the role of *PTPRG* in cancer affecting glial cells. The data report higher expression of the other member of R5 group *PTPRZ* in glioblastoma cells (Muller et al., 2003). Firstly, *PTPRZ*-knockdown has reduced some peculiar characteristics of the tumor such as proliferation, migration and growth as well as decreasing the expression of several transcription factors connected with the cancer-stemness, such as SOX2, OLIG2 and POU3F2. Secondly, the soluble portion of PTPRZ (sPTPRZ) constitutes a promising diagnostic biomarker present in the cerebrospinal fluid (CSF) which helps to identify different types of gliomas (Ulbricht et al., 2006; Fujikawa et al., 2016; Fujikawa et al., 2017; Yamanoi et al., 2020). In this tumor, both RPTPs members of R5 group were overexpressed possessing an oncogenic behavior. Noda group was able to synthesize specific inhibitors for the D1 domain of PTPRZ/PTPRG. NAZ2329 molecule increased the phosphorylation of some downstream targets of phosphatases (e.g. Y118-paxillin) limiting the proliferation and migration of some glioblastoma cell lines, confirming the effects produced by the knockout of the gene (Fujikawa et al., 2017). Nonetheless, data on these tumors are limited and further studies will be useful to confirm a specific oncogenic role for *PTPRG*.

CONCLUSION

PTPRG is emerging as a multifunctional protein with multiple roles in healthy and disease tissues with still poorly characterized, emerging issues. As an example, strong PTPRG expression has been reported in endocrine cells of the gastrointestinal tract, pancreatic islets of Langerhans, adrenal medulla and thyroid, only associative evidence indicate a potential role in normal cell homeostasis/differentiation in this site (Brenachot et al., 2017) and that a dysregulated PTPRG could be involved the development of neuroendocrine tumors (Vezzalini et al., 2007). Numerous contributions that we have tried to summarize in this review have provided compelling evidence of a relevant role in cancers. More specifically, while large amounts of data are available for the hematopoietic system, much work done on solid tumors highlights what may be a "tip of the iceberg". As we have seen, cancer cells implement different strategies to shutdown PTPRG, producing both the inhibition of expression and block the phosphatase activity. Altering the methylation state of the gene promoter appears to be one of the most commonly used systems in solid and non-solid cancer cells (Cheung et al., 2008; Della Peruta et al., 2010; van Roon et al., 2011; Chatterton et al., 2014; Stevenson et al., 2014; Xiao et al., 2014; Tomasello et al., 2020). Furthermore, with the advent of genomic technologies we have begun to understand additional post-translational regulatory mechanisms imposed by ncRNAs, such as miRNA and lncRNA. Surprisingly, the antisense RNA 1 of PTPRG (PTPRG-AS1) was found to be upregulated in different solid cancers representing a significant emerging predictor for tumor progression (Faghihi and Wahlestedt, 2009; Wang and Chang, 2011; Ge et al., 2021). Despite the fact that it has been clarified a suppressive role for *PTPRG* in various tumors, limited data suggesting an oncogenic role by the receptors PTPRZ/PTPRG in glioblastoma. Further studies will therefore be essential to explain this opposite pattern noted in CNS tumors (Fujikawa et al., 2016; Fujikawa et al., 2017; Yamanoi et al., 2020). Finally, besides to suggesting a potential pharmacological target that may be of interest in different types of malignancies, data currently available provide evidence to support a first possible clinical application as a "monitoring tool" for follow-up of CML patients. Considering the restoration of PTPRG expression in CML patients who reach the MMR (major molecular response) after TKI-treatment, as opposed to those who have not reached the MMR, can make the PTPRG a "tool" to monitor the recovery of normal hematopoiesis (Della Peruta et al., 2010; Vezzalini et al., 2017; Drube et al., 2018; Ismail et al., 2021). Clearly, a significant progress was done in the last decades since the initial cloning in 1993, with 143 publications mentioning *PTPRG* in PubMed database to date, with a trend to a year-by-year increase. However, much work is still be done to unveil the molecular details of this intriguing gene product, studies that are instrumental to exploit potential clinical application.

AUTHOR CONTRIBUTIONS

Manuscript design, CB and CS; resources, CS; writing—original preparation, CB; review and editing, CB: and CS; visualization, CB; project administration, CS; funding acquisition, CS.

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GLOSSARY

TSG tumor suppressor gene

RPTKs receptor protein tyrosine kinases

PTPs protein tyrosine phosphatases

RPTPs receptor protein tyrosine phosphatases

NRPTPs non-receptor protein tyrosine phosphatases

PTPRG-AS1 PTPRG antisense 1

CNS central nervous system

ncRNA non coding RNA

RRE ras-responsive element

LUAD lung adenocarcinoma

NPC nasopharyngeal carcinoma

ALL acute lymphoblastic leukemia

CLL chronic lymphocytic leukemia

CML chronic myeloid leukemia

PLCG1 Phospholipase C-gamma 1

CTCF CCCTC-binding factor

PTECs renal proximal tubular epithelial cells

ERK extracellular signal-regulated kinase

IkB inhibitor of nuclear factor kappa B

JNK c-Jun N-terminal kinase

MAPK mitogen-activated protein kinase

BTK Brutun's tyrosine kinase

JAKs janus tyrosine kinases

CXCR4 CXC motif chemokine receptor 4

BCRs breakpoint cluster regions

ABL1 ABL proto-oncogene 1

TAT-ICD cell-penetrating peptide/intracellular domain of PTPRG

CRKL CRK like protein

STATs signal transducer and activator of transcription

TKIs tyrosine kinase inhibitors

EGFR epidermal growth factor receptor

FGFR fibroblast growth factor receptor

PI3Ks phosphoinositide 3-kinases

AKT protein kinase B

CREB cAMP response element-binding protein

OPCML opioid binding protein/cell adhesion molecule like

AXL axl receptor tyrosine kinase

CSF cerebrospinal fluid

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