TGF-β AND BMP SIGNALING IN CANCERR

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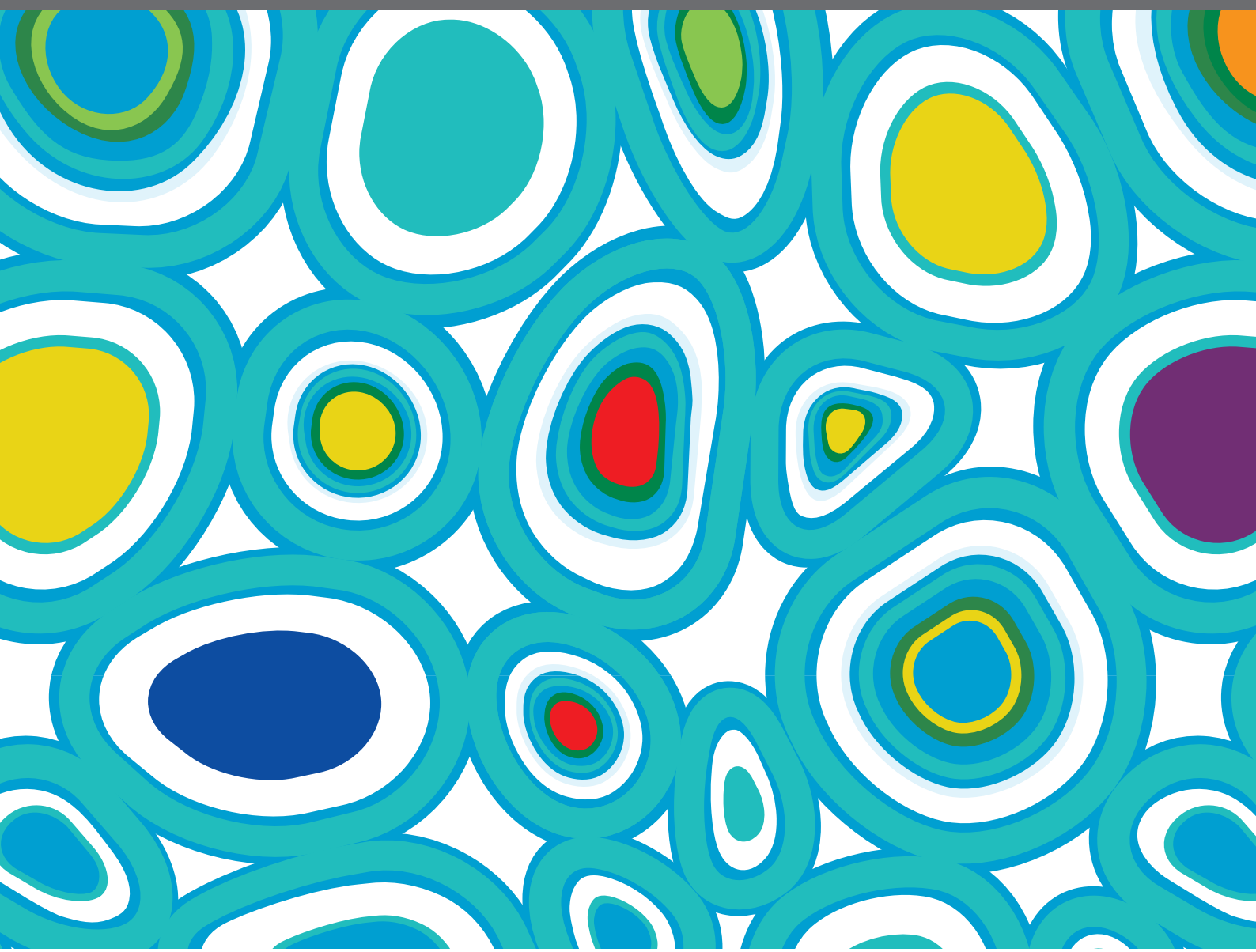

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TGF-β AND BMP SIGNALING IN CANCER

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Editorial: TGF-β and BMP signaling in cancer

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Editorial on the Research Topic
TGF-β and BMP signaling in cancer

Transforming growth factor-β (TGF-β) family members, which includes TGF-βs, activins and bone morphogenetic proteins (BMPs) are structurally related secreted cytokines that fulfil key roles during embryonic development and in maintaining tissue homeostasis (Siegel and Massagué, 2003). Perturbation of these cytokine actions may lead to various diseases, including cancer. TGF-β family members signal via specific transmembrane type I and type II serine/threonine kinase receptors and intracellular SMAD transcriptional effector proteins. The type I receptors are phosphorylated by type II kinases and determine the signaling specificity within the cell surface ligand-receptor complex. Whereas type I receptors for TGF-β and activin (i.e., TβRI or activin receptor-like (ALK)5 and ActR-IB or ALK4, respectively) signal via SMAD2 and SMAD3, two receptor-regulated SMADs (R-SMADs), BMP type I receptors (ALK1, ALK2, BMPRIA or ALK3, and BMPRIB or ALK6) activate three R-SMADs, i.e. SMAD1, -5 and -8 by direct phosphorylation at the C-terminus. Activated R-SMADs form complexes with the common mediator SMAD4, and then the Smad complexes accumulate within the nucleus. There they act in concert with transcriptional co-activators/co-repressors and epigenic regulators to regulate specific gene transcriptional responses. Next to the canonical SMAD pathway, TGF-β family receptors can also signal via non-SMAD signaling pathways (Heldin and Moustakas, 2016).

TGF-β family members are multifunctional cytokines and elicit effects that are highly dependent on cellular context (Morikawa et al., 2016; David and Massagué, 2018). In cancer, TGF-β family members have been attributed with both tumor suppressive and tumor promoting activities. Among all its family members, the action of TGF-β in cancer has been investigated the most. It is likely that many of the observations for TGF-β also apply (with some variation) to other family members. In normal cells, pre-malignant and even some malignant tumor cells, TGF-β restrains cell proliferation, induces apoptosis

and contributes to genome stability. Cancer cells can become insensitive to these tumor suppressive effects when receptors or SMADs become mutated or dysfunctional in other ways. As a result, these cells may undergo uncontrolled growth. Moreover, in advanced cancer cells, when proto-oncogenes and tumor suppressor genes have become activated or inactivated, respectively, cells not only become insensitive to the TGF-β-induced cytostatic and pro-apoptotic effects, but may also use the SMAD pathway to stimulate pro-oncogenic effects, such as induction of the epithelial-to-mesenchymal transition (EMT) programme and thereby promote cancer cell invasion and metastasis (Katsuno et al., 2013). Moreover, besides cancer cells, host cells can secrete high amounts of TGF-β, which acts not only on cancer cells but also cells from the tumor microenvironment, thereby stimulating tumor angiogenesis and immune evasion (Battle and Massague, 2019; Liu et al., 2021).

While targeting TGF-β signaling by interfering with TGF-β-receptor interaction or inhibiting the receptor kinase activity for cancer therapy has been pursued by many academic and company laboratories, still no TGF-β inhibitor has been clinically approved. Part of this can be attributed to the fact that the inhibitors tested in clinical trials do not act in a cell type specific manner, and when administered systemically lead to on target toxic side effects. Recently, however, we see a renewed interest in targeting this pathway by (selectively) interfering with the TGF-β-induced immune suppression as it may allow for more effective immune checkpoint inhibitor therapy (Battle and Massagué, 2019; Liu et al., 2021).

This Research Topic comprises five original research and six review (including one minireview) articles covering diverse and complementary aspects on the role of TGF-β and BMPs in cancer progression. Shuelten and Zhang provide an overview on the important role of TGF-β in the tumor microenvironment. In particular, they focus on how TGF-β acts as a potent differentiation factor for epithelial and endothelial cells and fibroblasts, a polarizing agent for macrophages and a mediator of metabolic changes, which all contribute to epithelial tumor formation. How the interplay between TGF-β signaling and metabolism controls cellular homeostasis and contributes to cancer progression is discussed in depth by Liu and Chen. A picture is emerging that many metabolic pathways are controlled in a highly cell type-dependent manner. Zhang et al. focus in their review on involvement of TGF-β as a pivotal driver of cancer therapy resistance. Combining TGF-β inhibitors with immune therapies has promise but will require careful selection of patients that benefit most from treatment. Extracellular vesicles (EVs) are emerging as an important mechanism for cell communication. Multiple TGF-β signaling components have been shown to be present within or associated with these vesicles, and were reported to play a pivotal role in cancer metastasis, immune evasion and therapy resistance. In addition, the TGF-β signaling components that are associated with EVs may have potential as biomarkers for prognosis, diagnosis and therapy prediction (Rodrigues-Junior et al.). Whereas the preceding papers mainly focused on TGF-β, Ehata and Miyazono and Guyot et al. present their reviews the roles of BMPs in cancer progression. Like TGF-β, BMPs have both pro-tumorigenic and tumor suppressive roles. Ehata and Miyazono discuss the application of BMP signaling inhibitors for cancer treatment. Guyot et al. focus on the parallel actions of BMP2/4 in myeloid leukemia and breast cancer.

In an original research paper, Shi et al. report on how TGF-β enriched CAFs act as a critical determinant for lung metastasis of squamous cell carcinoma (SCC). They propose that these cells may provide a prognostic marker and therapeutic target for metastasis of SCC to lungs. Trelford and Di Guglielmo delineate the critical involvement of SMAD4 and the TAK1-TRAF6-p38 MAPK pathway in TGF-β-induced autophagy in non-small cell lung cancer cells (NSCLCs). Using publicly available databases, Tu et al. analyse the potential role of SNAI family members in breast cancer prognosis and immune regulation. Using a similar bioinformatic approach, Gao and Zhou analyze mRNA expression levels of RUNX family members and observe their correlations with prognosis and immune cell infiltration in breast cancer. Huang et al. ascribe in their original research article a key effector role for a member of the Paraneoplastic Ma family, i.e. PNMA5, as a downstream BMP2 effector in mediating the bone metastasis of NSCLCs. It will be interesting to explore the therapeutic targeting of PNMA5 for the treatment of NSCLC bone metastasis.

Taken together, the current Research Topic provides valuable new insights into the role of TGF-β and BMPs in cancer progression. New directions of future research are offered on how to further explore the multifaceted role that TGF-β members play therein. We anticipate that soon the efforts in fundamental and translational research will lead to the clinical approval of a drug that targets a TGF-β family member for the treatment of specific cancer subtypes.

Author contributions

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Conflict of interest

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ORIGINAL RESEARCH
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PNMA5 Promotes Bone Metastasis of Non-small-Cell Lung Cancer as a Target of BMP2 Signaling

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Bone metastases frequently occur in NSCLC patients at the late stage, indicating poor survival. However, mechanisms about the initiation of NSCLC bone metastases remain largely unclear. In our previous reports, BMP2 signaling activation has been found to enhance NSCLC bone metastases through enhancing carcinoma cells migration, invasion, osteoclasts differentiation and osteoblasts immature differentiation. Nevertheless, downstream target genes of BMP2 contributing to those processes still remain unknown. In this project, we find that the expression of *Pnma5* is higher in metastatic bone tumors of Lewis lung carcinoma than in metastatic lung tumors and parental Lewis lung cells. *Pnma5* overexpression not only can promote cell migration and invasion of NSCLC cells but also tumor-induced osteoclasts differentiation. Interestingly, knockdown of *Pnma5* in Lewis lung cells blocks BMP2 signaling from inducing Lewis lung cells migration and invasion. Although BMP2 signaling can promote Lewis lung cells-induced osteoclasts differentiation from macrophages, this effect can also be blocked when *Pnma5* is knocked down in Lewis lung cells. Moreover, *Pnma5* can promote NSCLC bone metastases *in vivo* as the downstream target of BMP2. Those results above indicate that BMP2 signaling enhances NSCLC bone metastases via its direct downstream target gene *Pnma5*. This research reveals the detailed molecular mechanism about how BMP2 signaling contributes to NSCLC bone metastases via PNMA5 and provides a new potential therapeutic target for the treatment of NSCLC bone metastases.

Keywords: PNMA5, NSCLC, bone metastases, BMP2, target gene

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INTRODUCTION

Lung cancer is one of the most deadly cancers worldwide (Hanahan and Weinberg, 2011; Vargas and Harris, 2016). Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer. Nearly 80% of lung cancer cases are NSCLC, with subtypes of adenocarcinoma, squamous carcinoma, adenosquamous carcinoma, large cell carcinoma and sarcomatoid carcinoma

(Herbst et al., 2018). When NSCLC patients were at late stages, distant metastasis frequently occurs, resulting in poor prognosis. The median survival time is between 14 and 17 months when distant metastases occur in NSCLC patients (Juan and Popat, 2017). Thus, the therapy for metastatic NSCLC is still challengeable.

Several cancer types tend to cause bone metastases, such as breast cancer, prostate cancer and NSCLC (McAllister and Weinberg, 2014). About 30–40% of NSCLC patients accompany with bone metastases at late stages. Moreover, among NSCLC patients with bone metastases, lung adenocarcinomas are more commonly seen than squamous cancers (Santini et al., 2015; Oliveira et al., 2016). Previous reports have revealed some mechanisms presenting in NSCLC bone metastases (Popper, 2016; Sathiakumar et al., 2017). Parathyroid hormone-related peptide (PTHrP) and receptor activator of Nuclear Factor-kappaB ligand (RANKL), which play key roles in osteoclasts activation (Popper, 2016; Macedo et al., 2017), are found to have functions in NSCLC bone metastases (Nakamura et al., 2006; Kuo et al., 2013). Moreover, miRNA-33a, which targets PTHrP, has been reported to reduce bone metastatic burden in NSCLC (Kuo et al., 2013). In addition, matrix metalloproteinase (MMPs), are supposed to be involved in bone metastases (McAllister and Weinberg, 2014; Wood et al., 2014; Massague and Obenauf, 2016; Ganguly et al., 2020; Zhang et al., 2020), as they contribute to extracellular matrix degradation and interaction between cancer cells with stroma. Thus, signaling pathways that activate MMPs, like transforming growth factor (TGF-β), Wnt, CXCR4, and NFκB, may also play roles in NSCLC bone metastases via MMPs (Popper, 2016). However, in comparison with breast cancer and prostate cancer, the detailed mechanisms about how NSCLC bone metastasis occurs remain largely unclear (McAllister and Weinberg, 2014).

The high expression of bone morphogenic protein 2 (BMP2) has been reported in NSCLC (Langenfeld et al., 2003, 2005; Bieniasz et al., 2009; Choi et al., 2012; Fei et al., 2013). BMP2 signaling activation can enhance lung adenocarcinoma cell proliferation, migration, invasion and lung metastases (Langenfeld et al., 2003; Hsu et al., 2011; Chu et al., 2014). Moreover, BMP2 in the tumor microenvironment is also associated with NSCLC prognosis. High expression of BMP2 in the stroma may result in poor prognosis in NSCLC (Rajski et al., 2015). In our previous reports, BMP2 signaling is found to enhance NSCLC bone metastases via both osteolytic and osteoblastic mechanisms (Huang et al., 2020). However, the downstream target genes of BMP2 signaling associated with NSCLC bone metastases still remain unknown.

PNMA5 is a member of the Paraneoplastic Ma (PNMA) family. Members of the PNMA family have been reported to play roles in carcinoma development (Pang et al., 2018). Lee et al. (2016) has demonstrated that PNMA5 is highly expressed in colon cancer and can enhance the apoptosis of breast cancer cells *in vitro*. According to our RNA-seq data in the previous report (Huang et al., 2020), the expression of *Pnma5* is found to be higher in bone metastatic tumors of Lewis lung carcinoma than in lung metastatic tumors and parental Lewis lung cells. However, researches on the roles that PNMA5 play in NSCLC are rare.

In this project, we have found that PNMA5 can enhance the migration and invasion of NSCLC cells. In addition, PNMA5 can enhance the NSCLC-induced osteoclasts differentiation to promote bone metastasis of NSCLC. Interestingly, PNMA5 is found to be a downstream target of BMP2 signaling in NSCLC. BMP2 signaling enhances bone metastasis of NSCLC via PNMA5. Altogether, PNMA5 can be a downstream target of BMP2 signaling and plays roles in NSCLC bone metastases. Furthermore, PNMA5 can be a new potential therapeutic target for NSCLC patients with bone metastases at late stages.

MATERIALS AND METHODS

Antibodies, siRNAs, and Reagents

Antibodies used in this study: anti-PNMA5 (Abcam, ab150921); monoclonal anti-Smad1/5 (Cell Signaling Technology, 6944) and anti-β-Actin (Sigma, A1978). The sequences of siRNAs applied to knock down *Pnma5* in LLC cells were as follows. SiRNA 1# Target: GCAGAAACCTTATGTTAGA; siRNA 2# Target: CTAGAAATGATCCCAACAA; siRNA 3# Target: CTGACTACTTGCTACGTTT. Reagents: BMP2 (R&D, 355-BM-100); Tris-HCl, NaCl and other reagents were purchased from Sigma.

Mice

Female C57BL/6 mice (6–8 weeks of age) were used in this study, and they were bred and maintained in a specific pathogen-free animal facility at Fujian Medical University. Mice were euthanized with carbon dioxide asphyxiation at the end of observation. All animal experiments were approved by the Animal Ethical Committee of Fujian Medical University (2018-039).

Cells and Transfection

NSCLC cell lines: A549 (ATCC number: CCL-185) and Lewis lung cells (ATCC number: CRL-1642). The macrophage cell line: Raw 264.7(ATCC number: TIB-71). Lewis lung cells were cultured in RMPI1640 (Invitrogen, Carlsbad, CA, United States) which contained 10% fetal bovine serum (FBS) (Hyclone, Utah, United States); A549 and Raw 264.7 cells were cultured in DMEM (Invitrogen) with 10% FBS (Hyclone). Transfection of siRNAs into LLC and A549 cells were performed by using polyethylenimine (polysciences, Inc., PA, United States).

Lewis Lung Carcinoma Metastasis

For the tail veins injection model: 1×10^6 LLCs were injected into the tail veins of per C57/BL6 mice to make the lung and bone metastatic model. For the orthotopic model: 1×10^6 LLCs were pre-treated with the vehicle or 20 ng/mL BMP2 for 24 h. Then the tumor cells were injected into the left lung lobes of per C57BL/6 mice to make the orthotopic model. LLCs frequently tend to colonize in the lungs and bones. Mice were sacrificed with carbon dioxide asphyxiation and tumor tissues were harvested for further analyses after 35 days of injection. Bone metastatic tumor sizes were measured by tumor length and width by using clipper directly and lung metastatic tumor

sizes were measured via the HE staining photos. The tumor volumes were calculated via the formula V = (L × W × W)/2, where V is tumor volume, W is tumor width, L is tumor length (Huang et al., 2020).

Hematoxylin and Eosin Stain

The tissue sections were treated as what mentioned in the previous report (Huang et al., 2020). The sections were cut into 2.5 μm tissue sections after they were embedded in paraffin. Tissue sections were then dewaxed with xylene. 100, −95, and −75% alcohol gradients were used to rehydrate the sections. The tissue sections were stained in Hematoxylin for 20 min, and then differentiated with 1% hydrochloric acid for 30 s. After that, the sections should go through 15 min of PBS blue staining and 3 min of eosin staining. Sections were dehydrated with a gradient of 95–100% alcohol after rinsing. The sections were cleaned with xylene for two times, before the sections were finally mounted with a neutral resin. Photos were taken by Olympus microscope BX53.

Immunoblotting

This assay was conducted as what mentioned in the previous report (Huang et al., 2020). The TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, pH = 7.5) were used to lyse cells and tissues. Then, the cell lysates were mixed with 4 × loading buffer (40 mM Tris-HCl, 200 mM DTT, 4% SDS, 40% Glycerol, 0.032% Bromophenol Blue, pH = 8.0). The samples were run with 4% stacking gel and 10% separating gels. After that, proteins on the gels were transferred to nitrocellulose filter membranes. And then, antibodies were incubated. The membranes' exposure was done with thermo Pierce ECL and FluorChem E (Protein Simple).

Cell Migration Assays

Cells were treated as what mentioned in the previous report (Huang et al., 2020). Cells with density of 1×10^4 cells/insert were seeded on the upper layer of Corning cell culture insert with polycarbonate membrane (Transwell@, 8.0μm pore size, Corning) and cultured in media without FBS. The complete culture media (10% FBS) with or without 20 ng/mL BMP2 were placed below the cell permeable membrane in the well plates. After an incubation for 24 h in 37°C, 5% CO2, the cells migrating through the membrane were stained with 0.1% crystal violet and counted.

Cell Invasion Assays

Cells were treated as what mentioned in the previous report (Huang et al., 2020). 10:1 DMEM and matrigels (BD BioSciences) were utilized to pre-treat Corning cell culture insert with polycarbonate membrane (Transwell@, 8.0 μm pore size, Corning). Cells with density of 1×10^5 cells/insert were seeded on the pre-treated inserts and cultured in media without FBS. The complete culture media (10% FBS) with or without 20 ng/mL BMP2 were filled in the well plates below the cell permeable membrane. After incubated for 48 h in 37°C, 5% CO_2, the cells migrating through the membrane were stained with 0.1% crystal violet and counted.

Cell Proliferation Assays

Cells were treated as what mentioned in the previous report (Huang et al., 2019). Cells were seeded in 96 well plates at a density of about 3,000 cells/well. Relative cell intensity was measured with the cell counting kit-8 (CCK-8, Dojindo Molecular Technologies) after indicated time.

Tartrate-Resistant Acid Phosphatase (Trap) Staining

3×10^4 murine pre-osteoclast RAW 264.7 cells were seeded directly into each well of the 6-well co-culture plates, 3×10^4 lung cancer cells were seeded into each of the Corning cell culture inserts with polycarbonate membrane (Transwell@, 0.4 μm pore size) of the co-culture 6-well plates in triplicate. Lung cancer cells were treated with the vehicle or 20 ng/mL BMP2. The culture media were DMEM medium supplemented with 10% FBS and changed every 2 days. TRAP staining was performed on day 6 with a leukocyte acid phosphatase kit (Sigma, 387A). $TRAP^+$ cells were scored as mature osteoclasts and quantified (Huang et al., 2020).

Chromatin Immunoprecipitation Assay

Cells were treated as what mentioned in the previous report (Huang et al., 2019). After Lewis lung cells and A549 cells were cross-linked by 1% formaldehyde solution for 15 min, the cells were neutralized with 125 mM Glysine for 5 min. Then, cells were lysed using lysis buffer (1% SDS, 50 mM Tris pH 8.0, 5 mM EDTA, proteinase inhibitors). Subsequently, the cell lysates were sonicated to get DNA fragments (300–500 bp). The sonicated lysates were pre-absorbed with protein A beads for 30 min at 4°C and then incubated with 10 μg antibodies (control IgG and anti-HIF1α) overnight at 4°C. At Day 2, Protein A agarose beads were added into the cell lysates to bind the antibodies and targeted proteins for 3 h at 4°C. After the bound beads were washed four times sequentially with salt buffers, the bound immunocomplexes were eluted from the beads with elution buffer (25 mM Tris, pH 8.0, 10 mM EDTA, 0.5% SDS) by heating at 65°C for 15 min. 1 mg/mL protease K was used for reversing the crosslinking at 65°C overnight. The obtained DNAs were purified and subjected to quantitative real-time PCR. Sequences of ChIP primers were as follows. Mouse *Pnma5* promoter: Forward 5′- CAGGGATTAAAGATGTGC -3′, Reverse 5′- GAGTAGGATAGGGCAGAG -3′. Human PNMA5 promoter: Forward 5′- TCAGCCTTCAGAAACATG -3′, Reverse 5′- CAAAGTGCTGGGATTAGA-3′.

Quantitative Real-Time PCR

This assay was conducted as what mentioned in the previous report (Huang et al., 2019). Total cell RNA was extracted with TRIzol (Invitrogen). Then, cDNA was synthesized via reverse transcripts with Revertra Ace (Promega, Madison, United States). Quantitative real-time PCR was performed with

an ABI QuantStudio 5 system. The results were measured by the comparative Ct method. The relative expression values of targeted genes were normalized to GAPDH expression. The primer sequences were as follows. Mouse *Pnma5*: Forward 5′- GTGGTTGTCAAACCCCGTAG-3′, Reverse 5′-TTCCCTGTAGGAACAGTGCTAA-3′; human PNMA5: Forward 5′-AGATGAGGGCCGAAGTATGAC′, Reverse 5′-GCTCTAAAGGTGGGGATCTAACT-3′; Mouse and human Gapdh: Forward 5′-CATGGCCTTCCGTGTTCCTA-3′, Reverse 5′-CCTGCTTCACCACCTTCTTGAT-3′.

Statistical Analysis

The Student's *t*-test, one-way ANOVA test, Wilcox rank sum test and log-rank test were used as indicated in the figure legends. $P < 0.05$ were considered statistically significant.

RESULTS

Pnma5 Is Highly Expressed in Bone Metastasis of Lewis Lung Carcinoma

Lewis lung carcinoma originated from a C57BL/6 mouse was a spontaneous lung adenocarcinoma (Bertram and Janik, 1980; Zhu et al., 2018). Tail veins injection of carcinoma cells could be a classical way to make animal models of tumor metastasis (Brady et al., 2016; Lu et al., 2019; Wang et al., 2020). Lewis lung carcinoma cells (LLCs) were injected into tail veins of C57BL/6 mice to establish lung metastases and bone metastases models. In our previous reports, RNA-seq was carried out to analyze the transcriptome differences among metastatic bone tumors, metastatic lung tumors and parental Lewis lung cells (Huang et al., 2020). The results shown here were based upon data assessed online at the Gene Expression Omnibus (GEO) (NO. GSE148101). Some representative differential expressed genes were shown in **Figure 1A**. We majorly focused on the significant differential expressed genes (DEGs) which were expressed higher in metastatic bone tumors than in metastatic lung tumors and parental cells, because those genes were more likely to contribute to bone metastasis of Lewis lung carcinoma. Consistently, MMPs with high expression in metastatic bone tumors based on our RNA-Seq data had been reported to play roles in prostate or breast cancer bone metastasis (Larson et al., 2013; Colden et al., 2017; Ganguly et al., 2020; Zhang et al., 2020). *Pnma5* was another gene that was expressed higher in metastatic bone tumors than in metastatic lung tumors and parental cells (**Figure 1A**). Although, functions of *Pnma5* in cancer progression had been partially revealed in colon cancer and breast cancer, the roles of *Pnma5* playing in NSCLC bone metastases were still unclear (Lee et al., 2016). Thus, we went further to research whether *Pnma5* promoted NSCLC bone metastases. We examined the mRNA levels of *Pnma5* in four metastatic bone tumors, one metastatic lung tumor and parental cells by quantitative real-time PCR. In consistence with the RNA-seq data, the expression of *Pnma5* was also higher in bone metastases than in lung metastases and parental cells (**Figure 1B**). Besides, the protein levels of *Pnma5* were also increased in metastatic bone tumors of Lewis lung carcinoma in contrast with metastatic lung tumors (**Figure 1C**).

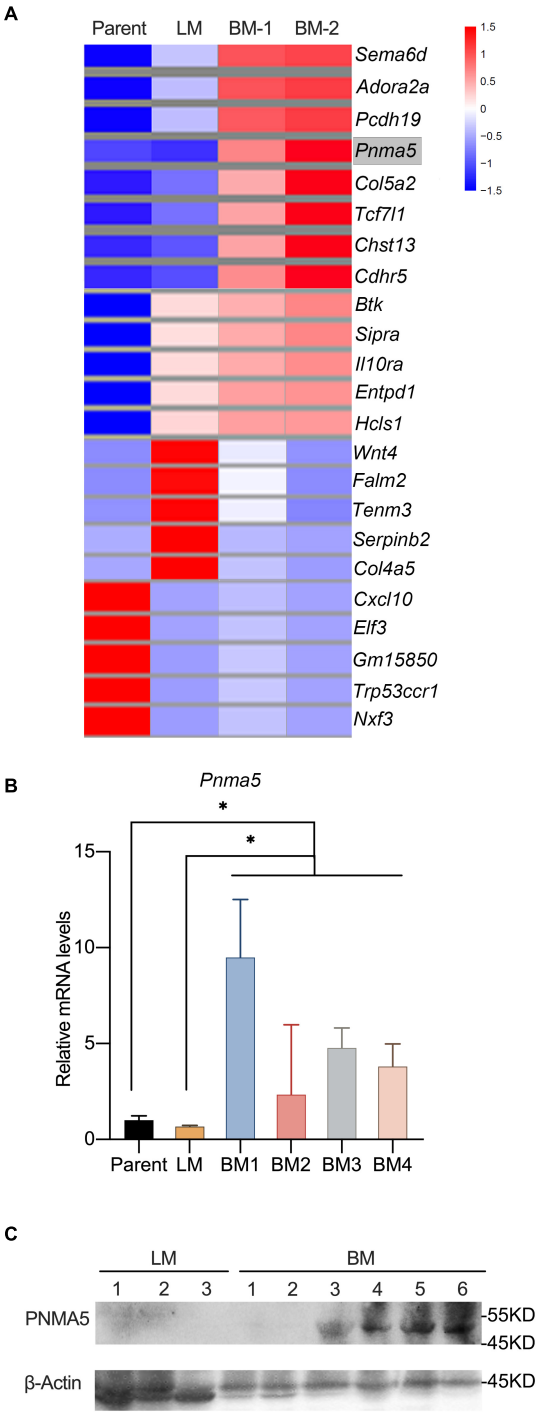

FIGURE 1 | *Pnma5* is highly expressed in bone metastasis of Lewis lung carcinoma. **(A)** Heatmap with mRNA-based expression of representative genes in bone metastasis (BM-1 and BM-2), lung metastasis (LM) and parental Lewis lung cells (Parent). The gene names were shown on the right of the heatmap. Values are normalized intensities, log2. **(B)** Comparison of relative *Pnma5* mRNA levels of bone metastasis (BM), lung metastasis (LM) and parental Lewis lung cells (Parent) by qPCR. *$P < 0.05$. **(C)** Lysates of the indicated tissues were harvested to be subjected to western blot for PNMA5. β-Actin was the reference for the blots. LM, lung metastasis; BM, bone metastasis.

BMP2 Signaling Activation Induced the Expression of *Pnma5*

In our previous report, BMP signaling was activated in bone metastatic tumors of NSCLC and BMP2 enhanced bone metastases of NSCLC (Huang et al., 2020). Thus, we proposed the hypothesis that BMP2 signaling activation could induce the expression of *Pnma5* in NSCLC cells. Interestingly, we found that BMP2 treatment could induce the upregulation of *Pnma5* mRNAs in both mice NSCLC cells LLCs and human NSCLC cells A549 (**Figures 2A,B**). Moreover, the protein levels of PNMA5 were increased when mice NSCLC cells LLCs and human NSCLC cells A549 were under BMP2 treatment (**Figures 2C,D**).

Pnma5 Was the Direct Target of BMP2 Signaling

When BMP2 binds to its receptor BMPRII and BMPRI, Smad1/5/8 can be phosphorylated by BMPRI and then translocated into nucleus with Smad4 to regulate the expression of downstream target genes (Miyazono et al., 2005; Wu et al., 2016). The Smad1/5/8 usually binds to the motif "CAGAC" or "GGCGCC" (Smads binding element sequences, SBEs) in the promoter of its target genes. Therefore, we analyzed the PNMA5 promoter sequences to find the "CAGAC" or "GGCGCC" motifs. We found several SBEs in the −3 kb∼ + 1 bp region of mice and human PNMA5 (**Figure 3A**). Interestingly, one of the SBEs was highly conserved in human, mice and rats (**Figure 3B**). To make sure whether Smad1/5/8 could bind this SBE of *Pnma5* promoter, we further conducted the ChIP-qPCR assay in LLC and A549 cells with the Smad1/5 antibody. Primers were designed on the conserved SBE region along the *Pnma5* promoter (**Figure 3A**). As the amplicon covered the conserved SBE in the promoter of *Pnma5*, we showed that BMP2 signaling activation could lead to the binding of Smad1/5 to SBE in LLC and A549 cells (**Figures 3C,D**). Thus, these data demonstrated that Smad1/5 bound to the SBE of *Pnma5* promoter to contribute to the upregulation of *Pnma5* when BMP2 signaling was activated. *Pnma5* was a direct target gene of BMP2 signaling.

BMP2 Signaling Induced the Migration and Invasion of NSCLC Cells via PNMA5

In our previous report, BMP2 signaling activation enhanced NSCLC cells migration and invasion (Huang et al., 2020). And PNMA5 was confirmed to be a direct target of BMP2 signaling in NSCLC cells based on results above. Thus, we further examined the role of PNMA5 played in migration and invasion of NSCLC cells by the transwell assay. We overexpressed PNMA5 in LLC cells and A549 cells (**Supplementary Figures 1A,C**). Interestingly, PNMA5 overexpression enhanced migration and invasion of LLC cells and A549 cells (**Figures 4A–D**). We further focused on whether BMP2 signaling promoted migration and invasion of NSCLC cells via PNMA5. The expression of *Pnma5*

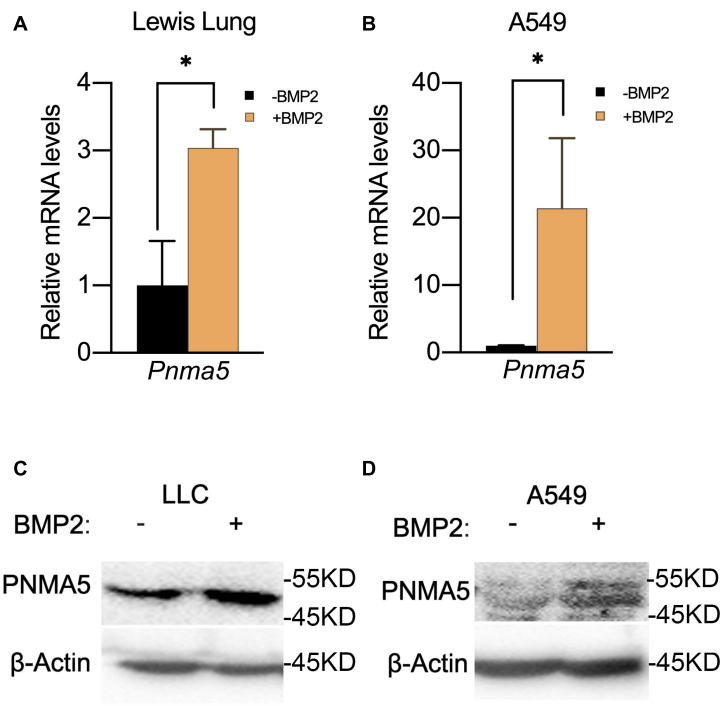

FIGURE 2 | BMP2 signaling activation induced the expression of PNMA5. **(A)** Comparison of relative *Pnma5* mRNA levels of LLC cells with or without BMP2 treatment by qPCR. *$P < 0.05$. **(B)** Comparison of relative *Pnma5* mRNA levels of A549 cells with or without BMP2 treatment by qPCR. *$P < 0.05$. **(C)** LLC cells treated with or without BMP2 for 1 h. Lysates of the indicated treated LLC cells were harvested to be subjected to western blot for PNMA5. β-Actin was the reference for all the blots. **(D)** A549 cells treated with or without BMP2 for 1 h. Lysates of the indicated treated A549 cells were harvested to be subjected to western blot for PNMA5. β-Actin was the reference for all the blots.

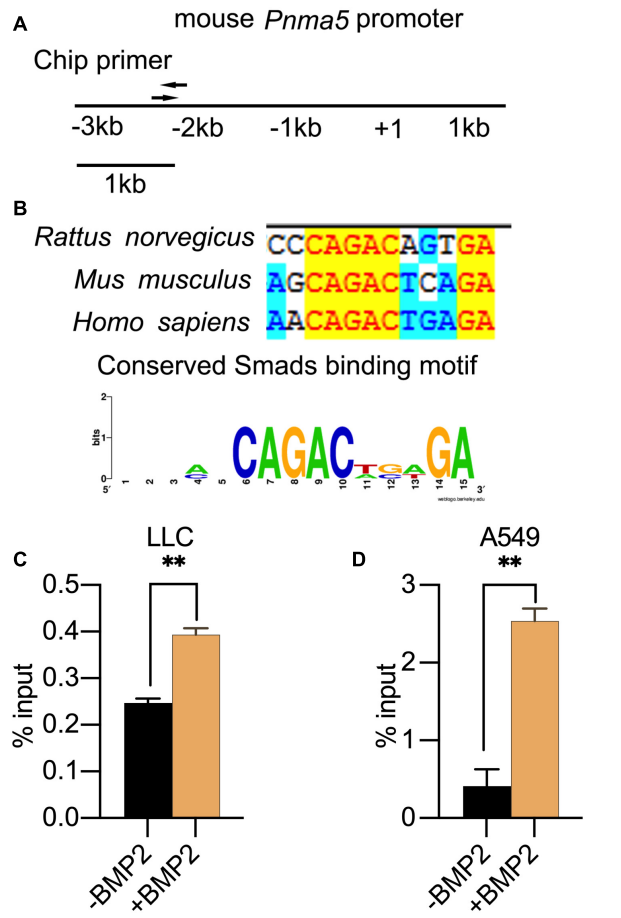

FIGURE 3 | PNMA5 was the direct target of BMP2 signaling. **(A)** The DNA sites where the ChIP primers were designed at the PNMA5 promoter. **(B)** The "CAGAC" element in the PNMA5 promote is conserved across species. The "CAGAC" Smad1/5 binding motif across species was analyzed by WebLogo. **(C)** ChIP assay was performed in LLC cells treated with or without BMP2 for 6 h using the Smad1/5 antibodies. The immunoprecipitated DNA was amplified by quantitative real-time PCR with the primers designed in **(A)** $^{**}P < 0.01$. **(D)** ChIP assay was performed in A549 cells treated with or without BMP2 for 6 h using Smad1/5 antibodies. The immunoprecipitated DNA was amplified by quantitative real-time PCR with the primers designed in **(A)** $^{**}P < 0.01$.

was knocked down in LLC cells (**Supplementary Figure 1B**). We found that BMP2 could no longer induce the migration and invasion of LLC cells with low expression of *Pnma5*, indicating that BMP2 signaling enhanced LLC cells' migration and invasion via *Pnma5* (**Figures 4E,F**).

BMP2 Signaling Mediated NSCLC-Induced Osteoclasts Differentiation via PNMA5

Bone metastases can be classified into osteolytic, osteoblastic or mixed subtypes based on the effect of cancers on normal bone remodeling (Selvaggi and Scagliotti, 2005; Quayle et al., 2015). The osteolytic mechanism has been reported to be associated with NSCLC bone metastases (Nakamura et al., 2006; Kuo et al., 2013). In osteolytic metastases, osteoclasts played an important role in the remodeling of bones (Mundy, 2002; Li et al., 2019; Brunetti et al., 2020). Interestingly, in our previous reports, BMP2 had been shown to stimulate NSCLC-induced osteoclast differentiation from macrophages (Huang et al., 2020). Thus, we went further to examined whether BMP2 signaling mediated NSCLC-induced osteoclasts differentiation via PNMA5. As shown in **Figures 5A,B**, overexpression of PNMA5 in NSCLC cells promoted the tumor-induced osteoclast differentiation of macrophages. Furthermore, if *Pnma5* was knocked down in LLC cells, BMP2 could not mediate NSCLC-induced osteoclast differentiation from macrophages (**Figure 5C**). The results above indicated that BMP2 signaling mediated NSCLC-induced osteoclasts differentiation via PNMA5.

BMP2 Signaling Enhanced Bone Metastases of Lewis Lung Carcinoma via PNMA5 *in vivo*

Cell migration, invasion and osteolysis are all key factors in bone metastases of carcinoma (Labelle et al., 2011; Macedo et al., 2017). According to the results above, PNMA5 enhanced the migration and invasion of NSCLC cells as a target gene of BMP2 signaling. It promoted NSCLC-induced osteoclasts differentiation via PNMA5 as well. Thus, we further focused on whether BMP2 signaling promoted NSCLC bone metastases via PNMA5 *in vivo*. LLCs were injected into the left lung lobes of C57BL/6 mice. We found that LLC cells could localize in the left lungs to form the primary tumors, but also the left shoulders to form bone metastatic lesions (**Figure 6A**). We transfected LLC cells with the empty vector or the PNMA5-carried vector to establish the stable empty vector or PNMA5 expressed LLC cell lines. After that, the EV overexpressed or PNMA5 overexpressed LLC cells were injected into the left lung lobes of C57BL/6 mice. We found that the EV overexpressed or PNMA5 overexpressed LLC cells could both localize in the left lungs and left shoulders of 57BL/6 mice (**Figure 6B**). There was no difference between the average volumes of primary tumors in left lungs of the EV group and the PNMA5 group (**Figure 6C**). However, the average volumes of bone metastatic tumors of the PNMA5 group were much larger than that of the EV group (**Figure 6D**). Besides, the metastatic lesions formed in the PNMA5 group showed a more invasive phenotype in contrast with the EV group (**Figure 6B**), as the bone destruction occurred in the PNMA5 group but not the EV group. Furthermore, mice in the PNMA5 group survived shorter than mice in the EV group (**Figure 6E**). Those results above indicated that PNMA5 promoted bone metastases of LLC cells *in vivo*. In our previous reports, BMP2 was shown to enhance the bone metastases of LLC cells *in vivo* directly (Huang et al., 2020). LLC cells were pre-treated with the vehicle or 20 ng/mL BMP2 for 24 h. After that, the pre-treated LLCs were injected into the left lung lobes of C57BL/6 mice. We found that BMP2 treated LLC cells could form larger bone metastatic lesions than the vehicle treated LLC cells (**Figure 6F**). However, if *Pnma5* was knocked down in LLC cells, BMP2 could no longer enhance the formation of bone

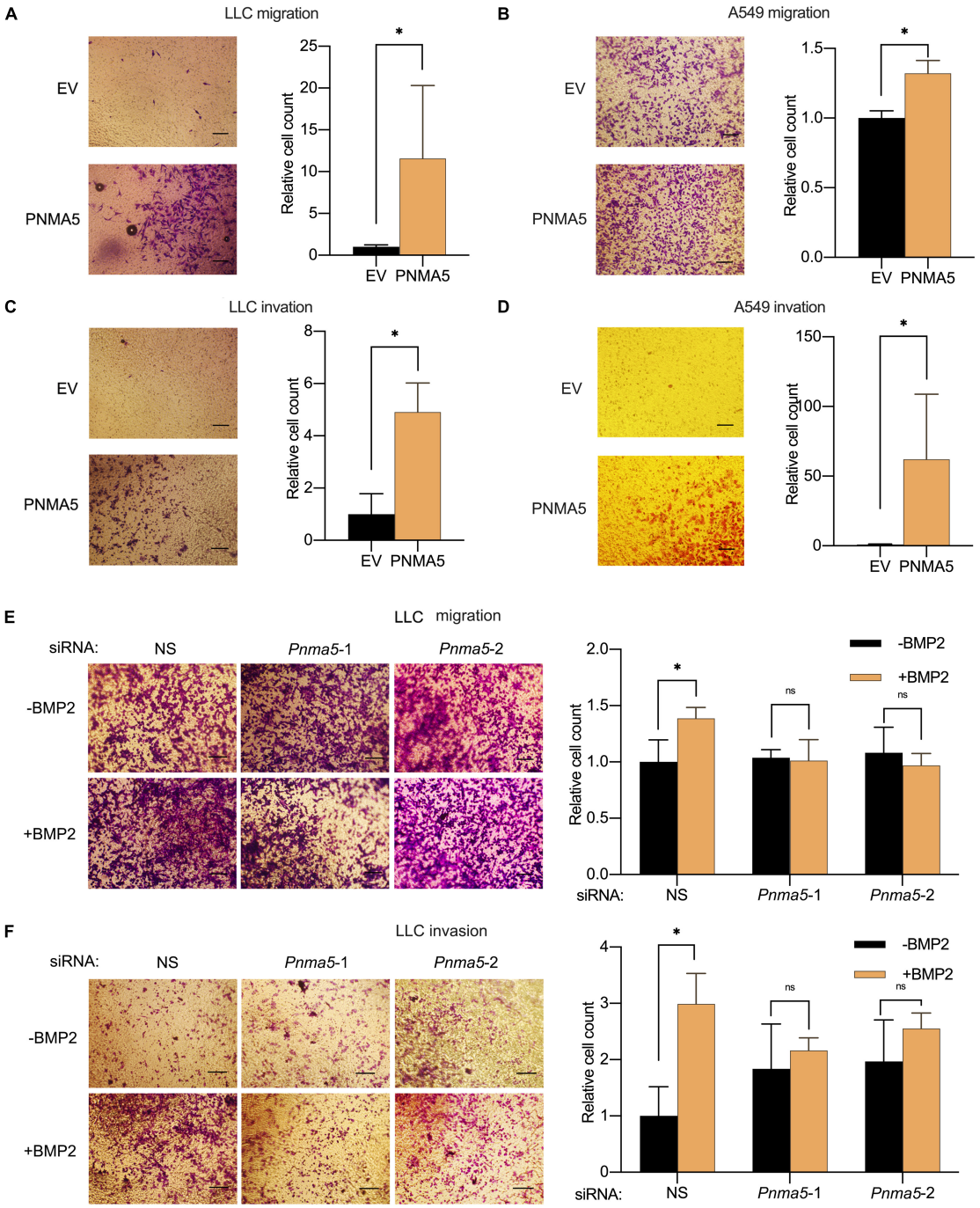

FIGURE 4 | BMP2 signaling induced the migration and invasion of NSCLC cells via PNMA5. **(A,B)** The 1×10^4 empty vector (EV) and PNMA5 overexpressed LLC **(A)** or A549 **(B)** cells were seeded and cultured in media without FBS on the upper layer of the Corning cell culture insert with polycarbonate membrane (Transwell@, 8.0μm pore size) for 24 h. The complete culture media were placed below the cell permeable membrane in the well plate. The migrating cells were stained with crystal violet and were taken photos. Average cell numbers of at least three fields were counted and shown on the right. *$P < 0.05$. **(C,D)** Corning cell culture insert with polycarbonate membrane (Transwell@, 8.0μm pore size) were pre-treated with 10:1 DMEM and matrigels (BD BioSciences). The 1×10^5 empty vector (EV) and PNMA5 overexpressed LLC **(C)** or A549 **(D)** cells were seeded and cultured in media without FBS on the upper layer of the pre-treated cell culture insert for 24 h. The complete culture media were placed below the cell permeable membrane in the well plate. The invading cells were stained with crystal violet and were taken photos. Average cell numbers of at least three fields were counted and shown on the right. *$P < 0.05$. **(E)** The 1×10^4 siRNA scrambler, siRNA *Pnma5*-1 and siRNA *Pnma5*-2 expressed LLC cells were treated as described in **(A)**. The migrating cells were stained with crystal violet and were taken photos. Average cell numbers of at least three fields were counted and shown on the right. *$P < 0.05$. **(F)** The 1×10^5 siRNA scrambler, siRNA *Pnma5*-1 and siRNA *Pnma5*-2 expressed LLC cells were treated as described in **(C)**. The invading cells were stained with crystal violet and were taken photos. Average cell numbers of at least three fields were counted and shown on the right. *$P < 0.05$.

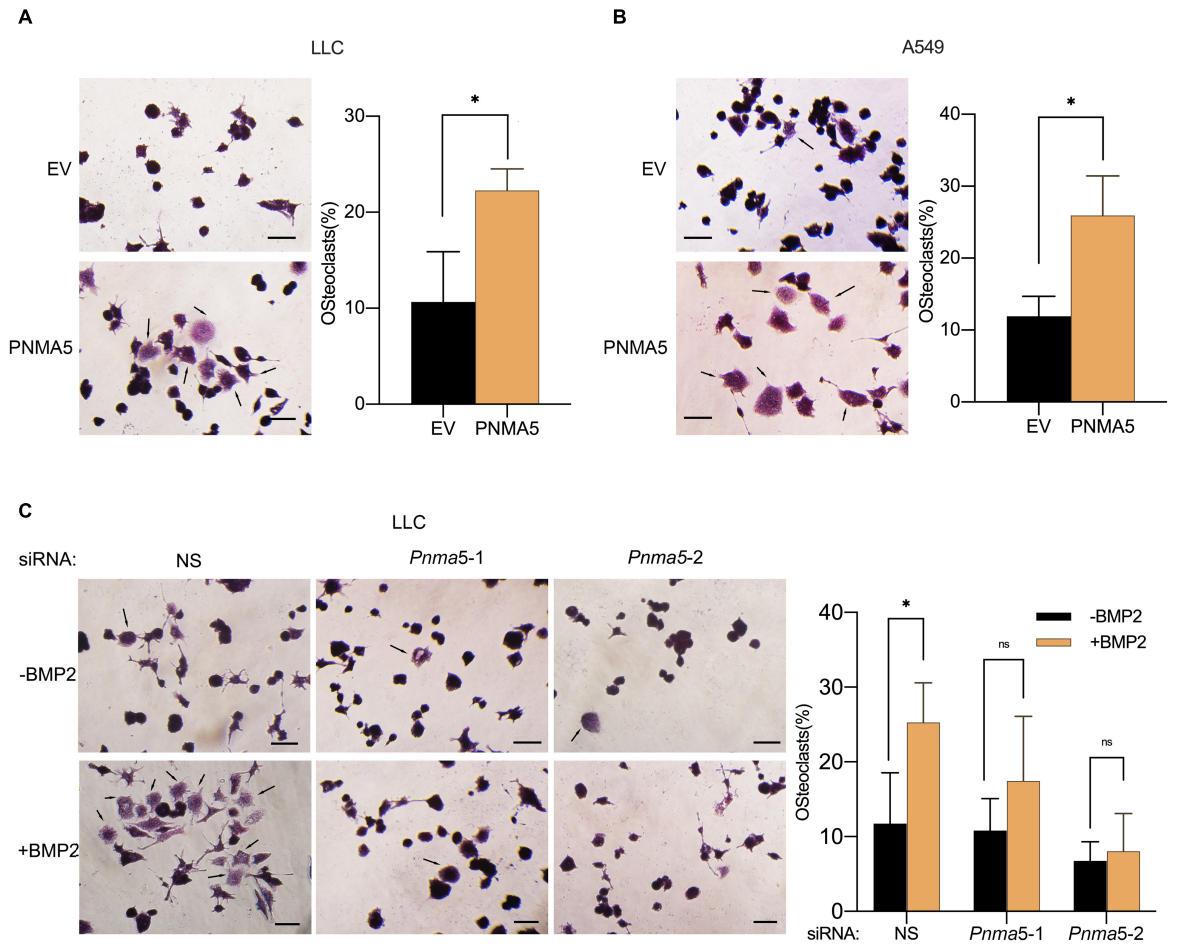

FIGURE 5 | BMP2 signaling enhanced NSCLC cells-induced osteoclasts differentiation via PNMA5. **(A,B)** 3×10^4 empty vector (EV) and PNMA5 overexpressed LLC **(A)** or A549 **(B)** cells were seeded into the Corning Cell Culture Inserts with polycarbonate membrane (Transwell@, 0.4 μm pore size) and 3×10^4 RAW 264.7 cells were seeded below the polycarbonate membrane into the wells of the 6-well co-culture plates (Corning). TRAP staining was conducted for RAW 264.7 cells cultured for 6 days by a leukocyte acid phosphatase kit. Representative photos were shown. Scale bars, 50μM. Average $Trap^+$ cell numbers of at least three fields were calculated and shown on the right. Black arrows showed the osteoclasts. The *p*-value was based on the Student's *t*-test. $^*P < 0.05$. **(C)** 3×10^4 siRNA scrambler, siRNA *Pnma5*-1 and siRNA *Pnma5*-2 expressed LLC cells were seeded into the Corning Cell Culture Inserts with polycarbonate membrane (Transwell@, 0.4μm pore size) and 3×10^4 RAW 264.7 cells were seeded directly into the wells of the 6-well co-culture plates (Corning). TRAP staining was conducted for RAW 264.7 cells cultured for 6 days by a leukocyte acid phosphatase kit. Representative photos were shown. Scale bars, 50μM. Average $Trap^+$ cell numbers of at least three fields were calculated and shown on the right. Black arrows showed the osteoclast. The *p*-value was based on the Student's *t*-test. $^*P < 0.05$.

metastatic lesions of LLC cells (**Figure 6F**). The same results also could be found when the survival curve of the mice was analyzed. BMP2 treated LLC cells injected mice could survive shorter than the vehicle treated LLC cells injected mice. However, if *Pnma5* was knocked down in BMP2 treated LLC cells, the mice could survive as long as the vehicle treated LLC cells injected mice (**Figure 6G**). Altogether, BMP2 signaling was observed to enhance bone metastases of Lewis lung carcinoma via PNMA5 *in vivo*.

DISCUSSION

Bone metastases frequently occur in NSCLC, resulting in poor prognosis (Kuchuk et al., 2013; Santini et al., 2015; Oliveira et al., 2016). As the destruction of bones mediated by osteoclasts plays key roles in the formation of bone metastatic lesions, denosumab targeting RANKL (the important factor for osteoclasts differentiation) has been used to treat patients with bone metastases in clinics (D'Antonio et al., 2014; De Castro et al., 2015; Nasser et al., 2019). However, the therapy efficacy is limited. Thus, research on mechanisms about how NSCLC bone metastases occur has been a hot spot in recent years. LncRNA MALAT1, miRNA-33, CXCR4, and TGF-β signaling have all been reported to contribute to bone metastases of NSCLC (Liu et al., 2016; Popper, 2016; Liao et al., 2018; Yang et al., 2019). Interestingly, in our present work, we firstly report that PNMA5 enhances NSCLC bone metastases as a target gene of BMP2 signaling. PNMA5 maybe a new potential therapeutic target for NSCLC bone metastases treatment.

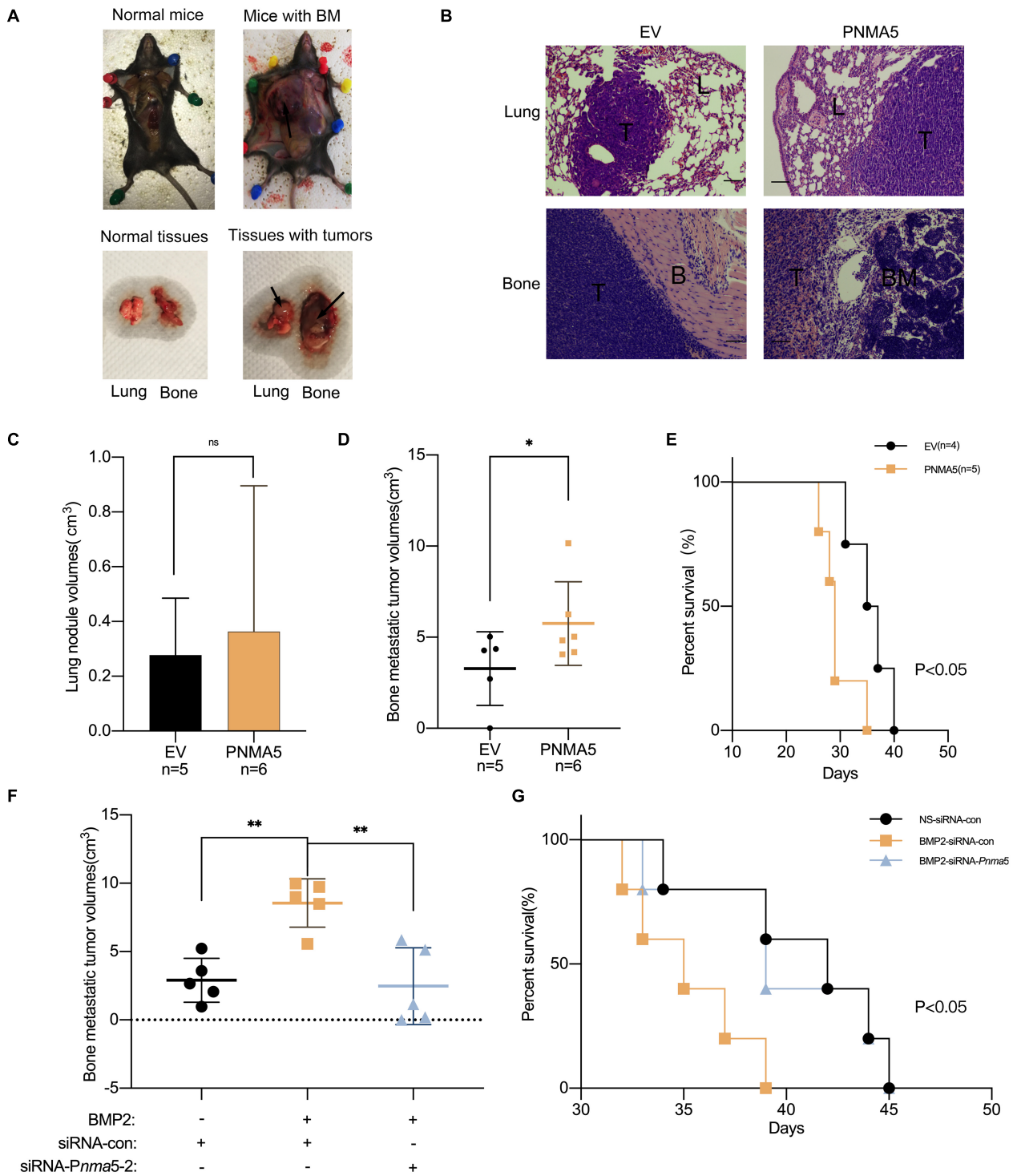

FIGURE 6 | BMP2 signaling enhanced bone metastasis of Lewis lung carcinoma via PNMA5 *in vivo*. **(A)** 1×10^6 empty vector (EV) and PNMA5 overexpressed LLC cells were injected into the left lung lobes of C57BL/6 mice. Graphs of tumor-burdened mice were shown. Black arrows showed the bone metastatic lesions. Normal and tumor burdened lung and bone tissues were shown. Black arrows showed the metastatic lesions. BM: metastatic bone tumors. **(B)** Representative HE staining of tissues from lungs and bones in **(A)**. Scale bars of the 100× photos were 100 μM. L, normal lung tissues; T, tumor tissues; B, normal bone tissues; BM, normal bone marrow tissues. **(C)** Tumor sizes of mice lungs in **(A)** were measured and tumor volumes were calculated. **(D)** Tumor sizes of mice bones in **(A)** were measured and tumor volumes were calculated. $^*P < 0.05$. **(E)** Death of mice in **(A)** were recorded and survival curves were drawn. $^*P < 0.05$. **(F)** 1×10^6 siRNA scrambler, siRNA *Pnma5*-1 and siRNA *Pnma5*-2 expressed LLC cells were injected into the left lung lobes of C57BL/6 mice. Tumor sizes of mice bones in **(A)** were measured and tumor volumes were calculated. $^{**}P < 0.01$. **(G)** Death of mice in **(F)** were recorded and survival curves were drawn. $^*P < 0.05$.

PNMA5 belongs to the PNMA protein family. The aberrant protein expression level and mutations of PNMA family members are associated with the human Paraneoplastic Disorder (PND). PND patients usually exhibit syndrome consisting of auto-immunity, neuro-degeneration, and cancer (Pang et al., 2018). High-throughput sequencing analysis reveals that PNMA5 may be associated with metastasis of colon cancer (Zhou et al., 2019). Moreover, PNMA5 has been reported to promote apoptosis in human cancers (Lee et al., 2016). However, there is still no direct evidence to show that PNMA5 contributes to cancer metastases. We have found that overexpression of PNMA5 can enhances migration and invasion of NSCLC cells but inhibits proliferation of NSCLC cells *in vitro* (**Supplementary Figures 2A,B**). Besides, high expression of PNMA5 enhances tumor-induced osteoclasts differentiation *in vitro* and promotes bone metastatic lesions formation *in vivo*. Our research provides direct evidence to show that PNMA5 contributes to NSCLC bone metastases rather than NSCLC cells growth. Moreover, we still observed a phenomenon, in which the migration and invasive ability of LLC cells seemed to be slightly increased but not significantly, when *Pnma5* was knocked down in LLC cells without BMP2 treatment. However, overexpression of PNMA5 could enhance the migration and invasion of LLCs. There are two potential reasons for this phenomenon. On one hand, when the expression of PNMA5 is at relative low levels, its effect on the migration and invasion of LLCs is not significant. On the other hand, PNMA5 could inhibit the proliferation of LLCs, thus knock-down of PNMA5 may increase the proliferation of LLCs which lead to the enhancement of migration and invasion of cells that we observed.

Osteoclastgenesis plays dominant roles in carcinoma bone metastases. When osteoclast activity is increased and osteoblast activity is decreased, bone resorption occurs to provide microenvironment for tumor colonization (Mathis et al., 2018). Cancer cells frequently regulate the osteoclast differentiation when bone metastases occur through secreting the key factors for osteoclastgenesis, like RANKL and PTHrP (Fornetti et al., 2018). Thus, cancer cells have been found to affect the osteoclast differentiation by some mechanisms. Previous experimental research has reported that low expression of SOSTDC1 in NSCLC cells promoted cancer cells-induced osteoclast differentiation (Chen et al., 2018). The gene expression changes of NSCLC cells can mediate the secreting factors of NSCLC cells, which subsequently regulate the osteoclast differentiation. High expression of CXCR4 in NSCLC cells have been shown to promote cancer cells-mediated osteoclast differentiation through secreting VCAM1 (Liao et al., 2018). Moreover, C5aR1 can also enhance NSCLC cells to induce osteoclast differentiation through secreting CXCL16 (Ajona et al., 2018). In recent years, exosomes, which can mediate the osteoclast differentiation, have also been found to be secreted by NSCLC cells (Taverna et al., 2017). In our research, we verified that BMP2 signaling activation could promotes LLC cells-induced osteoclast differentiation, and this effect could be blocked by knockdown of *Pnma5*. PNMA5 can be the downstream target of BMP2 signaling in mediating tumor associated osteoclasgenesis. Nevertheless, the downstream cytokines or exosomes of PNMA5 in NSCLC cells, which affect osteoclastgenesis still remains unclear, which needs further research.

In our previous work, we reported that BMP2 signaling activation could enhance NSCLC bone metastases. In the current research, we initially demonstrate that *Pnma5* is the downstream target gene of BMP2 signaling to enhance NSCLC bone metastases. There is no previous report showing the upstream signaling pathway that regulates the expression of PNMA5 until now. Thus, our work finds a new potential mechanism about how BMP2 signaling functions in regulating cancer metastases. However, the associated proteins of PNMA5 in regulating the NSCLC bone metastases still remain unknown, which needs further research in the future.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethical Committee of Fujian Medical University (2018-039).

AUTHOR CONTRIBUTIONS

FH: conceptualization, data curation, formal analysis, funding acquisition, roles, and writing–original draft. GW: data curation, formal analysis, and writing–review and editing. YC: conceptualization, data curation, and formal analysis. CW, RL, BW, XX, JH, and LF: investigation. 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.2021.678931/full#supplementary-material

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Comprehensive Analysis of RUNX and TGF-β Mediated Regulation of Immune Cell Infiltration in Breast Cancer

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Runt-related transcription factors (RUNXs) can serve as both transcription activators and repressors during biological development, including immune cell maturation. RUNX factors have both tumor-promoting and tumor-suppressive roles in carcinogenesis. Immune cell infiltration and the tumor immune microenvironment have been found to be key regulators in breast cancer progression, treatment response, and patient outcome. However, the relationship between the RUNX family and immune cell infiltration in breast cancer remains unclear. We performed a comprehensive analysis to reveal the role of RUNX factors in breast cancer. Analysis of patient data in the Oncomine database showed that the transcriptional levels of RUNX proteins in breast cancer were elevated. Kaplan–Meier plotter (KM plotter) analysis showed that breast cancer patients with higher expression of RUNX proteins had better survival outcomes. Through analysis of the UALCAN database, we found that the transcriptional levels of RUNX factors were significantly correlated with some breast cancer patient characteristics. cBio Cancer Genomics Portal (cBioPortal) analysis showed the proportions of different RUNX genomic alterations in various subclasses of breast cancer. We also performed gene ontology (GO) and pathway analyses for the significantly differentially expressed genes that were correlated with RUNX factors in breast cancer. TIMER database analysis showed that immune cell infiltration in breast cancer could be affected by the transcriptional level, mutation, and gene copy number of RUNX proteins. Using the Gene Set Cancer Analysis (GSCA) database, we analyzed the effects of RUNX gene methylation on the level of immune cell infiltration in breast cancer. We found that the methylation level changes of RUNX2 and RUNX3 had opposite effects on immune cell infiltration in breast cancer. We also analyzed the relationship between the methylation level of RUNX genes and the TGF-β signaling pathway using the TISIDB database. The results showed that the methylation levels of RUNX1 and RUNX3 were correlated with the expression of TGF-β1. In summary, our analysis found that the RUNX family members can influence the infiltration of various immune cells in breast cancer depending on their expression level, mutation, gene copy number, and methylation. The RUNX family is an important regulator of immune cell infiltration in breast cancer and may serve as a potential prognostic biomarker.

Keywords: RUNX, transcription factor, breast cancer, methylation, immune cell infiltration, TGF-β

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INTRODUCTION

Breast cancer is the most common cancer type among women and is one of the leading causes of cancer-related deaths worldwide (Siegel et al., 2021). With improvements in diagnostic techniques and precise treatments, the overall prognosis of breast cancer patients has substantially improved in recent years. However, not all patients respond favorably to current therapy, and relapse is common. This is due to the complex nature of breast cancer pathogenesis, development, and metastasis. Currently, the role of tumor-infiltrating immune cells in breast cancers and their effects on tumor progression and immunotherapy are gaining attention (Azizi et al., 2018). Clinical data-based analysis has indicated a significant impact of tumor-infiltrating immune cells on the clinical outcome of breast cancer patients, including treatment response, recurrence, and death (Adams et al., 2014; Ali et al., 2014; Loi et al., 2014). However, the regulatory mechanisms of immune cell infiltration in breast cancer are not fully understood. It is necessary to characterize the immune microenvironment of breast cancer, develop biomarkers to facilitate precise patient stratification, and provide potential therapeutic molecular targets to modulate the breast cancer microenvironment.

The Runt-related transcription factor (RUNX) family consists of three members in mammals: RUNX1, RUNX2, and RUNX3. All three types of RUNX factors are expressed in the mammary gland, and play a regulatory role in physiological and pathological states. RUNX1 is expressed in all subpopulations of murine mammary epithelial cells, with the exception of secretory alveolar luminal cells (Van Bragt et al., 2014). RUNX2 is expressed not only in embryonic mammary glands but also in adult luminal and basal cell lineages (Owens et al., 2014). With RUNX3, inactivation and protein mislocalization occur during the early stages of breast cancer progression (Subramaniam et al., 2009). The RUNX family is of critical importance in developmental processes and tumorigenesis (Blyth et al., 2005). RUNX factors serve as activators or repressors during developmental processes (Shin et al., 2021). Likewise, the role of RUNX factors in cancer biology has also been found to be two-sided. The RUNX family has been reported to exert both tumor-suppressive and tumor-promoting roles in breast cancer. For example, RUNX1 has been shown to regulate the estrogen receptor-positive luminal lineage, and RUNX1 mutations may present as an additional genetic predisposition in breast cancer development (Van Bragt et al., 2014). RUNX2 has been shown to have an antagonistic role in estradiol-induced breast cancer proliferation (Chimge et al., 2012). RUNX3 destabilizes estrogen receptor alpha and suppresses its transcriptional activation, thus supporting its tumor-suppressive role in breast cancer (Huang B. et al., 2012). In contrast, RUNX2 has also been demonstrated to promote tumorigenesis in breast cancer. RUNX2 deletion prolongs overall survival (OS) in mice with breast cancer (Owens et al., 2014). Moreover, RUNX2 contributes to the bone metastasis of breast cancer in an integrin-dependent pathway (Li X.Q. et al., 2016).

Given the complex roles of RUNX family members in breast cancer, a comprehensive analysis is needed to reveal the relationship between these transcription factors and breast cancer. In this study, we analyzed the transcriptional changes in RUNX family members, promoter methylation level, RUNX gene alteration, and their relationship to immune cell infiltration as well as breast cancer patient prognosis. Our study indicates the important role of RUNX family members in breast cancer pathophysiology and may provide potential biomarkers or therapeutic targets to facilitate early breast cancer diagnosis and treatment.

MATERIALS AND METHODS

Oncomine

The Oncomine database was used to compare the expression levels of the three RUNX members across a variety of cancer types. The *p*-value was set as 1E−4, and the fold change was set as 2.

Gene Expression Profiling Interactive Analysis

The RUNX expression profile was analyzed using the Gene Expression Profiling Interactive Analysis (GEPIA) tool, which was developed based on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases (Tang et al., 2017). The RUNX expression profiles were compared between different breast cancer stages.

UALCAN

The UALCAN portal (Chandrashekar et al., 2017) was used to analyze how the clinical characteristics of breast cancer patients were related to RUNX expression profiles and RUNX promoter methylation status. The pan-cancer analysis of RUNX factor expression was also carried out using the UALCAN web.

Kaplan–Meier Plotter

The relationship between RUNX expression levels and prognosis of breast cancer patients, was analyzed using the Kaplan–Meier plotter (KM plotter; Gyorffy et al., 2010). A total of 2,032 patients were analyzed, and they were split according to the median expression level of RUNX transcription factors. The two groups were compared to determine OS, distant metastasis-free survival (DMFS), relapse-free survival (RFS), and progression-free survival (PFS).

cBio Cancer Genomics Portal

The alterations of RUNX members in breast cancer subtypes were analyzed using the Breast Invasive Carcinoma (TCGA, PanCancer Atlas) dataset, which includes information from 1,084 samples. The alteration types include mutation, fusion, amplification, deep deletion, and multiple alterations.

LinkedOmics Analysis

LinkedOmics is a multi-omics database containing information on 32 cancer types from TCGA (Vasaikar et al., 2018). The significantly differentially expressed genes that were correlated with RUNX members were analyzed using the TCGA_BRCA

cancer cohort (HiSeq RNA platform) in LinkedOmics. Kyoto Encyclopedia Genes and Genomes (KEGG) pathway analysis was performed using gene set enrichment analysis (GSEA). The genes were also classified using gene ontology (GO) according to: biological processes, cellular components, and molecular functions.

TIMER Analysis

The TIMER web server (Li B. et al., 2016; Li et al., 2017) was used to analyze the infiltration of six types of immune cells in breast cancer, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells. The gene module was used to evaluate the relationship between target gene expression and immune cell infiltration, whereas the mutation module was used to analyze the gene mutation with an abundance of immune infiltrates. The SCNA module was used to analyze the correlation between RUNX gene somatic copy number alterations and tumor infiltration levels.

Gene Set Cancer Analysis

The Gene Set Cancer Analysis (GSCA) database (Liu C.J. et al., 2018) was used to analyze the relationship between RUNX factor methylation levels and the infiltration of six immune cell types: B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in breast cancer.

TISIDB Analysis

TISIDB is an integrated web portal for the analysis of tumor-immune system interactions (Ru et al., 2019). The correlations between RUNX gene methylation and the expression of TGF-β1 and TGFBR1, two of the critical immunomodulators in breast cancer, were analyzed using TISIDB.

RESULTS

Pan-Cancer Analysis of RUNX Family Member Expression

To study the role of RUNX family members in cancer, we performed a pan-cancer analysis using Oncomine. Transcription levels of RUNX1, RUNX2, and RUNX3 were all increased in most types of cancers, including breast, esophageal, head and neck, and pancreatic cancer (**Figure 1A**). Expression levels of RUNX factors were then compared across TCGA tumors. RUNX1 and RUNX2 expression in breast cancer was relatively high, ranking second after acute myeloid leukemia (LAML) for all the analyzed tumor types, indicating their potential role in breast cancer (**Figure 1B**).

Transcriptional Levels of RUNX Factors in Breast Cancer

The expression of RUNX members in breast cancer cells was analyzed using GEPIA. We found that the expression level of RUNX1 was significantly associated with the stage of breast cancer ($p = 0.0001$), while there was no significant difference in RUNX2 and RUNX3 expression (**Figure 2**). To further dissect the relationship between RUNX expression levels and breast cancer patient characteristics, we performed UALCAN analysis. The transcriptional level of RUNX1 in breast cancer was found to be associated with the TP53 mutation status. Breast cancer patients with mutant TP53 had significantly lower RUNX1 levels than normal controls, whereas patients with non-mutant TP53 had significantly higher RUNX1 levels (**Figure 3A**). There was a decreasing trend in RUNX1 transcriptional levels from stage one to stage four in breast cancer patients (**Figure 3B**). There was also a significant difference in RUNX1 levels between different breast cancer subclasses, with luminal type having the highest level and triple-negative showing the lowest level (**Figure 3C**). Furthermore, RUNX1 transcriptional levels were associated with different histological types (**Figure 3D**). Similarly, we also used the UALCAN database to further analyze the expression levels of RUNX2 and RUNX3 in breast cancer. We found that RUNX2 expression significantly varied in different stages, subclasses, and histological subtypes of breast cancer (**Supplementary Figure 1**). RUNX3 was down-regulated in luminal subclass but up-regulated in triple-negative subclass (**Supplementary Figure 2**). To validate these findings, we compared the levels of RUNX transcripts in different breast cancer subtypes to those of normal controls (**Table 1**). RUNX transcriptional levels have been shown to be significantly increased in various subtypes of breast cancer.

Survival Analysis

To study the relationship between RUNX transcription factor expression levels and the outcomes of patients with breast cancer, we performed KM plotter analysis. The results showed that patients with higher RUNX levels had longer DMFS, OS, and RFS (**Figure 4**). Specifically, higher RUNX1 expression was associated with longer DMFS, OS, and RFS. Higher RUNX2 expression was associated with longer RFS. Higher RUNX3 expression was associated with longer OS and RFS. These results indicate that RUNX1 and RUNX3 may play a more important role in the OS and RFS of breast cancer patients, and RUNX2 only affects the RFS of breast cancer patients.

Analysis of RUNX Alterations in Breast Cancer

The alterations in RUNX members in breast cancer subtypes were analyzed using cBio Cancer Genomics Portal (cBioPortal). Alteration types include mutation, fusion, amplification, deep deletion, and multiple alterations. Interestingly, the three RUNX members seemed to be altered in different subtypes of breast cancer with varying tendencies. The subtypes with the highest frequency of all alternate types were breast invasive lobular carcinoma, breast invasive carcinoma NOS, and breast invasive ductal carcinoma for RUNX1, RUNX2, and RUNX3, respectively (**Figure 5A**). For RUNX1, mutation ranked first in all alternate types in breast invasive lobular carcinoma (8.02%), followed by breast invasive carcinoma NOS (4.11%), and breast invasive ductal carcinoma (3.10%). RUNX1 was mainly distributed in the Runt domain and the linker region (**Supplementary Figure 3**). For RUNX2, the alteration with the highest frequency was amplification, with a percentage of 2.74% in breast invasive carcinoma NOS and 1.62% in breast invasive

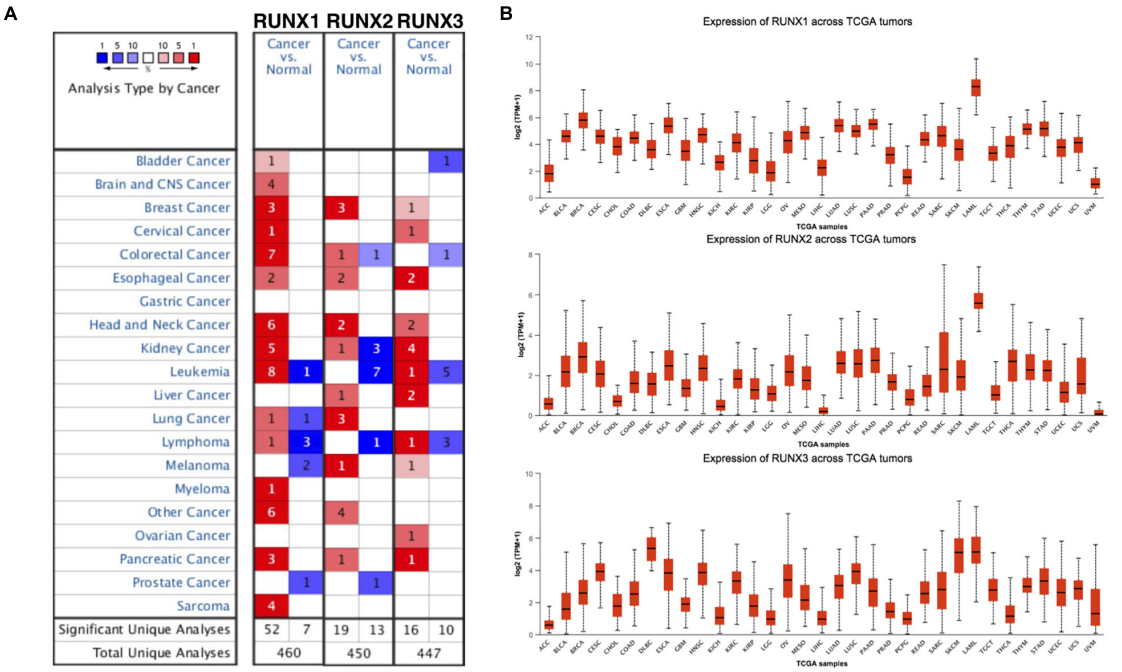

FIGURE 1 | The transcriptional levels of Runt-related transcription factors (RUNXs) in different types of cancers. **(A)** The transcriptional levels of RUNX1, RUNX2, and RUNX3 in various cancer tissues compared with normal tissues. **(B)** The transcriptional levels of RUNX1, RUNX2, and RUNX3 were compared across various The Cancer Genome Atlas (TCGA) tumors.

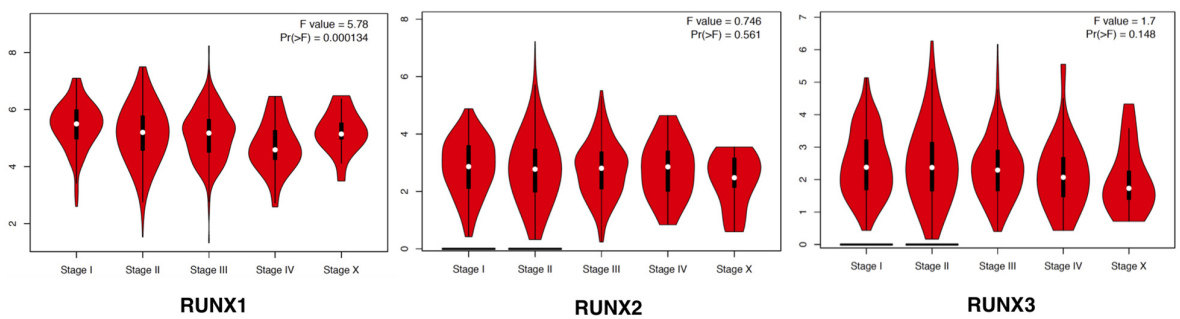

FIGURE 2 | Correlation between RUNX expression and tumor stage in breast cancer patients. The transcriptional levels of RUNX1, RUNX2, and RUNX3 in different stages of breast cancer were shown.

ductal carcinoma. For RUNX3, alterations were mainly found to be amplified in breast invasive ductal carcinoma (0.13%). RUNX1 showed the highest number of alterations among the three members (**Figure 5B**).

Analysis of Differentially Expressed Genes Correlated With RUNX Genes in Breast Cancer

We analyzed the differentially expressed genes that were correlated with RUNX1 in breast cancer using LinkedOmics (**Figure 6A**). The top 50 positively and 50 negatively correlated genes were visualized in heatmaps (**Figures 6B,C**). GSEA was performed to identify the enriched KEGG pathways for the top significantly differentially expressed genes (**Figure 6D**). Among them, ECM-receptor interaction and focal adhesion were significantly upregulated, whereas cell cycle, RNA transport, pyrimidine metabolism, and DNA replication pathways were significantly downregulated. These genes were also classified using GO (**Figure 6E**). The top three enriched biological process terms were biological regulation, metabolism, and responses to stimulus. The top three enriched cellular component terms were the membrane, nucleus, and membrane-enclosed lumen. The top three enriched molecular function terms were protein binding, ion binding, and nucleic acid binding. Similarly, differentially expressed genes correlated with RUNX2 (**Supplementary Figure 4**) and RUNX3 (**Supplementary Figure 5**) were also analyzed and enriched.

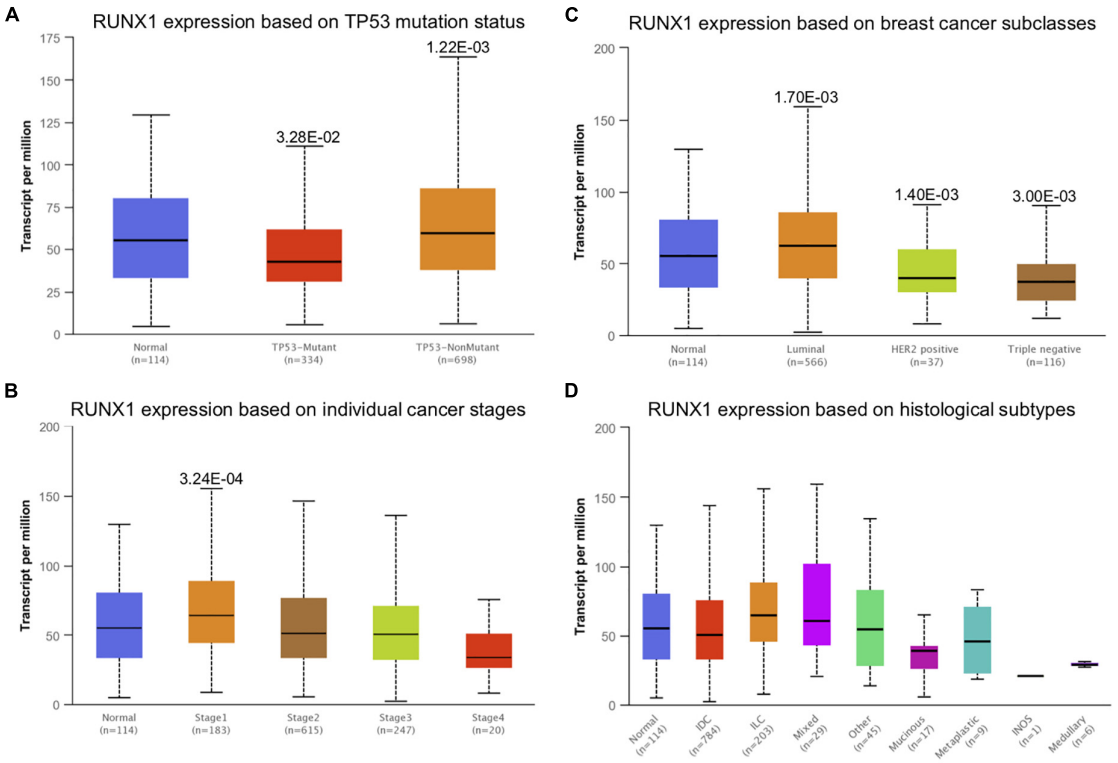

FIGURE 3 | Correlation analysis between RUNX1 expression and breast cancer patient characteristics. The RUNX1 expression levels in breast cancer patients classified by **(A)** TP53 mutation status, **(B)** individual cancer stages, **(C)** breast cancer subclasses, and **(D)** histological subtypes were shown.

TABLE 1 | The significant changes of Runt-related transcription factor (RUNX) transcriptional levels in different types of breast cancers vs. normal breast tissues (oncomine database).

	Type of breast cancer vs. normal breast tissue	Fold change	*p*-Value	*t* Test	Source and references
RUNX1	Invasive lobular breast carcinoma vs. normal	2.002	1.21E−11	7.745	TCGA
	Invasive breast carcinoma vs. normal	2.102	2.03E−17	9.804	TCGA
	Fibroadenoma vs. normal	2.494	0.016	4.036	Sorlie Breast 2 Statistics
	Fibroadenoma vs. normal	2.271	0.033	2.886	Sorlie Breast Statistics
	Invasive ductal breast carcinoma stroma vs. Normal	2.124	0.01	2.587	Karnoub Breast Statistics
RUNX2	Invasive breast carcinoma vs. normal	2.307	9.11E−14	8.192	TCGA
	Invasive lobular breast carcinoma vs. normal	2.053	3.62E−09	6.477	TCGA
	Invasive ductal breast carcinoma stroma vs. normal	4.617	1.54E−04	4.523	Karnoub Breast Statistics
	Invasive ductal breast carcinoma vs. normal	2.932	0.047	2.02	Radvanyi Breast Statistics
	Invasive mixed breast carcinoma vs. normal	3.08	0.044	2.014	Radvanyi Breast Statistics
RUNX3	Medullary breast carcinoma vs. normal	2.321	9.17E−08	6.396	Curtis Breast Statistics

TCGA, The Cancer Genome Atlas.

Runt-Related Transcription Factor 1 Promoter Methylation Level in Breast Cancer

Promoter methylation is an important regulator of gene expression. A previous study has shown that promoter methylation contributes to RUNX3 inactivation in breast cancer (Lau et al., 2006). We performed UALCAN analysis to evaluate RUNX1 promoter methylation levels and its relationship with breast cancer patient characteristics. Interestingly, we found that the RUNX1 promoter methylation level was significantly lower in breast cancer tissues than in normal tissues, regardless of stage, subclass, or histological type (**Figure 7**). This was consistent with the increased RUNX1 expression in breast cancer. Meanwhile, we also found that the RUNX2 promoter methylation level was significantly lower in breast cancer tissues than normal tissues, regardless of stage, subclass, or histological type (**Figure 8**). In contrast, RUNX3 promoter methylation level was significantly higher in breast cancer tissues than normal tissues, regardless of stage, subclass, or histological type

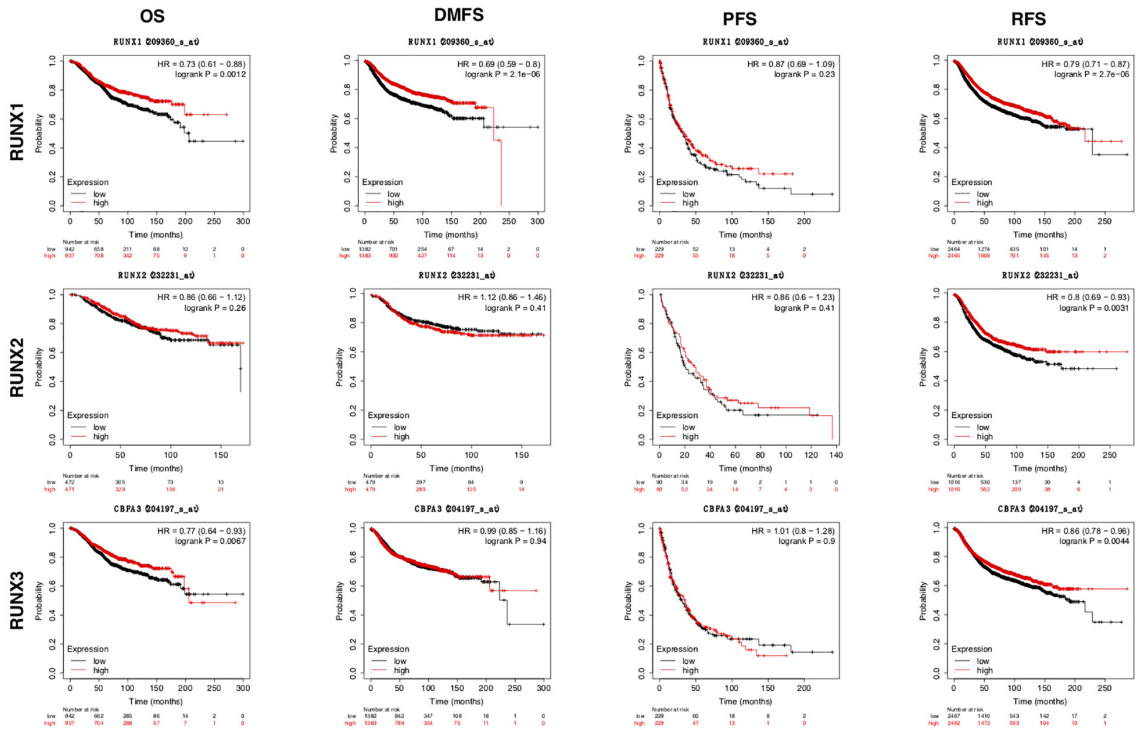

FIGURE 4 | The prognostic value of RUNX transcriptional level in breast cancer patients. The breast cancer patients were classified into two groups by the median expression levels of RUNX1, RUNX2, or RUNX3. The overall survival (OS), distant metastasis-free survival (DMFS), progression-free survival (PFS), and relapse-free survival (RFS) were compared between patients with high or low expression of RUNX transcription factors.

(**Figure 9**). Moreover, we used the MEXPRESS database to further verify the methylation level of the RUNX family in breast cancer. The results of the MEXPRESS database analysis were consistent with the UALCAN database analysis. These results indicate that different changes in the methylation levels of RUNX factors in breast cancer may account for the distinct effects of RUNX proteins on patient outcome.

Relationship Between the Expression Level of RUNX Factors and the Abundance of Immune Infiltrates in Breast Cancer

Runt-related transcription factors can regulate the development and maturation of immune cells, but the relationship between the expression levels of RUNX factors and immune cell infiltration in breast cancer is unclear. Therefore, we used the TIMER database to analyze the expression levels of RUNX factors and their relationship with the infiltration of six types of immune cells (B cells, $CD8^+$T cells, $CD4^+$T cells, macrophages, neutrophils, and dendritic cells) in breast cancer. The results indicated that the expression of RUNX1 was positively correlated with the infiltration of $CD8^+$T cells (COR = 0.183, P = 8.66E−09), $CD4^+$T cells (COR = 0.13, P = 5.24E−05), neutrophils (COR = 0.103, P = 1.43E−03), and macrophages (COR = 0.271, P = 5.77E−18), whereas it was negatively correlated with B cell infiltration (COR = −0.084, P = 8.26E−03) (**Figure 10A**). The expression level of RUNX2 was positively correlated with the infiltration of all types of immune cells that were analyzed: B cells (COR = 0.066, P = 3.87E−02), $CD8^+$T cells (COR = 0.294, P = 5.57E−21), $CD4^+$T cells (COR = 0.196, P = 8.40E−10), macrophages (COR = 0.473, P = 7.75E−56), neutrophils (COR = 0.303, P = 1.25E−21), and dendritic cells (COR = 0.298, P = 6.53E−21) (**Figure 10B**). The expression of RUNX3 was also positively correlated with the infiltration of B cells (COR = 0.434, P = 3.38E−46), $CD8^+$T cells (COR = 0.495, P = 1.81E−61), $CD4^+$T cells (COR = 0.582, P = 4.04E−88), macrophages (COR = 0.14, P = 1.1E−05), neutrophils (COR = 0.635, P = 1.13E−108), and dendritic cells (COR = 0.667, P = 2.99E−123) (**Figure 10C**).

Furthermore, we analyzed the relationship between the expression of RUNX1 and the infiltration of six types of immune cells in BRCA-basal, BRCA-HER2, and BRCA-luminal breast cancer subtypes. We found that RUNX1 expression was only positively correlated with $CD4^+$T cell invasion (COR = 0.227, P = 1.21E−02) in the BRCA-basal type. For the BRCA-HER2 subtype, RUNX1 expression was only positively correlated with macrophage infiltration (COR = 0.29, P = 2.72e−02). However, the expression level of RUNX1 in the BRCA-luminal subtype was positively correlated with the infiltration of $CD8^+$T cells (COR = 0.161, P = 1.84E−04), $CD4^+$T cells (COR = 0.208, P = 1.15E−06), macrophages (COR = 0.212, P = 6.26E−07), neutrophils (COR = 0.192, P = 7.81E−06), and dendritic cells (COR = 0.131, P = 2.39E−03) (**Supplementary Figure 6**). We

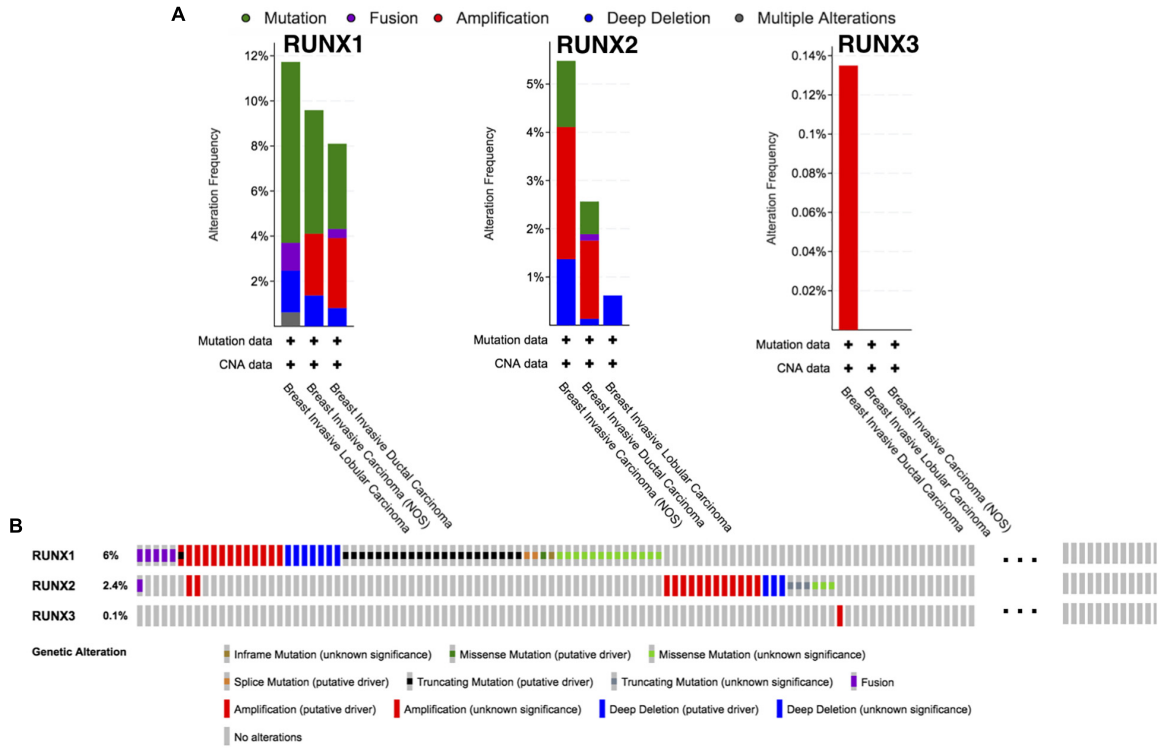

FIGURE 5 | Runt-related transcription factor alternation analysis in breast cancer. **(A)** The proportions of various RUNX alternations in different subclasses of breast cancer. **(B)** The genetic alternations of RUNX1, RUNX2, and RUNX3 in breast cancer patients.

also analyzed the relationship between the expression levels of RUNX2/RUNX3 and the infiltration of immune cells in BRCA-basal, BRCA-HER2, and BRCA-luminal breast cancer subtypes (**Supplementary Figures 7**, **8**).

Relationship Between RUNX Mutations and Immune Cell Infiltration in Breast Cancer

Various genes are mutated during breast cancer pathogenesis. Through analysis of the TIMER database, we found that RUNX1 had highly frequent mutations in breast cancer, while RUNX2 and RUNX3 had a lower frequency of mutation rate. Therefore, we analyzed the relationship between RUNX1 mutations and the infiltration of immune cells in breast cancer. The results showed that RUNX1 mutations significantly increased the levels of $CD8^+$T cells, $CD4^+$T cells, and macrophage infiltration in breast cancer (**Supplementary Figure 9**). This suggests that RUNX1 mutation may be used as a potential marker for detecting immune cell infiltration in breast cancer.

Correlation Between Gene Copy Number of RUNX Factors and Immune Cell Infiltration

Runt-related transcription factors not only affect breast cancer cells, but also modulate the function of immune cells. Through TIMER analysis, we found a positive correlation between RUNX1 gene copy number and the infiltration of B cells, $CD8^+$T cells, $CD4^+$T cells, macrophages, neutrophils, and dendritic cells in breast cancer (**Figure 11A**). The copy number change of the RUNX2 gene was only positively correlated with the infiltration of four types of immune cells in breast cancer: $CD8^+$T cells, $CD4^+$T cells, macrophages, and neutrophils (**Figure 11B**). In addition, the change in RUNX3 gene copy number was significantly correlated with the infiltration of $CD8^+$T cells, $CD4^+$T cells, macrophages, neutrophils, and dendritic cells (**Figure 11C**). These results suggest that changes in the copy number of RUNX genes may reflect the infiltration of multiple immune cells in breast cancer.

Furthermore, we analyzed the relationship between RUNX gene copy number and immune cell infiltration in breast cancer subtypes. We found that the infiltration of $CD4^+$T cells, macrophages, and neutrophils in BRCA-basal and BRCA-luminal subtypes was affected by changes in the RUNX1 gene copy number. However, the infiltration of immune cells into the BRCA-HER2 subtypes was not affected. In addition, the change in RUNX1 gene copy number in BRCA-luminal cells also affected the infiltration of B cells (**Supplementary Figure 10**). The change in RUNX2 gene copy number influenced the infiltration of multiple immune cells in the subtypes. In the BRCA-basal type, the RUNX2 gene copy number was associated with the infiltration of $CD8^+$T cells, $CD4^+$T cells, and neutrophils. The change in RUNX2 gene copy number in the BRCA-HER2 subtype was correlated with the infiltration of B cells, $CD8^+$T cells, $CD4^+$T

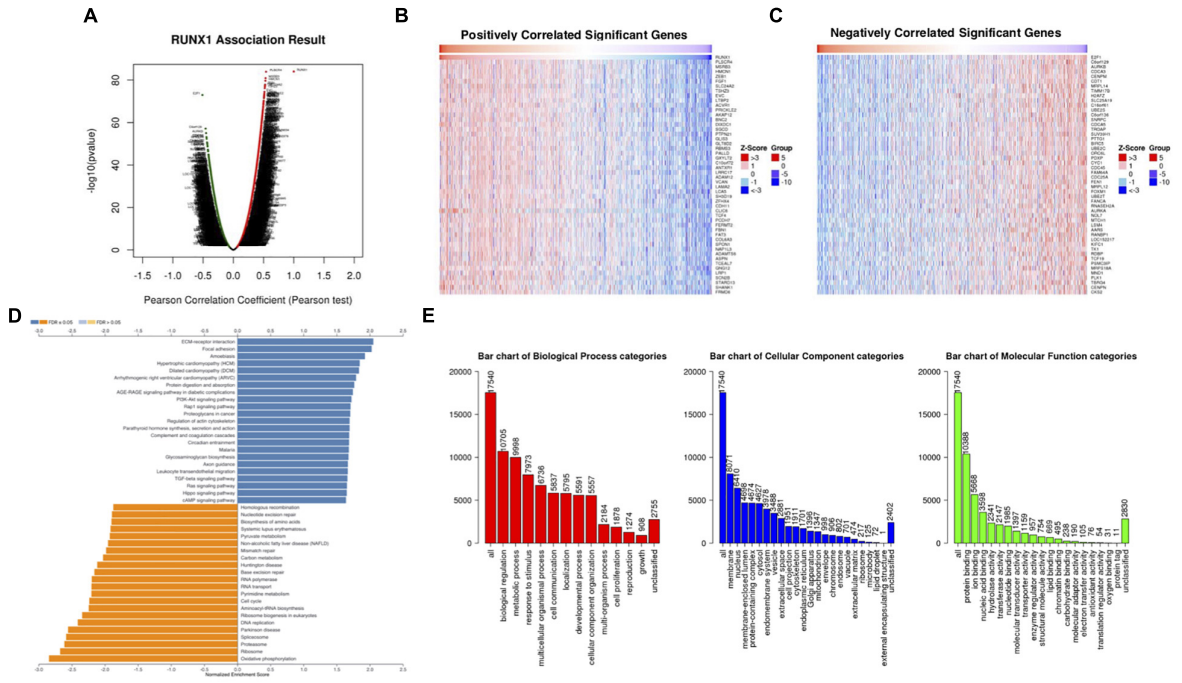

FIGURE 6 | Analysis of differentially expressed genes in correlation with RUNX1 in breast cancer. **(A)** Volcano plot showing the up-regulated and down-regulated genes correlated with RUNX1 expression (Pearson test). The significantly positively correlated **(B)** and negatively correlated **(C)** genes were shown in heatmaps. **(D)** Kyoto Encyclopedia Genes and Genomes (KEGG) pathway analysis of the significantly differentially expressed genes in correlation with RUNX1. **(E)** Gene ontology (GO) analysis of the significantly differentially expressed genes in correlation with RUNX1.

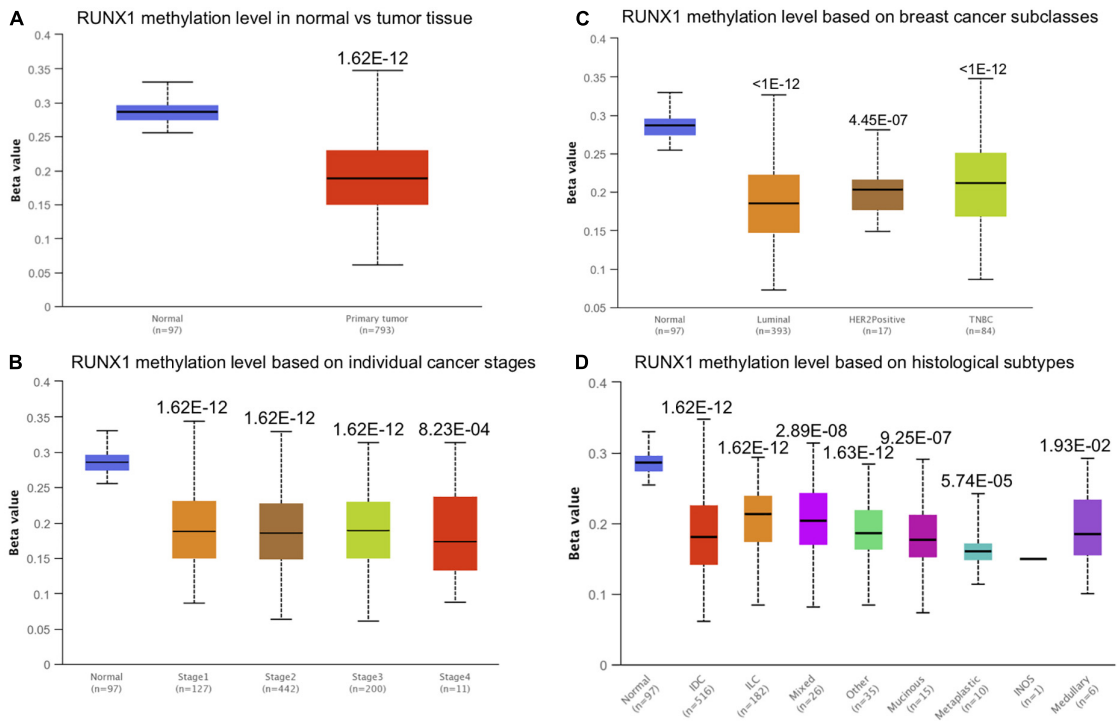

FIGURE 7 | Correlation analysis between RUNX1 methylation level and breast cancer patient characteristics. The RUNX1 methylation level in breast cancer patients classified by **(A)** sample types, **(B)** individual cancer stages, **(C)** breast cancer subclasses, and **(D)** histological subtypes were shown.

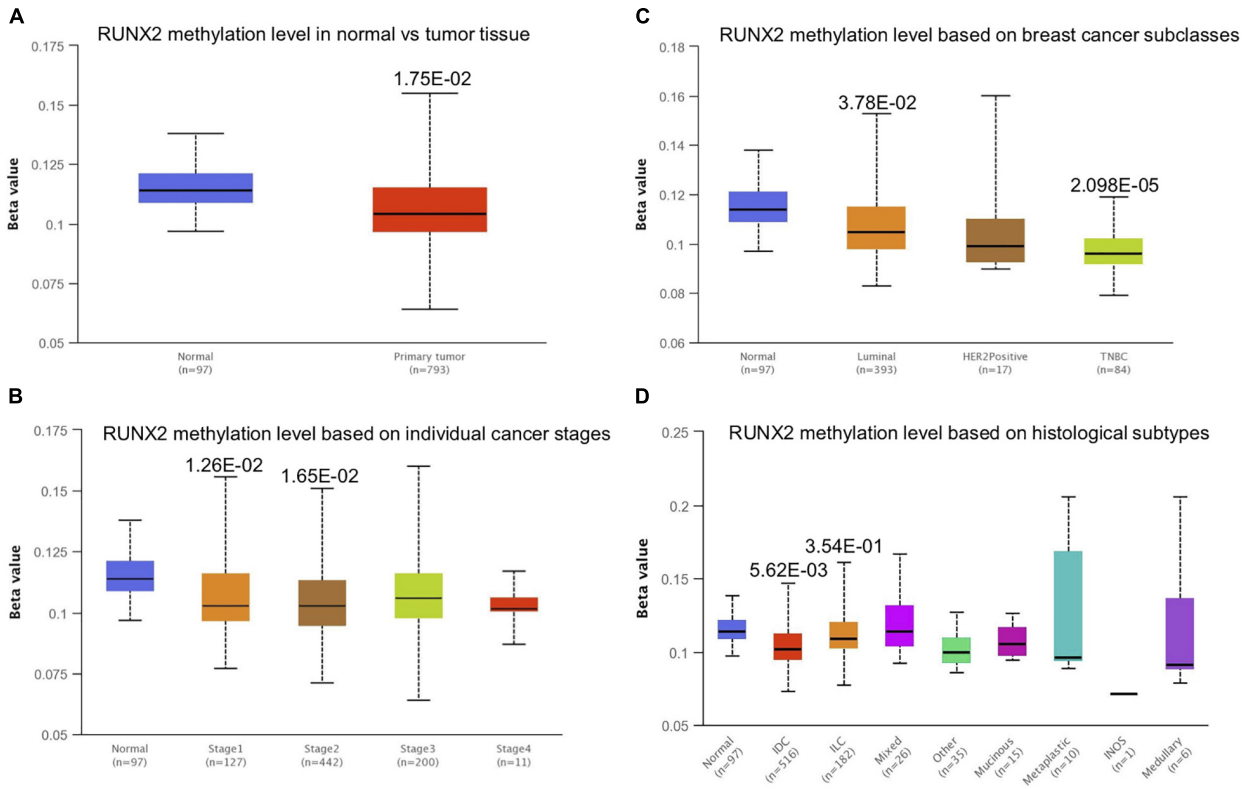

FIGURE 8 | Correlation analysis between RUNX2 methylation level and breast cancer patient characteristics. The RUNX2 methylation level in breast cancer patients classified by **(A)** sample types, **(B)** individual cancer stages, **(C)** breast cancer subclasses, and **(D)** histological subtypes were shown.

cells, macrophages, neutrophils, and dendritic cells. However, in the BRCA-luminal subtype, the change in RUNX2 gene copy number was only associated with the infiltration of B cells and $CD4^+$T cells (**Supplementary Figure 11**). For RUNX3, changes in gene copy number only affected the infiltration of $CD4^+$T cells in BRCA-HER2 and BRCA-luminal subtypes, whereas multiple types of immune cells were affected in the BRCA-basal subtype (**Supplementary Figure 12**). These results suggest that the change in RUNX1 gene copy number might mainly affect immune cell infiltration in BRCA basal and BRCA-luminal subtypes; the change in RUNX2 gene copy number might mainly affect immune cell infiltration in BRCA-basal and BRCA-HER2 subtypes; whilst the RUNX3 gene copy number change might mainly influence the change in immune cell infiltration in BRCA-basal type.

Relationship Between RUNX Gene Methylation and Immune Cell Infiltration

Through the above analysis, we have shown the methylation level change of RUNX factors in breast cancer, as well as the relationship between RUNX factor expression level and immune cell infiltration in breast cancer. However, it remains unclear whether changes in the methylation of RUNX genes also influence the infiltration of immune cells in breast cancer. Therefore, we further analyzed the association between the methylation levels of RUNX genes and immune cell infiltration (B cells, $CD8^+$T cells, $CD4^+$T cells, macrophages, neutrophils, and dendritic cells) in breast cancer using the GSCA database (**Figure 12**). The results showed that RUNX2 methylation was positively correlated with the infiltration levels of B cells, $CD8^+$T cells, and $CD4^+$T cells, and negatively correlated with the infiltration levels of macrophages and neutrophils (**Figure 12B**). In contrast, the methylation level of RUNX3 was negatively correlated with the infiltration of B cells, $CD8^+$T cells, and $CD4^+$T cells, whereas it was positively correlated with the infiltration levels of macrophages and neutrophils (**Figure 12C**). This suggests that the methylation levels of RUNX factors may have opposite effects on the same type of immune cell infiltration.

Correlation Between RUNX Methylation Level and TGF-β1 Expression in Breast Cancer

TGF-β is a critical negative immunoregulatory factor in immune balance. TGF-β not only inhibits various targets in the immune system, but also plays a role in tumor immune escape and adverse reactions to tumor immunotherapy (Batlle and Massague, 2019; Derynck et al., 2021). Through analysis of the TIMER database, we found that the expression of TGFB1, TGFBR1, and TGFBR2 are correlated with the infiltration levels of various immune cells in breast cancer (**Supplementary Figure 13**). We

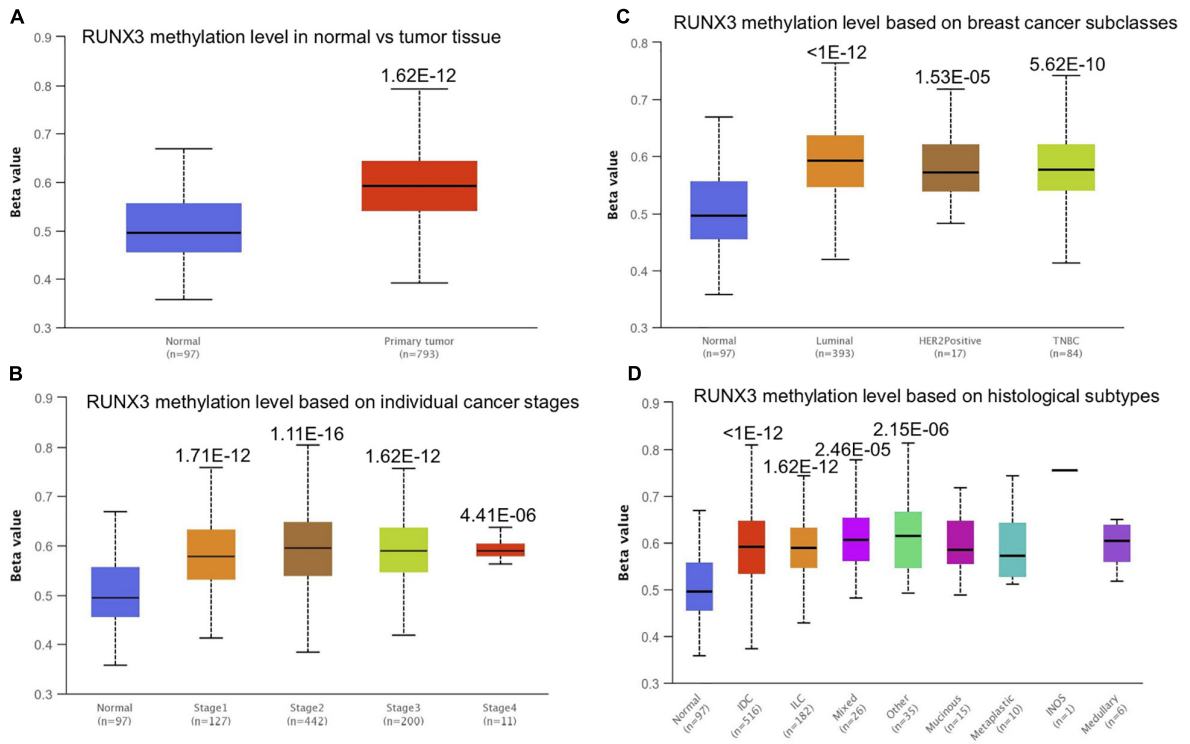

FIGURE 9 | Correlation analysis between RUNX3 methylation level and breast cancer patient characteristics. The RUNX3 methylation level in breast cancer patients classified by **(A)** sample types, **(B)** individual cancer stages, **(C)** breast cancer subclasses, and **(D)** histological subtypes were shown.

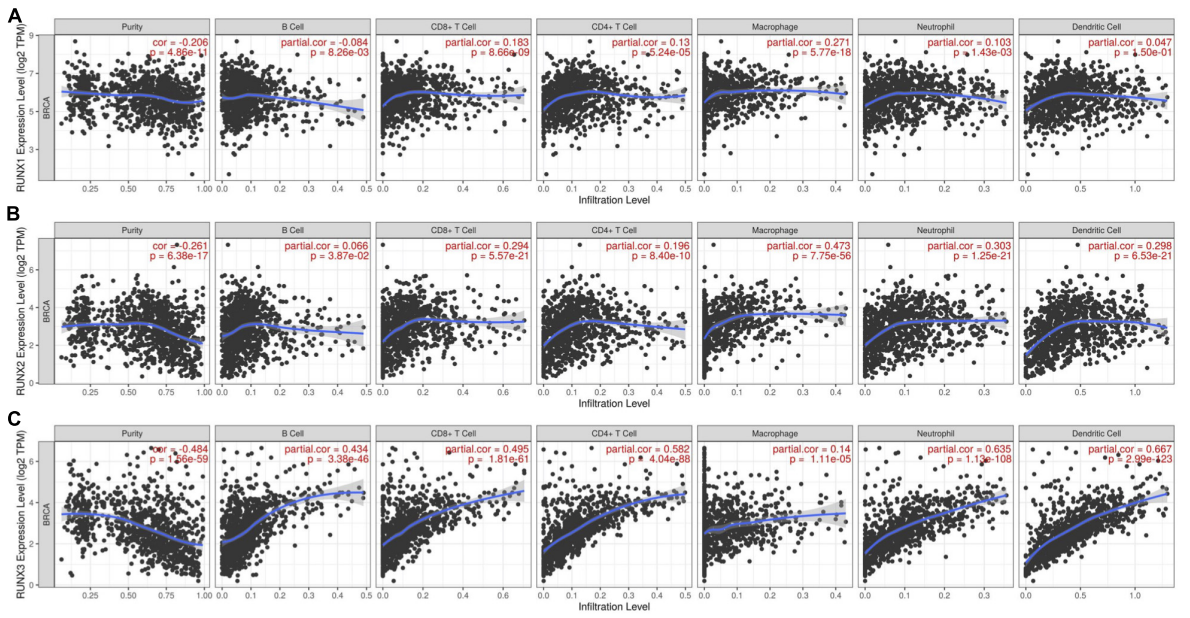

FIGURE 10 | The correlation between differentially expressed RUNXs and immune cell infiltration in breast cancer. The correlations between the transcriptional levels of **(A)** RUNX1, **(B)** RUNX2, and **(C)** RUNX3 with the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in breast cancer were shown.

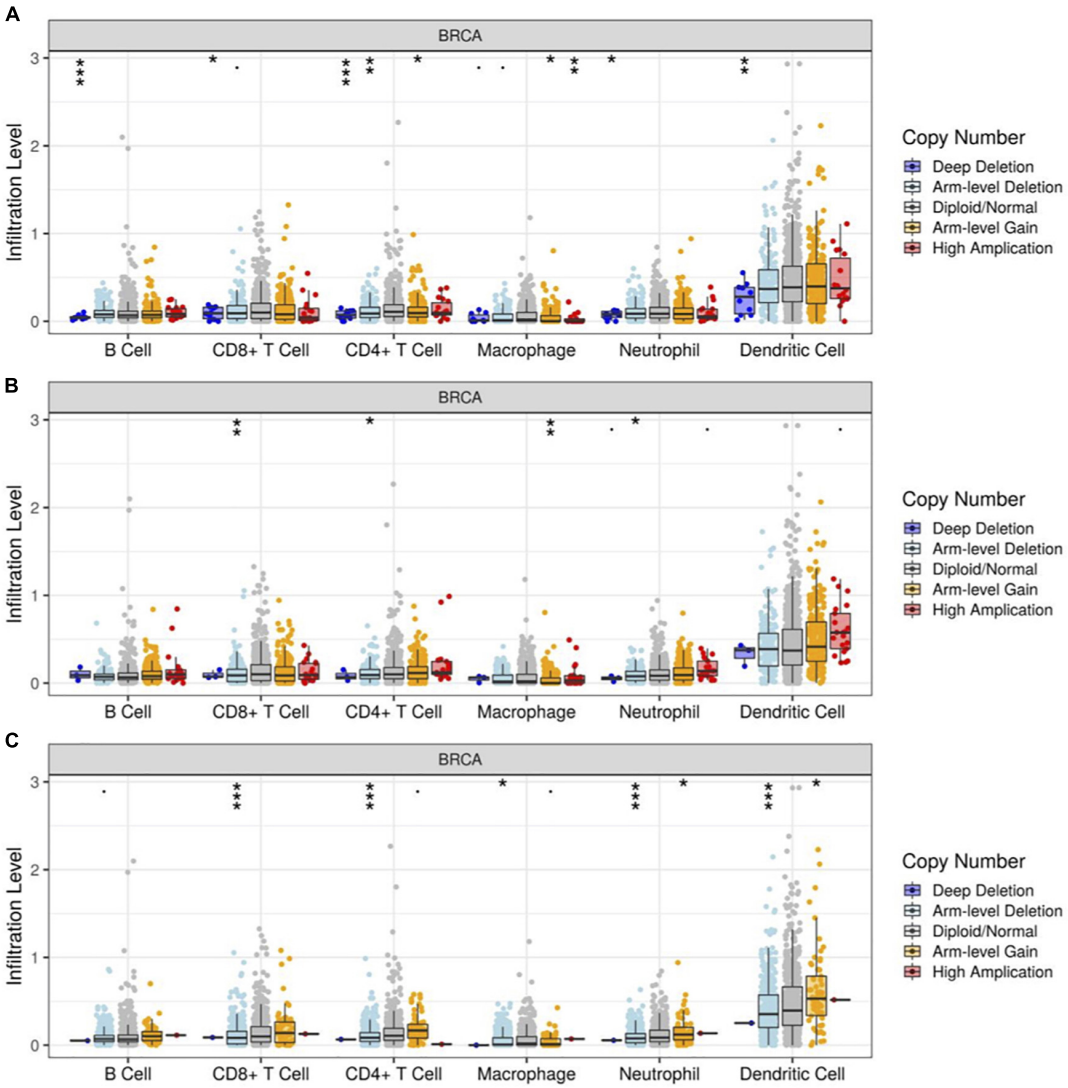

FIGURE 11 | Correlation between RUNX gene copy number and immune cell infiltration in breast cancer. The correlation between the gene copy number changes of **(A)** RUNX1, **(B)** RUNX2, and **(C)** RUNX3 with infiltration levels of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in breast cancer were shown. $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

used the TIMER database to further analyze the relationship between the expression level of TGFB1 and immune cell infiltration in different breast cancer subtypes. We found that the expression level of TGFB1 was positively correlated with the infiltration level of various immune cells in different breast cancer subtypes (**Supplementary Figure 14**). The relationship between the expression levels of TGFBR1 and TGFBR2 and immune cell infiltration in different breast cancer subtypes was also analyzed. We found that the expression level of TGFBR1 was mainly correlated with the level of macrophage infiltration in different breast cancer subtypes (**Supplementary Figure 15**). The expression level of TGFBR2 was positively correlated with the infiltration level of multiple immune cells in different subtypes of breast cancer (**Supplementary Figure 16**). These results indicate that the TGF-β signaling pathway is related to the infiltration level of multiple immune cells in different subtypes of breast cancer. However, it remains unclear whether the methylation levels of RUNX genes affect the TGF-β signaling pathway. To further evaluate the potential mechanisms underlying the effects of RUNX gene methylation on immune cell infiltration in breast cancer, we analyzed the relationship between the methylation level of RUNX genes and the TGF-β signaling pathway using the TISIDB database. The results showed that RUNX1 gene methylation level was positively correlated with the expression level of TGF-β1 (**Figure 13A**), while there was no correlation between RUNX2 methylation level and the expression of TGF-β1 (**Figure 13B**). However, RUNX3 methylation levels were negatively correlated with the expression levels of TGF-β1 (**Figure 13C**). The relationship between the methylation level of RUNX genes and the expression

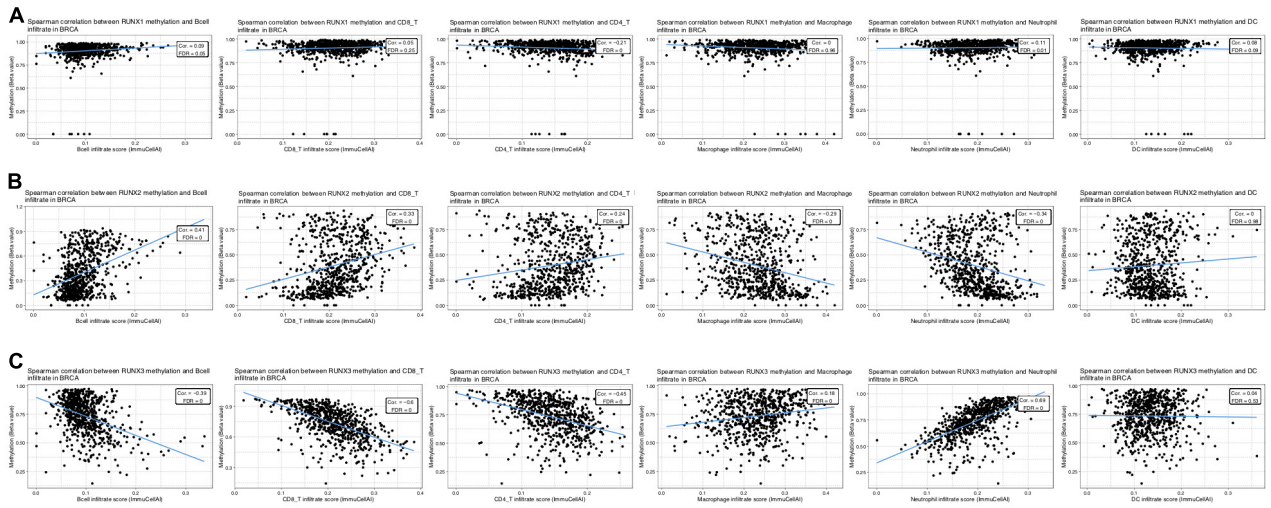

FIGURE 12 | Correlation between RUNX methylation level and immune cell infiltration in breast cancer. The correlations between changes in the methylation levels of **(A)** RUNX1, **(B)** RUNX2, and **(C)** RUNX3 with the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in breast cancer were shown.

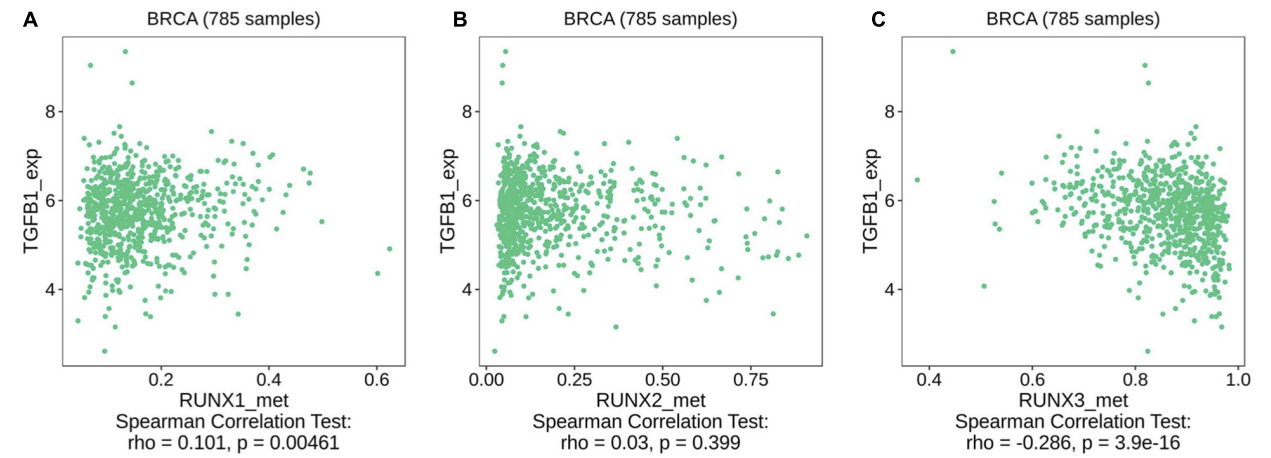

FIGURE 13 | Correlation between RUNX methylation level and TGF-β1 expression in breast cancer. The correlations between changes in the methylation levels of **(A)** RUNX1, **(B)** RUNX2, and **(C)** RUNX3 with the expression of TGF-β1 in breast cancer were shown.

of TGFBR1 was also analyzed, but no correlation was identified (**Supplementary Figure 17**). These results suggest that changes in the methylation level of RUNX genes may affect the expression of TGF-β1, which in turn might influence the activation of the TGF-β signaling pathway and immune cell infiltration in breast cancer.

DISCUSSION

The pathophysiology of breast cancer is multifaceted and is affected by both genetic and environmental factors. However, the clinical relevance of the immune system in breast cancer has not been adequately studied. The heterogeneity of breast cancer has been highlighted in the past decade, and this has stimulated the exploration of the relationship between the immune system and heterogeneity in different breast cancer subtypes. An increasing number of studies have shown that there are multiple immune cell infiltrates in breast cancer tissue, which could affect patient outcomes. These immune cells affect the pathogenesis and metastasis of breast cancer through various signaling pathways. For example, neutrophils can promote lung metastasis of breast cancer by secreting leukotriene B4 (Wculek and Malanchi, 2015), whereas podoplanin-expressing macrophages can contribute to the lymphoinvasion of breast cancer cells by binding with lymphatic endothelial cells to trigger matrix remodeling and lymphangiogenesis (Bieniasz-Krzywiec et al., 2019). In addition, lymphocyte infiltration in breast cancer is associated with clinical survival outcomes (Savas et al., 2016; Byrne et al., 2020). Although the essential role of immune cell infiltration in breast cancer has

gained widespread attention, the relationship between the RUNX family and immune cell infiltration remains poorly understood.

An increasing number of alterations have been identified in multiple tumor suppressor or activator genes in breast cancer patients, among which the RUNX1 gene has a high frequency of alterations. RUNX1 is a member of the RUNX family, which consists of RUNX1, RUNX2, and RUNX3 in mammals. They affect a variety of signaling pathways, including Wnt (Ito et al., 2008), Notch (Hilton et al., 2008), MST-YAP1 (Min et al., 2012), and receptor tyrosine kinase (Huang H. et al., 2012). These signaling pathways are of great importance during multiple stages of breast cancer pathogenesis and metastasis.

Multiple mechanisms have been studied to elucidate the role of the RUNX family in breast cancer. RUNX1 has been shown to inhibit the growth of breast cancer stem cells and promote tumor expansion (Hong et al., 2018). In addition, RUNX1 and RUNX3 may inhibit the YAP-regulated epithelial-mesenchymal transition process and improve breast cancer outcome (Kulkarni et al., 2018). In contrast, RUNX2 has been shown to increase the bone metastasis of breast cancer in an integrin-dependent way (Li X.Q. et al., 2016). However, a comprehensive analysis of the transcriptional changes in RUNX family members, promoter methylation level, RUNX gene alternation, and their relationship with immune cell infiltration as well as breast cancer patient prognosis, is still lacking.

Runt-related transcription factor members are critical during the regulation of immune cell development and function. For example, the differentiation of $CD4^{-}CD8^{-}$ T lymphocytes to $CD4^{+}CD8^{+}$ T lymphocytes depends on the expression of RUNX1 (Egawa et al., 2007). RUNX3 can synergistically regulate the transcription of $CD8^{+}$T cells with the T-box protein (Cruz-Guilloty et al., 2009). It is also important to analyze the correlation between RUNX expression levels and the infiltration of various immune cells in breast cancer. Using the TIMER database, we analyzed the correlation between the expression levels of RUNX protein family members and the infiltration of B cells, $CD8^{+}$T cells, $CD4^{+}$T cells, macrophages, neutrophils, and dendritic cells in different breast cancer subtypes. We also found that RUNX1 mutations in breast cancer significantly increased the infiltration of $CD8^{+}$T cells, $CD4^{+}$T cells, and macrophages in breast cancer.

Studies have shown that epigenetic modifications play an important role in the occurrence and development of breast cancer (Hinshelwood and Clark, 2008; Pasculli et al., 2018). It has been reported that promoters of more than 100 genes undergo hypermethylation in breast cancer (Jovanovic et al., 2010). The promoter region of RUNX3 is hypermethylated, resulting in its decreased expression in breast cancer (Jiang et al., 2008; Liu H. et al., 2018). To the best of our knowledge, our study provides the first detailed bioinformatic analysis of RUNX family gene methylation changes and its relationship with breast cancer characteristics, especially immune cell infiltration in breast cancer. We found that the methylation level of RUNX3 in breast cancer is completely opposite to that of RUNX1 and RUNX2. Interestingly, we also found opposite effects of RUNX2 and RUNX3 gene methylation changes on immune cell infiltration in breast cancer. Our findings suggest that changes in RUNX gene methylation may be a potential biomarker for immune cell infiltration in breast cancer.

Dysregulation of the growth factor signaling pathway is a significant characteristic of tumorigenesis and metastasis. The TGF-β signaling pathway not only regulates tumor cells but also modulates immune cells in the tumor microenvironment, thus playing an important role in the above process (Ikushima and Miyazono, 2010; Batlle and Massague, 2019; Derynck et al., 2021). For example, the TGF-β signaling pathway could downregulate the expression of TNF and IFN-γ, thereby inhibiting the proliferation of $CD4^{+}$T cells (Mangan et al., 2006). Through database analysis, we found that the expression levels of TGFB1 and TGFBR1 in breast cancer are correlated with the infiltration levels of multiple immune cells. To date, the relationship between RUNX gene methylation and the TGF-β signaling pathway remains unclear. Through analysis of the TISDB database, we found that the methylation levels of RUNX genes were correlated with the expression level of TGF-β1, which may affect TGF-β signaling pathway activation and immune cell infiltration in breast cancer.

In summary, our analysis deepens our understanding of the role of RUNX factors and TGF-β signaling pathway in breast cancer. This study will aid in elucidating the molecular mechanisms underlying the role of the RUNX family in breast cancer and provide potential therapeutic targets to improve breast cancer patient outcomes.

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.

AUTHOR CONTRIBUTIONS

FZ constructed the study and revised this manuscript. LG performed the data analysis and wrote the manuscript. Both 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.2021.730380/full#supplementary-material

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Cancer-Associated Fibroblasts Facilitate Squamous Cell Carcinoma Lung Metastasis in Mice by Providing TGFβ-Mediated Cancer Stem Cell Niche

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Cancer-associated fibroblasts (CAFs) have been shown to enhance squamous cell carcinoma (SCC) growth, but it is unclear whether they promote SCC lung metastasis. We generated CAFs from *K15.KrasG12D.Smad4*$^{-/-}$ mouse SCCs. RNA expression analyses demonstrated that CAFs had enriched transforming growth factor-beta (TGFβ) signaling compared to normal tissue-associated fibroblasts (NAFs), therefore we assessed how TGFβ-enriched CAFs impact SCC metastasis. We co-injected SCC cells with CAFs to the skin, tail vein, or the lung to mimic sequential steps of lung metastasis. CAFs increased SCC volume only in lung co-transplantations, characterized with increased proliferation and angiogenesis and decreased apoptosis compared to NAF co-transplanted SCCs. These CAF effects were attenuated by a clinically relevant TGFβ receptor inhibitor, suggesting that CAFs facilitated TGFβ-dependent SCC cell seeding and survival in the lung. CAFs also increased tumor volume when co-transplanted to the lung with limiting numbers of SCC cancer stem cells (CSCs). *In vitro*, CSC sphere formation and invasion were increased either with co-cultured CAFs or with CAF conditioned media (which contains the highest TGFβ1 concentration) and these CAF effects were blocked by TGFβ inhibition. Further, TGFβ activation was higher in primary human oral SCCs with lung metastasis than SCCs without lung metastasis. Similarly, TGFβ activation was detected in the lungs of mice with micrometastasis. Our data suggest that TGFβ-enriched CAFs play a causal role in CSC seeding and expansion in the lung during SCC metastasis, providing a prognostic marker and therapeutic target for SCC lung metastasis.

Keywords: squamous cell carcinoma, transforming growth factor-beta, cancer associated fibroblast, lung metastasis, cancer stem cell

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INTRODUCTION

Squamous cell carcinomas (SCCs) arise from stratified epithelia, and the most relevant organ sites are the skin and oral cavity where high UV irradiation, tobacco carcinogens, or human papillomavirus (HPV) infection are initiating events. The worst outcome of SCC is death caused by distant metastasis, most commonly to the lung (Bohnenberger et al., 2018; Alfieri et al., 2020). Lung metastasis is the process of cancer cells disseminating from a primary SCC, entering into blood vessels or lymphatic vessels (intravasation), survival and traveling, and moving out of vessels (extravasation) into the lung, and survival and expansion in the lung thereafter (Chaffer and Weinberg, 2011).

The major challenge for studying mechanisms of SCC lung metastasis is the shortage of spontaneous SCC lung metastasis models mimicking the entire metastatic process. We previously generated an aggressive SCC mouse model driven by $Kras^{G12D}$ mutation and *Smad4* deletion ($Smad4^{-/-}$) in keratin 15 (K15)-positive stem cells of stratified epithelial tissues, i.e., hair follicle bulge or tongue papillae (White et al., 2013). $K15.Kras^{G12D}.Smad4^{-/-}$ mice develop spontaneous SCCs, some of which metastasized to the lung (White et al., 2013). In that study, we identified that cancer stem cells (CSCs) derived from mutant stem cells in these SCCs have a higher invasive ability than non-CSCs (White et al., 2013). Taken together, CSCs are expected to have a higher chance than non-CSCs to invade from the primary site, survive through trafficking and engraft at the metastatic site. CSCs have self-renewal ability and the capacity to generate the progeny cells that constitute the tumor and are resistant to cell death (Chaffer and Weinberg, 2011; Malanchi et al., 2011). However, how CSCs as SCC metastasis "seeds" interact with their "soil" during metastasis, remains to be assessed.

Cancer stem cells depend upon the stromal niche to maintain their stem-like properties (Malanchi et al., 2011; Plaks et al., 2015). Among the most abundant cells in the stromal niche, cancer-associated fibroblasts (CAFs) communicate with cancer cells *via* cell–cell contacts and production of chemokines, cytokines, and factors that contribute to SCC progression (Markwell and Weed, 2015; Hogervorst et al., 2018; Peltanova et al., 2019). CAFs have been shown to enhance SCC cell proliferation, migration, and invasion *in vitro* (Li et al., 2015; Hogervorst et al., 2018). In an *in vivo* model, CAFs from skin SCC possess a proinflammatory gene signature that promotes tumor growth (Erez et al., 2010). In addition, we have previously shown that CAFs facilitate oral SCC (OSCC) tumor growth *in vivo* (Meng et al., 2014). Data from *in vitro* experiments have shown that CAFs enhance the self-renewal of CSCs in different cancers including HNSCCs (Chen et al., 2014; Álvarez-Teijeiro et al., 2018; Su et al., 2018; Le et al., 2019). However, it remains to be determined whether CAFs promote CSC invasion and whether enhancement of CSC properties (self-renewal and invasion) is sufficient to impact SCC metastasis *in vivo*, and if so, at what stage of metastasis and *via* what mechanisms.

In the current study, we transplanted metastatic SCC cells derived from $K15.Kras^{G12D}.Smad4^{-/-}$ mice and CAFs derived from the stroma of these SCCs into C57BL/6J or athymic mice. Using SCC-CAF co-transplantation, we sought to determine: whether CAFs enhance SCC lung metastasis; and if so, at which stage of lung metastasis; whether CAFs promote CSC self-renewal and invasion resulting in more lung metastasis; and, what signaling pathways drive CAF-influenced SCC lung metastasis. Our study revealed that CAF's primarily influence the distant metastatic site for CSCs to be seeded and expanded in the lung in a transforming growth factor-beta (TGFβ)-dependent manner. Use of a clinically relevant TGFβ inhibitor to inhibit CAF-promoted lung engraftment provides preclinical evidence of this critically important event and suggests that TGFβ from CAFs is a major contributor to metastasis. And, therapeutic targeting of SCC metastasis with TGFβ inhibitors is feasible and worthy of further research.

MATERIALS AND METHODS

Establishment of Cell Lines and Cell Culture

SP Flow Cytometry Sorting and Culture

Mouse SCC cell lines A223, B931 are derived from $K15.Kras^{G12D}.Smad4^{-/-}$ C57BL/6J mice as previously described (White et al., 2013). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Efflux of Hoechst 33342 dye to isolate the Hoechst-negative SP cells, a subpopulation of metastasis associated CSCs (White et al., 2013), was performed at the University of Colorado Cancer Center Flow Cytometry Shared Resource as previously described (White et al., 2013).

CAFs/Normal Tissue-Associated Fibroblasts Isolation, Culture, Purification

Cancer-associated fibroblasts were isolated from transplanted tongue SCC tumors and normal tissue-associated fibroblast (NAFs) were isolated from normal tongues of independent mice using enzymatic digestion as described in **Supplementary Materials and Methods** (Mazzocca et al., 2010; Yang et al., 2016). Two independent CAF cell lines and two independent NAF cell lines (from four different mice) were established and cultured in DMEM containing 10% FBS.

RNA-seq and Analysis

Total RNAs were extracted using RNeasy Plus Mini Kit (Qiagen, Germantown, MD, United States). Total RNA (100 ng) was used as input to construct mRNA libraries using the NuGEN Universal Plus mRNA-Seq protocol part no. 9133 (NuGEN, Redwood City, CA, United States). Sequencing was done on an Illumina NovaSEQ 6000 instrument using an S4 flow cell and 2 × 150 paired end sequencing (Illumina, San Diego, CA, United States). A custom computational pipeline consisting of the open-source gSNAP, Cufflinks, and R was used for alignment and discovery of differential gene expression (Presby et al., 2019). Briefly, each high-resolution sequencing read generated by each sample was mapped to the mouse genome (GMAPDBv2) using gSNAP, Cufflinks calculated the prevalence of transcripts from each known gene, and each gene was expressed as transcript

levels in fragments per kilobase of exon per million mapped reads (FPKM). From this, significant differentially expressed mRNA profiles were identified using ANOVA in R with an FDR of $P < 0.05$. These expression data were evaluated by GSEA against the Hallmark Gene Sets and Canonical Pathways KEGG Gene Sets using GSEA 4.0.3 software downloaded from gsea-msigdb.org. Raw sequencing files are available in the Sequence Read Archive (SRA[1]). Accession SRR13996315 and SRR13996316.

Tumor Transplantation and Treatment

Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus. C57BL/6J mice (Jackson Laboratory) or athymic nude (Charles River Laboratories) at 8- to 10-week of age were used as tumor and fibroblast transplantation recipients. A223 SCC cells were transplanted with or without fibroblasts to female C57BL/6J mice. SCC cells or their sorted CSCs from B931 were transplanted into athymic nude mice because they are incapable of tumor formation in immunocompetent C57BL/6J mice. A total of 1,000 total B931 were transplanted subcutaneously to the right flank of anesthetized mice. For SCC cell-fibroblasts tail vein co-injection and SCC cell-fibroblast subcutaneous co-transplantation, see **Supplementary Materials and Methods**. For SCC or CSC (SP) co-transplantation with fibroblasts to the lung, a total of 1,000 SCC cells or 100 SP cells with or without 5,000 CAFs/NAFs was injected into the mouse left lung (unless otherwise indicated). One lung tumor was initiated on the left side after cells were transplanted to the left lung, volume was calculated using the following formula: Volume (mm^3) = (length × width × depth)/2. Metastasis to the right lung was assessed by counting the number of metastases in H&E stained sections of the right lung. Complete methods are described in the **Supplementary Materials and Methods**.

For TGFβ inhibitor treatment, mice were treated with TGFβ inhibitor (LY2109761 or LY2157299, 150 mg/kg/day) by oral gavage or an equal volume of vehicle (Tran et al., 2017) (1% carboxymethylcellulose, 0.5% sodium lauryl sulfate, 0.085% povidone, and 0.05% antifoam) daily for 19–25 days before being sacrificed and lungs harvested. LY2109761 was used in early experiments and we later switched to the clinical drug version LY2157299 (galunisertib) to assure translational relevance. For more information, see **Supplementary Materials and Methods**.

Histology

Primary tumors and lungs harvested at the endpoint of the study were embedded in paraffin, sectioned, and stained with Hematoxylin and eosin staining (H&E). Histopathology of primary tumors, lung tissue, micrometastasis, and metastasis were evaluated on H&E sections.

Conditioned Media Collection

CAF/NAF CM Collection for CSC Sphere Formation

Cancer-associated fibroblasts/NAFs seeded at the same density (about 90%) were incubated in serum-free media and conditioned media (CM) were harvested after 36 h. CM were collected, centrifuged to remove cellular debris, and used immediately for CSC sphere formation assays (described below).

CM Collection and TGFβ1 ELISA

1×10^4 CSCs or SCCs with or without 5×10^4 CAFs or NAFs were cultured in the CSC media (serum-free media) for 36 h. CM were collected, centrifuged to remove the cells and debris, and used for TGFβ1 ELISA (R&D Systems, Minneapolis, MN, United States) following the manufacturer's instructions as reported previously (Li et al., 2004). The optical density (OD) of each well was detected using a microplate reader set to 450 nm. Tumor lysates were normalized to the same protein concentration prior to TGFβ1 ELISA.

CSC Sphere Assay

Squamous cell carcinoma cells were transduced with green fluorescence protein (GFP)-expressing lentivirus and selected by flow cytometry sorting as previously described (White et al., 2013). CAFs or NAFs were transduced with NucLight Red Lentivirus (Essen Biosciences, Ann Arbor, MI, United States) followed by selection with 2.5 μg/mL bleomycin to obtain cells stably expressing nuclear red fluorescence protein (RFP) (RFP^+ CAF/NAF). Cells were plated in ultra-low attachment (ULA) plates (Corning) to assess sphere-forming capacity. For direct co-culture, 100 GFP^+ CSCs with or without 500 RFP^+ CAFs/NAFs were seeded in each well of a 24-well ULA plate. To assess whether CM of CAFs/NAFs induced sphere formation, 100 μL CM of CAFs/NAFs were mixed with 50 GFP^+ CSCs (in 50 μL CSC media) in each well of a 96-well ULA plate. TGFβ inhibitor (LY2157299) was applied at a final concentration of 5 μmol/L or an equal volume of DMSO was added as a negative control. After culturing for 7–10 days, whole well imaging was performed using an IncuCyte Zoom live cell imaging instrument at the University of Colorado Cell Technologies Shared Resource. Spheres with diameter >100 mm were counted.

Invasion Assay

Invasion assay was performed as previously described (White et al., 2013). Transwell Matrigel-coated invasion chambers (BD Biosciences, 8 μm pore membranes) were prepared according to the manufacturer's instructions. A total of 50,000 RFP^+ CAFs/NAFs were seeded in the bottom well 24 h before 10,000 GFP^+ CSCs were added in the top chamber. TGFβ inhibitor (LY2109761) was applied at final concentration of 5 μmol/L or an equal volume of DMSO was added as a negative control. After 48 h, uninvaded cells were removed from the upper chamber with a moist cotton swab and invaded cells below the top chamber were fixed in 10% formalin and stained with hematoxylin. Three fields at 100× magnification were captured and counted in each of three replicates.

RT-qPCR

Unconditioned media and CAF CM were prepared as described above and applied to 100,000 recipient A223 cells in sphere culture and incubated 24 h. RNA was harvested as described above. RT-qPCR was performed using 40 ng RNA, Brilliant II QRT-PCR 1-Step Master Mix (Agilent,

[1]https://www.ncbi.nlm.nih.gov/sra

Santa Clara, CA, United States) and TaqMan gene expression assays for *Junb* (Mm04243546_s1), *Spp1* (Mm00436767_m1), *Vegfa* (Mm00437306_m1), and *Gapdh* (Mm99999915_g1) (ThermoFisher, Rockford, IL, United States). The expression of each gene relative to *Gapdh* was determined using the $2^{\Delta CT}$ method and normalized to the unconditioned media control.

IHC Staining

IHC of Mouse Tissues

IHC staining was performed as previously described (Lu et al., 2006; Luo et al., 2019). The primary antibodies used for mouse tissues were α-smooth muscle actin (αSMA, 1:500, CST), p-SMAD3 (1:250, Abcam, Cambridge, MA, United States), cleaved-caspase 3 (1:200, CST), CD31 (1:200, CST), Ki67 (1:400, Abcam), and TGFβ1 (1:300, Abcam). All sections that contained tumor tissue in each group were stained and quantified. For p-SMAD3, cleaved-caspase 3, CD31, and Ki67, five 200× fields/lung tumor sample were captured, and positive staining cell number or positive staining area for each field was quantified by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, United States). The positive staining fraction (%) or positive staining cell number value of each field/sample was averaged to obtain the positive staining fraction or cell number for each sample.

IHC of Human OSCC Samples

West China Hospital of Stomatology, Sichuan University, China approved the experiment as being human subject exempted. De-identified human tissue paraffin sections were used in this study. All tissues were from primary OSCC tumor biopsies from patients without prior cancer therapy. A total of 6–7 non-lung metastatic OSCCs and 7–10 lung metastatic OSCCs were used for IHC staining using primary antibodies against TGFβ1 (1:100, Abcam), p-SMAD2 (1:100, Invitrogen), or p-SMAD3 (1:100, Abcam). A total of 5–10 fields at 200× magnification were captured per OSCC sample. Quantification of area stained and the integrated optical density (IOD) of indicated markers in each image were measured by Image-Pro Plus 6.0 (Media Cybernetics). Average optical density (Hu et al., 2014) (AOD = IOD/Area) was used in this study for statistical analysis. The mean AOD of 5–10 fields was the AOD value for one OSCC sample.

Statistical Analyses

Statistical analyses for comparisons between two groups were performed using SPSS version 24.0 for Windows (IBM, New York, NY, United States). Normality test for group data sets was determined by Shapiro–Wilk normality test. Statistical differences between two groups were performed by unpaired parametric Student's *t*-test or non-parametric Mann–Whitney exact test, as appropriate. Statistical differences between more than two groups were determined by one way ANOVA with Tukey's multiple comparison test using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, United States). Individual data points represented values of technical or biological replicates, and they were shown as mean ± SEM. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

TGFβ Signaling Is Enriched in Mouse SCC CAFs

We transplanted SCC cells from A223 line into the tongues of female C57BL/6J recipients. Once the tumor was developed, we established CAF cell lines as previously reported (Yang et al., 2016). We confirmed that CAF lines are free of tumor cells that would harbor *Smad4* deletion, express *Cre* transcript, and $KRAS^{G12D}$ protein (**Supplementary Figures 1A,B**). We also established normal fibroblast cell lines from the tongue of female non-tumor bearing mice. CAFs and NAFs did not express keratins (**Supplementary Figures 1B,C**), which are still expressed in poorly differentiated SCC cells, indicating fibroblast cell lines are not contaminated with epithelial cells. The fibroblasts expressed fibroblast activation protein (FAP) and vimentin (**Supplementary Figure 1C**) demonstrating their fibroblast phenotype.

To understand the molecular landscapes of CAFs vs. NAFs, we performed mRNAseq with three technical replicates of one CAF cell line and one NAF cell line. Overall, CAFs were significantly different from NAFs with 2007 significantly differentially expressed genes identified between the two groups ($P < 0.05$) (**Figure 1A**). GSEA interrogation against the Hallmark Gene Sets and the KEGG Pathway Gene Sets was performed and both analyses identified enrichment of TGFβ signaling (**Figures 1B,C**). *Tgfb1*, *Tgfb2*, and TGFβ signaling mediators were highly expressed in CAFs whereas *Tgfb3* and inhibitory *Smads* (*Smad6* and *Smad7*) were reduced in CAFs (**Figure 1D**). Because TGFβ1 is the predominant TGFβ ligand in tumors (Martin et al., 2020), we further examined if CAFs are a major source of TGFβ1 protein. TGFβ1 ELISA analysis showed that CM from CAFs derived from A223 SCCs produced more TGFβ1 protein than cultured A223 cells, whereas CM from NAFs produced much less TGFβ1 protein (**Figure 1E**).

CAFs Increase SCC Engraftment and Expansion in the Lung in a TGFβ-Dependent Manner

We assessed if TGFβ activation in CAFs is sufficient to affect metastasis *in vivo*. We first co-transplanted 1,000 SCC cells (A223 or B931) with 5,000 CAFs, 5,000 NAFs or without fibroblasts to the flank skin, tail vein, or directly to the left lung of the recipient C57BL/6J or athymic nude mice. These routes of transplant mimic different stages of metastasis (intravasation, survival, extravasation, seeding, and expansion in the lung), respectively. CAFs, but not NAFs, increased SCC expansion in the lung of C57BL/6J recipients upon co-injection directly to the left lobe of the lung, and TGFβ inhibitor LY2109761 attenuated the effect of CAFs on SCC expansion in the lung (**Figures 2A,B**). CAFs had no effect on primary tumor growth, or lung metastasis when co-injected into the skin (from the primary site) or through tail vain injection (intravenous trafficking) (**Supplementary Figures 2A–C**). Additionally, CAFs only affected tumor volumes and lesions in the left lung but not metastasis to the right lung (**Supplementary Figure 3**), indicating that CAFs promoted

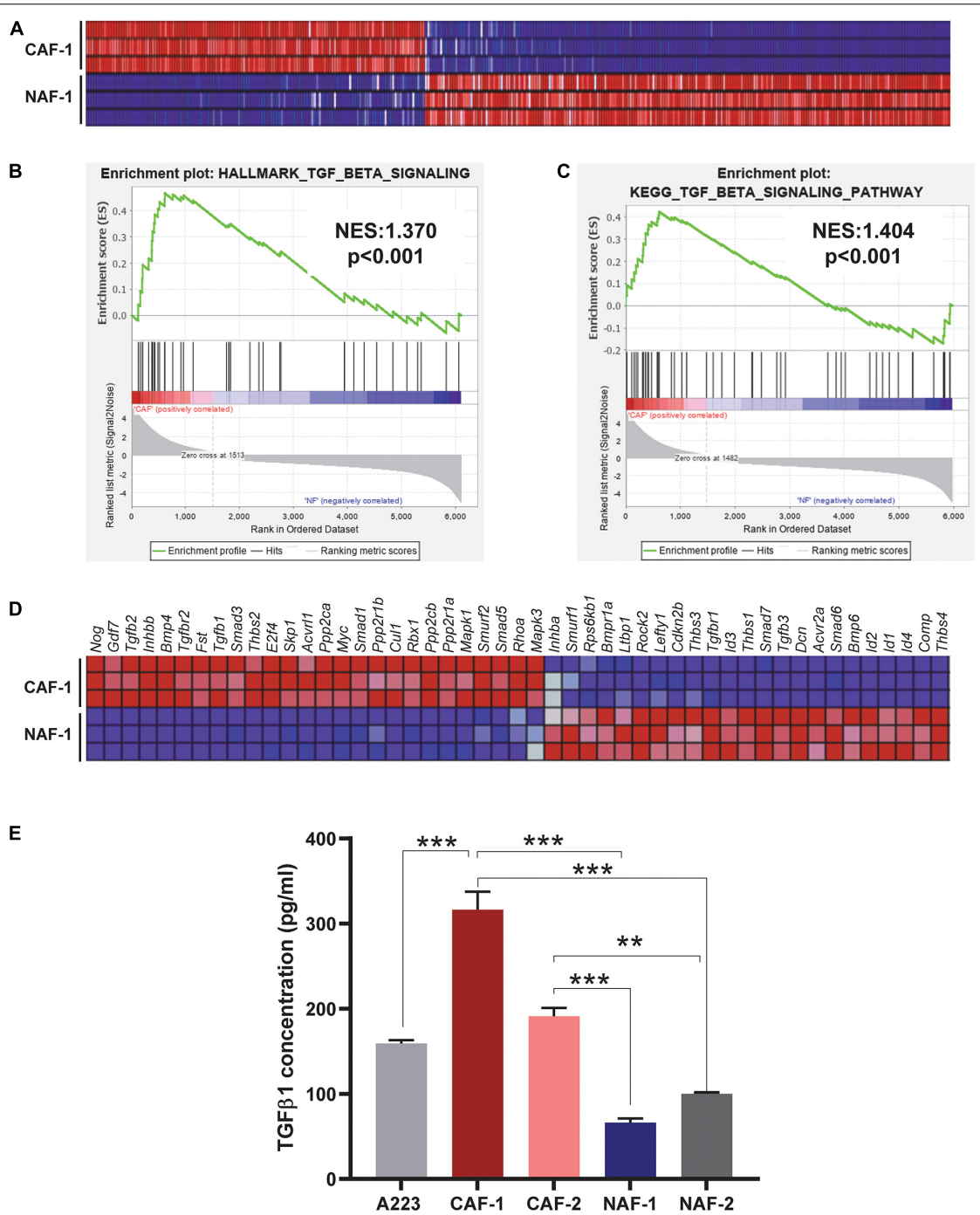

FIGURE 1 | TGFβ signaling is activated in CAFs. **(A)** Heat map of all differentially expressed genes comparing three technical replicates of the CAF-1 to NAF-1 cell lines ($P < 0.05$). **(B,C)** GSEA analysis of the Hallmark Gene Sets **(B)** and the KEGG Pathways Gene Sets **(C)** identified enrichment of TGFβ signaling in CAFs compared to NAFs. NES: normalized enrichment score. **(D)** Heat map of differentially expressed genes between CAFs and NAFs in the KEGG TGFβ signaling pathway. **(E)** Detection of TGFβ1 in conditioned media of SCCs, CAFs, and NAFs was performed using ELISA. Either three or four technical replicates were conducted for each cell type and two independent CAF cell lines and two independent NAF cell lines were utilized. CAF-1 and CAF-2 cell lines were derived from two different, independent SCC tumors. NAF-1 and NAF-2 cell lines were derived from the tongues of two different wild type non-tumor-bearing C57BL/6J mice. $^{**}P < 0.01$, $^{***}P < 0.001$.

SCC cell seeding and expansion but not trafficking in vessels or invasion within the lung. IHC staining of αSMA, a commonly used marker for activated fibroblasts (Sridhara et al., 2013; Luksic et al., 2015; Kalluri, 2016; Maqsood et al., 2020), was used to determine the position of activated fibroblasts or CAFs. αSMA positive fibroblasts were distributed among tumor cells when

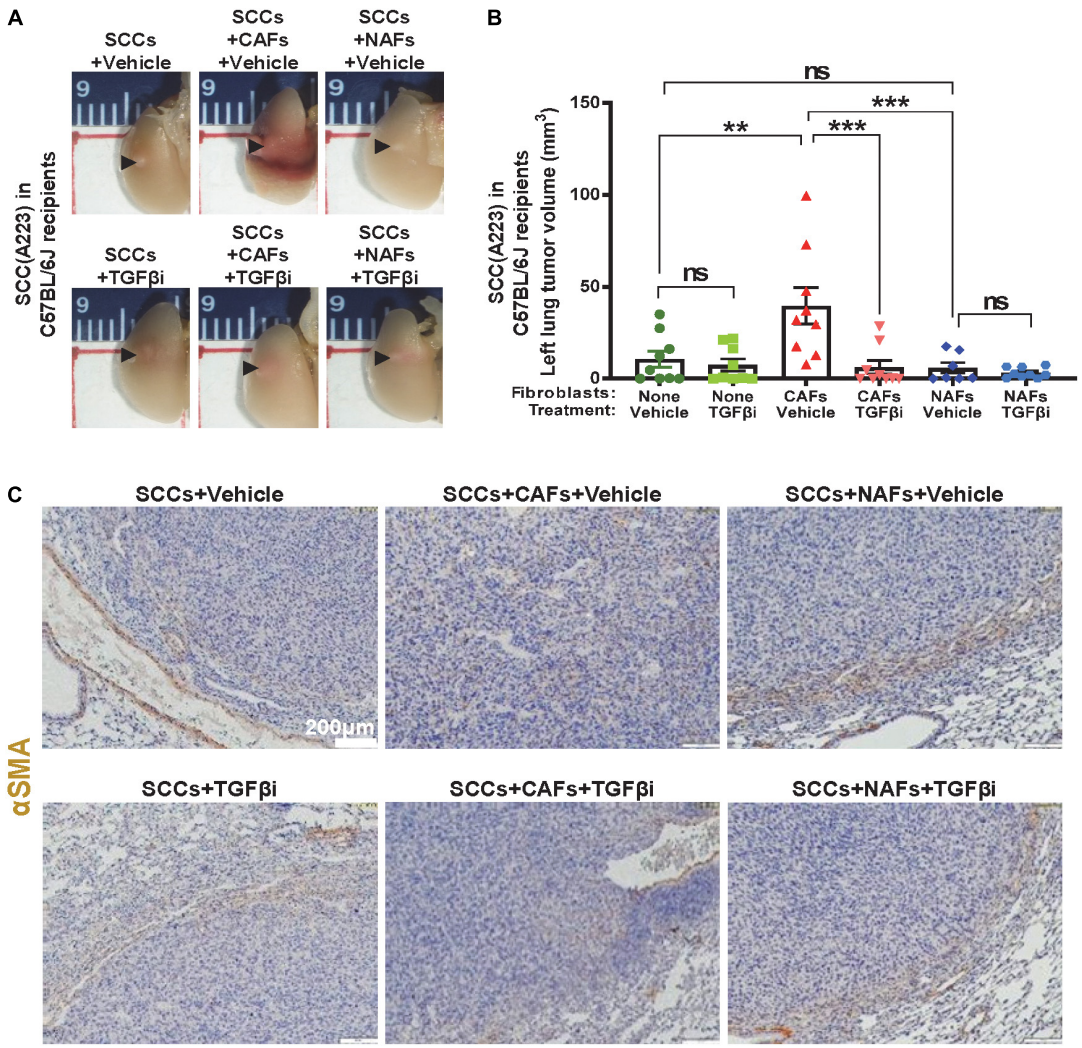

FIGURE 2 | Cancer-associated fibroblasts contributed to TGFβ-dependent SCC seeding to the lung in immunocompetent recipients. **(A)** 1,000 A223 SCC cells and 5,000 of the indicated fibroblast types were co-transplanted to left lung, mice were treated daily with vehicle or TGFβ inhibitor ("TGFβi") LY2109761 and lungs were harvested on day 21 and imaged under 1× magnification using a dissecting microscope. Representative gross images are presented. **(B)** Quantification of lung-seeded tumor volume (from gross samples) at the end point, $^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(C)** Representative αSMA IHC stained images.

CAFs were co-injected. However, αSMA positive fibroblasts were mainly located in the periphery of the tumor in other groups (**Figure 2C**). As αSMA also stains vessels, it is critical to note only the staining independent of vessels.

Compared to lung tumors derived from SCC + NAF co-transplantations, lung tumors derived from co-transplanted SCC + CAFs showed increased p-SMAD3, a surrogate marker of TGFβ activation (**Figures 3A,B**), decreased cleaved-caspase 3, a marker of apoptosis (**Figures 3C,D**), elevated CD31, a marker of endothelial cells (**Figures 3E,F**) and elevated Ki67 (**Figures 3G,H**) in vehicle-treated mice. Further, TGFβ inhibitor LY2109761 attenuated p-SMAD3 and CD31, and increased cleaved-caspase 3 in all groups (**Figures 3B,D,F**), validating on-target activity of the inhibitor and suggesting that apoptosis suppression and increased angiogenesis require TGFβ signaling and a positive relationship between CD31-marked angiogenesis and apoptosis suppression. TGFβ inhibitor only attenuated Ki67 in tumors derived from SCC + CAF co-injection (**Figures 3G,H**), suggesting that elevated TGFβ in CAFs contributes to SCC growth after seeding to the lung, but SCC proliferation is not driven by intrinsic SCC TGFβ signaling.

CAFs Increase CSC Seeding to the Lung in a TGFβ-Dependent Manner

To determine whether CAFs grow and expand with transplanted tumor cells, we labeled SCC cells with GFP and CAFs with RFP. After co-transplantation of 1,000 GFP^+ SCC cells with 5,000 RFP^+ CAFs directly to the left lung, we were unable to detect RFP^+ CAFs (data not shown). We therefore increased the cell transplant numbers 10-fold and monitored relative levels of RFP^+ CAFs and GFP^+ SCC cells at multiple time points

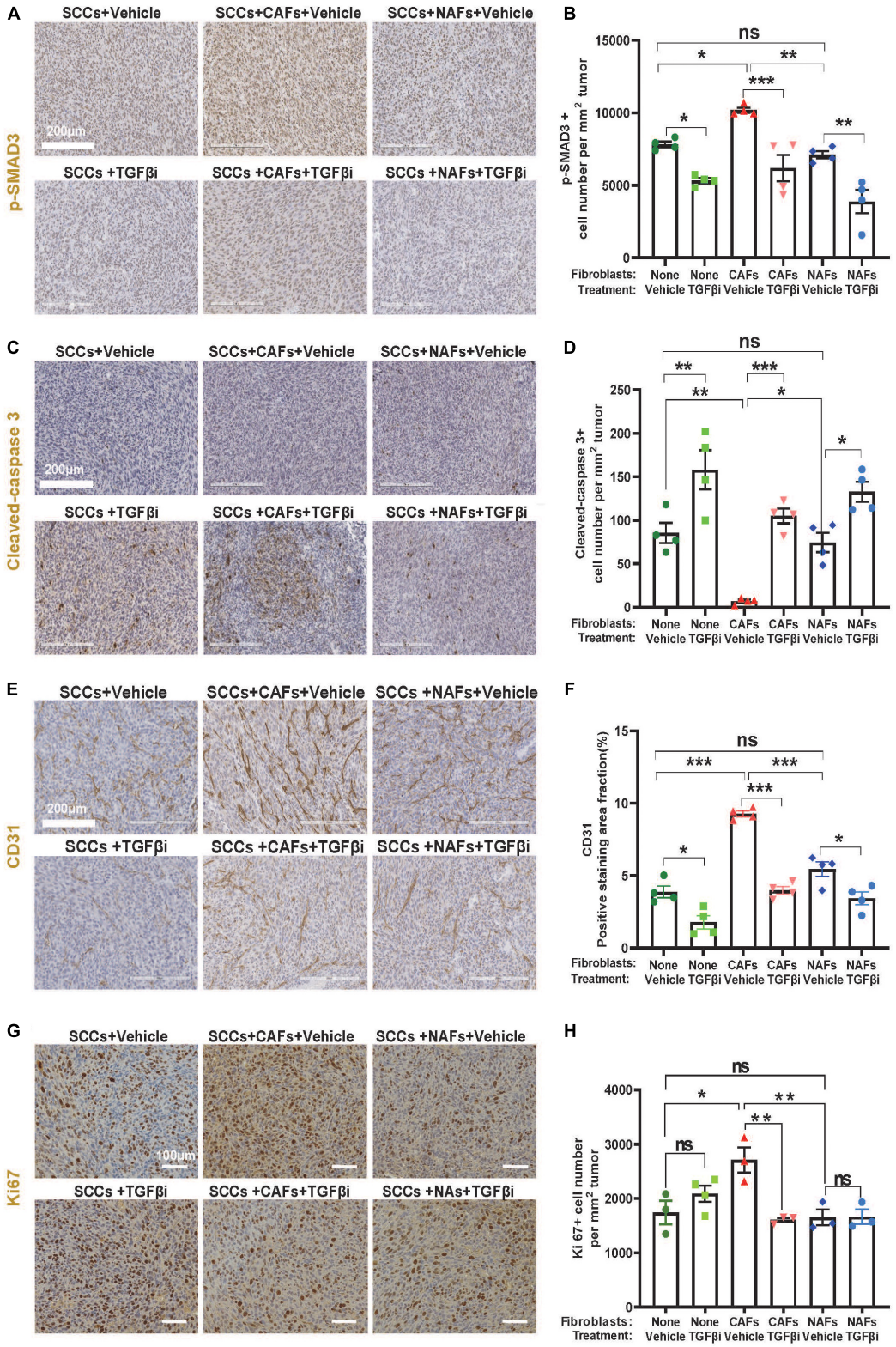

FIGURE 3 | Increased proliferation and angiogenesis and decreased apoptosis in CAF-co-transplanted, lung-seeded tumors is attenuated by TGFβ inhibition. **(A,B)** Representative p-SMAD3 IHC stained images and quantification in lung-seeded A223 tumors. Scale bar: 200 μm. **(C,D)** Representative cleaved-caspase 3 IHC stained images and quantification in lung-seeded A223 tumors. Scale bar: 200 μm. **(E,F)** Representative CD31 IHC stained images and quantification in lung-seeded A223 tumors. Scale bar: 200 μm. **(G,H)** Representative Ki67 IHC stained images and quantification in lung-seeded A223 tumors. Scale bar: 100 μm. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

post transplantation using fluorescent dissecting microscopy. Expansion of GFP^+ SCC cells was apparent as early as 6 days post-transplant and the intensity of the lesions continued to increase over time (**Figures 4A,B**). In contrast, the levels of RFP^+ CAFs were maintained at the same intensity level for the entire assay (**Figures 4A,B**), suggesting that transplanted CAFs do not appreciably expand with the tumor cells at this level of detection. To be clear, RFP^+ CAFs could be detected by traditional fluorescent microscopy (**Figure 4C**).

Since CAFs aid SCC expansion without themselves expanding, we next assessed if SCC cell expansion promoted by CAFs in the lung is due to CAFs' effects on CSC self-renewal and survival *in vivo*. We sorted the SP cells to define CSCs, a subpopulation of metastasis-associated CSCs, as previously described (White et al., 2013), and co-injected 100 SP cells with 5,000 CAFs or NAFs to the left lung. CAFs, but not NAFs, increased tumor volumes in either C57BL/6J or athymic recipients (**Figures 4D–G**), suggesting that CAFs' effect on lung CSC expansion does not require T-cells. Fluorescent dissecting microscopy confirmed that the gross volumes were primarily GFP^+ SCC cells (**Figure 4E**), suggesting that CAFs do not themselves expand but facilitate CSC cell expansion in the lung. Treating mice with the TGFβ inhibitor LY2157299 reduced lung SCC volumes in CSCs co-injected with CAFs (**Figure 5**), suggesting that TGFβ signaling is critical to CAFs influence on CSC survival and/or expansion in the lung.

CAF-Produced TGFβ Promotes CSC Self-Renewal and Invasion

To determine if CAFs directly affect CSC self-renewal, we performed sphere formation assays in ULA plates. A total of 100 GFP^+ SP cells/well were co-cultured with 500 RFP^+ CAFs or NAFs per well in six-well plates. Because CAFs or NAFs were integrated into the spheres, only spheres with >50 GFP^+ CSCs/sphere were counted 7–10 days after culture. Both NAFs and CAFs increased the abundance of spheres, but CAF co-cultures had higher sphere numbers than NAF co-cultures (**Figures 6A,B**). Adding TGFβ inhibitor LY2157299 to the culture media (5 μmol/L) attenuated sphere formation in CSC-CAF co-cultures, but not CSC alone or CSC-NAF co-culture (**Figure 6B**), suggesting that TGFβ supplied by CAFs is responsible for increased sphere formation induced by CAFs. To assess if cell–cell contact is required for CAFs to enhance CSC sphere formation, we measured CSC sphere formation as a function of CM from CAFs or NAFs. CM from CAFs but not NAFs significantly increased sphere formation that was attenuated by TGFβ inhibitor (**Figures 6C,D**).

Because we previously demonstrated increased metastasis in mice with higher numbers of SP CSCs (White et al., 2013), we assessed if CAFs affect CSC invasion using transwell invasion chambers. We plated SP cells in the top chamber of Matrigel-coated membranes, and plated CAFs or NAFs in the bottom chamber, and quantified CSC invasion to the underside of the membrane. CAFs, but not NAFs enhanced CSC invasion and the effect was attenuated by TGFβ inhibition (**Figure 6E**).

Since CAFs were not in direct contact with CSCs (**Figures 6C–E**), spheres represent SCC cells alone and factors released from CAFs must have contributed to CSC sphere formation and invasion. Indeed, TGFβ1 ELISA showed that CAFs are the primary source of secreted TGFβ1 in conditioned culture media (**Figure 6F**). Finally, to assess if CAFs drive TGFβ signaling in neighboring SCC cells, we applied the CM from CAFs to recipient SCC cells in a short-term sphere culture and found that CAF CM increased the expression of know TGFβ target genes, *Spp1*, *Junb*, and *Vegfa* (**Figure 6G**). These TGFβ target gene products are involved in self-renewal, metastasis, clonal expansion, and angiogenesis (Gokulnath et al., 2017; Kim et al., 2017; Sui et al., 2017; Huang et al., 2019; Kallergi et al., 2019).

TGFβ Activation and CAFs Correlates With Metastasis in Human Oral SCCs and Mice

To assess if our findings in mouse models apply to human SCCs, we performed IHC staining of TGFβ1, p-SMAD2, and p-SMAD3 in primary tumor oral SCC clinical specimens from patients with or without lung metastasis. The staining intensity of these proteins in primary SCCs in patients with metastasis were significantly higher than SCCs in patients without metastasis (**Figures 7A,B**), supporting the notion that TGFβ activation in primary SCC cells might be a critical aspect of metastasis. Additionally, in mouse lungs with SCC micrometastasis from implanted flank SCCs, activated αSMA+ fibroblasts were coincidental with strong TGFβ1 and p-SMAD3, which were not evident in lungs without micrometastasis in tumor-bearing mice (**Figures 8A,B**), further suggesting the critical role of TGFβ1 in activating fibroblasts to prepare the metastatic niche.

DISCUSSION

CAFs Co-transplanted With SCCs Did Not Promote Primary SCC Growth or Trafficking but Enhanced SCC Lung Seeding and Expansion

In this study, we assessed the effect of CAFs on SCC formation and metastasis. While CAFs are reported to promote SCC growth (Orimo et al., 2005; Erez et al., 2010), the primary SCC growth in our model of transplantation of bulk SCC cells was not affected by CAFs. It is possible that SCCs in our model are aggressive with $Kras^{G12D}$-dependent autonomous growth further sustained by homozygous *Smad4* loss. Because of their aggressive behaviors, these SCCs could either be self-sustained or rapidly educate normal fibroblasts in their natural location to form CAFs in the native microenvironment. Consistent with this, the CAF cell lines used in these studies were generated by harvesting the CAFs from transplanted SCCs, demonstrating the ability of the tumor to generate CAFs from the normal mouse microenvironment. Further, CAFs did not appear to protect and aid SCC cell trafficking and extravasation as i.v. co-injection of SCCs/CAFs did not significantly increase the number of lung nodules seeded by tumor cells (**Supplementary Figure 2**). Given these results, it

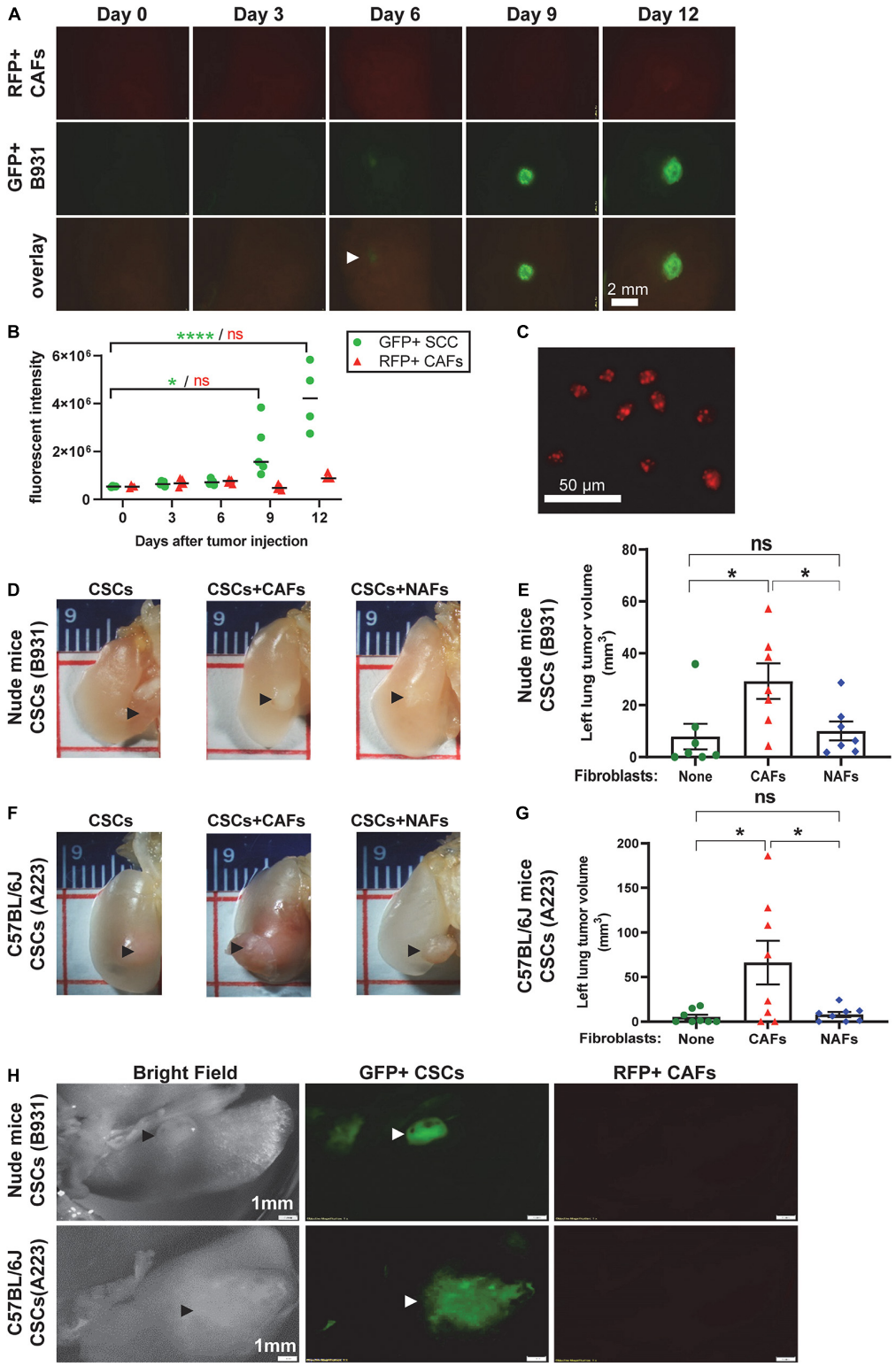

FIGURE 4 | Cancer-associated fibroblasts did not expand with CSC cells but facilitated CSC cell seeding and expansion in the left lung. Transplanted A223 and B931 CSCs were used in all experiments as indicated. **(A,B)** 10,000 GFP$^+$ B931 cells were co-transplanted with 50,000 RFP$^+$ CAFs directly to the left lung and 4–5 mice were sacrificed on days 0, 3, 6, 9, and 12 post-transplant and left lungs imaged as described in panel **(F)** to detect GFP and RFP positive lesions. Representative images of lungs at each time point are presented in panel **(A)** and quantification of GFP and RFP intensity is presented in panel **(B)**. **(C)** RFP$^+$ CAFs were imaged with an inverted fluorescent microscope using a 10× objective to demonstrate RFP positivity. **(D–G)** Representative images of left lung tumor, and

(Continued)

FIGURE 4 | Continued
quantification of left lung tumor volume in nude mice **(D,E)** and C57BL/6J mice **(F,G)** after 100 flow sorted side population (CSCs) of the indicated SCC cell lines were directly injected to the left lung with and without 5,000 CAFs or NAFs co-transplanted. Lungs were harvested and imaged from nude mice on day 19 and from C57BL/6J mice on day 25. **(H)** Brightfield and fluorescent imaging of gross tumors in the left lung tumor immediately after harvest was performed using a fluorescent dissecting microscope. SCCs were labeled with green fluorescence protein (GFP) and CAFs with red fluorescence protein (RFP). Only SCCs but not CAFs were detected. Scale bar: 1 mm. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{****}P < 0.0001$.

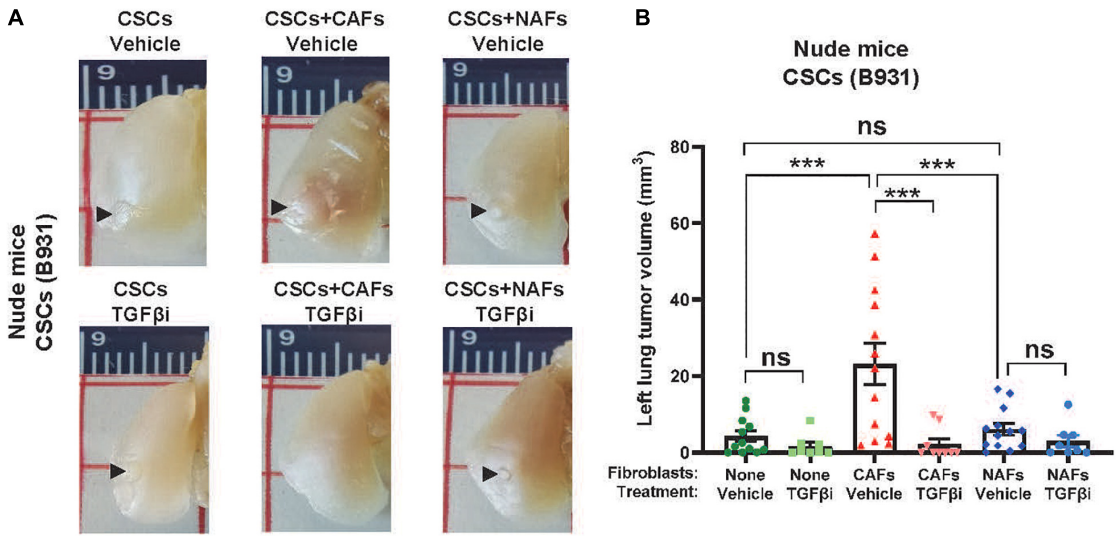

FIGURE 5 | Cancer-associated fibroblasts facilitated TGFβ-dependent CSC cell seeding and expansion in the left lung. **(A)** Representative images of left lung tumors formed after injecting 100 flow sorted B931 SP CSCs with 500 CAFs or NAFs. Mice were treated with vehicle or TGFβi as indicated. **(B)** Quantification of left lung tumor volumes at the time of harvest. $^{ns}P > 0.05$, $^{***}P < 0.001$.

is likely that SCC cells themselves have already acquired survival ability prior to dissemination and trafficking as we previously observed (Wu et al., 2019).

In contrast, CAFs promoted SCC cell expansion in the lung as compared to the same numbers of SCC and CAF cells co-transplanted at primary site. This contribution from CAFs appears to be linked to increased TGFβ1 (**Figure 1**) that could impact property changes of both SCC cells and CAFs. This notion is supported by data demonstrating that TGFβ inhibitor attenuated CAF's effects on SCC cell seeding to the lung (**Figures 2**, **5**), a foreign microenvironment for SCC cells to find their niche for establishment. This could explain the difference between no obvious effects of CAFs at the primary site but profound effects at the metastatic site. αSMA+ fibroblasts' presence in the lung-transplanted tumors (**Figure 2C**) further consolidated our notion that CAFs were a source of TGFβ1. It seemed that only co-injected CAFs instead of tumor cells alone can efficiently integrate more newly generated CAFs in the tumor, because only lung tumors derived from SCC/CAF co-injection have CAFs in the tumor core (**Figure 2C**). Since CAFs secreted higher TGFβ1 level than tumor cells or NAFs (**Figure 1E**) and TGFβ1 increased CAFs' migration (Karagiannis et al., 2014), we inferred one reason for this was that SCC/CAF secreted higher amount of TGFβ1 which increased fibroblast activation, and this enabled CAFs to establish in the tumor core.

The expansion of SCC volumes in the lung by CAFs could be due to reduced apoptosis, increased angiogenesis, and increased proliferation, all of which appeared to be contributed by TGFβ because TGFβ inhibition attenuated these CAF effects. Our results demonstrating that TGFβ inhibition reduced apoptosis in SCC cells are consistent with those reported in other cancer types. For example, TGFβ1 protects colon cancer cells from apoptosis (Moon et al., 2019) and TGFβ downregulation induced cancer cell apoptosis in melanoma and pancreas adenocarcinoma (Han et al., 2018). Our results suggested that elevated apoptosis could result from reduced angiogenesis, and this was in line with Folkman's finding (Folkman, 2003). In contrast, *Smad4* mutant SCC cells, including CSCs, have lost responsiveness to TGFβ-induced growth arrest which requires SMAD4 (Wu et al., 2018). This may explain why TGFβ inhibition did not affect SCC proliferation without CAFs. Therefore, the effect of TGFβ inhibition on reducing SCC proliferation induced by CAFs is likely to be mediated by additional growth factors produced in CAFs instead of in SCC cells.

Cancer-associated fibroblasts appeared to primarily affect CSCs, as co-injection of CAFs with CSCs have effects on CSC seeding to the lung similar to (or greater than) unselected SCC cells (**Figures 2A,B** vs. **Figures 4D–G**). Further, CAFs' effects were comparable in immune-compromised vs. competent background (**Figures 4D–G**). It is possible that these SCC cells

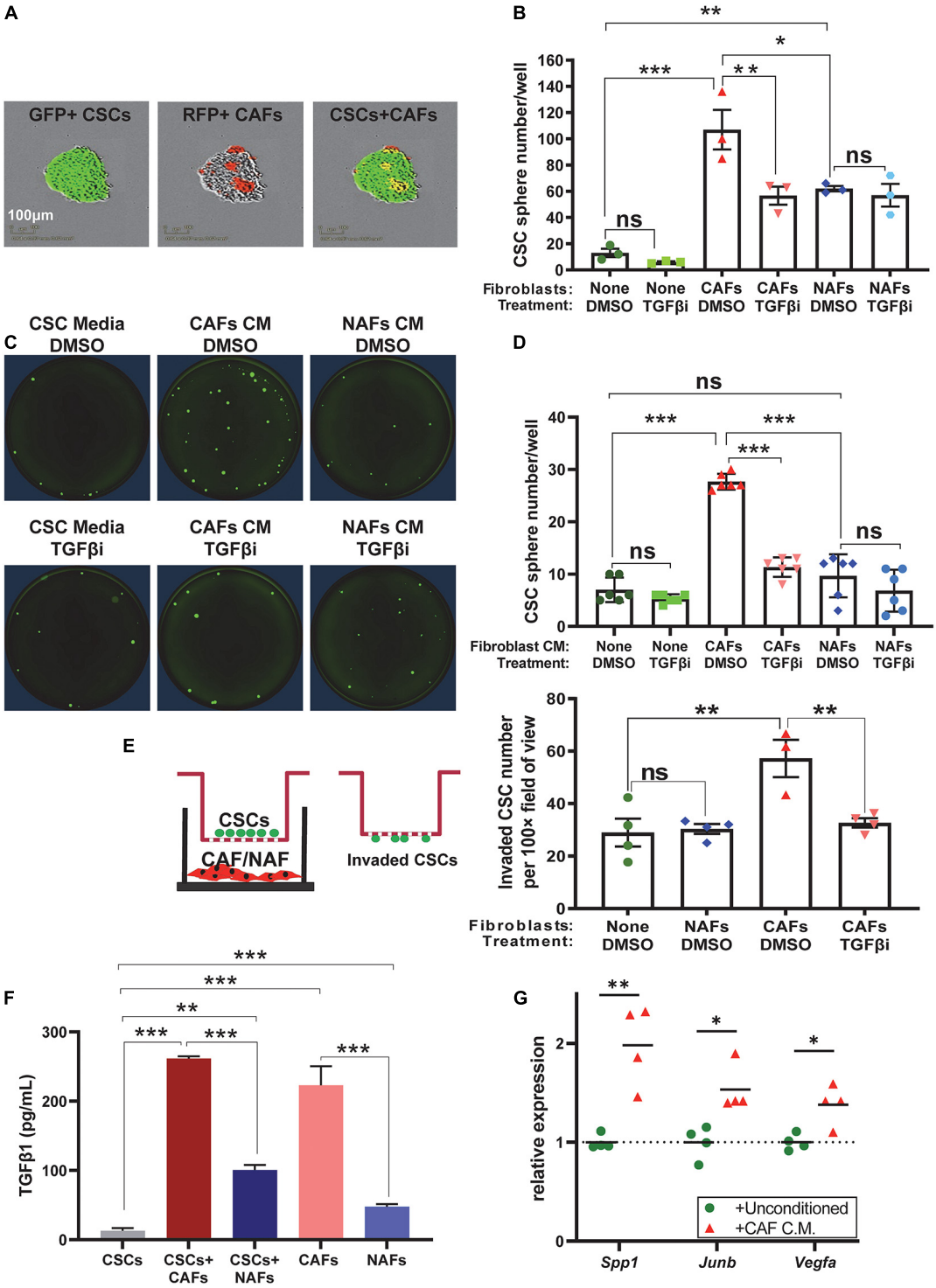

FIGURE 6 | Cancer-associated fibroblasts increased self-renewal and invasion of CSCs in a TGFβ-dependent manner *via* direct contact and paracrine effects. **(A)** Representative images of GFP$^+$ A223 CSCs and RFP$^+$ CAFs in spheroid co-culture. **(B)** Quantification of sphere number in each of the indicated A223 CSC treatment [vehicle (DMSO) or TGFβ inhibitor ("TGFβi")] ± fibroblast co-culture conditions. **(C,D)** Representative images and quantification of A223 CSC spheres cultured with the indicated conditioned media (CM) and vehicle (DMSO) or TGFβi. **(E)** Transwell invasion assay: A223 CSCs were seeded in the top Matrigel-coated chamber, and CAFs or NAFs were cultured in the bottom well. After 48 h, invaded CSCs attached to the underside of the upper chamber were quantified. **(F)** TGFβ1 concentration in each of the indicated CM was determined by ELISA. Either three or four technical replicates were conducted for each cell type. **(G)** The CM of CAFs or unconditioned control media was applied to recipient A223 cells in a 24 h sphere initiation assay. RNA harvested from A223 recipient cells was evaluated by RT-qPCR for the expression of TGFβ-target genes *Spp1*, *Junb*, and *Vegfa* normalized to the expression of *Gapdh*. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

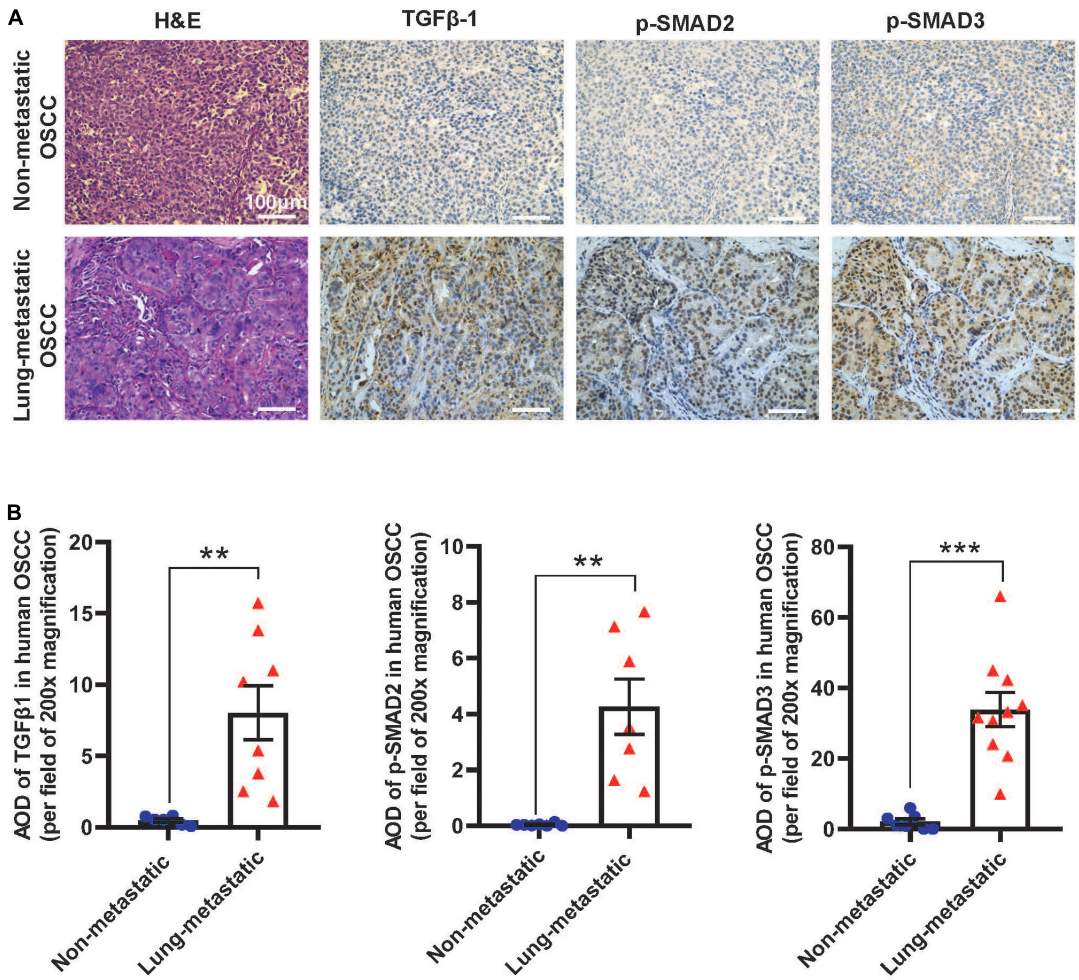

FIGURE 7 | TGFβ signaling is activated in primary human OSCC tumors in patients with lung metastasis. **(A)** Representative H&E and IHC images examining expression of the indicated markers in human OSCC. Upper panels are staining of the primary tumor of a representative patient without metastasis and lower panels are staining of the primary tumor from a representative patient with metastasis. Scale bar: 100 μm. **(B)** Quantification of TGFβ1, p-SMAD2, and p-SMAD3 staining by AOD value. $^{**}P < 0.01$, $^{***}P < 0.001$.

in the lung can rapidly induce immune suppression in a fashion similar to the primary tumor site as we previously observed (Mishra et al., 2016). Therefore, CAFs' effects are, to some extent, T cell-independent.

CAFs Promoted CSC Expansion and Invasion/Migration *via* Direct Contact and Paracrine Effects

The large absence of labeled CAFs in SCC lesions in the lung (**Figures 4A,H**) suggests that CAFs primarily provide a physical niche for CSCs and that CAFs do not proliferate with the tumor cells. This is evidenced by CSC self-renewal primarily expanding tumor cells with much fewer numbers of CAFs in CSC spheres (**Figure 6A**). Additionally, CAFs provide paracrine effects on promoting CSC expansion, evidenced by increased CSC spheres when CSCs were exposed to CAF conditioned culture media. The effects appear dependent upon TGFβ ligand, as TGFβ inhibitor attenuated this CAF-mediated CSC expansion. Our previous data have shown data that SP cells, but not CD49f+ CSCs, are a subpopulation of metastasis associated CSCs (White et al., 2013). Hence, we used sorted SP cells directly, but not CD49f+ CSCs, and our data provide mechanistic and *in vivo* validation that TGFβ signaling similarly promotes self-renewal of *Smad4* mutant SCC and does so *via* CAFs. CAFs also promoted TGFβ-associated CSC migration/invasion (**Figure 6E**), which could contribute to the increased sizes of lung SCC lesions by CAFs *in vivo* (**Figures 2**, **4**, **5**). In this specific model, TGFβ produced from CAFs can exert paracrine effects on CSCs *via* SMAD2/3-dependent, SMAD4-independent mechanisms (Oshimori et al., 2015; Yang et al., 2020), or non-canonical TGFβ signaling (Li et al., 2019; Woosley et al., 2019). Our data are consistent with previous reports that in several other cancer types, TGFβ activation promotes CSC properties including self-renewal (Woosley et al., 2019) and invasiveness (Oshimori et al., 2015). TGFβ responding-CSCs

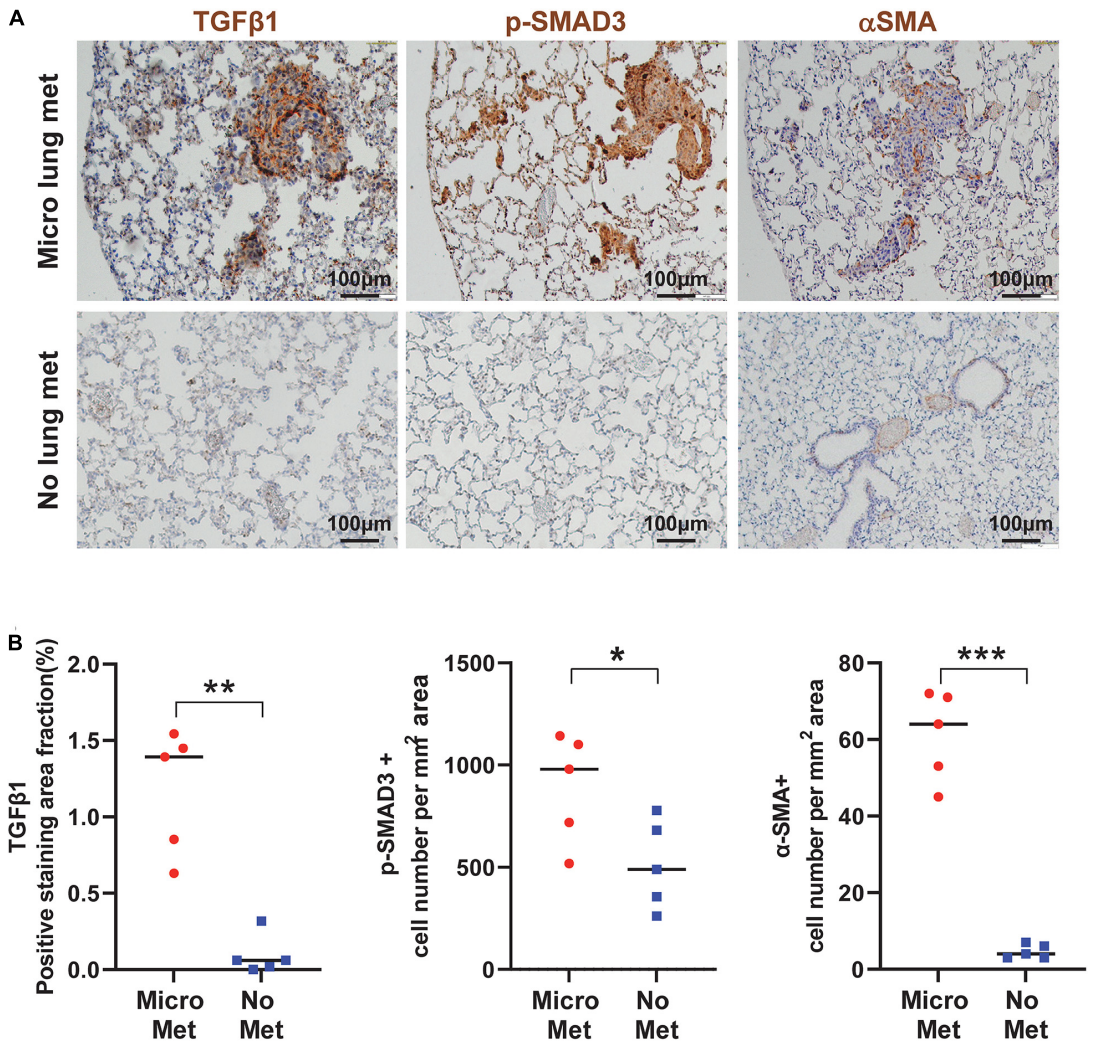

FIGURE 8 | TGFβ signaling is activated in the primary tumor associated with metastasis and the metastatic niche. **(A)** Representative IHC staining of TGFβ1, p-SMAD3, and αSMA in lungs from mice with or without micrometastasis at 4–5 weeks after B931 cell flank transplantation. Scale bar: 100 μm. **(B)** Quantification of TGFβ1, p-SMAD3, and αSMA in lungs with or without B931 micrometastasis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

are apoptosis-resistant (Oshimori et al., 2015) and induce the exhaustion of cytotoxic T cells (Miao et al., 2019), all of which benefit CSC expansion in the lung.

CAFs Could Be a Major Target of TGFβ Inhibition for Treating Advanced SCC

TGFβ1 is often overexpressed in cancer (Calon et al., 2012; Pickup et al., 2013; Wu et al., 2020; Yegodayev et al., 2020). Our current study demonstrated that CAFs could produce TGFβ1 at higher levels than tumor epithelial cells (**Figures 1E**, **6F**), and TGFβ signaling plays a major role in CSC expansion in a foreign (metastatic) microenvironment. Although these SCC models induce CAF formation in the primary tumor and we cannot discern the actions of co-transplanted CAFs vs. the actions of newly formed CAFs in the primary tumor or the metastatic microenvironment, the direct conditioning of the lung microenvironment by co-transplanted CAFs to increase lung colonization in a TGFβ-dependent manner suggests that CAF-induced TGFβ signaling is a critical step. The correlation between TGFβ pathway activation and metastasis status of human SCCs in this study further validates the translatability of our mouse SCC models, suggesting that targeting TGFβ signaling, even in severely immune-compromised SCCs and *Smad4* mutant SCC, could have two benefits: (1) inhibition of CSC properties; (2) inhibition of invasion/metastasis/niche preparation. These notions are further supported by previous findings that primary SCCs with metastasis possess more activated fibroblasts and CAFs than those without metastasis, and that TGFβ activation increases activated fibroblast/CAF numbers that in turn secrete more TGFβ1 (Sridhara et al., 2013; Luksic et al., 2015; Maqsood et al., 2020). Because biopsy of SCC lung metastasis in patients is generally not feasible, it is difficult to draw a definitive conclusion using human specimens whether TGFβ1 is sufficient to generate

a paracrine/systemic effect for metastatic niche preparation, but our mouse model demonstrates a correlation between TGFβ ligands and micrometastasis. Since i.v. CSCs/CAFs co-injection in our mouse model did not increase CSC seeding to the lung, it suggests that systemic TGFβ1 secreted from primary SCCs (including their resident CAFs) could facilitate CAF formation in the lung either at the pre-metastatic niche or metastatic niche after SCC are seeded. Future studies will identify molecular and cellular mechanisms of metastatic or pre-metastatic niche development contributed by CAFs and TGFβ.

In summary, our study identified that CAFs promote CSC properties for them to survive and expand at the foreign, metastatic microenvironment. TGFβ1 ligand produced by CAFs is the dominant driving force of these effects. Our findings compel future studies examining the premetastatic and metastatic niche promoted by CAFs so that treating SCC metastasis in patients can be explored.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found below: https://www.ncbi.nlm.nih.gov/biosample/18349411; https://www.ncbi.nlm.nih.gov/biosample/18349412; and https://www.ncbi.nlm.nih.gov/sra/PRJNA715402.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus.

AUTHOR CONTRIBUTIONS

XS, JL, and CY performed experiments, analyzed data, and generated figures. DD, FW, SH, and KW performed experiments. MR performed data analysis. X-JW, CY, and HZ designed and supervised the study. XS, JL, HZ, CY, and X-JW wrote 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.2021.668164/full#supplementary-material

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REVIEW
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Transforming Growth Factor-β: An Agent of Change in the Tumor Microenvironment

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Transforming Growth Factor-β (TGF-β) is a key regulator of embryonic development, adult tissue homeostasis, and lesion repair. In tumors, TGF-β is a potent inhibitor of early stage tumorigenesis and promotes late stage tumor progression and metastasis. Here, we review the roles of TGF-β as well as components of its signaling pathways in tumorigenesis. We will discuss how a core property of TGF-β, namely its ability to change cell differentiation, leads to the transition of epithelial cells, endothelial cells and fibroblasts to a myofibroblastoid phenotype, changes differentiation and polarization of immune cells, and induces metabolic reprogramming of cells, all of which contribute to the progression of epithelial tumors.

Keywords: TGF-β, tumor-stromal crosstalk, cancer associated fibroblast (CAF), EMT—epithelial to mesenchymal transition, EndMT—endothelial to mesenchymal transition, tolerogenic differentiation, metabolic reprogramming

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INTRODUCTION

Cellular communication is crucial during development, tissue maintenance and tissue repair, and miscommunication can result in loss of organismic integrity, disease and death of the organism. During tumorigenesis, cells start to proliferate uncontrollably and invade the surrounding tissues to the detriment of the organism. Although altered behavior of tumor cells is a major contributor to tumor growth, it is understood that the surrounding stroma not only tolerates but supports tumor growth. The stroma of solid tumors provides structural support and supplies nutrients to tumor cells, and when tumor cells metastasize to distant organs they might more easily grow in locations that provide suitable conditions. This seed-and-soil theory of metastatic growth was first coined by Paget (Paget, 1889). Fidler and Hart (Hart and Fidler, 1980) showed a century later that melanoma cells spread to lung or ovarian tissue but not to renal tissue independent of the primary tumor site and concluded that tumor growth indeed depends on properties of the tumor cells (seeds) and host (soil). We now understand that tumor cells affect stromal cells and vice versa, and that the crosstalk between different tumor compartments contributes to tumor progression (Bhowmick et al., 2004a,b; Kaplan et al., 2005; Stuelten et al., 2008; Van Hove et al., 2021).

Cells interact with each other and the surrounding acellular matrix by releasing and sensing regulatory molecules. One of the master regulators of tumor-stromal crosstalk is TGF-β. TGF-β

instructs cell proliferation and death, cell metabolism, cell motility and migration, tissue repair, and organ development (Morikawa et al., 2016). In tumors, TGF-β acts as a tumor suppressor during early stages of tumorigenesis by inhibiting cell proliferation and promoting cell death. As tumors progress, TGF-β promotes tumor growth and metastasis by inducing a mesenchymal transition of epithelial and endothelial cells, inducing myofibroblastoid differentiation, altering differentiation and proliferation of immune cells, modulating matrix composition, and reprogramming cell metabolism (Roberts and Wakefield, 2003; Seoane and Gomis, 2017; Hua et al., 2020; Derynck et al., 2021). Through highly regulated, local activation, TGF-β has varied and context-dependent effects including the activation of specific Smad signaling cascades and alternative signaling pathways like PI3K/AKT or MAPK signaling; in addition, cross-talking with a multitude of signaling networks such as SDF1-, FGF- HGF-, EGF- or Hippo-, Wnt-, or Rho-signaling occurs (Mu et al., 2012; Luo, 2017; Zhang, 2017; Kim et al., 2018; Miyazono et al., 2018).

TRANSFORMING GROWTH FACTOR-β SIGNALING: PATHWAYS AND MECHANISMS

TGF-β, which exists in three isoforms, is synthesized as a propeptide consisting of the active TGF-β and the latency associated protein (LAP). The propeptide is cleaved by furin or furin-like protease during maturation, but LAP and TGF-β remain strongly associated via non-covalent interactions. LAP is tethered to latent TGF-β binding protein (LTBP) or glycoprotein-A repetitions predominant proteins (GARPs) to form latent complexes that shield the active TGF-β and prevent it from binding to receptors (Robertson and Rifkin, 2016). As such, most of the TGF-β deposited in the extracellular space is inactive, although active TGF-β is observed in specific locations (Barcellos-Hoff et al., 1994). Bioavailability of TGF-β is additionally regulated by TGF-β-binding proteins like fibromodulin and decorin which sequester TGF-β and prevent it from binding to specific TGF-β receptors (Hinz, 2015; Khan and Marshall, 2016; Nastase et al., 2018; Aubert et al., 2021). Activation of latent TGF-β is a key step in the regulation of TGF-β-signaling activity. During activation, active TGF-β is released from the latent complex by local changes in pH or shear stress, TSP1-, tenascin- or integrin binding, or by proteolytic cleavage by matrix metallo- and other proteases. Of those, integrin-mediated TGF-β activation is of particular importance, and loss of integrin-mediated TGF-β1 activation mimics the phenotype of TGF-β1-null mice (Yang et al., 2007). Likewise, mice lacking αvβ6- and αvβ8-integrins mimic the abnormalities of TGF-β1- and TGF-β3-null mice (Aluwihare et al., 2009). Integrin-mediated TGF-β activation depends on the recognition and binding of LAP's RGD motif by integrin αv. Two mechanisms of integrin-mediated TGF-β activation are known: traction force mediated release of active TGF-β, typically seen for αvβ6 integrin (**Figure 1-1**), and release of TGF-β by proteolytic cleavage of LAP, observed for αvβ8 integrin (**Figure 1-2**; Nolte and Margadant, 2020). Integrin αvβ6 is tethered to the actomyosin cytoskeleton. After binding LAP, αvβ6 integrins link the latent complex to the actomyosin cytoskeleton. Because the latent TGF-β complex is also connected to the extracellular matrix, actomyosin generated traction forces pull on and lead to conformational changes of the latent complex and release of active TGF-β (Buscemi et al., 2011; Klingberg et al., 2014; Hinz, 2015). Notably, in this model of traction force-mediated TGF-β activation the extracellular matrix provides the counterforce for actomyosin contraction; therefore, changes in matrix stiffness should affect the traction-force mediated release of TGF-β. Indeed, integrin-mediated TGF-β activation is more efficient in stiff matrix with an elastic modulus $>$ 10 kPa than in soft matrix (Klingberg et al., 2014; Hinz, 2015; Hiepen et al., 2020). In contrast, integrin αvβ8 does not interact with the cytoskeleton and thus cannot release active TGF-β by mechanical force transduction. It instead requires a chaperone, GARP or LRRC33, and proteases such as MT1-MMP (MMP14) to proteolytically cleave LAP and release active TGF-β (Mu et al., 2002; Liénart et al., 2018).

Once activated, TGF-β binds to TGF-β receptor type 2 (TGFβR2) to initiate signaling downstream. Upon binding TGF-β, TGFβR2 heterodimerizes with and phosphorylates TGF-β receptor type 1 (TGFβR1, ALK5) (**Figure 1-3**). In the canonical pathway, the activated receptor complex binds and phosphorylates receptor-regulated Smads (R-Smads), Smad2 and Smad3, which in turn heterotrimerize with the common Smad (Co-Smad), Smad4, to regulate TGF-β dependent gene expression (Shi and Massagué, 2003). The activity of the Smad signaling cascade is modulated by inhibitory Smads (I-Smad), Smad6 and Smad7, and Smurf1 and Smurf2, E3 ligases which ubiquitinylate TGF-β receptors and Smad proteins (Kavsak et al., 2000; Zhang et al., 2001; Tang et al., 2011; Nicklas and Saiz, 2013; Miyazawa and Miyazono, 2017; Yan et al., 2018; Sinha et al., 2021).

Several alternative TGF-β signaling cascades branch off the canonical signaling pathway beginning at the receptor level. Endoglin (CD105) is an accessory TGF-β receptor expressed in endothelial cells (Gougos and Letarte, 1988). Endoglin cannot bind TGF-β itself but does bind TGF-β1 and TGF-β3 when these interact with TGFβR3. Endoglin does not bind to TGF-β2 in any form (Barbara et al., 1999; Pawlak and Blobe, 2021). It facilitates the interaction of TGF-β and TGFβR2 with the non-classic type-1 receptor, ACVRL1/ALK1 (Nogués et al., 2020) and leads to a shift from TGF-β/TGFβR1/R2/Smad2/3 to TGF-β/ACVRL1/Smad1/5/8 signaling (**Figure 1-4**). Similarly, TGF-β can induce Smad1/5/8-signaling via formation of mixed TGFβR1/ALK5/ALK2 complexes (Ramachandran et al., 2018; **Figure 1-5**). In addition to Smad-signaling cascades, the activated TGFβR2/ALK5 receptor complex can activate TRAF6-TAB1-TAK1 and downstream p38 and JNK signaling (Sorrentino et al., 2008; Yamashita et al., 2008). The receptor complex can also activate PI3K/AKT signaling and feed into Ras/MEK/Erk, Rho/Rock, CDC42/Rac/Pac and Jak/Stat signaling cascades (Lee et al., 2007; Mu et al., 2012; Zhang et al., 2013; Tang L.-Y. et al., 2017; Zhang, 2017; **Figure 1-6**). Further downstream, activated Smad3 in the

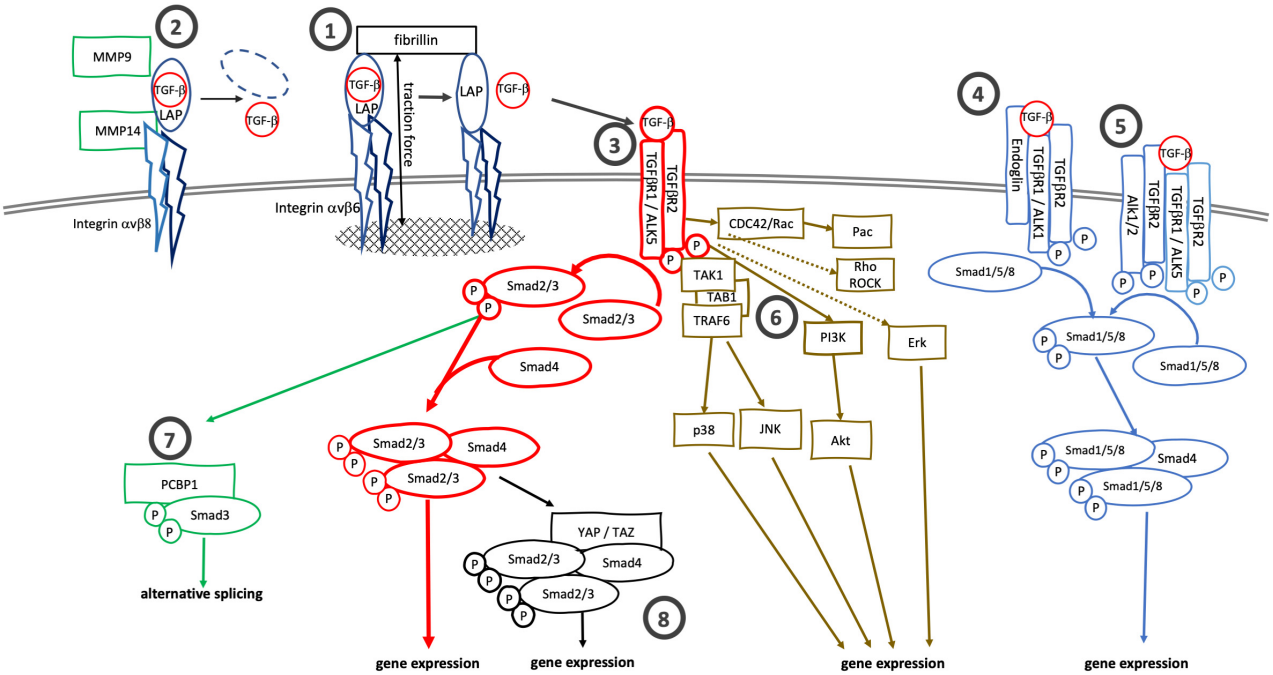

FIGURE 1 | Canonical and alternative TGF-β signaling cascades. TGF-β can be activated by **(1)** traction force mediated release from the latent complex after binding to integrin αvβ6 or by **(2)** proteolysis after binding of the latent complex to integrin αvβ8. **(3)** TGF-β binds to specific receptors, TGFβR2 and TGFβR1 (ALK5), to initiate canonical Smad2/3 signaling. Alternatively, TGF-β can bind to **(4)** TGFβR2/ALK1/endoglin complexes or **(5)** TGFβR2/ALK5—TGFβR2/ALK1/2 complexes to activate Smad1/5/8 signaling. Alternatively to the Smad2/3 signaling cascade TGFβR2/ALK5 complexes can also activate MAPK-, Rho/ROCK- and cdc42/Rac/Pac signaling cascaded. pSmad3 itself can bind to **(7)** PCBP1 to support alternative mRNA splicing. **(8)** The binding of Smad2/3/Smad4 complexes to YAP/TAZ also alters gene expression profiles.

presence of CDK-, MAPK-, AKT- or PAK1-signaling can bind to PCBP1 and mediate alternative splicing (Tripathi et al., 2016; **Figure 1**-7).

TRANSFORMING GROWTH FACTOR-β SIGNALING IN TUMORS

Transforming Growth Factor-β, an Agent of Change

TGF-β is known as a potent growth inhibitor of cells of epithelial orgin, but it was first described and isolated based on its ability to transform cells and found expressed in different tumors and cell types (de Larco and Todaro, 1978; Roberts et al., 1980). In fact, TGF-β is secreted by and can act on most cells. The effects of active TGF-β are context specific (Guido et al., 2012). During development, TGF-β induces epithelial-mesenchymal transition (EMT) and facilitates gastrulation and organismic development as well as tissue repair (Thiery et al., 2009). Similarly, endothelial-mesenchymal transition (EndMT) and fibroblast-myofibroblasts transition is observed during development and tissue repair. Dysregulated EMT, EndMT and myofibroblastoid differentiation are seen in fibrotic diseases, vascular malformations, epithelial dedifferentiation and tumor growth; in advanced stages of cancers, TGF-β-induced EMT promotes tumor invasion, metastasis, and chemo-resistance (Tsubakihara and Moustakas, 2018; Katsuno and Derynck, 2021).

Cancer-Associated Fibroblasts—Bystanders Turned Culprit

Originally considered a mere presence in tumors, CAFs are now appreciated as active partners in tumor development. CAFs can modulate stemness, proliferation, invasion and dissemination of tumor cells, ECM composition, inflammatory infiltration, angiogenesis and drug resistance. They are derived from various progenitors including resident fibroblasts, mesenchymal stem cells, adipose tissue derived stem cells, and endothelial cells. Such diverse origins confer a marked heterogeneity of CAF gene expression profiles (Calon et al., 2014; Mezawa and Orimo, 2021). Nevertheless, a core signature of TGF-β regulated ECM genes has been identified in many CAFs and goes along with poor prognosis (Navab et al., 2011; Calon et al., 2015; Chakravarthy et al., 2018).

The roles of TGF-β signaling in CAFs have been demonstrated in more detail in breast cancer models, in which TGF-β and SDF1 are part of two autocrine and cross-talking signaling loops that drive myofibroblast/CAF development at the invasive front (Kojima et al., 2010; Yu et al., 2014). Increased expression of the TGF-β target SNAI1 in fibroblasts leads to increased SDF-1 secretion (Blanco-Gómez et al., 2020). The CAF-secreted TGF-β and SDF-1 promote angiogenesis by recruiting endothelial progenitor cells, and increase growth and EMT of tumor cells

(Orimo et al., 2005; Yu et al., 2014; Matsumura et al., 2019). At the same time, autocrine myofibroblast TGF-β/SDF-1 signaling attenuates expression of CD26 (Dpp4), which can cleave SDF-1, such further increasing SDF-1 signaling (Mezawa et al., 2019). Thus, once triggered, this positive feedback loop maintains myofibroblast differentiation and supports tumor progression by targeting endothelial and tumor cells.

A consequence of fibroblast-myofibroblast transition, ECM secretion by CAFs changes such that matrix stiffness and density increases. This not only impacts migration of tumor cells through the matrix, immune infiltration, vascularization and drug delivery, it also affects cell differentiation and integrin signaling. Increased ECM stiffness directly impacts epithelial differentiation via increasing integrin clustering and Erk and Rho-signaling, and promoting a malignant phenotype (Wozniak et al., 2003; Paszek et al., 2005; Lu et al., 2012). *In vivo*, the elastic modulus of tumors increases as the tumor grows, and can reach 40–50 kPa (Samani et al., 2007; Kawano et al., 2015; Wang et al., 2017), making integrin-mediated TGF-β activation more effective (Klingberg et al., 2014; Hinz, 2015; Hiepen et al., 2020) and impacting tumor progression. Indeed, high αvβ6 expression correlates with worse prognosis in breast cancer, and integrin β6 neutralizing antibody decreased tumor growth in xenograft models of breast cancer (Moore et al., 2014). On a cellular level, integrin β1 signaling is necessary for TGF-β mediated p38-signaling and EMT in mammary epithelial cells (Bhowmick et al., 2001), and in basal carcinoma, αvβ6-mediated TGF-β activation in epithelial cells leads to fibroblast-myofibroblasts transition and secretion of HGF by myofibroblasts; HGF in turn promotes invasiveness of tumor cell (Marsh et al., 2008).

Cross-talk between activated TGF-β- and YAP/TAZ-signaling can further increase matrix stiffness via alternative signaling cascades (**Figure 1–8**). To this end, YAP associates with Smad7 to increase its affinity to the TGFβR1 and to increase its inhibitory effect on TGF-β signaling (Ferrigno et al., 2002). Further downstream, YAP can bind Smad3 to form a YAP-TEAD4–Smad3-p300 complex on the promotor of CTGF, a cytokine involved in EMT and tumor progression (Fujii et al., 2012; Sonnylal et al., 2013; Zhu et al., 2015). TAZ controls the nucleocytoplasmic localization of the Smad2/3-Smad4 complex by binding to Smad2/3-Smad4 and increasing nuclear accumulation of Smad2/3-Smad4 (Varelas et al., 2008). In both cases, the YAP/TAZ-Smad complexes increase the fibrotic response (Piersma et al., 2015). Matrix stiffness itself can increase YAP/TAZ activation (Dupont et al., 2011) as well as TGF-β activation, forming another positive feedback circle to drive tissue fibrosis and tumor progression.

Proteolytic degradation of the ECM, for example by MMPs, is also important for tumor progression. TGF-β regulates MMP expression and MMPs proteolytically activate TGF-β. For example, tumor cell derived TGF-β can increase MMP9-secretion by fibroblasts (Stuelten et al., 2005). MMP9 in turn can bind to CD44, and then proteolytically cleave LAP and release TGF-β in addition to remodeling the extracellular matrix (Yu and Stamenkovic, 2000). As MMPs are released into the extracellular space, activation of TGF-β by this mechanism is likely less localized than traction-force dependent αvβ6-mediated activation. Other differences between these two types of TGF-β activation are that αvbβ6-mediated activation is effective in ECM stiffness, while proteolytic activation might function in soft matrix and concurs with softening of the matrix as proteins like collagens are degraded. In turn, the degradation of ECM proteins by MMPs "opens" the matrix and might allow for smoother travel of tumor cells through the extracellular space.

In summary, CAFs contribute to tumor progression by changing ECM composition and stiffness as well as the cytokine microenvironment in the tumor. As CAF-mediated changes in matrix composition spread through the environment, one might hypothesize that the resulting changes in matrix stiffness and TGF-β activation contribute to the spread of malignant cell phenotypes through the surrounding environment.

Endothelia—More Than the Coating of the Vascular Wall

Tumors depend on blood supply for nutrients, and thus need to co-opt vessels in order to travel to distant sites. TGF-β can modulate neoangiogenesis and induce EndMT. TGF-β stimulates neoangiogenesis by inducing VEGF expression in tumor and stromal cells like macrophages in a Smad3-dependent manner (Donovan et al., 1997; Benckert et al., 2003; Kaminska et al., 2005; Sun et al., 2018). Further effects of TGF-β on endothelial cells are due the presence of the TGF-β Coreceptor Endoglin.

Endoglin has an important role in regulating angiogenesis and endothelial function (Cheifetz et al., 1992; Düwel et al., 2007; Albiñana et al., 2017). Endoglin is found to be overexpressed in the tumor neovasculature of brain, lung, breast, stomach and colon (Minhajat et al., 2006). In animal models, endoglin overexpression in tumor vasculature leads to leaky vessels with an incomplete mural coverage (Nogués et al., 2020; Ollauri-Ibáñez et al., 2020); on the other hand, haplo-insufficiency reduces the neovascularization and growth of Lewis lung tumors (Düwel et al., 2007). Mechanistically, endoglin shifts TGF-β signaling from canonical TGFβR2/ALK5-Smad2/3-signaling to the alternative TGFβR2/ALK1-Smad1/5/8 signaling cascade. While TGF-β/ALK5 signaling blocks cell proliferation, TGF-β/ALK1 signaling increases cell proliferation and motility (Lebrin et al., 2004). In addition, endoglin interacts with VEGFR2 in a VEGF-dependent manner to prevent its degradation to support tip cell formation (Tian et al., 2018). These observations support a general notion that increased endoglin expression shifts TGF-β signaling toward supporting tumor growth.

TGF-β-induced EndMT, similar to EMT, is characterized by upregulation of mesenchymal markers like α-SMA, FSP-1, vimentin and N-cadherin, by upregulation of transcription factors like Snail, Slug, Twist, and by downregulation of adhesion proteins like VE cadherin, CD31/PECAM-1 (Platel et al., 2019; Ma et al., 2020). This shift in gene expression results in endothelial cells undergoing EndMT. The loss of cell-cell contacts in the endothelial sheet during early EndMT facilitates the passing of tumor cells through the endothelial layer (Gasparics et al., 2016); later, endothelial cells acquire a pro-fibrotic phenotype with increased motility and a pro-inflammatory secretory profile, and finally convert into CAFs.

Indeed, up to 40% of total CAFs in a tumor can be derived from endothelial cells (Zeisberg et al., 2007).

Mechanistically, EndMT is triggered by canonical TGF-β-signaling via ALK5/Smad2/3 or alternative signaling via TGF-β/ALK5/PI3K/Ras/TAK1 (Platel et al., 2019; Ma et al., 2020). The three TGF-β isoforms play different roles in EndMT. In colon cancer, TGF-β2 is the most important TGF-β isoform to induce EndMT (Wawro et al., 2018). Effects of TGF-β1 and TGF-β3 on EndMT are mediated by increased TGF-β2 secretion in immortalized human dermal endothelial cells, and knockdown of TGF-β2 blocks TGF-β1/2-induced EndMT (Sabbineni et al., 2018). Interestingly, the affinity of TGF-β1 and TGF-β3 to TGFβR2 is about 200–300-fold higher than that of TGF-β2 (Pawlak and Blobe, 2021). Thus, TGF-β1/3 induced ALK5 signaling might be active at low TGF-β concentrations and drive neoangiogenesis in the presence of endoglin, while TGF-β2 signaling is activate when high concentrations of TGF-β2 out-compete TGF-β1/3-binding to TGFβR2.

Transforming Growth Factor-β and the Immune System—Suppression and Polarization

TGF-β affects the immune response to tumors on several levels: it modulates accessibility of tumors for immune cells by increasing matrix density and regulating neoangiogenesis, and it regulates proliferation, differentiation and migration of immune cells.

Generally, tumor-derived TGF-β can attract myeloid and lymphoid cells, but it also leads to immunosuppression and immune evasion of tumors by changing proliferation and differentiation of residential T cells, neutrophils and macrophages, dendritic cells and NK cells (Batlle and Massagué, 2019; Brown and Marshall, 2019). Specifically, TGF-β inhibits T-cell proliferation as well as Th1 differentiation by inhibiting IL-2 expression, and together with other cytokines promotes Treg and Th17 differentiation (Zhang, 2018). Smad3/E4BP4 signaling inhibits NK cell development and reduces immune surveillance of melanoma and lung tumors (Tang P. M.-K. et al., 2017). Furthermore, tumor derived TGF-β together with other cytokines shifts the balance of tumor associated macrophages (TAM) and neutrophils (TAN) from TAM1 toward pro-tumorigenic TAM2 (Gong et al., 2012) and from TAN1 toward pro-tumorigenic TAN2 (Fridlender et al., 2009). Together, the shift toward Treg, Th17, M2 and N2 differentiation lead to a tolerogenic immune response to tumors.

The polarization of immune cells can increase their capacity to activate TGF-β. It is worth noting that immune cells, which have high motility and are not well anchored into the extracellular matrix, often employ αvbβ8-mediated TGF-β activation which relies on proteolytic TGF-β activation, rather than αvβ6-mediated activation which relies on traction forces and requires robust cell-matrix contacts. Integrin αvb8 is found on monocytes, macrophages, dendritic cells and Tregs (Fenton et al., 2017; Nolte and Margadant, 2020). Tregs, in contrast to naïve T cells, express high levels of αvβ8 and require it to release active TGF-β from the LAP/GARP complex, which in turn leads to Treg-mediated immunosuppression (Edwards et al., 2014; Stockis et al., 2017). αvβ8-activated TGF-β is necessary to quench inflammation and auto-immunity, but also to prevent anti-tumor immunity through increased Treg activity (Brown and Marshall, 2019). Likewise, αvβ8 expression on dendritic cells leads to immunosuppression (Travis et al., 2007; Fenton et al., 2017). Furthermore, αvβ8 is upregulated on M2- and downregulated on M1-macrophages (Kelly et al., 2018). In mouse models blocking of αvβ8 by monoclonal antibodies suppresses growth of squamous cell carcinoma, mammary cancer, colon cancer and prostate cancer, emphasizing the role αvβ8/TGF-β mediated immune tolerance of tumors (Dodagatta-Marri et al., 2020).

Changes of TGF-β expression and signaling in immune cells can also contribute to tumor progression. CD2-driven overexpression of TGF-β in T lymphocytes leads to delayed tumor development in dextran sodium sulfate/azoxymethane-induced colonic tumorigenesis (Becker et al., 2004). Smad3 null mice show a variety of abnormalities of the immune system, including an activated phenotype of T-lymphocytes, impaired chemotactic response of neutrophils to TGF-β, and chronic intestinal inflammation which can concur with colon tumors in aging mice (Yang et al., 1999). Loss of Smad4 in T lymphocytes increases pro-inflammatory cytokine expression and leads to increased development of epithelial tumors (Hahn et al., 2011).

Although high TGF-β-signaling in tumors leads to immune tolerance, loss of epithelial or fibroblast TGF-β signaling increases inflammation and promotes tumorigenesis: Epithelial loss of Smad4 increases inflammatory infiltration and development of dextran-sulfate-induced colon tumors; and loss of fibroblast TGF-βRII has been associated with increased inflammation, DNA damage in epithelial cells, and tumor formation in the forestomach (Achyut et al., 2013; Means et al., 2018). Thus, dysregulation of TGF-β signaling in different tumor compartments can modulate the immune response to promote tumorigenesis.

In tumor immune microenvironment, upregulated immune checkpoints protect cancer cells from immune killing (Munn and Bronte, 2016). PD-1/PD-L1 is the currently most studied immune checkpoint pathway. TGF-β has been shown to increase PD-1 expression on immune cells, while anti-PD-1 increases tumor cell pSMAD3 and can induce immunosuppression (Baas et al., 2016; Park et al., 2016; Dodagatta-Marri et al., 2019; Wu et al., 2020). Thus, blockade of TGF-β signaling enhances the effects of PD-1 inhibitors or overcomes primary resistance to PD-1 blockade *in silico* and *in vivo* (Terabe et al., 2017; Strauss et al., 2018; Chen et al., 2021; Siewe and Friedman, 2021).

Tumor Metabolism—A Symbiotic Relationship of Parenchymal and Mesenchymal Cells

To compensate for restricted blood and nutrient supply in tumors, another property of TGF-β comes in handy: it can shift the metabolism of cells in the tumor environment such that a symbiotic relationship between tumor cells and stromal cells results (Yoshida et al., 2019; Angioni et al., 2021).

Early on, it was observed that TGF-β increases glucose uptake and lactate secretion of cells (Inman and Colowick, 1985;

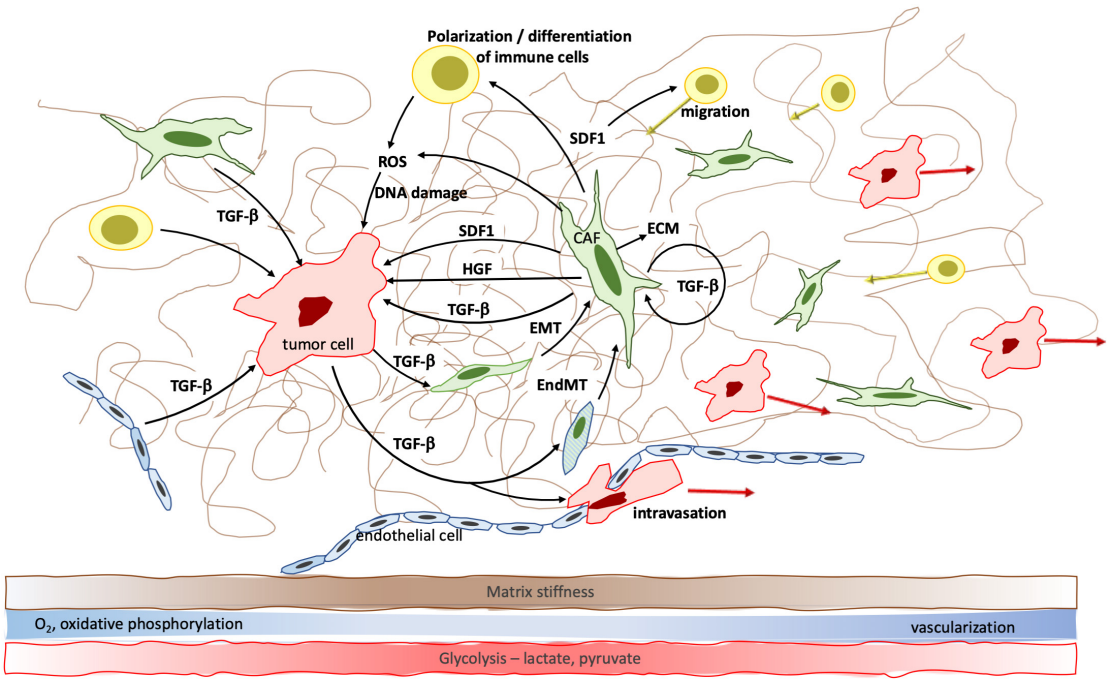

FIGURE 2 | TGF-β facilitates cell-cell communication within the tumor microenvironment and changes cell differentiation, polarization and metabolism to promote tumor growth. Specifically, TGF-β induces fibroblast—myofibroblast transition, epithelial—mesenchymal transition (EMT) and endothelial—mesenchymal transition (EndMT) which result in increased cancer associated fibroblast (CAF) density. CAFs significantly contribute to increased matrix stiffness. EndMT furthermore leads to reduced endothelial cell-cell contacts which facilitates transmigration of tumor cells and metastatic spread. The effect of TGF-β on cell metabolism leads to a shift from oxidative phosphorylation to anaerobic glycolysis and accumulation of lactate, pyruvate and genome-damaging ROS in the hypoxic tumor center.

Esposito et al., 1991). TGF-β signaling is now known to affect oxidative phosphorylation, the pentose phosphate pathway, glycolysis, fatty acid oxidation, and amino acid metabolism (Yadav et al., 2011; Angioni et al., 2021). In general, TGF-β shifts metabolism from mitochondrial oxidative phosphorylation toward a ketogenic metabolism, and EMT and EndMT, which are induced by TGF-β, can shift tumor and endothelial cell metabolism from oxidative phosphorylation toward anaerobic glycolysis (Angioni et al., 2021). Such a switching of the tumor metabolism from oxidative phosphorylation to anerobic glycolysis and lactate production was first described by Warburg (Warburg et al., 1927; Kim and Baek, 2021).

Mechanistically, auto- or paracrine TGF-β signaling reduces Cav-1 expression and concomitantly CD36 expression which leads to increased ROS production and HIF-1α stabilization. HIF-1α in turn increases glycolysis and increased lactate production (Guido et al., 2012; Heinzelmann et al., 2018; Yoshida et al., 2019). In tumor cells, TGF-β upregulates MCT1, increasing their capacity to uptake metabolites like lactate (Uddin et al., 2020).

The byproducts of anerobic glycolysis themselves have effects on cells and can further disturb cell and tissue physiology (Angioni et al., 2021). Specifically, lactate, which in tumors can be as high as 40 mM (Walenta et al., 2000), increases collagen production by fibroblasts and endothelial cells, endothelial cell migration and stimulates IL-8-dependent angiogenesis (Beckert et al., 2006; Végran et al., 2011). Lactate also has many effects on immune cells: it inhibits proliferation, cytokine production and cytotoxic activity of cytotoxic CD8 cells; increases ARG-1 expression in macrophages, such reducing T-cell activation and proliferation; and leads to differentiation of tolerogenic dendritic cells (Fischer et al., 2007; Nasi et al., 2013; Peter et al., 2015; Romero-Garcia et al., 2016).

In addition to its effects on energy metabolism, TGF-β-induced metabolic reprogramming of CAFs leads to increased reactive oxygen species (ROS) production and ROS accumulation by inactivation of CSK3 and the mitochondrial complex IV (Byun et al., 2012). The increased ROS levels in the tumor increase inflammation and DNA damage in tumor cells, and such further advance tumor progression.

TGF-β-mediated metabolic reprogramming of CAFs can spread to neighboring cells (Guido et al., 2012). Conceivably, once triggered, large parts of the tumor stroma might convert to a "Warburg-like" cancer metabolism. This metabolic flexibility would allow CAFs and other cells to better adapt to the changing demands of the tumor microenvironment to hypoxic and aerobic zones: in the fibrotic and hypoxic tumor core, tumor cells, fibroblasts and endothelial cells can utilize glucose by anaerobic glycolysis and secrete lactate and pyruvate, while at the oxygen-rich edges of the tumor lactate and pyruvate can be taken up by tumor cells, fibroblasts and endothelial cells and entered into the citrate cycle. In summary, TGF-β induces a metabolic plasticity that allows cells to successfully adapt to and thrive in the challenging and ever-changing tumor environment.

CONCLUSION

From its discovery 40 years ago to today, TGF-β has proven to be a major player in cell biology. The tightly regulated temporospatial activation of TGF-β as well as its wide network of canonical and alternative signaling cascades and cross-talking with other signaling networks lead to cell- and compartment specific effects. Aside from the suppression of tumor cell proliferation during the early phases of tumorigenesis, the effects of the universally present TGF-β on cells are many; in their core, they relate to cell metabolism and differentiation (**Figure 2**). It is these effects that explain TGF-β's unique and multifaceted role in tumor progression, from stiffening of the tumor matrix, to neoangiogenesis, to immune tolerance, and to metabolic changes throughout the varying tumor areas. As a consequence, tumor and other cells acquire increased adaptability that enables them to thrive in hypoxic, nutrient poor and stiff tumor areas as well as in the more pliable, well vascularized marginal areas, and to contribute to tumor progression.

While the mechanisms by which TGF-β exerts its functions are increasingly unraveled, many questions still remain. How are some of the effects of TGF-β compartment specific when cells are exposed to TGF-β from different sources, that is, when fibroblasts respond to tumor cell derived TGF-β but not their own, how do they sense the difference? And regarding the activation of TGF-β one wonders: Does integrin-binding of LAP merely serve the release of active TGF-β, or also lead to active integrin signaling? Does LAP have additional functions once TGF-β is released?

As research into the mechanism of TGF-β signaling is ongoing, several clinical studies exploring the effect of modifying TGF-β signaling on tumor growth have been launched in the past two decades, starting with the pan-TGF-β binding antibody ID11. Other strategies employed in modifying TGF-β signaling for therapeutic purposes include antisense oligonucleotides, small molecule receptor kinase inhibitors, and peptide aptamers (Xie et al., 2018; Liu et al., 2021). With targeting immune checkpoints as a major focus of current cancer therapies, several clinical trials with combined inhibition of PD1/PD-L1 and TGF-β are ongoing. In addition, bifunctional fusion proteins targeting PD-L1 or CTLA-4 and the TGFβR2 to inhibit TGF-β pathway and immune checkpoint simultaneously, were shown to be superior to PD-1 or CTLA-4 inhibitors in controlling tumor growth *in vitro* and *in vivo* (David et al., 2017; Lan et al., 2018; Ravi et al., 2018).

Future clarification of the cell- and context specific effects of TGF-β will help to further harness its signaling network for tumor therapy.

AUTHOR CONTRIBUTIONS

CS and YZ conceived and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Canonical and Non-canonical TGFβ Signaling Activate Autophagy in an ULK1-Dependent Manner

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The mechanism(s) in which transforming growth factor beta 1 (TGFβ) modulates autophagy in cancer remain unclear. Here, we characterized the TGFβ signaling pathways that induce autophagy in non-small cell lung cancer cells, using cells lines stably expressing GFP-LC3-RFP-LC3ΔG constructs that measure autophagic flux. We demonstrated that TGFβ1 increases Unc 51-like kinase 1 (ULK1) protein levels, 5′ adenosine monophosphate-activated protein kinase (AMPK)-dependent ULK1 phosphorylation at serine (S) 555 and ULK1 complex formation but decreases mechanistic target of rapamycin (mTOR) activity on ULK1. Further analysis revealed that the canonical Smad4 pathway and the non-canonical TGFβ activated kinase 1/tumor necrosis factor receptor-associated factor 6/P38 mitogen activated protein kinase (TAK1-TRAF6-P38 MAPK) pathway are important for TGFβ1-induced autophagy. The TAK1-TRAF6-P38 MAPK pathway was essential for downregulating mTOR S2448 phosphorylation, ULK1 S555 phosphorylation and autophagosome formation. Furthermore, although siRNA-mediated Smad4 silencing did not alter mTOR-dependent ULK1 S757 phosphorylation, it did reduce AMPK-dependent ULK1 S555 phosphorylation and autophagosome formation. Additionally, Smad4 silencing and inhibiting the TAK1-TRAF6-P38 MAPK pathway decreased autophagosome-lysosome co-localization in the presence of TGFβ. Our results suggest that the Smad4 and TAK1-TRAF6-P38 MAPK signaling pathways are essential for TGFβ-induced autophagy and provide specific targets for the inhibition of TGFβ in tumor cells that utilize autophagy in their epithelial-mesenchymal transition program.

Keywords: macroautophagy, ULK1, autophagic flux, mTOR, tumorigenesis, lung cancer, LC3B

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Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; aPKC, atypical protein kinase C; ATG, autophagy related; DMSO, dimethyl sulfoxide; EMT, epithelial-mesenchymal transition; F-12K, Kaighn's modification of Hams F-12; FBS, fetal bovine serum; GFP, green fluorescent protein; HRP, horseradish-peroxidase; K, lysine; LC3, microtubule-associated membrane protein light-chain 3; NSCLC, non-small cell lung cancer; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide-3 kinase; P-Smad2, phosphorylated Smad2; R-Smad, receptor-Smad; RFP, red fluorescent protein; RPMI, Roswell Park Memorial Institute; SARA, smad anchor for receptor activation; S, serine; SDS-PADE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TAK1, TGFβ-activated kinase 1; TGFβ1, transforming growth factor beta 1; TGFβR, transforming growth factor beta receptor; TRAF6, tumor necrosis factor receptor-associated factor 6; ULK, unc 51-like kinase.

INTRODUCTION

Macroautophagy, hereafter referred to as autophagy, is a catabolic process facilitated by lysosomes and acidic late endosomes that degrade macromolecules and organelles to replenish the building blocks for nucleic acids, proteins, carbohydrates, and lipids (Feng et al., 2014). Virtually all cells increase the rate of autophagy (autophagic flux) to eliminate the influx of damaged cellular materials mediated by cell stress to survive (Ding et al., 2007). However, cells have mechanisms to dampen autophagic flux because excessive degradation may initiate cell death (Shi et al., 2012). For example, cells modulate autophagic flux through post-translational modifications of autophagy related protein 1 (ATG1) (Yang and Klionsky, 2020). The phosphorylation status and activation of ATG1—Unc 51-like kinase 1 (ULK1) in mammals—is determined by a balance between mechanistic target of rapamycin (mTOR) and 5′ adenosine monophosphate activated protein kinase (AMPK) activity (Hosokawa et al., 2009; Makhov et al., 2014). When the rate of autophagy is detrimental to cells, mTOR phosphorylates ULK1 at serine (S)757 to disrupt ULK1-AMPK interactions (Kim et al., 2011). Alternatively, cell stressors impede mTOR and activate AMPK to directly phosphorylate ULK1 at S317, S555 and S778 (Dorsey et al., 2009). AMPK-dependent phosphorylation of ULK1 results in the formation of the ULK1 complex (Zachari and Ganley, 2017).

Autophagic degradation requires multiple ATG proteins downstream of the ULK1 complex to generate double membrane vesicles known as autophagosomes that engulf cellular materials prior to fusing with lysosomes or late endosomes (Bernard and Klionsky, 2013). Briefly, the ULK1 complex initiates autophagy by phosphorylating beclin-1 at S30 to assemble a phosphoinositide-3 kinase (PI3K) complex (Russell et al., 2013; Park et al., 2018), which inserts phosphatidylinositol lipids into membranes to recruit ATG proteins responsible for autophagosome formation (Matsunaga et al., 2010). Autophagosome growth is facilitated via ATG12-ATG5-ATG16L1 complexes incorporating lipids and ATG8—microtubule-associated light-chain 3 (LC3) in mammals—into autophagosome membranes (Sakoh-Nakatogawa et al., 2013). Prior to membrane incorporation, LC3 is post-translationally modified into LC3-I and LC3-II, which involves an ATG4-dependent cleavage to expose a C-terminal glycine residue (LC3-I) that is conjugated to phosphatidylethanolamine (LC3-II) by ATG7 and ATG3 (Satoo et al., 2009). As autophagosomes develop, autophagy cargo receptors tether cellular materials destined for degradation to LC3-II (Dooley et al., 2014). Once autophagosomes fully form, they migrate via microtubules and kinesin toward lysosomes in perinuclear regions of cells (Cheng et al., 2016). Autophagosomes fuse with lysosomes to generate autophagolysosomes (Mackeh et al., 2013) that contain lysosomal enzymes responsible for degrading autophagosomes and their cellular cargo (Klionsky et al., 2014).

Although autophagy is important for cellular homeostasis and survival, the protective functions of autophagy act as a double-edged sword in tumorigenesis (Eskelinen, 2011; Moscat and Diaz-Meco, 2012). For example, autophagy has been linked to drug resistance (Zou et al., 2012), epithelial-mesenchymal transition (EMT) (Alizadeh et al., 2018), cell migration (Tuloup-Minguez et al., 2013), metastasis (Qin et al., 2015), anoikis resistance (Peng et al., 2013), and aggressive tumor phenotypes (Mathew et al., 2007). As such, there is a need to understand the signaling pathways that may activate autophagy to promote tumorigenesis. In the past decade, several reports have suggested that transforming growth factor beta (TGFβ) activates autophagy (Kiyono et al., 2009; Suzuki et al., 2010; Xu et al., 2012; Fu et al., 2014; Alizadeh et al., 2018; Trelford and Di Guglielmo, 2020). Interestingly, like autophagy, TGFβ signaling impedes tumor formation in normal cells, yet promotes metastatic potential in tumor cells (Katsuno et al., 2013). In particular, TGFβ ligands are upregulated in several tumor microenvironments to induce angiogenesis, EMT and compromise immune cell surveillance (Thomas and Massagué, 2005; Jung et al., 2017; Muppala et al., 2017).

TGFβ signaling is initiated when transforming growth factor beta receptor type III (TβRIII) presents TGFβ ligands to transforming growth factor beta receptor type II (TβRII). TβRII transphosphorylates the transforming growth factor beta receptor type I (TβRI) that phosphorylates receptor Smads (R-Smads) and non-Smad proteins (Gunaratne et al., 2014). In Smad-dependent (canonical) TGFβ signaling, once R-Smads (Smad2 and Smad3) are phosphorylated by TβRI, they are released from the Smad Anchor for Receptor Activation (SARA) proteins. R-Smads then enter the nucleus in the presence of the co-Smad, Smad4, where they regulate gene expression (Weiss and Attisano, 2013). In Smad-independent (non-canonical) TGFβ signaling, TβRI or TβRII phosphorylate non-Smad proteins such as TGFβ activated kinase 1 (TAK1), atypical protein kinase C (aPKC), Par6, and PI3K complexes that regulate several cellular processes such as apoptosis, migration, proliferation, adhesion, differentiation, post-translational modifications, transcription and motility (Zhang, 2009).

Depending on the cell type, some studies have suggested that TGFβ-dependent autophagy relies on Smad transcription factors to upregulate ATG genes (Kiyono et al., 2009) whereas others emphasize that TGFβ activates autophagy by impeding mTOR (Fu et al., 2014; Chang et al., 2020). However, the specific TGFβ signaling pathway responsible for autophagy remain(s) unclear. Furthermore, many studies investigating TGFβ-dependent autophagy relied on LC3 protein levels as a readout for autophagy, which provides an incomplete picture (Klionsky et al., 2016; Trelford and Di Guglielmo, 2020). For this reason, our previous work verified that TGFβ increased autophagic flux using cells stably expressing green fluorescent protein (GFP)-LC3-red fluorescent protein (RFP)-LC3ΔG (Trelford and Di Guglielmo, 2020). After ATG4 cleaves GFP-LC3-RFP-LC3ΔG to generate GFP-LC3 and RFP-LC3ΔG, RFP-LC3ΔG cannot be conjugated to a phosphatidylethanolamine nor be incorporated into the autophagosome membrane. Therefore, during autophagy, the GFP-LC3 is degraded whereas the RFP-LC3ΔG is resistant to autophagic degradation (Kaizuka et al., 2016). Here, using non-small cell lung cancer (NSCLC) cell lines expressing GFP-LC3-RFP-LC3ΔG, we evaluated the role of specific components of

the TGFβ signaling pathway on autophagy. The purpose of this work was to identify TGFβ signaling pathways responsible for activating autophagy in NSCLC cell lines to highlight molecular targets for cancer therapy.

MATERIALS AND METHODS

Antibodies and Reagents

Primary antibodies were purchased from the following vendors: anti-GAPDH (Cell Signalling Technology, 2118S), anti-phospho-S465/467-Smad2 (P-Smad2; Cell Signalling Technology, 3108L), anti-Smad2/3 (BD Transduction laboratories, 562586), anti-LC3B (Cell Signalling Technology, 9236S), anti-ULK1 (Cell Signalling Technology, 8054S), anti-phospho-S555-ULK1 (Cell Signalling Technology, 5869S), anti-phospho-S757-ULK1 (Cell Signalling Technology, 6888S), anti-ULK2 (Santa Cruz, sc-293453), anti-SARA (Cell Signalling Technology, 13285S), anti-Smad4 (Cell Signalling Technology, 38454S), anti-mTOR (Cell Signalling Technology, 2972S), anti-phospho-S2448-mTOR (Cell Signalling Technology, 2971S), anti-adenosine monophosphate-activated protein kinase α (AMPKα; Cell Signalling Technology, 2532S), anti-phospho-T172-AMPKα (P-AMPK; Cell Signalling Technology, 50081S), anti-aPKCζ (Santa Cruz, sc-17781), anti-aPKCι (Santa Cruz, sc-17837), anti-TAK1 (Cell Signalling Technology, 5206S), anti-TRAF6 (Cell Signalling Technology, 8028S), anti-cleaved PARP (Cell Signalling Technology, 5625S) and anti-TGFβRIII (Santa Cruz, sc-74511). Secondary antibodies used for western blot analysis were as follows: Horseradish-peroxidase (HRP)-conjugated goat anti-rabbit-IgG (Thermo Fisher Scientific, 31460) and goat anti-mouse-IgG (Thermo Fisher Scientific, 31430). Fluorescently conjugated donkey anti-rabbit or donkey anti-mouse antibodies (Life Technologies) were used for immunofluorescence studies. Hoechst stain (Invitrogen, H3569) was used to label nuclei prior to live cell imaging. The pharmacological agents used to inhibit signaling pathways were SB431542 (TGFβ receptors; Selleckchem, S1067), LY294002 (PI3K; Sigma Aldrich, L9908-1MG), P38 MAPK Inhibitor (Calbiochem, 506126), Compound C (AMPK; Calbiochem, 171260) and ULK-101 (ULK1 and ULK2; Sellechchem, S8793).

siRNA Studies

si-Control (4457289) or two different human siRNA constructs were purchased from Thermo Fisher Scientific (silencer select) for each knockdown experiment. The siRNA targets were si-SARA (s17932 and s17933), si-Smad4 (s534708 and s8404), si-TGFβRIII (s24 and s26), si-TAK1 (s13766 and s13767), si-TRAF6 (s14388 and s143789), si-PKCζ (s11128 and s71714), si-PKCι (s11110 and s71706), si-ULK1 (s15963 and s15965) and si-ULK2 (s18704 and s18705). Every experiment was conducted using both siRNAs; the first siRNA listed for each target was used in the main Figures and the second siRNA listed for each target was used in the **Supplementary Figures** as described.

Cell Culture and Transfections

A549 cells and H1299 NSCLC cell lines were cultured in a humidified tissue incubator at 37°C under 5% CO_2. A549 cells and H1299 cells were incubated with Kaighn's Modification of Hams F-12 (F-12K; Corning, 10-025-CV) and Roswell Park Memorial Institute (RPMI; Corning, 10-043-CVR) media, respectively. Cells were treated with 250 pM TGFβ1, 10 μM ULK-101, 10 μM Compound C, 20 μM SB431542, 40 μM LY294002 and 10 μM P38 MAPK Inhibitor in media supplemented with 10% FBS. Transient siRNA knockdowns were performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific, 13778150) and optimem media (Thermo Fisher Scientific, 22600134) as per the manufacturer's protocol. Stable GFP-LC3-RFP-LC3ΔG expressing cells were generated using PolyJet transfection reagent (Froggabio, Toronto, ON, Canada) and a cDNA pMRX-IP-GFP-LC3-RFP-LC3ΔG vector (Addgene, 84573). Transfected cells were isolated using growth media supplemented with 10% FBS and 1 μg/mL puromycin (Thermo Fisher Scientific, A1113802).

Immunoblotting

TNTE lysis buffer (50 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 1 mg/mL pepstatin, 50 μM phenylmethylsulfonyl fluoride, 2.5 mM sodium fluoride, and 10 mM sodium pyrophosphate phosphatase inhibitor) was used to lyse cells for 20 min prior to protein collection. Following lysis, cell lysates were centrifuged at 21,000 g_{av} at 4°C for 10 min. Protein concentration was determined using the DC™ protein assay (Bio-Rad, Hercules, CA, United States) and a Victor 3V Multi-Detection Microplate Reader (PerkinElmer, Waltham, MA, United States). Prior to immunoblotting, Laemmli loading buffer was added to the protein lysates and the samples were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following a standard wet transfer protocol, proteins were transferred onto a nitrocellulose membrane and blocked with 5% skim milk for 1-h, rocking at room temperature. Primary antibodies were incubated overnight with the nitrocellulose membranes, rocking at 4°C. On the following day, nitrocellulose membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1-h at room temperature. Enhanced chemiluminescent substrate (Bio-Rad, 1705060) was added prior to visualizing using a Versa-doc Imager (Bio-Rad) and QuantityOne® 1-D Analysis software (Bio-Rad) analyzed the relative intensity of protein bands.

Immunofluorescence Microscopy

A549 cells cultured on glass coverslips were treated with 0 or 250 pM TGFβ for 24 h. Following treatment, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized after 5 min of 0.1% Triton X-100 and blocked for 1 h. Antibodies against ULK1 were diluted to a final concentration of 1:100. The cells were left in 4°C rocking with the antibody overnight. The following day, the cells were washed with PBS and incubated with an anti-rabbit secondary antibody for 1 h. The cells were washed with PBS and incubated with DAPI dissolved in a PBS solution for 10 min. Coverslips were then mounted onto microscope slides using Immu-mount (Thermo Fisher Scientific, 9990402) and were left in the dark overnight. The coverslips were visualized and imaged using an inverted Olympus IX81 fluorescence microscope or a Nikon Eclipse Ti2

(Nikon Instruments) confocal microscope. ImageJ (version 2.0) was used to quantify relative nuclear ULK1 intensity/Total ULK1 intensity. This experiment was repeated in A549 cells treated with si-RNA against Smad4 and A549 cells treated with si-RNA against TAK1 and TRAF6 in combination with a p38 MAPK inhibitor. Each data point represents quantitation from ≥100 cells from each condition.

Autophagic Flux Assay

A549 cells and H1299 cells were transfected with a cDNA pMRX-IP-GFP-LC3-RFP-LC3ΔG vector developed by the Mizushima laboratory (30; Addgene). Successfully transfected cells express two forms of LC3: GFP-LC3 and a mutant LC3 with a C-terminal glycine deletion (RFP-LC3ΔG). Immunoblotting using LC3 specific antibodies could distinguish the RFP-LC3ΔG, GFP-LC3-I and GFP-LC3-II bands, which are quantified using QuantityOne® 1-D Analysis software to determine the GFP/RFP ratio. Furthermore, using a 63x objective of an Olympus IX 81 inverted fluorescence microscope, we imaged the Hoechst, green and red channels. The GFP/RFP ratio was determined by ImageJ version 2.0, which quantified the average pixel intensity for green and red channels.

Assessing Autophagosome and Lysosome Co-localization

A549 cells stably expressing GFP-LC3 were treated with si-RNA against Smad4 or si-RNA targeting TRAF6 and TAK1 in combination with a p38 MAPK inhibitor for 24 h. Each experiment was conducted in the presence and absence of TGFβ1 for 24 h. LysoTracker Deep Red labeled lysosomes and Hoechst stain labeled the nucleus 2 h and 10 min prior to imaging, respectively. Imaging and quantitation was performed as previously described (Trelford and Di Guglielmo, 2020).

LC3 Puncta

A549 cells expressing GFP-LC3 that were subjected to live imaging using an Olympus IX 81 inverted fluorescence microscope to assess autophagic flux. This also allowed for the determination of the relative LC3 puncta per cell. Image J version 2.0 was used to quantify the number of puncta/cell utilizing puncta size, pixel count and circularity.

Statistical Analysis

A Student's *t*-test and One-way or Two-way ANOVA followed by a Dunnett's multiple comparisons test were used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism Software version 9.0 and P-values < 0.05 were considered to be statistically significant.

RESULTS

TGFβ1 Activates Autophagy by Regulating the mTOR-ULK1 Pathway

We previously reported that TGFβ1 induced ULK1 protein levels and stimulated autophagy in NSCLC cells (Trelford and Di Guglielmo, 2020), however the mechanism of how this was achieved remained unknown. To this end, we first investigated if TGFβ1 alters AMPK and mTOR activity by following site-specific ULK1 phosphorylation. Briefly, we measured ULK1 S555 phosphorylation to assess AMPK-dependent activity, ULK1 S757 to measure mTOR-dependent phosphorylation of ULK1 and mTOR S2448 phosphorylation to assess active mTOR (Klionsky et al., 2016). A549 cells and H1299 NSCLC cells were treated with TGFβ1 for 24 h prior to lysis and immunoblotting, and we observed that in response to TGFβ1, ULK1 phosphorylation of S555 tripled in A549 cells (**Figure 1A**) and doubled in H1299 cells (**Figure 1B**). Although there was a twofold increase in ULK1 protein levels in both cell lines, the ratio of phospho-S555-ULK1/ULK1 rose significantly (**Figures 1A,B**). Furthermore, we observed that TGFβ1 had little effect on mTOR protein levels but produced a slight, yet significant, decrease in P-mTOR in both A549 and H1299 cells (**Figures 1A,B**). Since a low P-mTOR/mTOR ratio increases the amount of ULK1 available for AMPK-dependent S555 phosphorylation and a high phospho-S555-ULK1/ULK1 ratio indicates an increase of active ULK1, we postulated that TGFβ1 increases the amount of post-translationally modified ULK1 to initiate autophagy. One hallmark of autophagy is the cellular redistribution of ULK1 to omegasomes (Karanasios et al., 2013). Therefore, to investigate if TGFβ1 alters the subcellular localization of ULK1 and ULK2, we carried out immunofluorescence microscopy (**Figure 1C**). We observed that TGFβ1 treatment induces a co-localization of both ULK1 and ULK2 in cytoplasmic puncta (**Figure 1C**). Interestingly, in response to TGFβ1, we also observed a small, but reproducible decrease in the nuclear signal for both ULK1 and ULK2. To confirm this observation, we carried out confocal microscopy and observed an approximate 20% decrease in nuclear ULK1 and ULK2 in response to TGFβ1 (**Supplementary Figure 1**).

To assess the role of ULK1 and/or ULK2 in TGFβ-dependent autophagy, we used ULK-101, a pharmacological inhibitor of both ULK1 and ULK2 (Martin et al., 2018). For this analysis, we utilized A549 cells and H1299 cells stably expressing a GFP-LC3-RFP-LC3ΔG construct that measures autophagic flux, as previously described (Trelford and Di Guglielmo, 2020). These cells were treated with ULK-101 in the presence and absence of TGFβ1, and in both cell lines we observed that TGFβ1 increased ULK1 and LC3B-II protein levels whereas it decreased ULK2 protein levels and the GFP/RFP ratio. Interestingly, ULK-101 decreased ULK1, ULK2 and LC3B-II protein levels and inhibited TGFβ-dependent autophagy, as measured by the GFP/RFP ratio (**Figures 2A,B**). To further assess autophagic flux in control or ULK-101-treated A549 cells in the presence or absence of TGFβ1 we carried out fluorescence microscopy analysis (**Figure 2C**). We observed that TGFβ1 significantly decreased the GFP/RFP ratio by 50 ± 10% and that ULK-101 restored the GFP/RFP ratio to control levels (**Figure 2D**). Furthermore, we quantified LC3-puncta/cell and observed that although TGFβ1 increased the number of LC3-puncta/cell, ULK-101 decreased the ratio of LC3-puncta/cell in the presence and absence of TGFβ1 (**Figure 2E**).

Since ULK-101 inhibits the kinase activity of both ULK1 and ULK2, we next specifically targeted ULK1 or ULK2 using

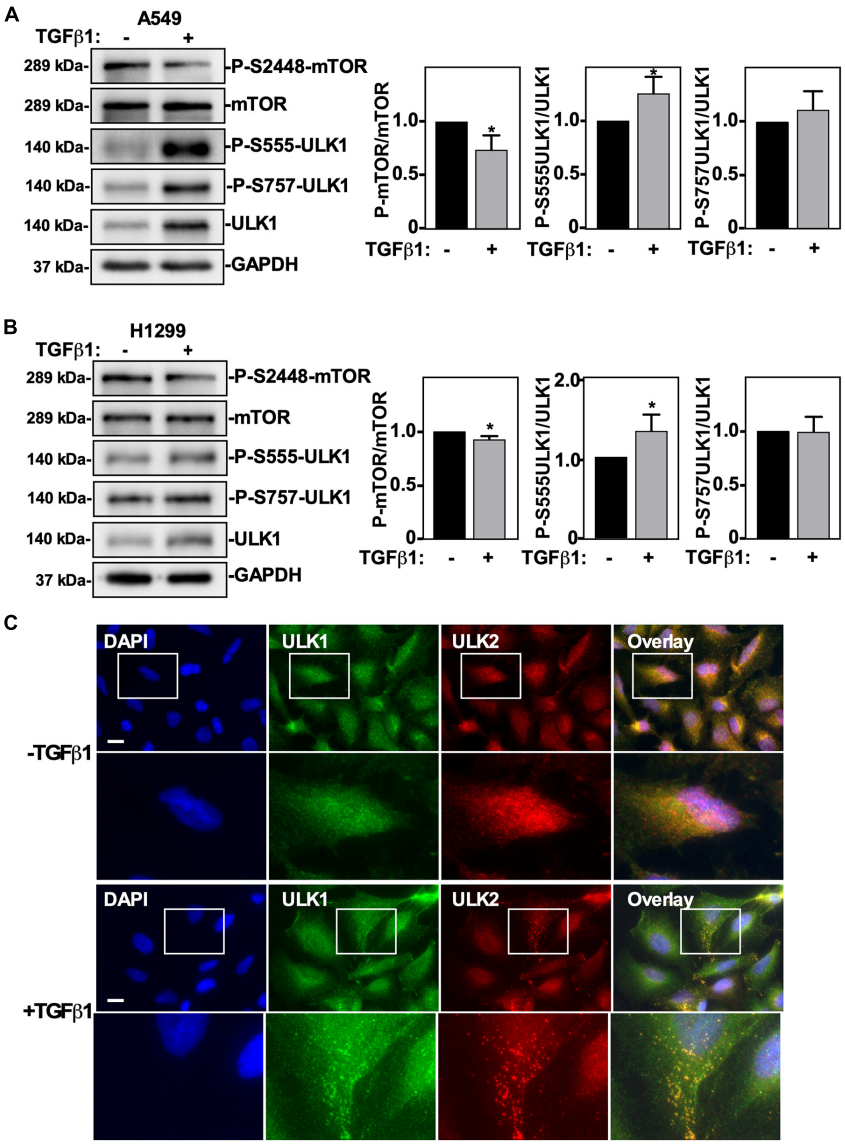

FIGURE 1 | The effect of TGFβ1 on mTOR and ULK1 activity in NSCLC cells. A549 **(A)** or H1299 **(B)** cells were treated with 250 pM TGFβ1 for 24 h. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-mTOR, anti-phospho-S2448-mTOR, anti-ULK1, anti-phospho-S555-ULK, anti-phospho-S757-ULK1, or anti-GAPDH (loading control) antibodies. The steady-state levels of phospho-S555-ULK1, ULK1, phospho-S2448-mTOR and mTOR were quantified using QuantityOne software and the phospho-mTOR/mTOR and phospho-ULK1/ULK1 ratios were graphed ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$. **(C)** A549 cells were treated with 250 pM TGFβ1 for 24 h. Cells were fixed and stained with DAPI (blue), antibodies against ULK1 (green) and ULK2 (red), and imaged using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm.

small interfering RNA (siRNA). A549 cells or H1299 cells were treated with control siRNA (si-Control), siRNA targeting ULK-1 (si-ULK1) or ULK-2 (si-ULK2), or a combination of both si-ULK1 and si-ULK2 followed by TGFβ1 stimulation. Western blotting indicated that in A549 cells, two different siRNAs targeting ULK1 significantly decreased ULK1 protein levels by >80% and increased ULK2 and LC3B-II protein levels by 150 ± 18% and 140 ± 9%, respectively (**Figure 3A** and **Supplementary Figure 2A**). Furthermore, in the presence of TGFβ1, the two ULK1 siRNAs increased the GFP/RFP ratio compared to the TGFβ1 treatment, suggesting that ULK1 activity is necessary for TGFβ1-induced autophagy in A549 cells (**Figure 3A** and **Supplementary Figure 2A**). In H1299 cells, the ULK1 siRNAs also reduced ULK1 protein levels by >80% and increased ULK2 protein levels by 150 ± 21% (**Figure 3B** and **Supplementary Figure 2B**). Additionally, the ULK1 siRNAs had no effect on LC3B-II protein levels but consistent with A549 cells, increased the GFP/RFP ratio, suggesting that ULK1 activity is important for TGFβ1-induced autophagy in H1299 cells as well (**Figure 3B** and **Supplementary Figure 2B**). In both cell lines, the ULK2 siRNAs decreased ULK2 protein levels, increased LC3B-II and ULK1 protein levels but had no

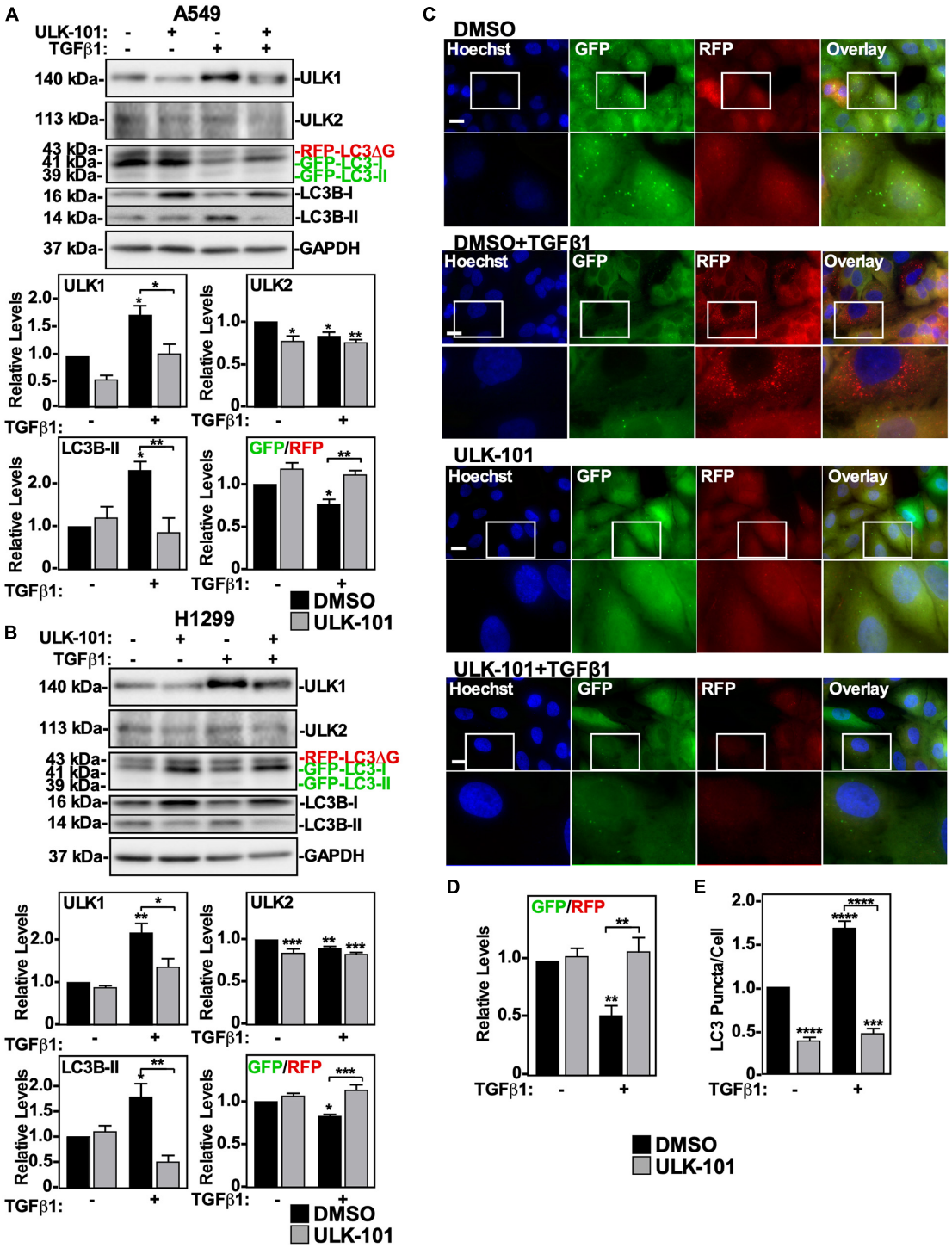

FIGURE 2 | The effect of inhibiting ULK1 on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were treated with 10 μM of the ULK1/2 inhibitor, ULK-101, or DMSO (vehicle control) in the presence and absence of 250 pM TGFβ1 for 24 h. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-ULK1, anti-ULK2, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state ULK1, ULK2, and LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. **(C)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images ($n = 3 \pm$ SD). Significance is indicated as *** = $P < 0.001$ and **** = $P < 0.0001$.

effect on the GFP/RFP ratio (**Figures 3A,B** and **Supplementary Figures 2A,B**). Taken together, these results suggest that ULK1 but not ULK2 is involved with TGFβ1-induced autophagy. As a parallel approach, we carried out fluorescence microscopy on GFP-LC3-RFP-LC3ΔG expressing cell lines (**Figure 3C**). A549 cells transfected with two different siRNA to ULK1 significantly increased the GFP/RFP ratio by 35 ± 9%, whereas si-ULK2 had little effect (**Figure 3D** and data not shown). Finally, quantifying LC3 puncta/cell revealed that in the presence of TGFβ1, all treatments with siRNAs targeting ULK1 had fewer LC3 puncta/cell (**Figure 3E** and data not shown). Taken together, our results suggest that TGFβ1 activates autophagy by increasing AMPK-dependent ULK1 S555 phosphorylation.

TGFβ1-Induced Autophagy Relies on TβRI Kinase Activity

Although we determined that TGFβ1-dependent autophagy is facilitated by ULK1, the signaling pathway that effects ULK1 protein levels and phosphorylation are unknown. We therefore assessed which TGFβ receptors are essential to TGFβ1-dependent autophagy. We first inhibited the TβRII/TβRI complex (Miyazawa and Miyazono, 2017) using the a pharmacological inhibitor, SB431542, which blocks the kinase activity of TβRI (Inman et al., 2002). A549 cells and H1299 cells were treated with SB431542 in the presence and absence of TGFβ1 and immunoblotted for ULK1, phospho-Smad2, Smad2 and LC3B. We observed that SB431542 inhibited TGFβ1-dependent Smad2 phosphorylation in both cell lines (**Figures 4A,B**). In A549 cells, SB431542 blocked the TGFβ1-dependent decrease of the GFP/RFP ratio and increase of ULK1 and LC3B-II protein levels (**Figure 4A**). In H1299 cells, SB431542 disrupted the TGFβ1-dependent decrease of the GFP/RFP ratio and increase of ULK1 protein levels. However, SB431542 treatments significantly increased LC3B-II protein levels by 210 ± 29% compared to control (**Figure 4B**). To confirm that TβRI kinase activity is necessary for TGFβ1-induced autophagy, we next utilized fluorescence microscopy to visualize cells expressing GFP-LC3-RFP-LC3ΔG as described above (**Figure 4C**). Quantifying the GFP and RFP channels revealed that SB431542 increased the TGFβ-dependent GFP/RFP ratio by 30 ± 5%, indicating that SB431542 inhibited TGFβ-dependent autophagic flux (**Figure 4D**). After analyzing the LC3 puncta/cell using the fluorescence images, we determined that SB431542, in the presence of TGFβ1, decreased the amount of LC3 puncta/cell with respect to the TGFβ1 treatment (**Figure 4E**). Finally, to assess any involvement of the type III TGFβ receptor (TβRIII), we used an siRNA approach, as this receptor does not have any intrinsic enzymatic activity. Interestingly, A549 cells and H1299 cells expressing two different siRNAs targeting TβRIII exhibited a slightly higher basal level of autophagic flux, but TGFβ-dependent autophagy remained unperturbed by TβRIII silencing (data not shown). Taken together, these results confirm that the activity of the TβRII/TβRI TGFβ receptor complex is necessary for the TGFβ1-dependent increase of autophagic flux in both NSCLC cell lines.

Smad4-Dependent TGFβ1 Signaling Activates Autophagy

After TGFβ1 binds to TβRII/TβRI complexes, it initiates canonical and non-canonical signaling (Gunaratne et al., 2012). Since we observed that inhibiting TGFβ receptor kinase activity and Smad2 phosphorylation resulted in inhibition of autophagy (**Figure 4**), we assessed if reducing the accessibility of Smad2 to the TGFβ receptor complex would affect TGFβ-dependent autophagy. This was carried out by siRNA-mediated silencing of the Smad Anchor necessary for Receptor Activation (SARA). Interestingly, reducing SARA levels in both A549 or H1299 cells did not inhibit TGFβ-dependent induction of LC3B-II protein levels, or inhibit autophagy (data not shown). These results suggest that if the canonical TGFβ signaling pathway results in autophagy, removing a major member of the pathway, such as Smad4, may be necessary to alter TGFβ-dependent autophagy. We therefore evaluated if Smad4 silencing via siRNA targeting (si-Smad4) influenced TGFβ1-dependent autophagy. A549 cells and H1299 cells were transfected with si-Control or two different siRNAs targeting Smad4 in the presence or absence of TGFβ1, lysed and immunoblotted for Smad4, P-Smad2, Smad2, and LC3B. In both cell lines, we observed that TGFβ1 increased LC3B-II protein levels and decreased the GFP/RFP ratio (**Figures 5A,B** and **Supplementary Figures 3A,B**). Interestingly, Smad4 silencing increased the proportion of phosphorylated Smad2, which suggested that TGFβ-dependent autophagy relies on the presence of Smad4 (**Figures 5A,B**). Indeed, although Smad4 silencing had differing effects on LC3B-II protein levels in A549 *vs.* H1299 cells, both cell lines showed attenuated TGFβ1-dependent GFP/RFP ratio in the absence of Smad4, suggesting that Smad4 is necessary to induce TGFβ-dependent autophagic flux (**Figures 5A,B** and **Supplementary Figures 3A,B**). To investigate this further, we used fluorescence microscopy to image the GFP/RFP autophagic flux ratio in A549 cells (**Figure 5C**). Quantifying the GFP/RFP ratios indicated that TGFβ1 decreased the GFP/RFP ratio compared to the si-Control treatment by 60 ± 5%. Alternatively, siRNAs targeting Smad4 in the presence of TGFβ1 did not significantly alter the GFP/RFP ratio with respect to the si-Control treatment (**Figure 5D** and data not shown). Lastly, we examined the influence that Smad4 had on relative LC3 puncta/cell. Although the TGFβ1 treatment significantly increased the relative number of LC3 puncta/cell, we observed that TGFβ1 treatment in Smad4-silenced cells did not significantly increase the ratio of LC3 puncta/cell compared to control cells (**Figure 5E** and data not shown). These results support the conclusion that TGFβ1 induces autophagy via Smad4. Having ascertained that the canonical pathway is important for promoting TGFβ1-dependent autophagy, we next assessed the contribution of non-canonical TGFβ pathways.

Non-canonical TGFβ1 Signaling Upregulates Autophagy

We first investigated the role of the PI3K non-canonical TGFβ signaling pathway on TGFβ1-induced autophagy using LY294002, an inhibitor of the PI3K-mTOR pathway (Zhang et al., 2005; Ding et al., 2010). A549 cells and H1299 cells were treated

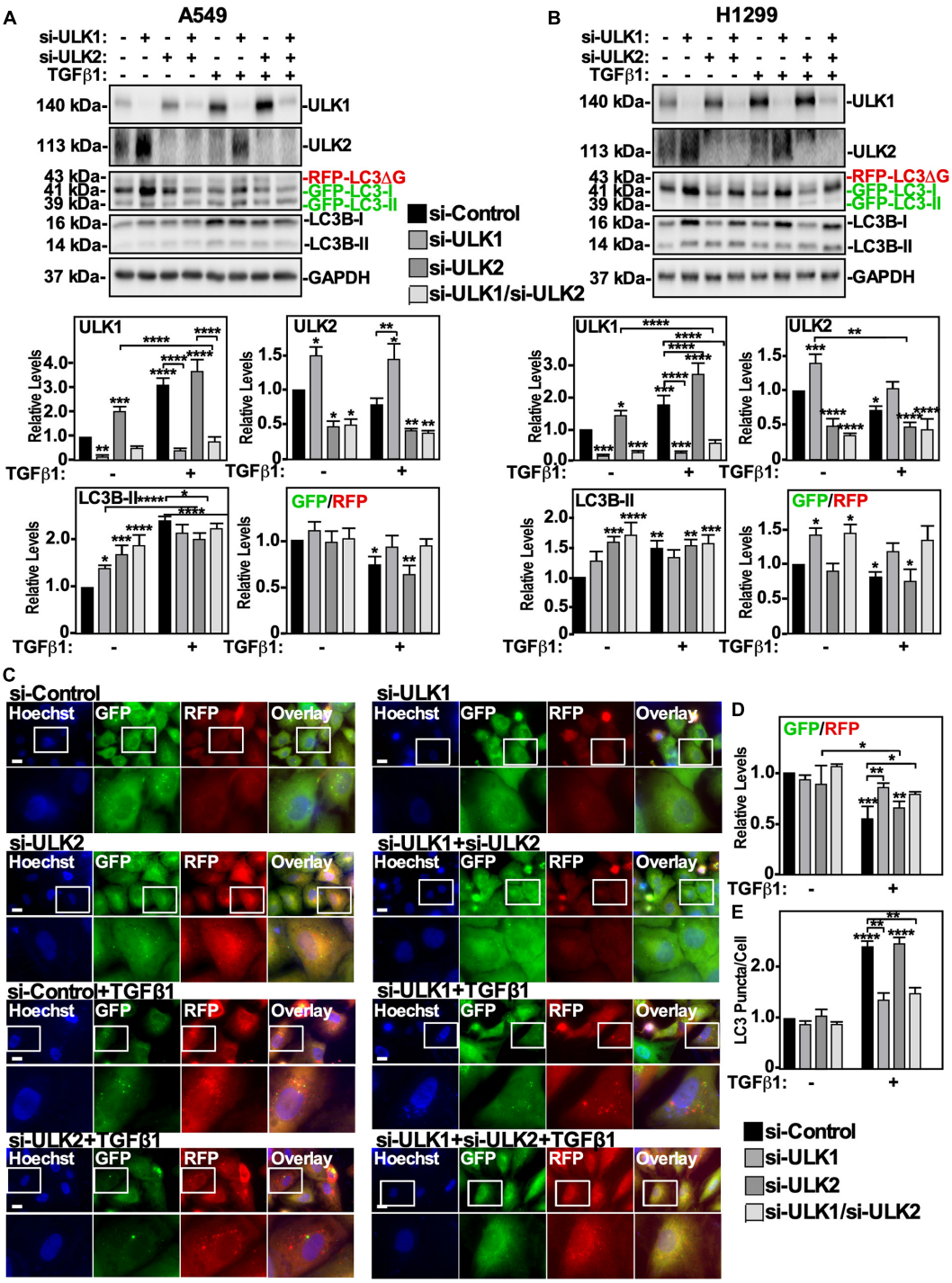

FIGURE 3 | Assessing ULK1 and ULK2 silencing on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with control siRNA (si-Control), siRNA targeting ULK1 (si-ULK1; s15963) or siRNA targeting ULK2 (si-ULK2; s18704) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-ULK1, anti-ULK2, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state ULK1, ULK2 and LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$. **(C)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$ and **** = $P < 0.0001$.

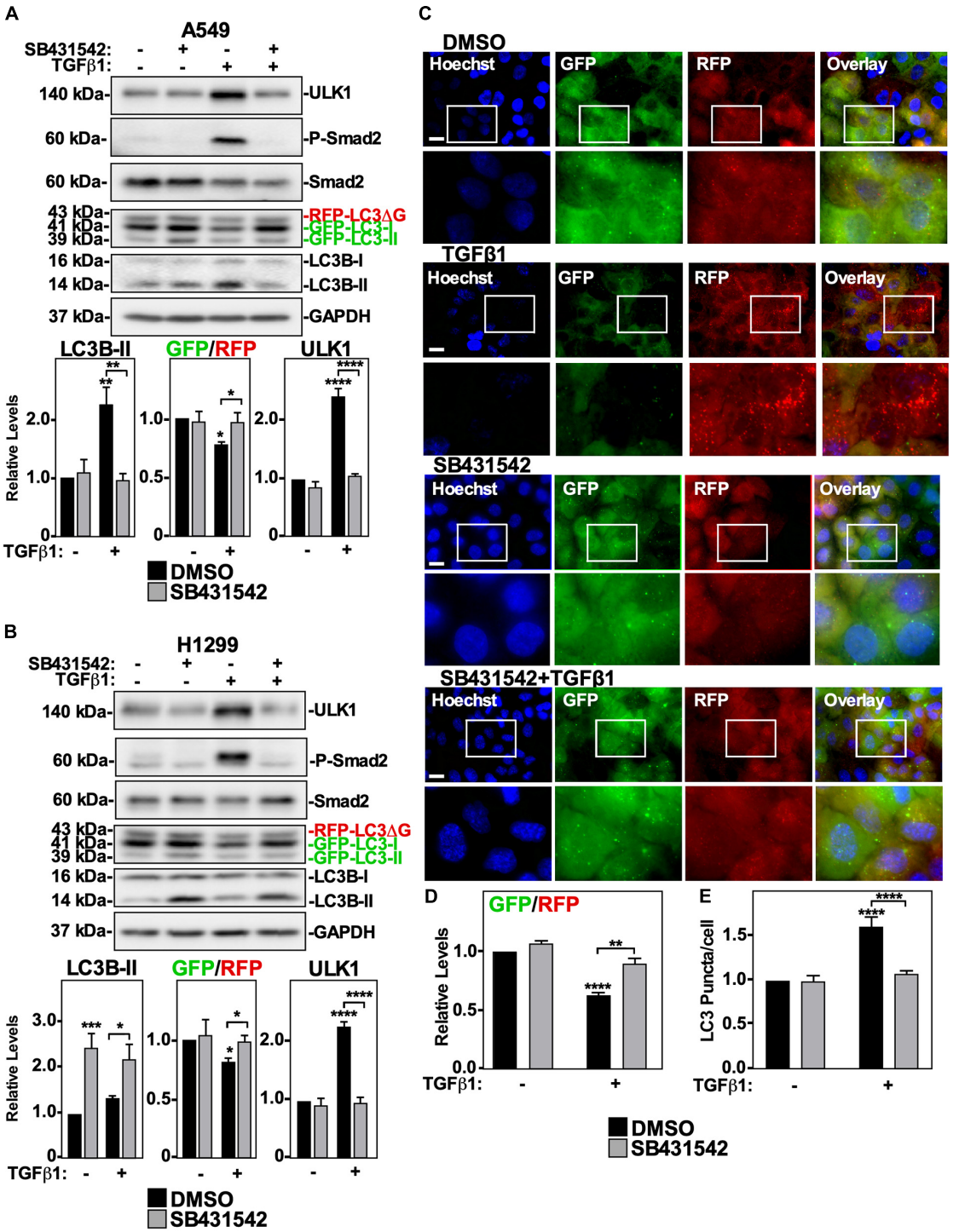

FIGURE 4 | The effect of SB431542 on TGFβ1 induced autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were treated with 20 μM SB431542 or DMSO (vehicle control) in the presence and absence of 250 pM TGFβ1 for 24 h. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-ULK1, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state ULK1 and LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$. **(C)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$ and **** = $P < 0.0001$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images ($n = 3 \pm$ SD). Significance is indicated as **** = $P < 0.0001$.

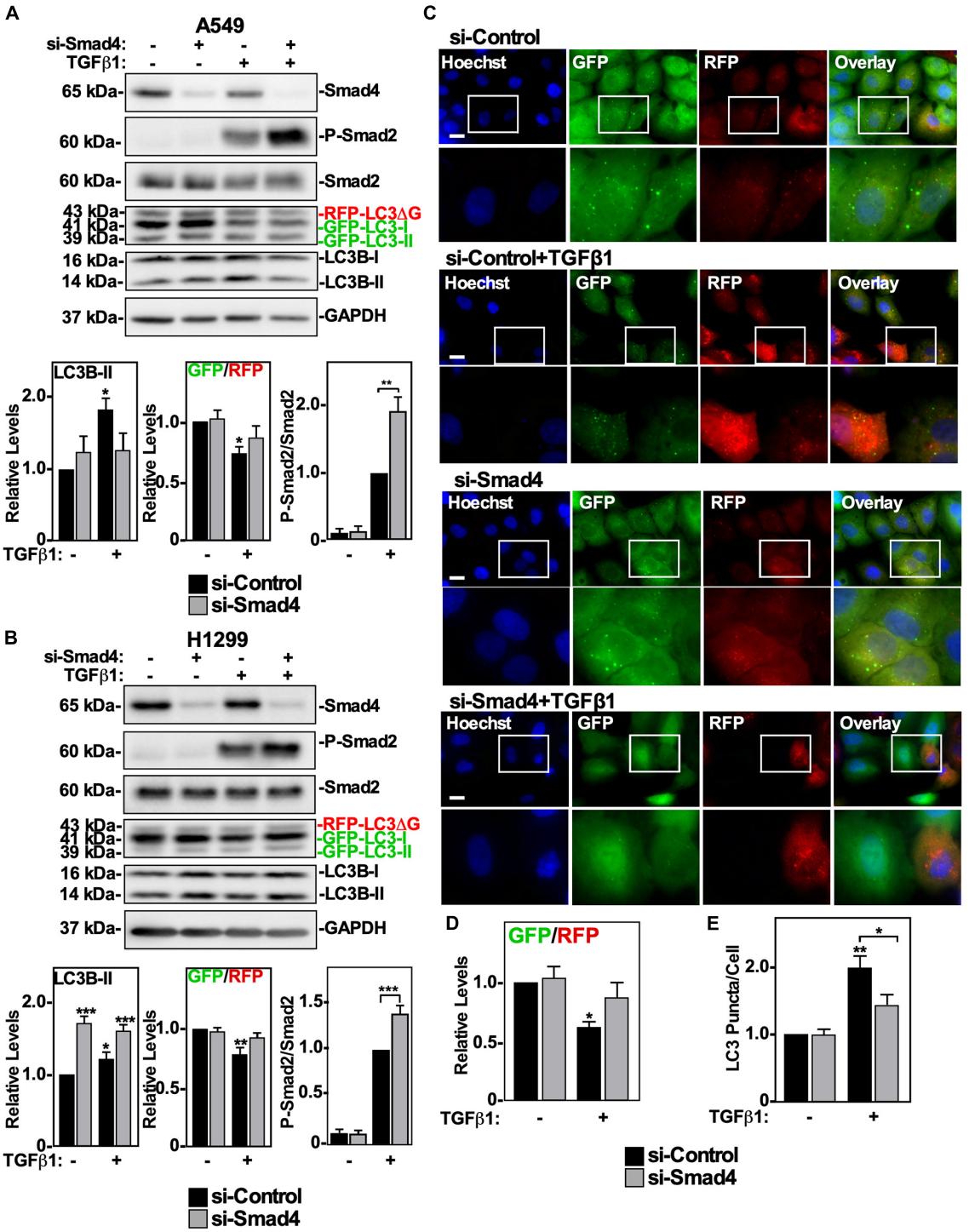

FIGURE 5 | The effect of Smad4 silencing on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or siRNA targeting Smad4 (si-Smad4; s534708) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-Smad4, anti-P-Smad2, anti-Smad2, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. **(C)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and ** = $P < 0.01$.

with LY294002 in the presence and absence of TGFβ1, lysed and immunoblotted for mTOR, P-mTOR, and LC3B. We observed that LY294002 treatment increased LC3B-II protein levels and reduced the GFP/RFP ratio to a greater extent than the TGFβ1 treatment alone (**Figures 6A,B**). These results suggest that the PI3K pathway and autophagic flux are inversely proportional to one another. We verified that the PI3K pathway does not facilitate TGFβ1-dependent autophagy by treating A549 cells with LY294002, with and without TGFβ1, prior to fluorescence microscopy imaging (**Figure 6C**). In all cases where the cells were treated with LY294002, we observed a marked decrease in GFP-LC3 signal, and the quantitation of the GFP/RFP ratios suggested that LY294002 decreased the GFP/RFP ratio in the presence and absence of TGFβ1 (**Figure 6D**). Finally, we observed that both TGFβ1 and LY294002 increased the amount of LC3 puncta/cell, however LY294002 significantly increased (>50%) the number of LC3 puncta/cell compared to the TGFβ1 treatment alone (**Figure 6E**). Since these results suggest that any PI3K activity that is stimulated by TGFβ would impede autophagy, we next turned our attention to another non-canonical TGFβ pathway, the aPKC pathway.

Both aPKCζ and aPKCι have been shown to be involved with TGFβ-dependent processes such as EMT and apoptosis (Gunaratne and Di Guglielmo, 2013; Gunaratne et al., 2014). To investigate if this pathway is involved with autophagy, we utilized siRNAs selective for aPKCζ (si-aPKCζ) or aPKCι (si-aPKCι). Since we had previously observed that aPKCι silencing increases aPKCζ protein levels (Gunaratne et al., 2014), we utilized a double si-aPKCι and si-aPKCζ knockdown approach. A549 cells and H1299 cells were treated with si-Control or si-aPKCζ/si-aPKCι in the presence or absence of TGFβ1, lysed and immunoblotted for aPKCζ, aPKCι, LC3B, and GAPDH. In A549 cells, we observed that TGFβ1 increased LC3B-II protein levels and decreased the GFP/RFP ratio in the presence of si-Control and si-aPKCζ/si-aPKCι treatments (**Supplementary Figure 4A**). In H1299 cells, we found that si-aPKCζ/si-aPKCι and TGFβ1 increased LC3B-II protein levels compared to the si-Control treatment, however, in the presence of TGFβ1, si-aPKCζ/si-aPKCι significantly reduced LC3B-II protein levels by 25 ± 5%. Additionally, in the presence of TGFβ1, the GFP/RFP ratio of the si-aPKCζ/si-aPKCι treatment was not statistically different compared to the si-Control treatment (**Supplementary Figure 4B**). Based on these results, aPKCs may not be involved with TGFβ1-dependent autophagy. To confirm this, we treated A549 cells with si-Control or si-aPKCζ/si-aPKCι and used fluorescence microscopy to image GFP-LC3 and RFP-LC3ΔG (**Supplementary Figure 4C**). Quantitation of the GFP/RFP ratios indicated that all TGFβ1 treatments had reduced GFP/RFP ratios with respect to the si-Control treatment (**Supplementary Figure 4D**), and that TGFβ1 increased the number of LC3 puncta/cell regardless of aPKC knockdown (**Supplementary Figure 4E**). Having observed that TGFβ1 may not require aPKCζ or aPKCι to activate autophagy, we next directed our attention to the TAK1-tumor necrosis factor receptor-associated factor 6 -P38 mitogen activated protein kinase (TAK1-TRAF6-P38 MAPK) pathway.

To assess if the TAK1-TRAF6-P38 MAPK pathway was involved with TGFβ1-dependent autophagy, we first inhibited each component of the pathway separately. The effects of pharmacologically inhibiting p38 MAPK in A549 cells and H1299 cells was assessed by immunoblotting for cleaved PARP, as TGFβ1 increases PARP cleavage via P38 MAPK (Gunaratne et al., 2015). In both cell lines, we observed that the P38 MAPK inhibitor blocked TGFβ1-dependent PARP cleavage, however it did not alter TGFβ-dependent autophagy, as assessed by western blotting and fluorescence microscopy (**Supplementary Figure 5**). We next assessed the involvement of TRAF6 in TGFβ-dependent autophagy using siRNA specific for TRAF6 (si-TRAF6). A549 cells and H1299 cells treated with si-Control or si-TRAF6 in the presence or absence of TGFβ1 showed that TRAF6 silencing did not affect TGFβ1 mediated changes to LC3B-II protein levels or the GFP/RFP ratio (**Supplementary Figures 6A,B**). To verify that TRAF6 silencing had no effect on TGFβ1-induced autophagy, we used fluorescence microscopy on A549 cells treated as described above (**Supplementary Figure 6C**). In the presence and absence of TGFβ1, si-TRAF6 did not alter the GFP/RFP ratios or impact the number of LC3 puncta/cell (**Supplementary Figures 6D,E**). Finally, we used siRNA specific for TAK1 (si-TAK1) to silence TAK1 in A549 cells and H1299 cells. In both cell lines, si-TAK1 decreased LC3B-II protein levels and the partially reversed TGFβ-dependent autophagic flux, as assessed by western blotting (**Supplementary Figures 7A,B**). Although this observation was not seen by fluorescence microscopy (**Supplementary Figures 7C–E**), the promising results from the western blot analysis prompted us to try a combination of inhibitors of this pathway. We therefore inhibited TAK1, TRAF6, and P38 MAPK activity simultaneously to achieve maximal blockade of this non-canonical TGFβ signaling pathway (**Figure 7**). A549 cells and H1299 cells were treated with si-TRAF6, si-TAK1 and P38 MAPK inhibitor in the presence and absence of TGFβ1, lysed and immunoblotted for TAK1, TRAF6, cleaved PARP, and LC3B. In both cell lines, we observed that using two sets of siRNAs to TAK1 and TRAF6, in combination with a P38 MAPK inhibitor decreased LC3B-II protein levels and increased the GFP/RFP ratio (**Figures 7A,B** and **Supplementary Figure 8C**). To verify the role of this pathway in TGFβ1-induced autophagy, we used fluorescence microscopy to image A549 cells treated as described above (**Figure 7C**). Quantitation revealed that inhibiting the TAK1-TRAF6-P38 MAPK pathway, in the presence of TGFβ1, significantly increased the GFP/RFP ratio by 20 ± 5% compared to the TGFβ1 treatment (**Figure 7D** and data not shown). Additionally, in the presence and absence of TGFβ1, inhibiting the TAK1-TRAF6-P38 MAPK pathway reduced the relative number of LC3 puncta/cell (**Figure 7E**). Taken together, these results suggest that TGFβ1 relies on the TAK1-TRAF6-P38 MAPK to upregulate autophagy.

TGFβ1-Induced Autophagosome-Lysosome Co-localization Is Regulated by Smad4 and TAK1-TRAF6-P38 MAPK Signaling Pathways

Above we observed that the Smad4 and TAK1-TRAF6-P38 branches of the canonical and non-canonical TGFβ

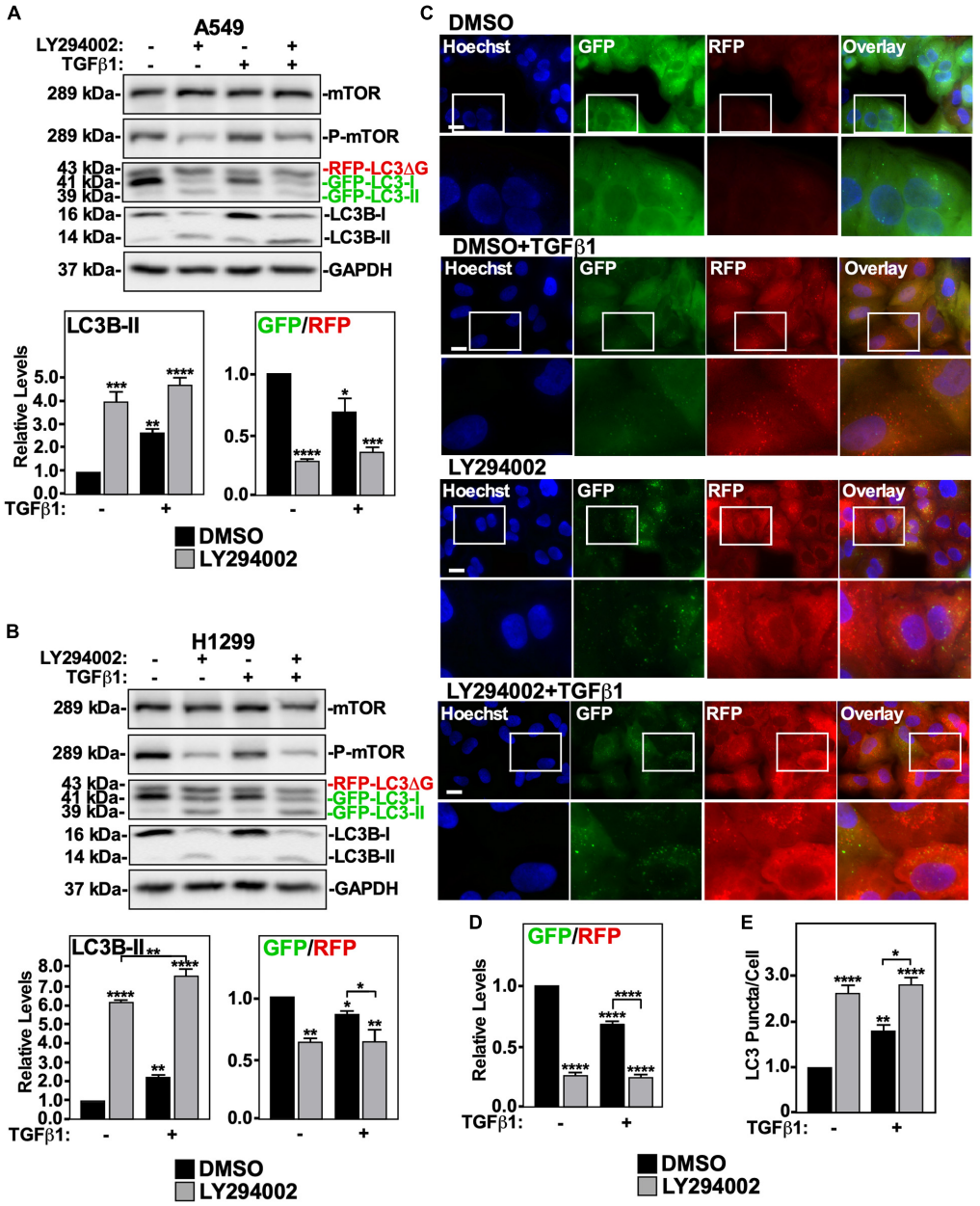

FIGURE 6 | The effect of LY294002 on TGFβ1 induced autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were treated with 40 μM LY294002 or DMSO (vehicle control) in the presence and absence of 250 pM TGFβ1 for 24 h. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-ULK1, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state ULK1 and LC3B-II protein levels and the GFP/RFP ratio are shown below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$. **(C)** A549 cells stably expressing a cDNA GFP-LC3-RFP-LC3ΔG construct were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images ($n = 3 \pm$ SD). Significance is indicated as **** = $P < 0.0001$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and **** = $P < 0.0001$.

signaling pathways influence TGFβ-dependent autophagy. To gain more mechanistic insight, we next utilized A549 cells stably expressing GFP-labeled LC3 protein to determine if either Smad4 silencing or inhibiting the TAK1-TRAF6-P38 MAPK pathway disrupted the TGFβ1-dependent increase of GFP-LC3-lysosome co-localization. Briefly, A549 cells expressing GFP-LC3 were transfected with si-Control or si-Smad4, in the presence and absence of 250 pM TGFβ1 for 24 h and labeled with LysoTracker Deep Red to identify lysosomes (**Figure 8A**). We observed that in the absence of TGFβ

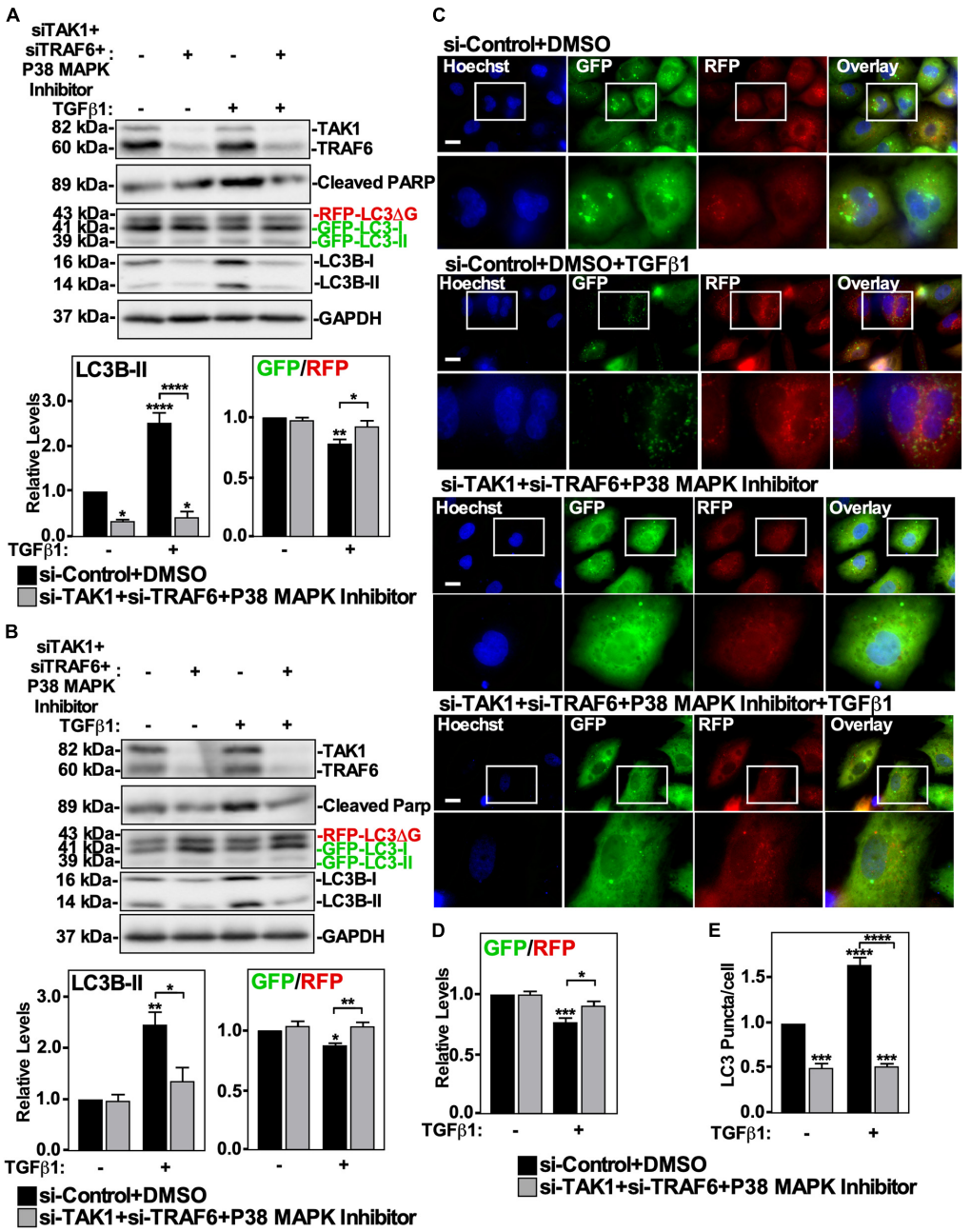

FIGURE 7 | The effect of TAK1-TRAF6-P38 MAPK pathway on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or siRNA targeting TAK1 (si-TAK1; s13766), siRNA targeting TRAF6 (si-TRAF6; s14388) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM P38 MAPK inhibitor for 24 h. The cells were then lysed, subjected to SDS-PAGE and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state LC3B-II protein levels and the GFP/RFP ratio are shown below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and **** = $P < 0.0001$. **(C)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and *** = $P < 0.001$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images ($n = 3 \pm$ SD). Significance is indicated as *** = $P < 0.001$ and **** = $P < 0.0001$.

there was little GFP-LC3 co-localizing with lysosomes, however TGFβ induced the accumulation of GFP-LC3 into Lysotracker-positive puncta. Interestingly, Smad4 silencing reduced both GFP-LC3 accumulation within cells and the co-localization with lysotracker puncta (**Figure 8A**). Inhibiting the TAK1-TRAF6-P38 pathway using a combination of si-TAK1, si-TRAF6

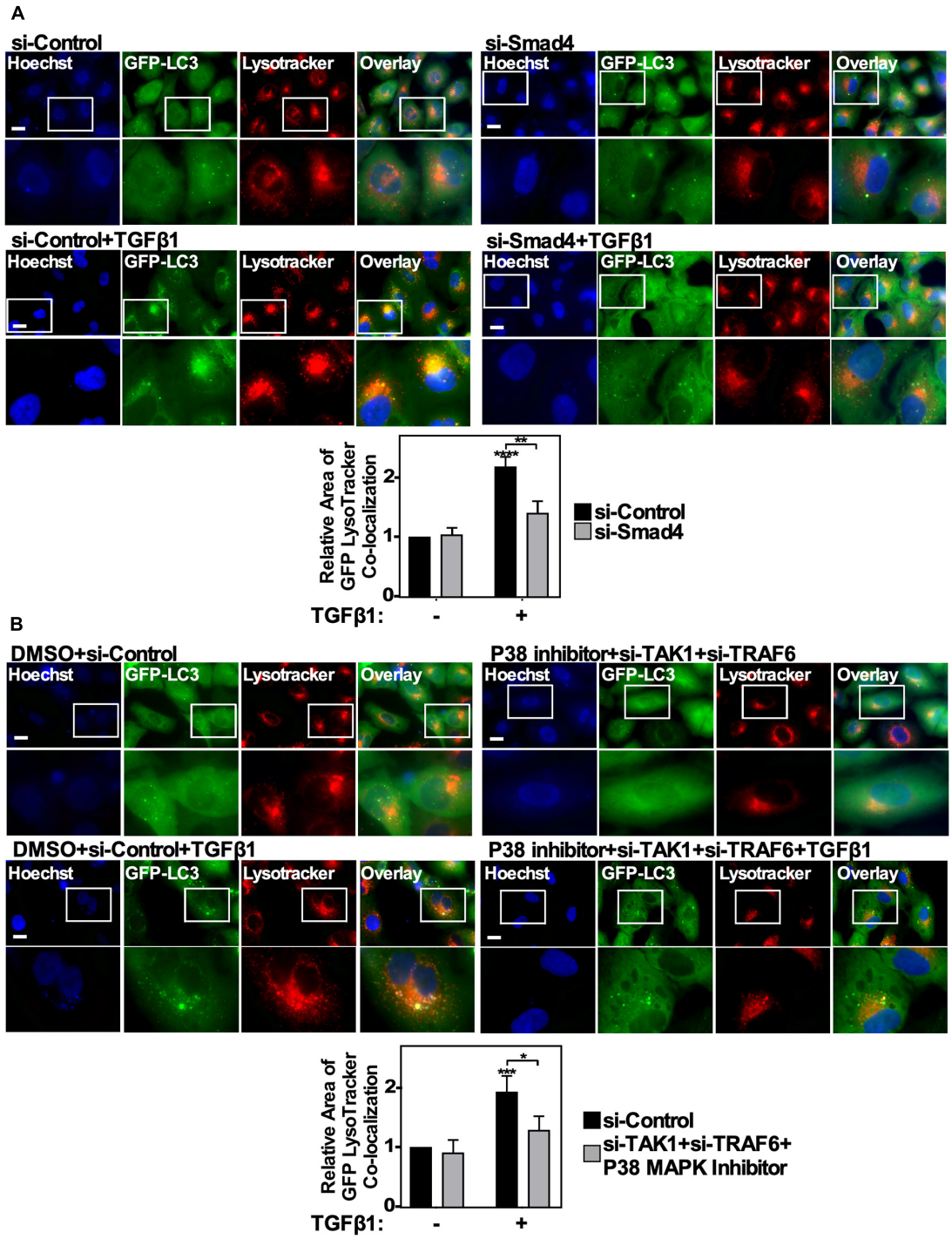

FIGURE 8 | The effect of canonical and non-canonical TGFβ signaling on autophagosome/lysosome co-localization. **(A)** A549 cells expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-Smad4 (s534708) for 48 h. The cells were then incubated in the absence or presence of 250 pM TGFβ1 for 24 h. LysoTracker Deep Red (red) and Hoechst stain (blue) were added 2 h and 10 min, respectively, prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Scale bars = 10 μm. Image J version 2.0 was used to quantify the number of yellow pixels per cell area for each treatment. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as ** = $P < 0.01$ and **** = $P < 0.0001$. Bar = 10 μm. **(B)** A549 cells expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-TAK1 (s13766) and si-TRAF6 (s14388) for 48 h. The cells were then incubated in the absence or presence of 250 pM TGFβ1 and 10 μM P38 MAPK inhibitor for 24 h. LysoTracker Deep Red (red) and Hoechst stain (blue) were added 2 h and 10 min, respectively, prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Scale bars = 10 μm. Image J version 2.0 was used to quantify the number of yellow pixels per cell area for each treatment. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as * = $P < 0.05$ and *** = $P < 0.001$. Bar = 10 μm.

and P38 MAPK inhibitor yielded similar results, as inhibiting the TAK1-TRAF6-P38 pathway blocked the TGFβ1-dependent increase in GFP-LC3-lysosome co-localization (**Figure 8B**). In summary, these results verified that both Smad4 and the TAK1-TRAF6-P38 MAPK signaling pathways are necessary for TGFβ1 to induce autophagosome and lysosome co-localization, which temporally occurs immediately prior to lysosomal-dependent degradation.

Smad4 Regulates ULK1 Phosphorylation and TAK1-TRAF6-P38 MAPK Activation Inhibits the mTOR-ULK1 Pathway

Since TGFβ1 activates autophagy using Smad4 and TAK1-TRAF6-P38 MAPK signaling pathways, we next investigated if these pathways influenced mTOR and ULK1 phosphorylation. A549 cells and H1299 cells were treated with si-Control or two siRNAs targeting Smad4 in the presence or absence of TGFβ1, lysed and immunoblotted using phospho-specific antibodies for mTOR and ULK1. In both cell lines, Smad4 knockdown had no effect on the P-mTOR/mTOR or phospho-S757-ULK1/ULK1 ratios (**Figures 9A,B**). However, in the presence of TGFβ1, Smad4 silencing decreased the phospho-S555-ULK1/ULK1 in A549 cells by 50 ± 15% and in H1299 cells by 50 ± 19% (**Figures 9A,B**). To assess if this could be due to increased AMPKα activity, we analyzed AMPKα T172 phosphorylation status and observed that P-AMPKα levels remained constant in the presence or absence of TGFβ and/or Smad4 (**Figures 9A,B**).

Next, A549 cells and H1299 cells were treated with si-TRAF6, si-TAK1 and P38 MAPK inhibitor in the presence of TGFβ1, lysed and immunoblotted for P-mTOR, mTOR, phospho-S555-ULK1, phospho-S757-ULK1, ULK1 and GAPDH. In both cell lines, inhibiting the TAK1-TRAF6-P38 MAPK pathway had no effect on the phospho-S757-ULK1/ULK1 ratios (**Figures 9C,D**). In A549 cells treated with TGFβ1, inhibiting the TAK1-TRAF6-P38 MAPK pathway increased the P-mTOR/mTOR ratio by 25 ± 12% and decreased the phospho-S555-ULK1/ULK1 ratio by 20 ± 5% (**Figure 9C**). In H1299 cells treated with TGFβ1, inhibiting the TAK1-TRAF6-P38 MAPK pathway increased the P-mTOR/mTOR ratio by 20 ± 5% and decreased the phospho-S555-ULK1/ULK1 ratio by 30 ± 10% (**Figure 9D**). Interestingly, inhibiting the TAK1-TRAF6-P38 pathway increased the basal level of AMPKα-T172 phosphorylation, however the P-AMPKα/AMPKα ratio was unchanged in response to TGFβ (**Figures 9C,D**). Since these results suggested that AMPKα activity may not be necessary for TGFβ1-dependent autophagy, we inhibited AMPKα activity in A549 cells using Compound C and observed that while Compound C altered basal autophagy, it did not affect TGFβ1-dependent autophagy (**Supplementary Figure 9**). Finally, to assess if canonical and/or non-canonical pathways would induce the nuclear export of ULK1 in response to TGFβ, we carried out confocal microscopy in cells treated with siRNAs targeting Smad4 or TAK1 + TRAF6, in combination with a P38 inhibitor. We observed that perturbing either pathway inhibits TGFβ1-dependent ULK1 cellular re-localization from the nucleus (**Supplementary Figure 10**).

Taken together, our results show that by using various pharmacological and/or siRNA mediated approaches, we have observed that TGFβ1 induces autophagy by increasing ULK1 activity, which is dependent on TβRI kinase activity, the canonical Smad4 signaling pathway and the non-canonical TAK1-TRAF6-P38 MAPK signaling pathway (**Figure 10**).

DISCUSSION

We previously uncovered several aspects of TGFβ1-dependent autophagy in NSCLC cells, and observed that TGFβ1 increased *ULK1*, *ATG9A*, *ATG16L1* and *LC3* gene expression, but only the protein levels of LC3B-II and ULK1 (Trelford and Di Guglielmo, 2020). We also observed that LC3B-II protein levels are limited in measuring autophagy and therefore methods that investigate autophagic flux should be used to measure TGFβ1-dependent autophagy. Finally, we reported that siRNA-mediated ATG5/7 knockdown decreases TGFβ-dependent autophagic flux in NSCLC cells (Trelford and Di Guglielmo, 2020). Therefore, although macroautophagy can be activated independently of ATG5/7 or ULK1 activity (Arakawa et al., 2017), our results suggest that the majority of autophagic degradation initiated by TGFβ1 is mediated by canonical macroautophagy (Trelford and Di Guglielmo, 2020).

Here, using pharmacological inhibitors and siRNA to target specific TGFβ1 signaling pathways, we mechanistically characterized TGFβ1-dependent autophagy in two NSCLC cell lines. We observed that TGFβ1-dependent autophagy was diminished in the absence of Smad4 protein or the disruption of the TAK1-TRAF6-P38 MAPK pathway. Further analysis revealed that Smad4 knockdown did not alter P-mTOR/mTOR ratios, suggesting that it affects autophagy downstream of mTOR. Consistent with this hypothesis, we found that Smad4 upregulated AMPK-dependent ULK1 S555 phosphorylation. However, due to the fact that Smad4 knockdown did not disrupt the increase of ULK1 protein levels, TGFβ may alter *ULK1* expression or degradation via a Smad4-independent mechanism. Alternatively, the TAK1-TRAF6-P38 MAPK pathway may influence autophagy by impeding mTOR S2448 phosphorylation. This would explain why inhibiting the TAK1-TRAF6-P38 MAPK pathway increased the P-mTOR/mTOR ratio, decreased autophagic flux and reduced the phospho-S555-ULK1/ULK1 ratio.

The link between Smad4 and TGFβ1-dependent autophagy that we observed in NSCLC cells was consistent with studies investigating TGFβ-dependent autophagy in pancreatic ductal adenocarcinoma cell lines (Liang et al., 2020) and breast cancer cell lines (Cheng et al., 2018). However, the role of Smad4 in TGFβ-dependent autophagy is complex and remains an area that needs to be further investigated. This is because Smad4 was observed to not be essential for TGFβ-dependent autophagy in Smad4 negative cell lines (Liang et al., 2020). Also, the presence of Smad4 may not be sufficient to drive TGFβ-dependent autophagy. For example, when we inhibited the TAK1-TRAF6-P38 MAPK pathway, Smad4 did not sustain TGFβ1-dependent autophagy in NSCLC cell lines. Finally, there is some evidence

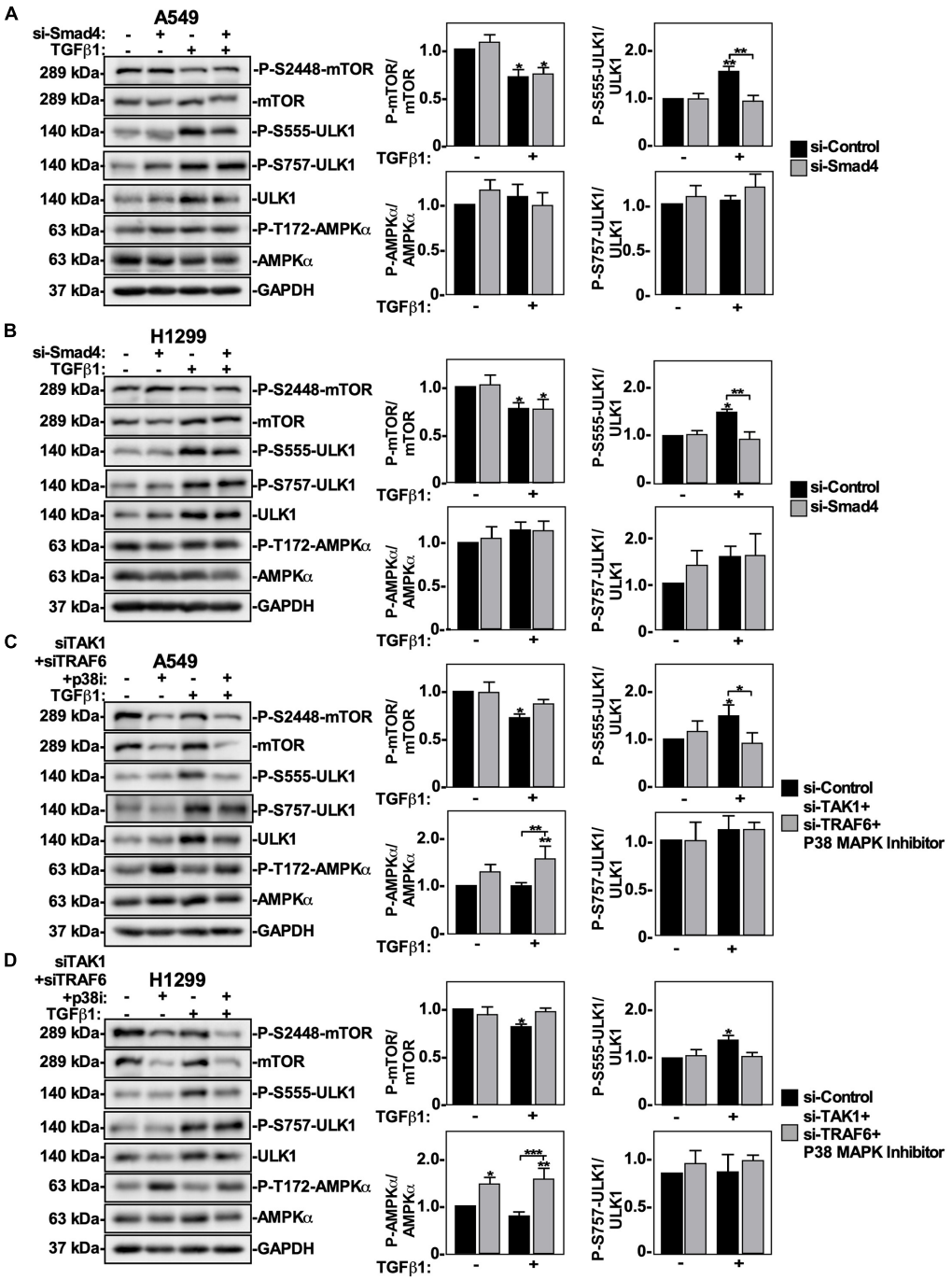

FIGURE 9 | The effect of canonical and non-canonical TGFβ signaling on mTOR and ULK1 activity in NSCLC cells. A549 **(A)** or H1299 **(B)** cells were transfected with si-Control or si-Smad4 (s534708) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-phospho-S757-ULK1, anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα and anti-GAPDH antibodies. The steady-state levels of phospho-S555-ULK1, ULK1, anti-phospho-S757-ULK1, phospho-T172-AMPKα, AMPKα, phospho-S2448-mTOR, and mTOR were quantified using QuantityOne software and the phospho-mTOR/mTOR, phospho-ULK1/ULK1 and phospho-AMPKα/AMPKa ratios were graphed ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and ** = $P < 0.01$. A549 **(C)** or H1299 **(D)** cells were transfected with si-Control or si-TAK1 (s13766) and si-TRAF6 (s14388) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM P38 MAPK inhibitor (p38i) for 24 h. The cells were then lysed, subjected to SDS-PAGE and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-ULK1, anti-phospho-S757-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα, and anti-GAPDH antibodies. The steady-state levels of phospho-S555-ULK1, ULK1, phospho-T172-AMPKα, AMPKα, phospho-S2448-mTOR and mTOR were quantified using QuantityOne software and the phospho-mTOR/mTOR, phospho-AMPKα/AMPKα and phospho-ULK1/ULK1 ratios were graphed ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

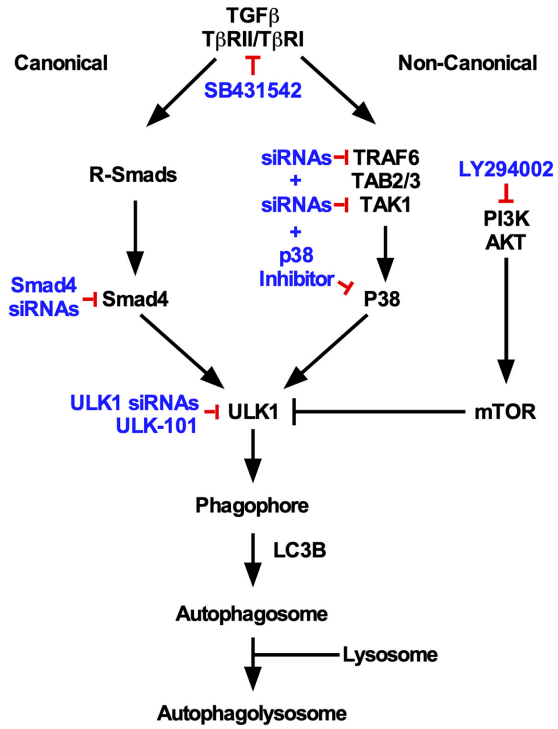

FIGURE 10 | Summary of TGFβ1-dependent autophagy in NSCLC cells. Based on the inhibitory strategies used in this study (indicated in blue), specific canonical and non-canonical TGFβ pathways were observed to regulate TGFβ-dependent autophagy. Both pathways were observed to converge on ULK1 activity and were necessary for lysosomal targeting of LC3B.

to suggest that Smad4 impedes autophagy. For instance, in orthotopic pancreatic tissue samples, *Smad4* expression was inversely correlated to autophagy (Wang et al., 2018). Therefore, the role of Smad4 in TGFβ-dependent autophagy is likely cell type dependent. In support of this, miRNA targeting of Smad4 in breast cancer cells attenuated autophagy (Cheng et al., 2018) whereas Smad4 depletion protected pancreatic cancer cells from radiotherapy by inducing autophagy (Wang et al., 2018). Less unclear is the importance of Smad4 in tumorigenesis. To date, Smad4 is known as the most common Smad family gene mutated in cancer (Sarshekeh et al., 2017). Smad4 mutations are found in approximately 50% of pancreatic adenocarcinomas (Howe et al., 1998), 20% of colorectal cancers (Chu et al., 2004) and 5% of head and neck squamous cell carcinomas (Lin et al., 2019). Currently, more research is needed to characterize the relationship between Smad4, autophagy and cancer to determine if Smad4 genetic targeting in cancer cells could impede tumorigenesis by hindering both TGFβ and autophagy-dependent drivers of cancer.

Since members of the TAK1-TRAF6-P38 MAPK pathway have been shown to affect autophagy, we decided to study this pathway in TGFβ1-dependent autophagy. A possible explanation for the lack of knowledge with respect to how it is involved in TGFβ1-dependent autophagy, is that this pathway is accessed by numerous stimuli. For instance, TAK1 is activated by tumor necrosis factors, toll-like receptors, interleukins and TGFβ ligands prior to activating P38 MAPK and c-Jun N-terminal kinase, which regulate metabolism, growth, survival and tumorigenesis (Landström, 2010). Polyubiquitination and activation of TRAF6 is initiated by interleukins and toll-like receptors during innate proinflammatory responses; nucleotide-binding and oligomerization domain containing protein 2 receptors recognizing bacteria; recognition of viral RNAs; TGFβ receptors; receptor activator of nuclear factor kappa-B ligands during osteoclast differentiation; and several cell surface receptors on B-lymphocytes and T-lymphocytes (Dainichi et al., 1107). Therefore, due to the broad spectrum of stimuli that induce TAK1, TRAF6, or P38 MAPK activation, we knew little about their respective roles in autophagy and even less with regards to TGFβ1-dependent autophagy.

TAK1 functions as an upstream AMPK kinase by phosphorylating AMPK at threonine172 (Aashaq et al., 2019). For this reason, the increase in P-AMPKα/AMPKα ratio in cells subject to TAK1-TRAF6-P38 MAPK pathway inhibition was surprising. Since AMPK stimulates autophagy by phosphorylating ULK1 to form the ULK1 complex and by suppressing mTOR activity (Liu et al., 2018), TAK1 has become a target of interest to suppress autophagy. For instance, TAK1 inactivation in mice has resulted in the accumulation of dysfunctional mitochondria in skeletal muscle (Hindi et al., 2018). Furthermore, compared to their wild-type counterparts, mice with hepatocyte depletion of *TAK1* developed hepatosteatosis due to autophagy suppression in which further analysis indicated that TAK1 depletion suppressed AMPK activity and increased mTOR activity. However mTOR inhibition restored autophagy, therefore, consistent with our findings TAK1 may influence autophagy at the level of or upstream of mTOR (Inokuchi-Shimizu et al., 2014).

Experiments investigating TAK1 have highlighted a relationship between TGFβ signaling, autophagy and cancer. For example, the genetic deletion of *TAK1* blocked growth and migration of hepatocellular carcinoma (Inokuchi-Shimizu et al., 2014). Likewise, TAK1 knockdown experiments attenuated tumor growth in xenograft models (Inokuchi-Shimizu et al., 2014; Hindi et al., 2018). One possible explanation for this is that TAK1 expression is positively correlated with mTOR expression and phosphorylation. Therefore, as the activity of TAK1 increases, autophagic flux decreases and disrupts the tumor promoting properties of autophagy in cancer cells (Cheng et al., 2019). In support of this, inhibition of TAK1 in Kras-dependent NSCLC cell lines induced apoptosis by inhibiting protective autophagy (Yang et al., 2018).

TRAF6 is an E3 ubiquitin ligase proven to be essential for toll-like receptor 4-dependent autophagy. TRAF6 stabilizes beclin-1 by conjugating it to lysine(K)63-linked polyubiquitin chains (Shi and Kehrl, 2010). Furthermore, TRAF6 in partnership with autophagy and beclin-1 regulator 1 tethers ULK1 to K63-linked polyubiquitin chains to promote its stability, self-association and kinase activity (Nazio et al., 2013; Zhao and Zhang, 2016). Interestingly, TRAF6 may be a suitable therapeutic target for the pro-tumorigenic properties of autophagy. For instance, peroxiredoxin 1, an antioxidant enzyme, was observed to inhibit TRAF6 ubiquitin-ligase activity, downregulate autophagy and

inhibit cancer cell migration (Min et al., 2018). Additionally, blocking TRAF6 in mice models of cancer cachexia attenuated autophagy-dependent muscle wasting (Paul and Kumar, 2011). For this reason, future work is needed to explore how silencing TRAF6 influences TGFβ-dependent autophagy and the pro-tumorigenic properties of TGFβ.

To date, P38 MAPK has been implicated in augmenting cancer cachexia by upregulating autophagy. For example, stimulating toll-like receptors in mice upregulated *ATG6*, *ATG7,* and *ATG12* expression in a P38 MAPK-dependent manner to promote muscle wasting. When P38 MAPK activity was blocked with SB202190, *ATG* genes were downregulated and mice were rescued from muscle wasting phenotypes (McClung et al., 2010). However, the role of P38 MAPK in autophagy is cell type dependent. For instance, in microglial cells, after lipopolysaccharide stimulate toll-like receptors, P38 MAPK is activated and phosphorylates ULK1, which disrupts ULK1 from recruiting ATG13 and other components of the ULK1 complex (He et al., 2018). Furthermore, another study identified that when SB202190 blocked P38 MAPK activity, the p53-dependent apoptotic response is interrupted and autophagy was upregulated, which promoted cancer cell resistance to 5-fluorouracil (De La Cruz-Morcillo et al., 2012). Recently, evidence has emerged that flavopereirine, a chemotherapeutic agent that decreases the proliferation and viability of cancer cells largely through unknown mechanisms, inhibited autophagy by upregulating the P38 MAPK pathway (Chen et al., 2020). Although these forms of autophagy are independent of TGFβ, they are still important to understanding a potential relationship between TGFβ, cancer and autophagy.

In summary, TGFβ1 regulates autophagy using Smad4 and TAK1-TRAF6-P38 MAPK pathways to influence AMPK-dependent ULK1 S555 phosphorylation. Future work will evaluate how silencing Smad4 and the TAK1-TRAF6-P38 MAPK pathway impacts pro-tumorigenic properties of TGFβ and autophagy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CT carried out the work presented and finalized the writing of the manuscript. GD supervised the studies, helped design the overall experimental approach, and helped prepare the final manuscript. Both 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.2021.712124/full#supplementary-material

Supplementary Figure 1 | The effect of TGFβ1 on the nuclear intensity of ULK1 and ULK2. A549 cells were treated with 250 pM TGFβ1 for 24 h. The cells were fixed and stained with DAPI (blue), anti-ULK1 (green) and anti-ULK2 (red). A Nikon Eclipse Ti2 confocal microscope was used to visualize the cells and an optical slice through the nucleus was imaged. ImageJ (version 2.0) quantified relative nuclear ULK1 intensity/Total ULK1 intensity, which are graphed below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and **** = $P < 0.0001$. Bar = 10 μm.

Supplementary Figure 2 | The effect of a second series of ULK1 and ULK2 siRNAs on TGFβ1-dependent autophagy. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with control siRNA (si-Control), siRNA targeting ULK1 (si-ULK1; s15965) or siRNA targeting ULK2 (si-ULK2; s18705) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-ULK1, anti-ULK2, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$.

Supplementary Figure 3 | The effect of a second Smad4 specific siRNA on TGFβ1-dependent autophagy. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or siRNA targeting Smad4 (si-Smad4-1; s534708 or si-Smad4-2; s8404) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-Smad4, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and ** = $P < 0.01$.

Supplementary Figure 4 | The effect of aPKC knockdown on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or siRNA targeting aPKCζ (s11128) and aPKCι (s11110) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-aPKCζ, anti-aPKCι, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady state LC3B-II protein levels and the GFP/RFP ratio are shown below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and **** = $P < 0.0001$. **(C)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$.

Supplementary Figure 5 | The effect of P38 MAPK on TGFβ1 induced autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were treated with 10 μM P38 MAPK or equivalent volumes of DMSO in the presence and absence of 250 pM TGFβ1 for 24 h. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-cleaved parp, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state ULK1 and LC3B-II protein levels and the GFP/RFP ratio are shown graphically

below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. **(C)** A549 cells stably expressing a cDNA GFP-LC3-RFP-LC3ΔG construct were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$.

Supplementary Figure 6 | The effect of TRAF6 silencing on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-TRAF6 (s14388) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-TRAF6, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and **** = $P < 0.0001$. **(C)** A549 cells stably expressing a cDNA GFP-LC3-RFP-LC3ΔG construct were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as *** = $P < 0.001$.

Supplementary Figure 7 | The effect of TAK1 silencing on TGFβ1-dependent autophagy in NSCLC cell lines. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-TAK1 (s13766) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-TAK1, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. **(C)** A549 cells stably expressing a cDNA GFP-LC3-RFP-LC3ΔG construct were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. **(D)** ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$. **(E)** Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically below representative images ($n = 3 \pm$ SD). Significance is indicated as *** = $P < 0.001$ and **** = $P < 0.0001$.

Supplementary Figure 8 | The influence of a second TAK1 and TRAF6 series of siRNAs in combination with a P38 MAPK inhibitor on TGFβ1-dependent autophagy. A549 **(A)** or H1299 **(B)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-TRAF6 (s14389) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-TRAF6, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and **** = $P < 0.0001$. A549 **(C)** or H1299 **(D)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-TAK1 (s13767) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, lysed and subjected to SDS-PAGE and immunoblotted for anti-TAK1, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$. A549 **(E)** or H1299 **(F)** cells stably expressing GFP-LC3-RFP-LC3ΔG were transfected with si-Control or si-TAK1 (s13767) and si-TRAF6 (s14389) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM P38 MAPK inhibitor for 24 h. The cells were then lysed, subjected to SDS-PAGE and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of GFP/RFP ratios are shown to the right of representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and ** = $P < 0.01$.

Supplementary Figure 9 | The effect of Compound C on TGFβ1 induced autophagy in NSCLC cell lines. **(A)** A549 cells stably expressing GFP-LC3-RFP-LC3ΔG were treated with 10 μM Compound C or DMSO (vehicle control) in the presence and absence of 250 pM TGFβ1 for 24 h. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα anti-LC3B and anti-GAPDH antibodies. Quantitative analysis of steady state LC3B-II protein levels and the GFP/RFP, P-AMPKα/AMPKα, P-ULK1/ULK1 ratios are shown below representative immunoblots ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$. **(B)** A549 cells stably expressing a cDNA GFP-LC3-RFP-LC3ΔG construct were treated as described above. Hoechst stain (blue) was added 10 min prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 μm. ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

Supplementary Figure 10 | The effect of canonical and non-canonical TGFβ signaling on the cellular distribution of ULK1. **(A)** A549 cells were transfected with si-Control or two different siRNAs targeting Smad4 (si-Smad4-1; s534708 and siSmad4-2; s8404) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 h, fixed and stained with DAPI (blue) or anti-ULK1 (green). A Nikon Eclipse Ti2 confocal microscope was used to visualize the cells and an optical slice through the nucleus was imaged. ImageJ (version 2.0) quantified relative nuclear ULK1 intensity/Total ULK1 intensity, which are graphed below representative images ($n = 3 \pm$ SD). Significance is indicated as ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$. Bar = 10 μm. **(B)** A549 cells were transfected with si-Control or two different siRNAs against TAK1 (si-TAK1-1; s13766 and TAK1-2; s13767) or TRAF6 (si-TRAF6-1; s14388 and TRAF6-2; s14389) for 48 h. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM P38 MAPK inhibitor for 24 h. The cells were then fixed and stained with DAPI (blue) or anti-ULK1 (green). A Nikon Eclipse Ti2 confocal microscope was used to visualize the cells and an optical slice through the nucleus was imaged. ImageJ (version 2.0) quantified relative nuclear ULK1 intensity/Total ULK1 intensity, which are graphed below representative images ($n = 3 \pm$ SD). Significance is indicated as * = $P < 0.05$ and ** = $P < 0.01$. Bar = 10 μm.

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TGF-β Signaling and Resistance to Cancer Therapy

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The transforming growth factor β (TGF-β) pathway, which is well studied for its ability to inhibit cell proliferation in early stages of tumorigenesis while promoting epithelial-mesenchymal transition and invasion in advanced cancer, is considered to act as a double-edged sword in cancer. Multiple inhibitors have been developed to target TGF-β signaling, but results from clinical trials were inconsistent, suggesting that the functions of TGF-β in human cancers are not yet fully explored. Multiple drug resistance is a major challenge in cancer therapy; emerging evidence indicates that TGF-β signaling may be a key factor in cancer resistance to chemotherapy, targeted therapy and immunotherapy. Finally, combining anti-TGF-β therapy with other cancer therapy is an attractive venue to be explored for the treatment of therapy-resistant cancer.

Keywords: TGF-β pathway, TGF-β, chemotherapy resistance, targeted therapy resistance, immunotherapy resistance

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INTRODUCTION

Relationship Between TGF-β Signaling and Cancer Therapy Resistance

Cancer is a leading cause of death globally and there has been on-going efforts to find cures for it. In addition to surgical removal of tumors as well as radiotherapy, a plethora of chemical compounds and/or biological agents have been employed for the treatment of cancer. Chemotherapy, consisting of cytotoxic agents that aim to target highly proliferative cancer cells, was first introduced in the 1940's (Goodman and Wintrobe, 1946; Farber et al., 1948; Falzone et al., 2018). Since then, chemotherapeutic drugs such as paclitaxel, cisplatin, and doxorubicin have become first-line treatments for a variety of cancers (Falzone et al., 2018). However, chemotherapy acts not only on tumor cells but also on normal cells, which often leads to severe side effects. In search for anti-tumor drugs with higher selectivity for tumor cells and fewer adverse effects towards normal cells, scientists designed inhibitors against key molecular targets involved in driving cancer progression; such therapeutic strategies belong to the category of targeted therapy. For example, kinase inhibitors against the epidermal growth factor receptor (EGFR), like gefitinib and erlotinib, are used for the treatment of non-small cell lung cancer (NSCLC) patients with activating mutations in the EGFR gene (Antonicelli et al., 2013).

In the past 10 years, a new class of anti-cancer therapy has emerged with great promise in inducing prolonged responses in cancer patients with advanced or metastatic cancers (Sharma et al., 2017). Using biological agents such as monoclonal antibodies against immune checkpoints, as well as genetically engineered T cells, cancer immunotherapy harnesses the patient's immune system to recognize and eradicate tumors. In addition, researchers have been testing different combinations of cancer therapies to

optimize therapeutic efficacy while minimizing unwanted side effects. Despite advancements in anti-cancer therapies, achieving relapse-free survival remains challenging, due to the emergence of primary or acquired resistance in response to treatment (Oppermann et al., 2016). In some cases, patients fail to respond to cancer treatment in the first place, suggesting that primary resistance, which often arises from pre-existing genetic mutations or epigenetic alterations in the tumor, is impeding therapeutic response. In other cases, patients respond initially to drug treatment but its efficacy diminishes over-time, which indicates the development of acquired resistance. In this scenario, recurrent tumors are often more aggressive and resistant to treatments. Like primary resistance, acquired resistance can be attributed to a number of factors including genetic mutations that allow tumors to evade attacks by cancer therapy and/or to activate alternative survival pathways. Drug resistance is associated with increased expression of drug efflux transporters, activated proliferation and anti-apoptotic signaling, enhanced cancer stemness, as well as evasion of immunosurveillance (Nussinov et al., 2017). A number of recent studies have shown that activation of transforming growth factor β (TGF-β) signaling was associated with drug resistance in a variety of cancers including melanoma (Sun et al., 2014), NSCLC (Soucheray et al., 2015), breast cancer (Palomeras et al., 2019), hepatocellular carcinoma (HCC) (Bhagyaraj et al., 2019), colorectal cancer (CRC) (Quan et al., 2019), squamous cell carcinoma (SCC) (Brown et al., 2017), osteosarcoma (OS) (Wang et al., 2019), prostate cancer (Song et al., 2018a), as well as in tumor-initiating cells of a few types of cancer (Yu et al., 2018; Batlle and Massagué, 2019; Tang et al., 2020; Taniguchi et al., 2020). Moreover, high levels of TGF-β in patients with breast cancer, NSCLC, HCC, CRC predicted a poor prognosis (Calon et al., 2015; Okada et al., 2018; Zhuang and Wang, 2018; Tauriello, 2019; Guo et al., 2020). As a result, extensive research has been conducted to explore the potential role of TGF-β signaling inhibitors as means to overcome cancer treatment resistance (Huang et al., 2012; Sun et al., 2014; Jenkins et al., 2015; Koetz-Ploch et al., 2017; Li et al., 2019a; Wang et al., 2019).

The TGF-β superfamily, which comprises TGF-βs, Activins (Acts), Nodal, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and anti-Müllerian hormone (AMH), is implicated in embryonic development, cell proliferation, differentiation, apoptosis, and immune responses (Yang et al., 2010; Moses et al., 2016). Importantly, TGF-β is a regulator of tissue homeostasis and cancer may result from dysregulated TGF-β signaling. For instance, during the embryo implantation period, TGF-β signaling is active in the endometrium to balance apoptosis and proliferation of endometrial cells (Dimitriadis et al., 2005; Latifi et al., 2019). In stratified rectal and genital epithelia lacking type II TGF-β receptor (TβRII) expression, TGF-β signaling is disrupted, leading to destabilized tissue homeostasis and the development of spontaneous SCCs in stratified epithelia (Guasch et al., 2007). There are three TGF-β receptor ligands: TGF-β1, TGF-β2, and TGF-β3. TGF-β signaling is activated when activated TGF-β ligands bind to TβRII to recruit type I TGF-β receptor (TβRI), leading to phosphorylation and activation of TβRI, which phosphorylates downstream mediators SMAD2 and SMAD3. SMAD4 then binds to SMAD2 and SMAD3 to form heterotrimeric complexes that translocate to the nucleus to regulate the transcription of target genes (Derynck and Zhang, 2003; Yang et al., 2010). Furthermore, SMAD6 and SMAD7 are part of a negative feedback loop that regulates the TGF-β pathway. The versatility in TGF-β receptor-ligand interaction is thoroughly discussed in a number of reviews (Derynck and Zhang, 2003; Zhang et al., 1007). In addition to the canonical signaling pathway described above, there are several SMAD-independent TGF-β pathways, which consist of RHO GTPases, P38, jun N-terminal kinase (JNK), mitogen-activated protein kinase (ERK or MKK), and phosphoinositide 3-kinase (PI3K)-AKT (Lee et al., 2007; Sorrentino et al., 2008; Heldin and Moustakas, 2016; Principe et al., 2017), as shown in **Figure 1**.

The functions of TGF-β are cell type- and context-dependent. Increasing evidence suggests that TGF-β signaling acts like a double-edged sword in tumor progression (Bierie and Moses, 2006; Massagué, 2008). In healthy cells and early-stage cancerous cells, activation of TGF-β signaling pathway promotes cell-cycle arrest and apoptosis; while in late-stage cancers, TGF-β signaling acts as an oncogene to induce metastasis and drug resistance (Bardeesy et al., 2006; Morikawa et al., 2016). For example, SMAD4 is phosphorylated by anaplastic lymphoma kinase (ALK) at Tyr95 in ALK-positive gastrointestinal, pancreatic and lung tumors, resulting in the inhibition of tumor suppressor activity of TGF-β (Zhang et al., 2019a). SMAD4 deletion accelerates the transformation from premalignant to malignant phenotype in pancreatic progenitors harboring Kirsten rat sarcoma virus (KRAS) mutations (Bardeesy et al., 2006; Zhang et al., 2019a). On the other hand, in advanced pancreatic ductal adenocarcinomas (PDAC), intact TGF-β/SMAD4 pathway facilitates cancer progression; in advanced prostate cancer, bone-borne TGF-β induces osteoclastogenesis and bone metastasis by activating chemokine (C-X-C motif) receptor 4 (CXCR4) (Bardeesy et al., 2006; Zhang et al., 2021). These studies provided concrete evidence for the tumor suppressive role of the TGF-β pathway in pre-malignant cells and oncogenic role in advanced cancers. In the past few decades, the dual role of TGF-β in tumorigenesis and tumor-suppression have been extensively studied (Roberts and Wakefield, 2003; Levy and Hill, 2006; Massagué, 2008) and a growing body of literature elucidated that TGF-β/SMAD pathway was activated in multi-therapy resistance. However, the mechanisms underlying TGF-β mediated-drug resistance are still being explored and existing evidence lacks consistency. In this review, we mainly focus on the role of TGF-β signaling in drug resistance. Here, we provide an overview of pre-clinical and clinical studies of TGF-β signaling in regulating cancer drug resistance, and offer our perspective on potential strategies to target TGF-β-mediated drug resistance in cancer patients.

TGF-β SIGNALING AND RESISTANCE TO TARGETED THERAPY

Targeted therapy acts by interfering with oncogenic cellular processes to selectively eradicate cancer cells, mainly including specific enzymes, growth factor receptors, and signal transducers.

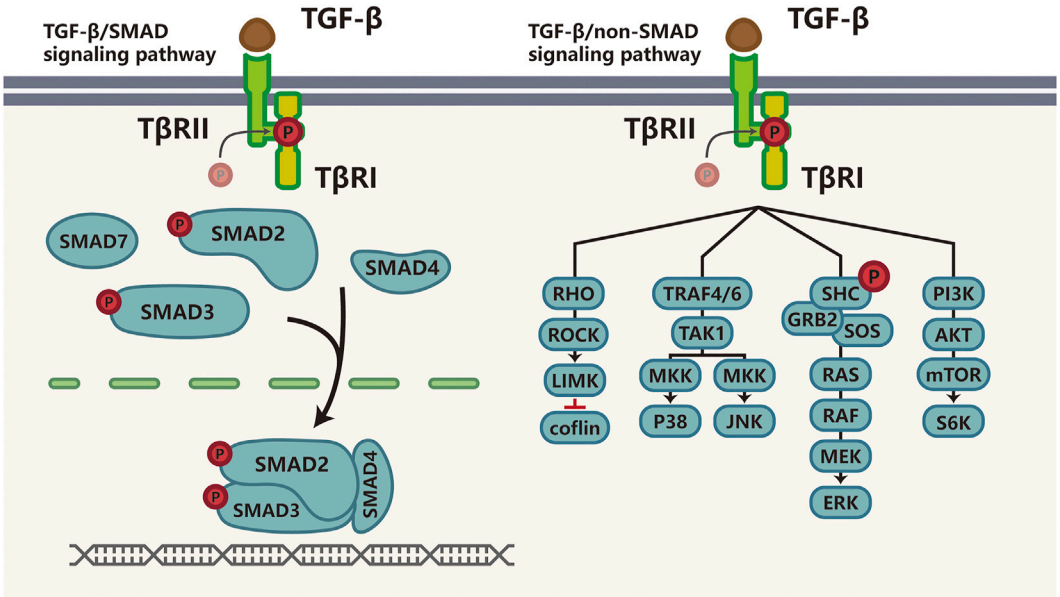

FIGURE 1 | TGF-β signaling pathway TGF-β transduces signaling through SMAD or non-SMAD signaling pathways. Actived TGF-β binds to TGF-β ligand, Once TGF-β binds to TβRII, TβRI is recruited, phosphorylated and activated to phosphorylate the downstream mediators-SMAD2 and SMAD3; then SMAD4 binds to activated SMAD2 and SMAD3 to form heterotrimeric transcriptional complexes that translocate and relay this signaling into the nucleus to further regulate transcription. This is called canonical TGF-β/SMAD signaling pathway (right). The non-SMAD-dependent activation of the TGF-β pathway involves signaling via RHO GTPases, P38, JNK, ERK or MEKK, and PI3K-AKT (left). Abbreviations: P, phosphorylation; TβR, transforming growth factor (TGF)-β receptor; ROCK, RHO-associated coiled-coil containing protein kinase; LIMK, LIM kinase; TRAF, TNF receptor-associated factor; TAK1, TGF-β-activated kinase-1. JNK, c-Jun N-terminal kinase; SHC, SRC homology 2 domain-containing transforming protein; GRB2, growth factor receptor-bound protein 2; SOS, son of sevenless; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-4,5-bisphosphate; mTOR, mechanistic target of rapamycin.

The first effective example of targeted therapy is the inhibition of the BCR-ABL1 oncogene in chronic myeloid leukemia (CML) (Salesse and Verfaillie, 2002). Subsequently, EGFR inhibitors (EGFRi) such as cetuximab, erlotinib and gefitinib were developed to treat EGFR-mutant NSCLC (Kazandjian et al., 2016); BRAF/MEK inhibitors (BRAFi/MEKi) were developed for BRAF-mutant melanoma; and epidermal growth factor receptor 2 inhibitors (HER2i) were developed for the treatment of HER2 positive ($HER2^+$) breast cancer (Harbeck and Gnant, 2017). However, the efficacy of targeted therapy is often compromised by drug resistance and studies found that up-regulation of TGF-β signaling was a major driver of targeted therapy resistance (Brunen et al., 2013). Next, we summarize recent findings describing how TGF-β signaling helps tumor cells bypass pathway inhibition by activating alternative survival pathways or anti-apoptotic signaling pathways (**Figure 2**).

One example of TGF-β signaling-mediated resistance to targeted therapy was reported in cancer treated with BRAFi/MEKi (Sun et al., 2014; Lu et al., 2017; Bugide et al., 2020). The MAPK signaling pathway consists of kinases RAS, RAF, MEK, and ERK, which are essential for cell proliferation and survival. Hyper-activation of MAPK signaling occurs frequently in human cancers, such as melanoma, colorectal cancer, thyroid carcinoma, and hepatic cancer (Fang and Richardson, 2005; Santarpia et al., 2012; Lee et al., 2020). Treatment with BRAFi/MEKi, such as vemurafenib, sorafenib and trametinib, often results in remarkable disease regression initially, followed by the development of BRAFi/MEKi resistance (Rizos et al., 2014; Sun et al., 2014; Lu et al., 2017). Studies found that TGF-β signaling was frequently up-regulated in BRAFi-treated cancer cells (Faião-Flores et al., 2017; Bugide et al., 2020). Screening with a short hairpin RNA (shRNA) library focusing on chromatin regulators, Sun and his colleagues (Sun et al., 2014) discovered that TGF-β signaling was activated by the suppression of SRY-box transcription factor 10 (SOX10), thereby causing an up-regulation of EGFR and platelet-derived growth factor receptor-β (PDGFRB) signaling to confer resistance to MAPK inhibitors. In addition, TGF-β signaling was reported to mediate the up-regulation of microRNA-125a (miR-125a) expression and suppression of pro-apoptotic pathway, which accounted for the acquisition of BRAFi resistance in BRAF-mutant melanoma patients (Koetz-Ploch et al., 2017). Prete and others (Prete et al., 2018) demonstrated that in cancer cells with BRAF mutations, therapeutic escape from BRAFi/MEKi was facilitated by pericytes that secreted thrombospondin-1 (TSP-1) and TGF-β1, both of which led to a rebound of pERK1/2, pAKT and pSMAD3 (Fedorenko et al., 2015).

In addition to cancers with BRAF mutations, TGF-β signaling is also associated with therapy resistance in cancers with hyperactive EGFR. EGFR mutation or amplification are frequently detected in lung cancer; and studies suggest that activation of TGF-β pathway is associated with EGFRi/EGFR tyrosine kinase inhibitor (TKI)/cetuximab resistance (Yao et al., 2010; Bedi et al., 2012; Kurimoto et al., 2016; Li et al., 2016; Du

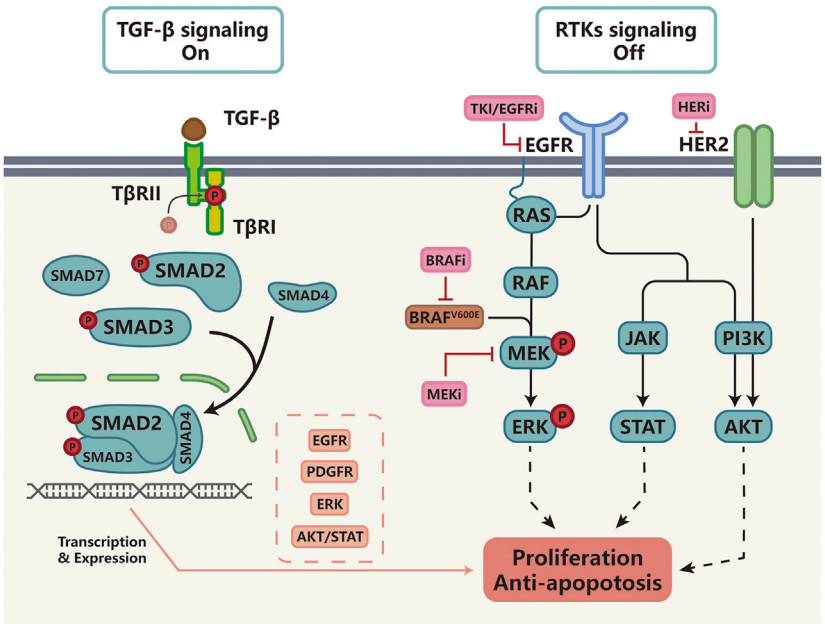

FIGURE 2 | TGF-β signaling and resistance to targeted therapy Cancers with activating BRAF-mutations or EGFR-mutations as well as HER2-positive cancer are often treated with small molecular inhibitors against these molecular targets. For example, $BRAF^{V600E}$ is often targeted by BRAFi such as vemurafenib, MEK by MEKi such as tramelinib, and HER2 by trastuzumab, Upon kinase inhibitor treatment, receptor tyrosine kinase (RTK) signaling is turned off. In cells that activate TGF-β-induced drug resistance, TGF-β signaling functions by increasing the expression of EGFR, PDGFR, ERK, AKT/STAT to activate alternative survival pathways and suppress apoptosis, protecting tumor cells from targeted therapy.

et al., 2020; Kuo et al., 2020; Qiu et al., 2020). Approximately 30% of NSCLC patients with EGFR-mutations have no response to TKIs; such primary resistance can be attributed to mutations in the transforming growth factor beta receptor 1 (TGFBR1) gene and the resulting activation of TGF-β/SMAD signaling pathway-mediated mesenchymal-epithelial transition (EMT) (Zhong et al., 2017; Zhang et al., 2019b). Suppression of TGF-β signaling and down-regulation of Slug expression enhanced the gefitinib-sensitivity in TKI-resistant lung cancer cells (Qiu et al., 2020). Mechanistically, in EGFRi-resistant cancer cells, TGF-β signaling can be regulated by the binding of transcriptional factors to the promoter of TGFBR, or directly to the receptor itself. For example, zinc finger protein 32 (ZNF32) binds to the TβRII promoter to promote the expression of TβRII, while mediator complex subunit 12 (MED12) negatively regulates TβRII through physical interaction in the cytoplasm. Elevated expression of ZNF32 or reduced expression of MED12 up-regulate TGF-β signaling, resulting in MEK/ERK pathway activation to promote EGFRi-resistance in lung cancer (Huang et al., 2012; Li et al., 2016). Yao et al (2010) showed that both tumor cell-autonomous mechanisms and changes in the tumor microenvironment (TME) could activate the TGF-β–SMAD/IL6 signaling axis to drive erlotinib resistance, as IL6-induced STAT3 expression protected tumor cells from apoptosis (Yao et al., 2010). Moreover, TGF-β could activate AKT in an EGFR-independent fashion to inhibit cell apoptosis in EGFR-mutant cancers when treated with cetuximab and TKIs (Bedi et al., 2012; Wang et al., 2019). Further studies demonstrated that TGF-β down-regulated the expression of the stemness factor SOX2 to promote TKI tolerance (Kuo et al., 2020). In addition to the regulation of downstream targets to drive drug resistance, TGF-β regulates the alternative splicing of TGF-β-activated kinase 1 (TAK1) transcript into two isoforms: the short isoform TAK1ΔE12 supports TGF-β-induced EMT and nuclear factor kappa B (NF-κB) signaling to confer resistance to afatinib (EGFR inhibitor), whereas the full-length isoform promotes TGF-β-induced apoptosis. Selective blockade of the expression of the short isoform by blocking TGF-β-induced alternative splicing of TAK1 may be potential avenue to overcome TGF-β-induced drug resistance (Tripathi et al., 2019).

Another example of TGF-β signaling-mediated resistance was reported in HER2 targeted therapy for HER2-positive cancers. Overexpression of HER2 occurs in 20–25% of human breast cancers; it is also observed in other types of cancers such as advanced gastric or gastroesophageal junction cancer (Boku, 2014). Trastuzumab is a humanized monoclonal antibody targeting HER2. Although it was approved for the treatment of metastatic cancers, a large cohort of the patients eventually developed trastuzumab resistance (Esteva et al., 2002). Studies found that TGF-β signaling pathway was consistently overexpressed in trastuzumab-resistant breast cancer cells and gastric cancer cells (Bai et al., 2014; Zhou et al., 2018). Treatment with exogenous TGF-β conferred insensitivity to trastuzumab in HER2-positive breast cancer cell lines, through up-regulating the expression of EMT and cancer stem cell (CSC) markers (Chihara et al., 2017). Growth differentiation factor 15 (GDF15)-mediated activation of TGF-β receptor-Src-HER2 signaling was also identified as a mechanism of trastuzumab resistance (Joshi

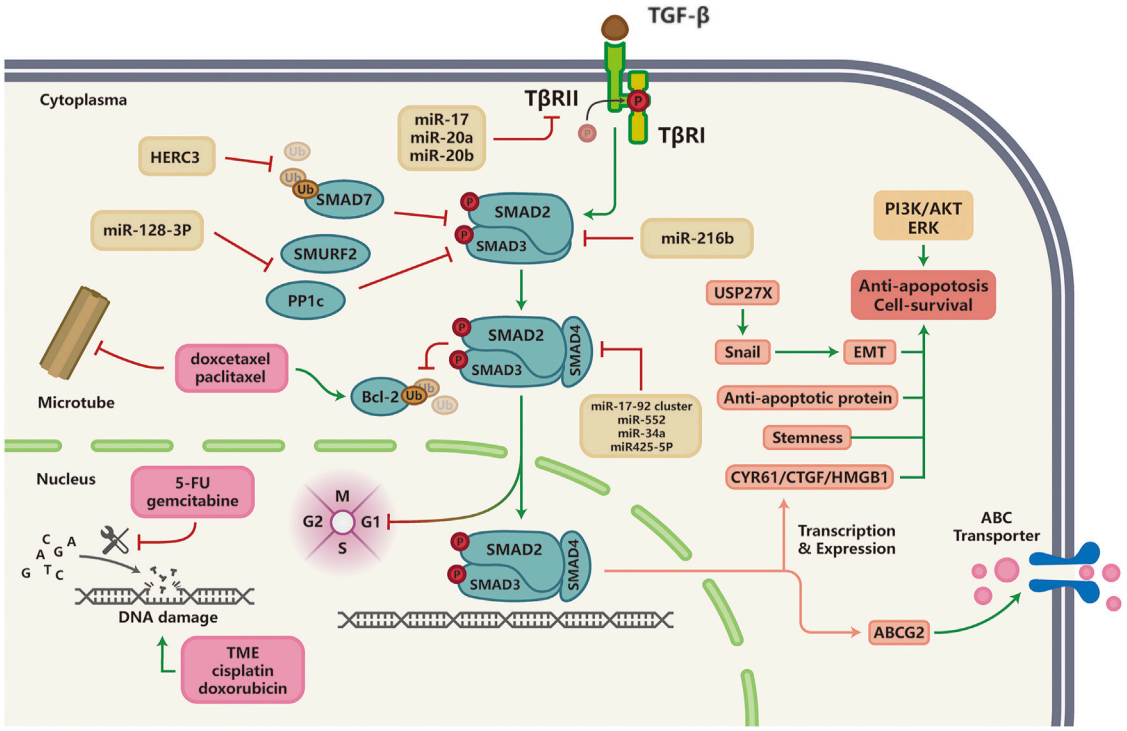

FIGURE 3 | TGF-β signaling and resistance to chemotherapy; Multiple miRNAs are implicated in TGF-β-induced chemotherapy resistance in various cancer types by targeting components of the TGF-β pathway (SMAD2, SMAD3, SMAD4). Anti-microtubule drugs promote Bcl-2 protein ubiquitination, which could be inhibited by TGF-β signaling to induce taxane resistance in malignancies. Hyperactivation of TGF-β signaling pathway induces resistance to DNA damaging agents and anti-metabolites through the activation of alternative survival pathways or anti-apoptotic signaling such as PI3K/AKT and ERK pathways, as well as elevated expression of ABC multi-drug transporters to facilitate cancer cell survival and drug efflux, respectively.

et al., 2011). Combined with the activation of Src-focal adhesion kinase (FAK), TGF-β integrated HER2 and integrin signaling to promote cell survival and invasion to escape trastuzumab-induced apoptosis (Wang et al., 2009).

Other targeted therapy with resistance mechanisms associated with TGF-β signaling include CD4/6 inhibitor (Palbociclib), FAK inhibitor (VS-4718), androgen receptor inhibitors (enzalutamide), and BET inhibitor (Liu and Korc, 2012; Lin et al., 2013; Shi et al., 2016; Liu et al., 2017; Song et al., 2018b; Cornell et al., 2019; Paller et al., 2019). Taken together, these studies clearly demonstrated that TGF-β signaling pathway had an essential role in the development of resistance to targeted therapy against a variety of oncogenic pathways across different malignancies.

TGF-β SIGNALING AND RESISTANCE TO CHEMOTHERAPY

The goal of chemotherapy is to eliminate highly proliferative cells but are non-specific compared with targeted therapy. Chemotherapy can be further divided into a few sub-categories based on their molecular mechanisms, including DNA damaging agents, anti-metabolites, and anti-microtubule agents. Emerging literature suggests that TGF-β signaling contributes to chemotherapy resistance in a variety of solid tumors. Here, we will summarize studies that reveal how TGF-β signaling induces chemotherapy resistance (**Figure 3**).

DNA Damaging Agents

DNA damaging agents, such as cisplatin, temozolomide (TMZ), oxaliplatin (OXA), doxorubicin, and etoposide, can cause cell cycle arrest and apoptosis through directly alkylating DNA, inhibiting topoisomerases and impairing DNA repair. However, like many other types of cancer treatments, chemotherapy efficacy is often compromised by the development of drug resistance. Drug resistance can arise from mutations, epigenetic changes, and other cellular and molecular mechanisms that are not yet fully elucidated (Chen et al., 2010; Ali et al., 2013; Cai et al., 2017; Li et al., 2019b; Li et al., 2019c; Lambies et al., 2019; Taniguchi et al., 2020; Vu et al., 2020). Because of the important roles of TGF-β signaling in acquired resistance against DNA damaging agents in cancer patients, the mechanisms underlying these processes are of high interest as they can direct novel drug development.

Accumulating evidence suggests that resistance to DNA damaging agents is often associated with activation of TGF-β signaling through various mechanisms, particularly by miRNA-mediated regulation of TGF-β signaling (Cai et al., 2017; Sun et al., 2017; Chuang et al., 2019; Zhu et al., 2019; Chen et al., 2020;

Huang et al., 2020; Vu et al., 2020). miR-128-3p, which was markedly up-regulated in cisplatin-resistant NSCLC cell lines, induced mesenchymal and stem-like properties by inhibiting two negative regulators of the TGF-β pathway, SMAD-specific E3 ubiquitin protein ligase 2 (SMURF2) and protein phosphatase 1c (PP1c), which resulted in the activation of TGF-β pathway, eventually leading to EMT and the development of cisplatin resistance (Cai et al., 2017). In colorectal cancer, miR-34a directly targets the 3′-UTR of SMAD4 and represses signaling via TGF-β/SMAD4. In OXA-resistant CRC patients, miR-34a is downregulated to enhance macroautophagy by activating the TGF-β/SMAD pathway (Sun et al., 2017). Another example of miRNA-mediated regulation of TGF-β signaling came from cigarette smoke condensate treated lung cancer cell lines, where miR-216b overexpression increased resistance to platinum-based therapy by downregulating SMAD3 to further restrain TGF-β-induced tumor suppression, as well as by overexpressing Bcl-2 to escape from apoptosis (Vu et al., 2020). Besides, other researchers found that the miR17 family (miR-17, miR20a, miR20b) mediated up-regulation of TGF-β/SMAD signaling pathway to confer cisplatin resistance in NSCLC (Jiang et al., 2014).

It is well known that TGF-β plays an essential role in EMT; therefore, people started to investigate if there was a link between EMT and acquired drug resistance in cancer. Recent studies demonstrated TGF-β regulated EMT and autophagy in chemotherapy-resistant cells (Fischer et al., 2015; Zheng et al., 2015; Jiang et al., 2016; Li et al., 2019c; He et al., 2019; Jiang et al., 2019; Ungefroren, 2019; Feng et al., 2020; Chen et al., 2021). Analysis of The Cancer Genome Atlas database (TCGA) and clinical data showed that in TMZ and X-ray treated-glioblastoma, the expression of HERC3 (the E3 ubiquitin ligase) was significantly up-regulated by autophagy inducers to promote degradation of SMAD7, thereby activating the TGF-β/SMAD signaling to promote EMT, cell survival, migration and chemoradio-resistance (Li et al., 2019c). In addition to promoting EMT, TGF-β also regulates the expression of autophagy-associated genes. For instance, TGF-β signaling was up-regulated in leptin-treated mesenchymal stem cells (MSC) to enhance the expression of autophagy-associated genes, which promoted cisplatin-resistance in OS cells (Feng et al., 2020). Similarly, in breast and pancreatic cancer cell lines, TGF-β signaling during EMT contributes to cisplatin resistance by up-regulating the expression of USP27X, which increases Snail1 protein stability (Lambies et al., 2019). *In vitro*, sustained TGF-β treatment induced cathepsin B (CTSB)-mediated degradation of Disabled-2 (Dab2), which activated autophagy and inhibited apoptosis by destabilizing the pro-apoptotic Bim protein, thereby modulating doxorubicin-resistance and tumor metastasis (Jiang et al., 2016). Interestingly, recent studies have unveiled that TGF-β signaling plays an important role in CSCs to mediate chemoresistance. Using an *in vitro* reporter system for lineage tracing, Oshimori and his colleagues (Oshimori et al., 2015) showed that very few TGF-β-responding squamous cell carcinoma stem cells (SCC-SCs) were sensitive to cisplatin treatment, suggesting that TGF-β signaling pathway mediated primary resistance in CSCs. In cisplatin-resistant oral squamous cell carcinoma (OSCC), TGF-β regulated cancer cell stemness through a SMAD-independent pathway: TGF-β inhibited the function of the tumor suppressor FOXO3a through the AKT pathway, which resulted in increased expression of stemness markers, such as SOX2 and ABCG2 (Li et al., 2019d); the same phenomenon was also observed in epirubicin-resistant three negative breast cancer (TNBC) cells (Xu et al., 2018).

Anti-Metabolites and Anti-Microtubule Drugs

Fluorouracil (5-FU) and gemcitabine, two anti-cancer agents belonging to the anti-metabolite category, are widely used to obstruct critical metabolic pathways that are necessary for cancer cell proliferation and survival. Studies showed that TGF-β signaling was involved in resistance to anti-metabolite drugs. Similar to what was observed in cases of chemo-resistance against DNA damaging agents, miRNAs are involved in the activation of TGF-β signaling in cells treated with anti-metabolites and anti-microtubule drugs. Examples of miRNA regulators of TGF-β signaling include miR-423-5p, miR-552, and miR-17–92 cluster (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) in prostate cancer, colorectal cancer, and pancreatic cancer (Cioffi et al., 2015; Zhao et al., 2019; Shan et al., 2020). Intriguingly, TGF-β/SMAD signaling activation directly induced G1 cell-cycle arrest in SCC cells, leading to the entry of tumor-propagating cancer cells (TPCs) into quiescence, which protected cancer cells from DNA damage caused by 5-FU treatment by adopting a condensed heterochromatic state (Brown et al., 2017). Activation of TGF-β signaling also indirectly promotes gemcitabine resistance through reduced expression of nucleoside transporters hENT1 and hCNT3, which are two critical genes that promote cellular uptake of drugs (Hesler et al., 2016). Moreover, hypoxia-inducible factor (HIF-1α) and cancer-associated fibroblasts (CAFs)-secreted TGF-β2 converge to activate the expression of hedgehog transcription factor GLI2 in CRC-CSCs, resulting in increased stemness/dedifferentiation and resistance to 5-FU (Tang et al., 2018). In addition, TGF-β induces the expression of cysteine-rich 61 (CYR61), connective tissue growth factor (CTGF) and high-mobility group box-1 (HMGB1), which facilitates chemotherapy resistance in cancers by promoting the expression of anti-apoptotic proteins (Hesler et al., 2016; Xian et al., 2017; Zhuang et al., 2017).

Microtubules are important components of almost all eukaryotic cells. Drugs such as colchicine, nocodazole, and taxane can cause cell cycle arrest by directly affecting the assembly and disassembly of microtubules in cells. Taxanes including docetaxel and paclitaxel are extensively used in the treatment of various solid tumors to disrupt microtubule function in tumor cells (Li et al., 2020a). Similar to the mechanism of resistance to anti-metabolic drugs, resistance to taxanes is associated with dysregulation of the TGF-β signaling pathway. For example, aurora-A kinase (AURKA) is overexpressed in TNBC to mediate TGF-β-induced EMT in docetaxel-resistant and paclitaxel-resistant breast cancer cells (Jalalirad et al., 2021). In breast cancer and ovarian cancer, TGF-β/SMAD signaling up-

regulates the expression of obg-like ATPase 1 (OLA1) and ST3GAL1 (a sialyltransferase), leading to accelerated EMT, enhanced cancer stem-like features, and the expression of anti-apoptotic proteins such as cleaved caspase 3, Bcl2-associated protein X (Bax) and Bcl-2 (Wu et al., 2018; Jalalirad et al., 2021). Moreover, it was reported that bone-borne TGF-β induced acetylation of human Krüppel-like factor 5 (KLF5) by activating CXCR4, which resulted in osteoclastogenesis, bone metastases, and the development of docetaxel resistance, on the other hand, the inhibition of TGF-β and CXCR4 signaling promoted cell cycle arrest and apoptosis in advanced prostate cancer cells (Zhang et al., 2021).

TGF-β SIGNALING AND RESISTANCE TO IMMUNOTHERAPY

The immune system has developed a precise mechanism to recognize and purge malignant cells. However, in response to immune surveillance, some tumor cells evolve to escape the attack from the immune system by changing or decreasing the expression of tumor-specific antigens, up-regulating immune checkpoint proteins, and altering the expression of certain cytokines to facilitate immune evasion (Kennedy and Salama, 2020). To date, clinically approved cancer immunotherapy includes immune-checkpoint inhibitors, which target immune checkpoints such as cytotoxic lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1), as well as chimeric antigen receptor T cell (CAR-T) therapy. These strategies aim to alleviate the suppression of the immune system by tumor cells, thereby reactivating anti-tumor responses and preventing immune escape (van den Bulk et al., 2018). Although cancer immunotherapy has made impressive progress in the treatment of a number of solid tumors and hematologic malignancies (Tumeh et al., 2014; Cristescu et al., 2018; Rodig et al., 2018), challenges persist as only a subset of patients with solid tumors are able to benefit from immunotherapy, owing to multiple factors such as the development of therapy resistance and interference from the intricate tumor microenvironment (TME). TGF-β is one of the most critical regulators of the TME; it is secreted by not only tumor cells but also multiple types of stromal cells including CAFs, tumor-associated macrophages (TAM), blood endothelial cells, MSC, lymphatic epithelial cells, and pericytes (Turley et al., 2015; Ganesh and Massagué, 2018). Interestingly, accumulating evidence suggests that TGF-β has an adverse role in immunotherapy response (Ganesh and Massagué, 2018; Batlle and Massagué, 2019; Larson et al., 2020). Here, we will provide a synopsis of studies on how TGF-β signaling modulates cancer immunotherapy response and discuss potential strategies to overcome TGF-β-induced immunosuppression (**Figure 4**).

TGF-β has been shown to regulate cellular functions of immunocytes including macrophages, neutrophils, bone marrow derived suppressor cells (MDSC), natural kill (NK) cells, dendritic cells (DCs) and T cells, by abolishing their cytotoxic function (Batlle and Massagué, 2019; Larson et al., 2020). TGF-β suppresses cellular functions of a variety of innate immune cells including macrophages, neutrophils, MDSC and NK cells, acting as an immune-suppressor in the TME; hyperactivation of the TGF-β signaling pathway polarizes macrophages to the pro-tumorigenic M2 phenotype by increasing Snail expression (Draghiciu et al., 2015; Zhang et al., 2016). In addition, activated TGF-β signaling converts N1 neutrophils to the immunosuppressive, pro-tumorigenic N2 phenotype by up-regulating production of arginine, CC chemokine ligand 2 (CCL2), CCL5 (Fridlender et al., 2009), while promoting the expansion of MDSCs resulting in immune tolerance (Batlle and Massagué, 2019). In addition, TGF-β was shown to block NK cell function by silencing the expression of NKG2D and NKp30 (Castriconi et al., 2003). TGF-β secreted by tumor cells facilitates the escape of tumor cells from immune surveillance by directly driving the conversion of NK cells into innate lymphoid cells type 1 (ILC1), which lacks cytotoxic function, or by impairing NKG2D-mediated cytotoxicity (Cortez et al., 2017; Gao et al., 2017; Lazarova and Steinle, 2019). DCs are the cardinal antigen presenting cells and the messenger between innate and adaptive immunity. By suppressing the expression of major histocompatibility complex II (MHC-II), TGF-β inhibits the ability of DCs to present antigens *in vitro* (Nandan and Reiner, 1997; Piskurich et al., 1998).

Other than the inhibition of cytotoxic functions of innate immunity as described above, TGF-β can also antagonize the adaptive immunity; and increasing evidence suggests that TGF-β signaling suppresses anti-tumor immunity by blocking the differentiation and functions of T helper1 (T_H1), T helper 2 (T_H2) $CD4^+$ T cells and cytotoxic $CD8^+$ T cells, while promoting the differentiation, function and survival of $CD4^+CD25^+$ forkhead box P3 (FoxP3) regulatory T cells (Tregs) cells (Nakamura et al., 2001; Thomas and Massagué, 2005; Tone et al., 2008). In healthy tissues, Tregs are present at a low level and suppress the function of T cells to maintain immune homeostasis. In activated Treg cells, the transmembrane glycoprotein A repetitions predominant (GARP) Protein is highly expressed and directs latent TGF-β to link with integrin avβ8 on the cell membrane to release active TGF-β, which contributes towards an immunosuppressive TME (Bouchard et al., 2021). Specific inhibition of TGF-β1 in GARP-expressing Treg cells was able to overcome resistance to PD-1/PD-L1 blockade in cancer patients (de Streel et al., 2020). Furthermore, to inhibit the release of active TGF-β in the TME, neutralizing antibodies were devised to target GARP or integrin avβ8, effectively reversing the adverse effect of TGF-β on T cells (Rachidi et al., 2017; Seed et al., 2021). Researchers also demonstrated that TGF-β suppressed T_H2-mediated cancer immunity. Blocking TGF-β signaling in $CD4^+$ T cells but not $CD8^+$ T cells restrained tumor growth by remodeling the TME and inducing tumor vasculature reorganization, leading to cancer cell death; this process was dependent on the T_H2 cytokine interleukin-4 (IL-4), but not the T_H1 cytokine interferon-γ (IFN-γ). In TβRII-deficient $CD4^+$ T cells, IL-4 promoted T_H2 cells gene expression program to induce T cell activation and T_H2 cells differentiation (Li et al., 2020b; Liu et al., 2020). The level of

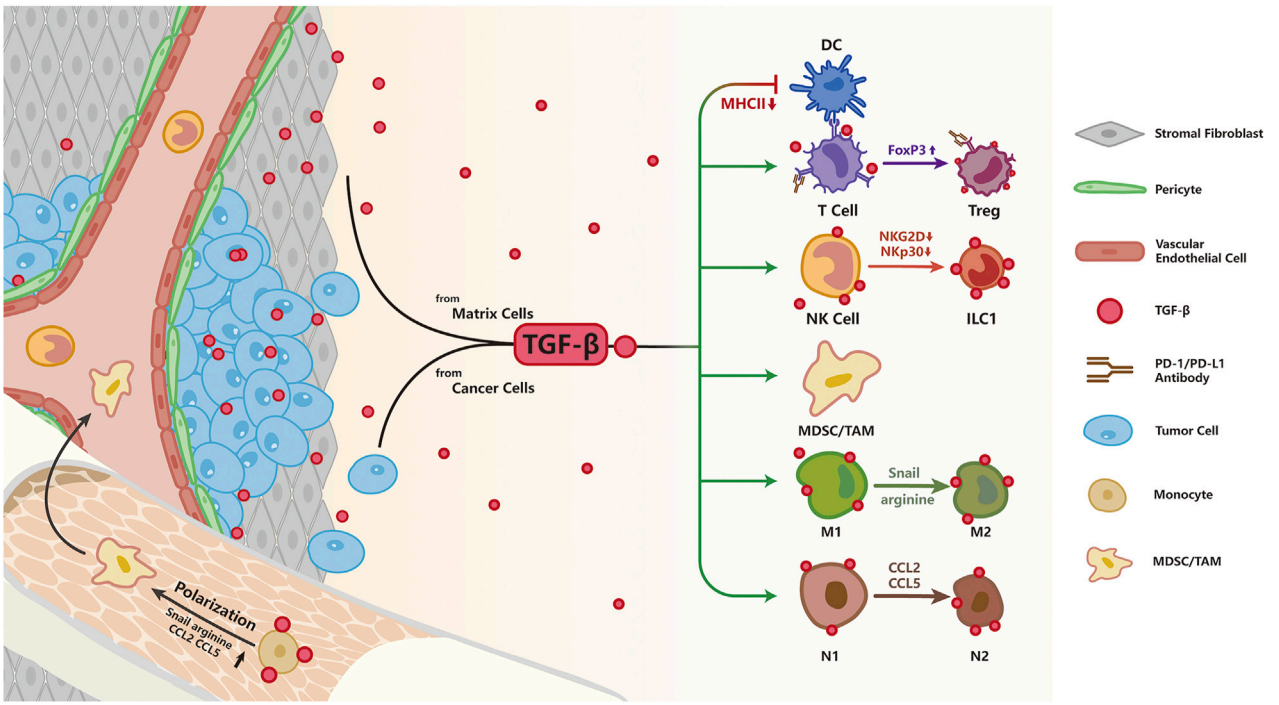

FIGURE 4 | TGF-β signaling and resistance to immunotherapy; As an immunosuppression cytokine, TGF-β is secreted by both tumor and stromal cells. TGF-β signaling pathway directly inhibits T cell function by up-regulating the expression of FoxP3, converting cytotoxic T cells to Treg cells to restrain immune response. Besides, TGF-β impairs NK function by down-regulation of NKG2D and NKp30, two surface receptors directing NK cells to eliminate abnormal cells. TGF-β impairs antigen presentation in DC cells by decreasing MHCII expression. TGF-β signaling pathway polarizes macrophages to the pro-tumorigenic M2 phenotype by increasing Snail, converts N1 neutrophils to an N2 phenotype by up-regulation of arginine, CCL2, CCL5, and facilitates expansion of MDSCs leading to enhanced immune tolerance.

$CD8^+$ T cells in the tumor parenchyma is a crucial factor in immunotherapy efficacy; TGF-β signaling in the TME has been implicated in the suppression of T-cell infiltration into tumors to compromise the efficacy of anti-PD-L1 antibody (Ganesh and Massagué, 2018). Other studies reported that TGF-β1 induced high expression of PD-1 and PD-L1 in T cells and tumor cells, respectively, to impair the anti-tumor activities of T cells and facilitate cancer immune evasion (Park et al., 2016; David et al., 2017; Tang et al., 2020). In addition to acquired resistance by activating alternative pathways of immune evasion, the lack of response to immunotherapy can sometimes be attributed to the presence of primary resistance in the tumor immune landscape. While mechanisms underlying primary resistance to cancer immunotherapy are yet to be fully characterized, inhibition of TGF-β signaling has been shown to overcome primary resistance to PD-1 blockade by altering the immunosuppressive TME (Martin et al., 2020; Siewe and Friedman, 2021).

To target TGF-β-mediated resistance to immunotherapy, several groups have tested the combination of TGF-β inhibitors with anti-PD-1/PD-L1 antibodies that were approved by the FDA for the treatment of multiple advanced cancers, including atezolizumab, durvalumab, and avelumab; results from these studies showed that combination treatment elicited higher anti-tumor activity in murine model and human cancer cell lines, such as breast cancer, colon cancer, SCC (Lan et al., 2018; Mariathasan et al., 2018; Tauriello et al., 2018; Dodagatta-Marri et al., 2019; Principe et al., 2019; Lind et al., 2020). Co-administration of TGF-β inhibitors and anti-PD-L1 antibody effectively reduced TGF-β signaling in stromal cells, resulting in improved T-cell penetration and more vigorous anti-tumor immunity to suppress urothelial tumor growth (Mariathasan et al., 2018). Similarly, the combinations of anti-CTLA4-TβRII or anti-PD-L1-TβRII elicited more pronounced anti-tumor responses than single treatments (Ravi et al., 2018). Upon expression of dominant-negative TβRII in CAR-T cells targeting prostate specific membrane antigen (PSMA), increased lymphocyte proliferation and exhaustion resistance were observed. This resulted in long-term *in vivo* persistence and enhanced infiltration of CAR-T cells into tumor sites, leading to improve tumor eradication in prostate cancer patient derived xenograft (PDX)-mouse model (Kloss et al., 2018). However, studies in mouse models of colon or pancreatic tumor demonstrated that combining anti-PD-1 and anti-TGF-β therapies improved long-term survival and delayed tumor growth in the MC38 murine colon carcinoma model, while failing to do so in the CT26 colon carcinoma model and KPC1 pancreatic tumor model (Sow et al., 2019; Bertrand et al., 2021). The above results suggest that special attention might be needed in selecting patients who would benefit the most from combination therapy.

TABLE 1 | Overview of combination anti-TGF-β therapy with other cancer therapies in clinical trials.

Drug (target)	Clinical trial (Phase)	Status	Cancer type	Arms	Outcomes
AP 12009 (TGF-β2)	NCT00431561 (Phase II)	Completed	Glioblastoma and anaplastic astrocytoma	AP 12009 (10 mM) AP 12009 (80 mM) Temozolomide or procarbazine, lomustine, and vincristine	Improved PFS Improved OS (Results for responders regardless drug concentration administered)
Cilengitide also called EMD 121974 (integrins αvβ3 and αvβ5)	NCT00705016 (Phase III)	Completed	Head and Neck Squamous Cell Carcinoma	Cilengitide (2000 mg) cetuximab+5-FU + cisplatin Cilengitide (2000 mg) cetuximab+5-FU + cisplatin	No improvement in PFS No improvement in OS
Cilengitide (integrins αvβ3 and αvβ5)	NCT00689221 (Phase III)	Completed	Glioblastoma	Cilengitide + temozolomide + radiotherapy Temozolomide + radiotherapy	No improvement in PFS No improvement in OS
M200 (integrin α5β1)	NCT00635193 (Phases I/II)	Completed	Ovarian cancer and primary peritoneal cancer	Liposomal doxorubicin (40 mg/m^2) M200 (7.5 mg/kg) Liposomal doxorubicin (40 mg/m^2) M200 (15.0 mg/kg) Liposomal doxorubicin (40 mg/m^2)	NA
LY2157299 also called galunisertib (TβRI)	NCT01220271 (Phases I/II)	Completed	Glioblastoma	Phase I LY2157299 (160 mg) + radiotherapy + temozolamideLY2157299 (300 mg) + radiotherapy + temozolamide Phase II LY2157299 (established dose) radiotherapy + temozolamideRadiotherapy + temozolamide	NA
LY2157299 (TβRI)	NCT02154646 (Phase I)	Completed	pancreatic cancer	galunisertib + gemcitabine	NA
LY2157299 (TβRI)	NCT01373164 (Phases I/II)	Completed	pancreatic cancer	galunisertib + gemcitabine vs placebo + gemcitabine	Improved OS
LY2157299 (TβRI)	NCT02734160 (Phase I)	Completed	pancreatic cancer	galunisertib + durvalumab (PD-L1 antibody)	NA
LY2157299 (TβRI)	NCT02423343 (Phases I/II)	Completed	NSCLC and HCC	galunisertib + nivolumab (anti-PD-1)	NA
LY2157299 (TβRI)	NCT02178358 (Phases I/II)	Completed	HCC	monotherapy vs combination with sorafenib or placebo + sorafenib	NA
TEW-7197 (TβR1)	NCT03074006 (Phases I/II)	Completed	pancreatic cancer	combination with FOLFOX in pancreatic cancer patients	NA
NIS793 (TGF-β)	NCT02947165> (Phase I)	Completed	Breast Cancer Lung Cancer Hepatocellular Cancer Colorectal Cancer Pancreatic Cancer Renal Cancer	NIS793 + PDR001 (anti-PD-1)	NA

5-FU, 5-fluoracil; PFS, progression-free survival; OS, overall survival; NA, not available (results are not publicly available).

Combination Therapy: Opportunities and Challenges

Despite great improvements in the clinical application of chemotherapy, targeted therapy, as well as immunotherapy over the past few decades, the development of drug resistance has been proven inevitable. As aforementioned, multiple studies have suggested that TGF-β signaling was associated with enhanced drug resistance and tumor metastasis. As a result, researchers have started to explore the possibility of using TGF-β inhibitors in combination with other anti-cancer agents to treat patients with metastatic or recurrent tumors. So far, pre-clinical studies have demonstrated that combination therapy effectively blocked cancer cell proliferation and invasion *in vitro* (cell lines), *in vivo* (mouse models), and *ex vivo* (patient tumor explants). For example, combined regimens of sorafenib or erlotinib with TGF-β inhibitor effectively potentiated sorafenib by increasing HCC cells apoptosis and suppressed the motility of erlotinib-resistant NSCLC cells, respectively (Serizawa et al., 2013; Serova et al., 2015). TGF-β signaling was found to be activated in cells that survived paclitaxel treatment; and combining TGF-β pathway inhibitors with paclitaxel potently prevented recurrences of basal-like breast tumors *in vivo* (Bhola et al., 2013). Combining TGF-β inhibitor with immunotherapy has also shown promise in a number of pre-clinical studies (Mariathasan et al., 2018; Tauriello et al., 2018; Lind et al., 2020).

Although encouraging advances in treatment efficacy were observed in combining TGF-β pathway inhibitors with other anti-cancer agents in pre-clinical studies, successes in clinical trials remained rare and results were inconsistent to say the least. Treatment combinations involving a number of TGF-β inhibitors that were designed to bind to TGF-β receptors and inhibit

receptor kinase activity, including AP12009, cilengitide, M200, LY2157299, NIS793, TEW-7197, have been tested in clinical trials (**Table 1**). Published results from these trials showed that the combination of LY2157299 with gemcitabine in metastatic pancreatic cancer (NCT01373164), as well as the combination of AP12009 with TMZ in Glioblastoma and anaplastic astrocytoma (NCT00431561) yielded encouraging outcomes. However, using cilengitide in combination with cisplatin and 5-FU to treat recurrent and/or metastatic hand and neck squamous cell carcinoma (HNSCC) resulted in no improvement in progression-free survival (PFS) and overall survival (OS) (NCT00705016) (NCT00689221). Furthermore, the efficacy of combining anti-TGF-β therapy with immunotherapy for the treatment of advanced solid tumor remains an unanswered question as data from clinical trials are not yet publicly available (NCT02423343) (NCT02947165).

A few factors might account for the suboptimal outcomes of anti-TGF-β therapies in a number of clinical trials. First of all, the animal models used in pre-clinical studies might not reflect the complexity of the disease in human patients; for instance, in models using patient-derived tumor xenografts, the TME in the mouse model can habour dramatic differences from the human TME, such that drugs might be effective in treating tumors in animal models but fail to do so in human patients. Second, TGF-β signaling is highly dynamic; feedback loops that regulate the activity of TGF-β signaling have been reported and oscillations in TGF-β signaling have been modeled and tested *in vitro* (Zi et al., 2011; Warmflash et al., 2012; Wegner et al., 2012). As a result, the effectiveness of antagonizing TGF-β signaling in an attempt to suppress cancer cell survival and drug resistance might be complicated by the innate fluctuations in TGF-β signaling. Furthermore, the heterogeneity in tumor cells can also contribute to heterogeneity in the response towards anti-TGF-β therapies. A study by Giampieri et al. demonstrated that single tumor cells activated TGF-β signaling locally and transiently, such that single cell motility, rather than collective movement, was enhanced (Giampieri et al., 2009). Importantly, inhibition of TGF-β signaling prevented single cell motility but not collective movement of tumor cells; cells expressing the dominant negative TβRII were incapable of metastasizing to the lung via blood vessels, while still being able to disseminate to lymph nodes via collective invasion (Giampieri et al., 2009). In addition, with TGF-β being a key regulator in the maintenance of tissue homeostasis, on-target cardiovascular toxic side effects and formation of benign tumors in response to the targeting of TGF-β signaling have been reported (Colak and Ten Dijke, 2017). Generally, although TGF-β inhibitors in combination with other anti-cancer treatments have yielded encouraging results in pre-clinical studies, thorough characterization of the mode of action and response to these inhibitors, as well as a better understanding of the pleitropic nature of TGF-β signaling are important to optimize the survival benefits from using TGF-β inhibitors and to facilitate the bench-to-bedside transition for anti-TGF-β therapy (Ciardiello et al., 2020).

CONCLUSION

The aberrant activation of TGF-β signaling plays a complex role in tumor progression, especially in the development of resistance towards cancer therapy. TGF-β induces drug resistance in targeted and chemotherapy by activating alternative survival pathways or anti-apoptotic signaling. On the other hand, other than activating TGF-β signaling pathways to induce drug resistance as delineated above, under certain circumstances, down-regulation of TGF-β signaling pathway has also been associated with enhanced drug resistance (Faião-Flores et al., 2017; Bugide et al., 2020; Vu et al., 2020). For example, down-regulation of TGF-β signaling through the inhibition by MITF (Microphthalmia-associated transcription factor) can confer MEKi resistance in melanoma (Smith et al., 2013). Reduced levels of SMAD3 or loss of SMAD4 suppressed the function of TGF-β-induced expression of tumor suppressor genes, resulting in the expression of anti-apoptotic proteins Bcl2 and Bcl-W, and enhanced cancer cell survival to confer platinum-resistance in NSCLC and 5-FU resistance in CRC, respectively (Zhang et al., 2014; Vu et al., 2020). Furthermore, some researchers suggested that TGF-β could serve as an important immune checkpoint in subverting "hot tumors," which had more infiltrating T-cells, into "cold tumors," which had lower immune infiltrates (Larson et al., 2020).

Therapeutic strategies using TGF-β inhibitors are making a tardy progress because of the dichotomous functions of TGF-β signaling in cancer. One of the two main concerns is that inhibitors of TGF-β signaling may impede cancer progression in the later stages of cancer but fail to suppress tumors at early stages. Another concern is that in clinical trials, the application of TGF-β inhibitors may result in off-target toxicity, especially cardiac toxicity (Turley et al., 2015) and dose-limiting toxicities (NCT01646203). In conclusion, although TGF-β inhibitors in combination with cancer therapy especially immunotherapy have shown great promise, thorough characterization of these inhibitors, as well as careful stratification and selection of patients are still required for cancer patients to truly benefit from it.

AUTHOR CONTRIBUTIONS

HL and QW concept formation. MZ and YZ wrote the manuscript. YC and JW editing and revision. All authors contributed to the article and approved the submitted version.

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Altered BMP2/4 Signaling in Stem Cells and Their Niche: Different Cancers but Similar Mechanisms, the Example of Myeloid Leukemia and Breast Cancer

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Understanding mechanisms of cancer development is mandatory for disease prevention and management. In healthy tissue, the microenvironment or niche governs stem cell fate by regulating the availability of soluble molecules, cell-cell contacts, cell-matrix interactions, and physical constraints. Gaining insight into the biology of the stem cell microenvironment is of utmost importance, since it plays a role at all stages of tumorigenesis, from (stem) cell transformation to tumor escape. In this context, BMPs (Bone Morphogenetic Proteins), are key mediators of stem cell regulation in both embryonic and adult organs such as hematopoietic, neural and epithelial tissues. BMPs directly regulate the niche and stem cells residing within. Among them, BMP2 and BMP4 emerged as master regulators of normal and tumorigenic processes. Recently, a number of studies unraveled important mechanisms that sustain cell transformation related to dysregulations of the BMP pathway in stem cells and their niche (including exposure to pollutants such as bisphenols). Furthermore, a direct link between BMP2/BMP4 binding to BMP type 1 receptors and the emergence and expansion of cancer stem cells was unveiled. In addition, a chronic exposure of normal stem cells to abnormal BMP signals contributes to the emergence of cancer stem cells, or to disease progression independently of the initial transforming event. In this review, we will illustrate how the regulation of stem cells and their microenvironment becomes dysfunctional in cancer via the hijacking of BMP signaling with main examples in myeloid leukemia and breast cancers.

Keywords: BMP, stem cells, cancer, microenvironment, mesenchymal, bisphenol, environmental exposure, resistance

INTRODUCTION

Cancer is a major public health issue considering its high mortality rate, increasing incidence, and cost to society. Despite tremendous progress in the development of targeted therapies, most cancers relapse owing to cancer stem cell (CSC) survival and treatment escape (Clarke, 2019). Two major axes remain to be solved to decrease the impact of cancer, one is to identify early and reliable signs of

tumor onset to prevent further transformation, taking into account the origin and properties of the niche, the other is to counteract CSC resistance before tumor progression and relapse (Saygin et al., 2019). The existence of spatially defined areas (called niches) essential for stem cell (SC) maintenance in adults was demonstrated in the bone marrow, and later in epithelial tissues and cancers. The microenvironment is a dynamic and complex milieu critical for the delivery of signals orchestrating cell proliferation, differentiation and apoptosis. Following the identification of different subsets of SCs, the composition and functions of adult SC niches began to be elucidated in the hematopoietic system and in solid tissues (Batsivari et al., 2020). Mesenchymal stromal cells (MSCs), a population of long-lived stem/progenitor cells, that contribute to cellular diversity and architecture of the niche and which play a central role in growth, survival and resistance of SCs and tumor cells. MSCs also secrete many morphogens, growth factors and cytokines, including BMPs. The definition of the tumor niche *per se* is still unclear, as its properties related to cancer onset, evolution and resistance have not been identified. Main hypotheses suggest that CSC resistance reflect the preservation of intrinsic protective mechanisms unique to the SC compartment or the re-emergence of these properties in cancer cells (O'Brien-Ball and Biddle, 2017). In addition, CSCs and their niche are engaged in a crosstalk regulating several SC-signaling pathways, as well as niche features (Arora and Pal, 2021). BMP2 and BMP4, produced within the SC microenvironment, are master regulators of the functions of tissue-specific SC and their surrounding MSC and emerge as key players of SC transformation. Here, we will focus on the role of BMP2 and BMP4 signaling in human hematopoietic and breast epithelial SC regulation, transformation, maintenance and drug resistance, in association with their microenvironment.

BMP2 and BMP4 Have Distinct Effects in Human Stem Cells

Hematopoiesis is supported by hematopoietic SCs (HSCs) and controlled by soluble factors, as well as cell/cell and cell/ECM interactions. Alterations of these processes induce various pathologies including leukemia that can directly affect HSCs. The influence of TGFβ, BMP and activin signaling on human HSCs can be investigated by studying regulators of the follistatin family, such as FLRG (FoLlistatin Related Gene). FLRG interacts with several members of the TGFβ family (Activin A and BMP2, BMP4, BMP6 or BMP7) (Tsuchida et al., 2000; Tortoriello et al., 2001; Maguer-Satta et al., 2003), and regulates HSCs during hematopoietic differentiation (Maguer-Satta et al., 2001). BMP2 fosters commitment of human HSCs toward erythroid cells, whereas BMP4 controls HSC self-renewal or megakaryocytic lineage engagement. Both FLRG and follistatin regulate erythroid commitment of human HSCs induced by activin or BMP2 (Maguer-Satta et al., 2003). Additionally, FLRG and follistatin molecules binding to the fibronectin type I domains of the fibronectin protein (outside of the integrin β1 binding domains) regulates HSCs by inducing their adhesion to fibronectin (Maguer-Satta and Rimokh, 2004; Maguer-Satta et al., 2006). This illustrates how BMP signaling can regulate HSCs by modulating their interaction with the microenvironment. Unlike BMP2, a strong cooperation between BMP4 and other cytokines related to HSC maintenance and megakaryopoiesis, such as Stem Cell factor or thrombopoietin, was identified. In the absence of thrombopoietin, BMP4 induces HSC commitment toward the megakaryocytic lineage, as well as terminal differentiation, leading to platelet production. BMP4 also induces a higher level of adhesion of human HSCs and progenitors to fibronectin than thrombopoietin (Jeanpierre et al., 2008). The importance of BMP4 in controlling HSC functions, through alpha4 integrin-mediated adhesion, was further documented in a murine model (Khurana et al., 2013). Therefore, BMP2 and BMP4 appear to play different regulating roles on human HSCs, whereas their function in murine hematopoiesis was reported to be more redundant (Bhatia et al., 1999; Borges et al., 2013; Khurana et al., 2013; Singbrant et al., 2020).

Epithelial cells and cells of the normal mammary gland environment (fibroblasts, adipocytes, hematopoietic cells) produce BMP2 and BMP4, suggesting a role in mammary SC regulation (Chapellier et al., 2015). The function of BMPs in normal breast was explored by isolating primary human epithelial cells, SCs and progenitors. Immature epithelial cells (SCs and progenitors) express different elements of the BMP pathway, indicating that BMPs could play a role in normal SC regulation. As for HSCs, BMP2, and BMP4 have distinct effects on SC regulation. Whereas BMP4 modulates the SC compartment and myoepithelial progenitors, BMP2 fuels commitment and proliferation of luminal progenitors (Chapellier et al., 2015; Clement et al., 2017). This is consistent with results reported for mammary gland development in mice, showing that BMP2 is involved in the regulation of the luminal lineage (Forsman et al., 2013). Despite their similarities, BMP2 and BMP4 thus exert different functions on human SCs in the mammary gland and hematopoietic system. These data indicate that BMP2 might preferentially affect lineage-committed progenitors, whereas BMP4 may have a broader effect on SCs and less tissue specific cells (megakaryocytic or myoepithelial progenitors). Likewise, BMPR1b and BMPR1a play distinct roles in MSCs fate regulation. Unlike BMPR1b, BMPR1a initiates both osteogenesis and chondrogenesis (Kaps et al., 2004). Conversely, alteration of BMPR1b expression in MSCs has been reported to reduce the bone mass and alter their osteo-differentiation (Shi et al., 2016). Despite these controversial data, it appears that BMPR1a and BMPR1b pathways, as well as those implying BMP2 and BMP4, are likely distinct and may not substitute to each other even if their respective roles in various SC cell fate remains unclear.

Abnormally Persistent BMP Signaling Initiates Transformation or Reprogramming Toward a SC-like Phenotype

Abnormalities of the BMP pathway have been reported at advanced stages of various cancers. Recent investigations reported its importance in early transformation steps of hematopoietic and breast tissues. Chronic Myeloid Leukemia

(CML) represents the reference model for SC transformation, whereas breast tumors contain CSC of debated origin. CML arises from a SC transformation event, induced by a single translocation generating the BCR-ABL oncogene. Patient samples at diagnosis revealed a dysregulation of several actors of the BMP pathway during chronic phase of the disease, with clear differences between mature ($CD34^-$) and immature ($CD34^+$) compartments (Laperrousaz et al., 2013). Alteration of BMP receptor type 1b (BMPR1b) expression at the HSC surface is induced by the expression of BCR-ABL and these molecular changes led to altered responses of leukemia cells to BMP2 and BMP4 as compared to normal bone marrow cells. Leukemic $BMPR1b^{high}$ cells respond to BMP4 by amplifying and maintaining their CSCs population, whereas BMP2 favors the expansion of myeloid CML progenitors. A similar role for BMP2 in the maintenance of CSCs was recently reported in hepatocellular carcinoma (Guo et al., 2021). In addition, BMPR1b mutations were linked to Brachydactyly type A1 characterized by bones hyperplasia, indicating that BMPR1b alterations could affect both the HSCs and MSCs in different physiopathological contexts by affecting their response to BMP4 (Laperrousaz et al., 2013; Racacho et al., 2015). In CML, dysregulation of intracellular BMP signaling mediated by BCR-ABL corrupts and amplifies the response to exogenous morphogens released by the niche, which are abnormally abundant and directly influence CSC fate. Indeed, concomitantly to intrinsic SC alterations, an increase in soluble BMP2 and BMP4, compared to healthy individuals, was detected within the tumor microenvironment of leukemia (Zylbersztejn et al., 2018) and breast cancer (Chapellier et al., 2015). Abnormal BMP2 production by SCs microenvironment together with BMP2-driven alterations of epithelial SC fate are involved in the emergence of luminal breast cancer cells (Chapellier et al., 2015). Chronic exposure of human immature mammary cells to high BMP2 levels initiates SC transformation toward a luminal tumor phenotype. Dysregulation of BMPs within the SC niche could then promote early steps of luminal transformation of resident epithelial cells through the following sequence of events: BMP2 binds to BMPR1b, and change the transcription factor balance FOXA1/FOXC1 in favor of FOXA1, concomitant to an upregulation of GATA3. Transformation then proceeded from an aberrant amplification of the natural response to BMP2, driving SC commitment toward luminal lineage and further expansion of luminal progenitors (Chapellier et al., 2015; Clement et al., 2017). A similar mechanism was observed in ovarian cancer (Choi et al., 2015). In breast and hematopoietic cancers, these data demonstrate that niche-secreted BMP2/BMP4 promote SC transformation through the amplification of a normal SC response, linked to their chronic exposure to high levels of these morphogens (Chapellier et al., 2015; Clement et al., 2017). Importantly, the presence of an inflammatory signal (such as IL6) appeared to be important to stabilize and maintain the transformed phenotype (Reynaud et al., 2011; Chapellier et al., 2015). Previous studies have shown that up-regulation of BMP4 could also be involved in transformation initiation in an inflammatory context, for example by promoting a metaplastic condition in which normal squamous esophageal epithelium is replaced by columnar epithelium (Barrett esophagus) (Milano et al., 2007). Altogether, these data also highlight the ability of the SC niche to deliver cues dictating the tumor phenotype.

Another mechanism to generate cancer stem cells could emerge from a "reprograming" process (O'Brien-Ball and Biddle, 2017) and results from a continuous exposure of mature cells to BMP2 and BMP4. Notably, BMP2 and BMP4 are overexpressed in the bone marrow of Acute Myeloid Leukemia (AML) patients (Voeltzel et al., 2018), supporting the aggressive clonal malignancy through excessive proliferation of immature cells blocked in their differentiation process. At the molecular level, alterations of the BMP pathway favor survival of chemotherapy-resistant immature-like leukemic cells. Binding of BMP4 to BMPR1a leads to ΔNp73 expression in mature AML blast cells, which in turn induces Nanog, reminiscent of cell "reprogramming" toward a CSC-like phenotype (Voeltzel et al., 2018). These features are associated with poor patient prognosis and treatment response. This was the first demonstration of a niche-driven AML leukemia cell reprogramming toward an SC-like phenotype, featuring BMP4 as a key stemness regulator. Interestingly, in many other tissues like brain, gastro-intestinal tissues, colon or liver, the importance of BMP4 and BMPR1a to predispose, initiate or maintain stemness of transformed cells was also evidenced (Brosens et al., 2011; Hover et al., 2016; Wang et al., 2019; Schwarzmueller et al., 2020). Nevertheless, for a given normal tissue, BMP signaling can display opposite functions such as preventing epithelial cells de-differentiation like in the intestinal murine tissue (Koppens et al., 2021).

At diagnosis of leukemia and breast cancer, BMPR1b was evidenced as an important transducer of BMP signals, acting as an amplifier of the normal SC response to BMP (**Figure 1**). This likely contributes to CSC survival independently of the initial oncogenic event. It also reveals the importance of simultaneous intrinsic and extrinsic alterations of BMP signaling to fuel transformation and demonstrates the direct implication of in the emergence and expansion of CSCs by inducing an over-amplified and persistent response of SC to the BMP signal. Altogether, it provides a proof of the "seed and soil" concept in the context of BMP-driven cancers that require two complementary events, one taking place within the niche and the other directly acting on (stem) cells. Due to the broad involvement of BMP signaling in cancer, this mechanism might be common to different cancers, and sheds light on the involvement of BMPs in cancer cells stemness (Kim et al., 2015; Huang et al., 2017; Sachdeva et al., 2019; Sun et al., 2020).

BMP Signaling as a Driver of Cancer Stem Cell Resistance to Treatment

CSCs constitute a reservoir likely involved in cancer recurrence in many tumors as they resist to several treatments and sustain disease for years. In CML, the first anti-cancer targeted therapy was developed and paved the way to the family of Tyrosine Kinase Inhibitors (TKIs). It efficiently eliminates most cycling

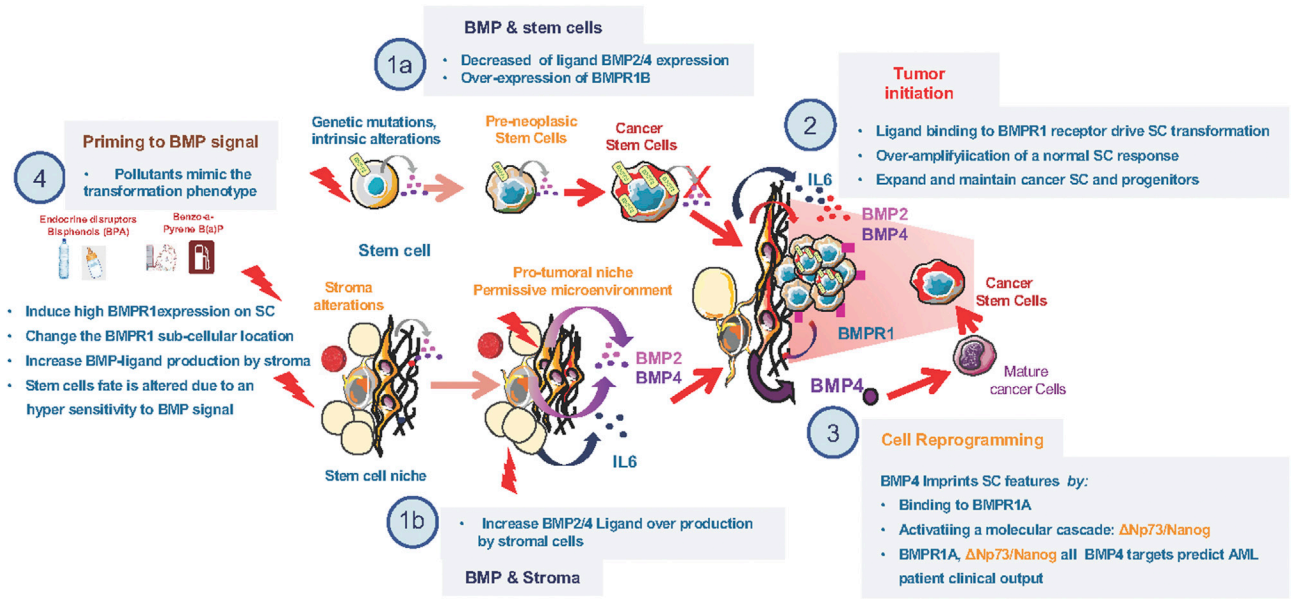

FIGURE 1 | Alterations of the BMP pathway involved in early steps of transformation. **(1a)** Decrease in ligand (BMP2 and/or BMP4) and increase in type1 BMP-receptor at time of diagnosis in stem cells of diferent leukemia and breast cancer types. **(1b)** Increase in ligand (BMP2 and/or BMP4) production by stromal cells of the tumor microenvironement at time of diagnosis in diferent leukemia and breast cancer types. **(2)** Cooperation between BMP2 overproduction by the stroma and IL6 to induce BMPR1b overexpressing stem-cells transformation. **(3)** BMP4 mediated reprogrammation of mature cells toward an imature stem-like cell. **(4)** Effect of environmental polluant on the expression of both ligand and BMPR1 expression in healthy cells.

progenitors and has become the standard-of-care for CML. However, some CSCs remain treatment-insensitive and prolong disease recurrence for years leading to a proportion of patients that develop primary or secondary resistance to TKIs, of unknown mechanism in 30% of the cases. The embryonic gene *Twist-1,* a context-dependent target or a regulator of BMP signaling depending on the cellular context, is overexpressed in various tumors and associated with poor prognosis and resistance. In CML, *Twist-1* is an early predictive molecular marker of resistance to TKIs at diagnosis, especially in patients with unidentified resistance mechanisms (Cosset et al., 2011). Following long-term TKI treatment, CSCs persist and escape through an intrinsic BMP4 autocrine loop that induce Twist-1 expression (Grockowiak et al., 2017) and impacts on G1-S progression during cell cycle regulation (Savona and Talpaz, 2008; Toofan et al., 2018). Moreover, resistance to TKIs is accompanied by an further dysregulation of the CSC microenvironment, producing excessive BMP4 (Grockowiak et al., 2017; Jeanpierre et al., 2021). Consequently, many patients likely retain treatment-resistant CSCs within their primary tumor or secondary metastatic site. *De facto*, most therapies achieve remission but patients would relapse because of the re-activation of a quiescent/dormant clone in several cancers (Risson et al., 2020). The SC environment create a permissive niche for the emergence, survival, re-activation, and resistance against therapy-induced apoptosis of CSCs and has become an important target for anti-cancer therapy (Batsivari et al., 2020; Risson et al., 2020). CSCs include quiescent cells resistant to standard therapies, differing from their normal counterparts in both hematopoietic and solid tumors (White and Lowry, 2015; Batsivari et al., 2020). Several studies unraveled the importance of TGFβ/BMP signaling in SC dormancy and adaptation to treatment, in association with the tumor microenvironment (Risson et al., 2020; Nobre et al., 2021). In CML, single cell RNA-Seq analysis of TKI-resistant CSCs showed a co-enrichment of BMP and Jak2 signaling targets, quiescence and SC signatures (Jeanpierre et al., 2021). Using a new model of persisting CML CSCs, BMPR1b-expressing cells displayed co-activation of Smad1/5/8 and Stat3 pathways (Jeanpierre et al., 2021). Treatment-induced quiescence of residual CSCs relies on the activation of Jak2-Stat3 signaling, mediated by BMP4 released from surrounding mesenchymal cells (Jeanpierre et al., 2021). Targeting of BMPR1b and Jak2 efficiently reversed this TKI-induced quiescence of the BMPR1b^{+} CSCs adhering to the stroma, and allowed them to re-enter a differentiation process. Such a strategy may contribute to eliminate dormant CSCs. However, in brain tumors the role of the BMP signaling in CSCs resistance to radiotherapy or chemotherapy remains controversial. While BMP4 could efficiently directly reduce glioma stemness by inducing their differentiation and death (Nayak et al., 2020), other groups reported that BMP inhibition only reduce cell proliferation without affecting stemness properties (Sachdeva et al., 2019). Moreover, methylation of BMPR1a appears to be of particular importance for Glioma SCs and their quiescence (Lee et al., 2008; Mira et al., 2010). Therefore, BMP is a key pathway involved in the dialogue between CSCs and the microenvironment to maintain a sub-fraction of CSCs in a quiescent/dormant stage through non-canonical BMP signaling pathways (Risson et al., 2020; Jeanpierre et al., 2021) (**Figure 2**).

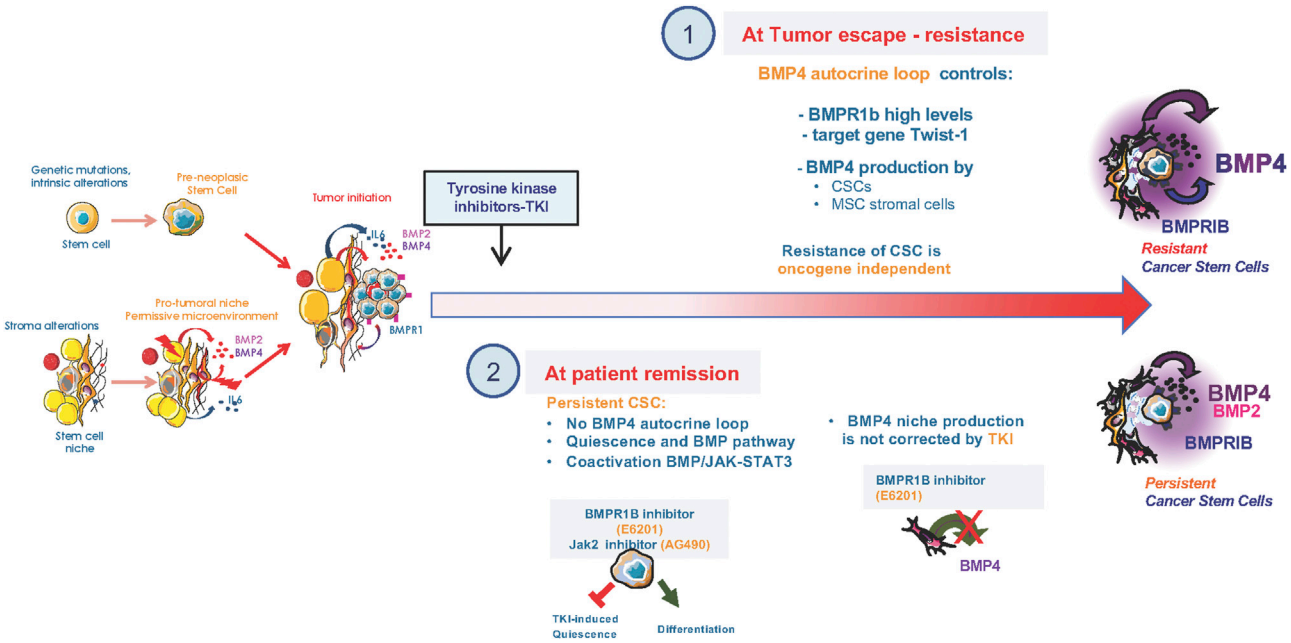

FIGURE 2 | Alterations of the BMP pathway upon treatment of CML cancer stem cells. **(1)** Setting of a BMP4 autocrine loop in CSCs that controls Twist1 expression to promote resistance of CML CSCs to tyrosine kinase inhibitors (TKIs) and retained high production of BMP4 by bone marrow mesenchymal stromal cells of resistant patients. **(2)** At patients remission under TKI treatment, CSCs showed a co-enrichment in BMP and Jak2-signaling, quiescence and SC signatures, as identified by single cell RNA-Seq analysis of TKI-persisting cells. In persisting CSCs, BMPR1b-cells displayed co-activated Smad1/5/8 and Stat3 pathways that are targeted by blocking BMPR1B/Jak2 signal using, for example, a specific BMPR1b inhibitor (E6201) or Jak2 (AG490) inhibitor. The TKI-induced quiescence of residual CSCs relies on a BMP4 signal delivered by surrounding mesenchymal cells and inhibited by the BMPR1B inhibitor (E6201). Dual targeting of BMP and Jak2 efficiently reverses TKI-dependent induction of quiescence and allowed re-entry into a differentiation process of the BMPR1b^{+} CSC sub-fraction that was adherent to the stroma.

Intrinsic and Extrinsic Origins of BMP Signaling Alterations

`BMP signaling alterations occurring at an early stage could constitute a recurrent driving event in many cancer types as demonstrated for CML, AML and breast cancer. In this context, CSCs could emerge from various tissues through different initiation events converging to initiate both intrinsic and extrinsic BMP signaling alterations. In CML, the link between the BCR-ABL translocation and BMPR1b dysregulation is established (Laperrousaz et al., 2013), but the origin of abnormal BMP production by stromal cells is unknown. BMP2 overproduction by the microenvironment may arise through exposure to carcinogens due to environmental contamination (Casey et al., 2015; Goodson et al., 2015), radiation (Sun et al., 2012), ultrasounds (Yang et al., 2014) or magnetic fields (Bloise et al., 2018). Our group identified that mammary SC transformation could be mediated by environmental pollutants, such as bisphenols (BPA and BPS), through the dysregulated expression of BMPs or their receptors. Indeed, exposure to environmental cues (radiations, BPA or Benzo-a-Pyrene:BaP) induces a higher production of BMP2 by healthy stromal cells or fibroblasts on the one hand, and stem cell-specific alterations of BMPR1 expression and localization on the other. Both alterations over-amplify BMP2/BMPR1b signaling in epithelial SCs, ultimately leading to transformation (Chapellier et al., 2015; Chapellier and Maguer-Satta, 2016; Clement et al., 2017). In this context, an emerging field of investigation aims at identifying alterations that appear in the bone marrow microenvironment, due to non-genetic events such as inflammation, hormones, ECM, cytokines or environmental cues (Yang et al., 2014; Bloise et al., 2018), and that could contribute to altered hematopoiesis. Altered MSCs could imprint microenvironment plasticity via the imbalanced generation of different cell types, which would in turn produce abnormal ECM or amounts of soluble molecules. This could have a major impact on cancer prevention and regulatory definition of endocrine-disruptors. It also highlights the importance of monitoring the BMP signaling for early detection of cancer initiation, and its potential relevance for cancer prevention (Jung et al., 2019; Lefort and Maguer-Satta, 2020).

CONCLUSION

From a clinical perspective, eradication of CSCs, involved in resistance and relapse, is critical. Abnormalities of BMP signaling are reported in many cancers, with studies mainly focusing on advanced stages while its roles in early transforming events are now emerging. Evidences in many cancers suggest the following

model of SC transformation and resistance through BMP signaling: 1) BMP type 1 receptor expression, localization, and/or activation is perturbed in SCs chronically exposed to exogenous signals (during tissue ageing, chronic inflammation, metabolic disorders or exposure to pollutants); 2) these chronic signals modify tissue SC microenvironmental properties, leading to increased BMP production. Excessive production of BMP (like BMP2 and BMP4) by the altered microenvironment continuously transduces signals of SC self-renewal, quiescence, expansion or survival, through the binding to overexpressed BMPR1b on pre-tumoral SCs. These signals ultimately lead to SC transformation. Following transformation, CSCs modify their dialogue with their microenvironment, leading to a dynamic and reciprocal remodeling of the tumor ecosystem through BMP signaling that simultaneously controls SCs and neighboring cells. In the presence of treatments, this abnormal BMP signaling is further altered, enabling persistence and/or survival of rare and specific subsets of CSCs hidden in a permissive niche. When the treatment pressure is released or when new signals are triggered, following *de novo* genetic events or additional abnormalities, this reservoir of persistent CSC subset is stimulated or re-expands, thereby driving relapse or treatment escape.

The BMP pathway is therefore likely to constitute a very early marker as well as a signaling target in terms of prevention and therapeutic strategies. Despite these remarkable advances, one of the remaining unresolved questions in the field is how does the BMP and TGFβ signaling compete, cooperate or synergize to regulate normal stem cells and participate in their transformation to initiate, maintain and promote cancers. Elucidating the molecular coordination between these two majors signaling pathways is then of the utmost importance at both fundamental and clinical level. Interfering with BMP receptor recognition by neutralizing molecules could restore a normal SC behavior and avoid further tumor progression. Moreover, targeting ligand production by stromal cells could induce an arrest of the transforming signal at early steps. Indeed, inhibition of BMP signaling leads to CSC death, and interrupts BMP production by surrounding stromal cells, in cooperation with other current treatments which remain inefficient as monotherapies (Jeanpierre et al., 2021). This proof-of-concept established in the hematopoietic system is likely to be extended to other cancers (Jung et al., 2019; Lefort and Maguer-Satta, 2020). Collectively, data imply a major role for the BMP pathway in orchestrating the dynamics of the CSC niche ecosystem in different cancers and at all stages of tumor progression. Targeting both BMPR1 and the tumor microenvironment might efficiently impact early transformed cells as well as residual persistent CSCs.

AUTHOR CONTRIBUTIONS

VM-S supported researches at administrative, financial and scientific level and wrote the manuscript. All authors contributed to research support, writing of the manuscript and final approval.

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REVIEW
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The Interplay Between TGF-β Signaling and Cell Metabolism

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The transforming growth factor-β (TGF-β) signaling plays a critical role in the development and tissue homeostasis in metazoans, and deregulation of TGF-β signaling leads to many pathological conditions. Mounting evidence suggests that TGF-β signaling can actively alter metabolism in diverse cell types. Furthermore, metabolic pathways, beyond simply regarded as biochemical reactions, are closely intertwined with signal transduction. Here, we discuss the role of TGF-β in glucose, lipid, amino acid, redox and polyamine metabolism with an emphasis on how TGF-β can act as a metabolic modulator and how metabolic changes can influence TGF-β signaling. We also describe how interplay between TGF-β signaling and cell metabolism regulates cellular homeostasis as well as the progression of multiple diseases, including cancer.

Keywords: TGF-β signaling, Smad, glucose metabolism, lipid metabolism, amino acid metabolism

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INTRODUCTION

Comprising 33 members in mammalian cells, the transforming growth factor-β (TGF-β) superfamily is distinct from other cytokines owning to its more widespread and pleiotropic effects (Morikawa et al., 2016). The TGF-β signaling pathway contributes to a broad range of physiological and pathological processes, and its key roles in development, immunity, wound healing, cancer, fibrosis, skeletal and cardiac diseases have been extensively studied (Massague, 2008; Wu and Hill, 2009; Dobaczewski et al., 2011; Travis and Sheppard, 2014; Meng et al., 2016; Morikawa et al., 2016; Salazar et al., 2016; Kiritsi and Nystrom, 2018; Derynck et al., 2020). A plethora of cellular activities, including cell proliferation, differentiation, apoptosis, adhesion and migration, are controlled by TGF-β superfamily members in a context-dependent manner (Feng and Derynck, 2005; Moustakas and Heldin, 2009; Massague, 2012; David and Massague, 2018). Although cellular responses to TGF-β signaling are mainly induced via its transcriptional regulation of genes (Massague and Chen, 2000; Massague and Wotton, 2000), other means have been recognized for TGF-β signaling to shape cell behavior, such as epigenetic modification, mRNA splicing and miRNA expression (Derynck and Budi, 2019). In addition, accumulating evidence indicates that TGF-β signal can also remodel cell metabolism.

As a network of chemical reactions essential for sustaining life, metabolism has long been centered in energy provision, building of blocks for biomacromolecules and elimination of compounds that are otherwise toxic to the organism. Studies in the past decades, especially with the aid of metabolomics, have further unraveled the profound interactions between metabolism and the regulation of protein activity and genes expression (Rinschen et al., 2019). Metabolic substrates, beyond serving as "ingredients" or biomarkers, are able to modify the chromatin structure and regulate gene expression (Li et al., 2018). On the other hand, metabolic enzymes, in response to signaling cues, can fulfill many moonlighting functions other than catalyzing (Xu et al., 2021). Therefore, these non-metabolic roles of metabolites and metabolic enzymes have been shown to play a critical role in signal transduction.

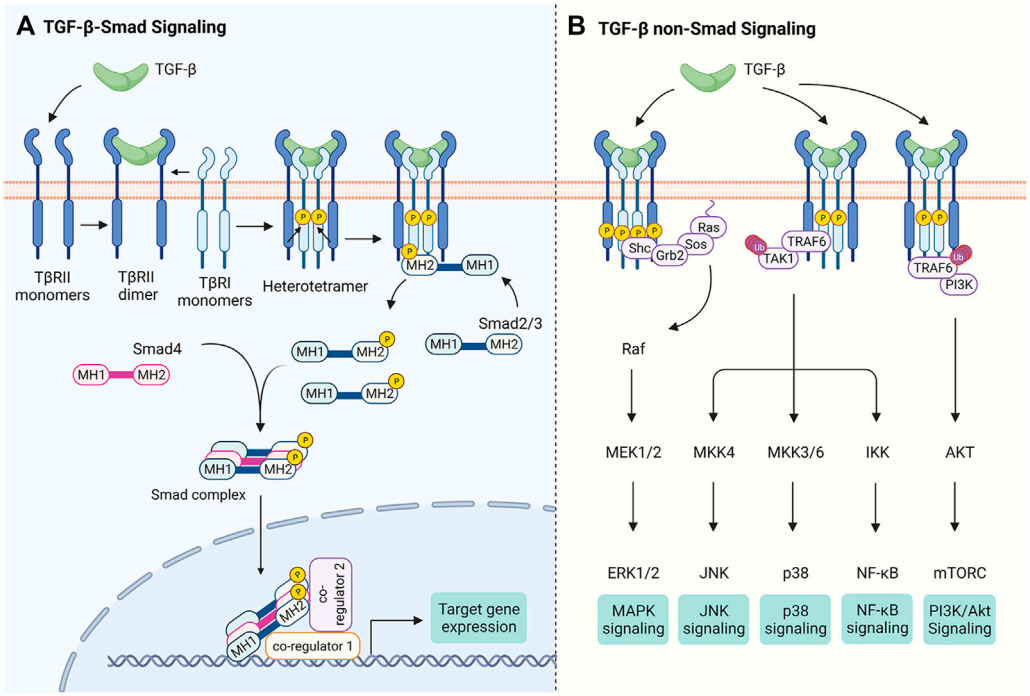

FIGURE 1 | The TGF-β Signaling Pathways. Binding of TGF-β to TβRII leads to the tetramer assembly of monomeric TβRII and TβRI receptors. **(A)** In Smad-dependent TGF-β signal transduction, TβRII trans-phosphorylates TβRI and activates its kinase activity, which in turn phosphorylates Smad2/3 at the C-terminal tail. Phosphorylated Smad2/3 form a trimeric complex with Smad4 and is translocated into the nucleus. The Smad complex binds DNA via their MH1 domains and controls gene expression through interacting with other transcription co-regulators. **(B)** TGF-β receptors trigger non-Smad signaling pathways. For instance, TGF-β receptors have been reported to recruit Shc, Grb2 and Sos to activate Ras, thereby initiating MAPK signaling. TGF-β receptors also activate TAK1 through TRAF6, which is required for TGF-β-induced JNK, p38 and NF-κB activation. It has been proposed that interaction of TRAF6 with TβRI also leads to PI3K/Akt activation. Figure is created with Biorender.com.

In this review, we discuss the current knowledge of how TGF-β signaling functions by altering various facets of cell metabolism and how metabolic changes can result in modulation of TGF-β signaling, thereby affecting an array of cellular processes. Such interplay between TGF-β signaling and cell metabolism is thought to be instrumental in maintaining homeostasis, and its aberration contributes to disease development. Due to the large number of TGF-β superfamily members, the scope of this review is restricted to the TGF-β ligands (TGF-β1, 2 and 3), which have been most extensively studied.

BASICS OF THE TGF-B SIGNALING PATHWAY

Based on the similarities in protein sequence and structure, the mammalian TGF-β members, with a few exceptions, can be classified into three major groups: the TGF-β family, the inhibin/activin family and the BMP (bone morphogenic protein)/GDF (growth and differentiation factor) family (Morikawa et al., 2016). The TGF-β family consists of TGF-β1, 2 and 3 that have largely redundant functions. Each isoform contains nine highly conserved cysteine residues, mediating the formation of inter- or intramolecular disulfide bonds that interlock two TGF-β polypeptides as a dimer (Hinck et al., 2016). The dimeric TGF-β ligand associates with the pro-region-derived latency-associated peptide (LAP) and a latent TGF-β binding protein (LTBP) and forms a large latent complex (LLC), which is trapped in the extracellular matrix (ECM) (Robertson and Rifkin, 2016). Activation of TGF-β ligands is mediated by different proteins in various tissues, serving as a way to ensure the precision of signal presentation (Rifkin, 2005).

Once activated, the dimeric TGF-β initiates signaling by promoting the assembly of two type I (TβRI) and two type II (TβRI) transmembrane receptors (Hata and Chen, 2016) (**Figure 1**). In the absence of TGF-β ligands, both TβRI and TβRII exist as monomers (Zhang et al., 2009; Zhang et al., 2010), although early studies reported that they exist as homodimers (Chen and Derynck, 1994; Henis et al., 1994; Gilboa et al., 1998), most likely due to their overexpression. Both of TβRI and TβRII possess Ser/Thr kinase activity in the cytoplasmic domain. Ligand binding results in the tetramer receptor complex formation with two TβRI and two TβRII, in which TβRI is activated via phosphorylation of Thr and Ser residues in its GS domain (TTSGSGSG) by the constitutively active TβRII (Wrana et al., 1994). The phosphorylation-induced conformational change activates the TβRI kinase that relays the signal to the effector Smad proteins (Huse et al., 1999; Huse et al., 2001; Chaikuad and Bullock, 2016; Hata and Chen, 2016) (**Figure 1A**).

There are three subgroups of Smad proteins: the receptor-activated Smads (R-Smads, Smad2/3 for TGF-β/activin/inhibin receptors and Smad1/5/8 for BMP/GDF receptors), the common-mediator Smad (Co-Smad, i.e., Smad4) that interacts with R-Smads, and the inhibitory Smads (I-Smads, Smad6 and Smad7). Both R- and Co-Smads propagate signals, while I-Smads dampen the signal transduction (Hata and Chen, 2016). All R-Smad proteins contain a highly conserved C-terminal MH2 domain that, via an inner L3 loop, engages in Smad-receptor and Smad-Smad interactions (Lo et al., 1998). The conserved N-terminal MH1 domain in R-Smads and Co-Smad has a nuclear localization signal and a DNA-binding β-hairpin (Hata and Chen, 2016; Chaikuad and Bullock, 2016; Shi et al., 1998; Hill, 2016). Upon activation of TβRI kinase activity, Smad2/3 is phosphorylated at two serine residues in the SSXS motif and subsequently is dissociated from the TβRI kinase domain, forming a trimeric Smad complex composed of two Smad2/3 and one Smad4 (Hata and Chen, 2016; Chaikuad and Bullock, 2016; Kawabata et al., 1998; Chacko et al., 2004; Xu et al., 2016). This Smad complex is then accumulated in the nucleus and acts as a transcription factor to regulate contextual expression of target genes through collaboration with diverse co-factors (Moustakas and Heldin, 2009; Massague et al., 2005) (**Figure 1A**). TGF-β ligands can also signal independently of Smad proteins through crosstalk with other signaling pathways (see Zhang, 2017; Derynck and Budi, 2019) (**Figure 1B**).

While it is clear that TGF-β signaling targets genes related to cell cycle progression, ECM production and epithelial-mesenchymal transition (EMT), a panoramic view of metabolic genes whose transcription directly controlled by TGF-β signaling are not attained. It remains even more obscure precisely how metabolic changes regulate the TGF-β signal transduction. In the following sections, we will illustrate the interplay between TGF-β signaling and multiple aspects of cell metabolism with a discussion on their important physiological or pathological roles in mammalian cells.

TGF-B SIGNALING AND GLUCOSE METABOLISM

The first evidence that TGF-β regulates glucose metabolism perhaps comes from work on Swiss mouse 3T3 cells demonstrating TGF-β treatment upregulates *Glut1* (glucose transporter type 1) mRNA level and increases glucose uptake (Kitagawa et al., 1991). This observation is later reproduced in rat glomerular mesangial cells and is associated with excessive glucose uptake-induced overproduction of ECM proteins (Inoki et al., 1999), which is a hallmark of diabetic nephropathy. In a different model using mouse normal mammary gland (NMuMG) cells to study TGF-β-induced EMT, however, Glut1 expression is reduced at both the protein and mRNA levels during short-term TGF-β exposure but is later restored, which may be explained by differential effects of TGF-β on proliferation of epithelial and mesenchymal cells through regulation of glucose uptake (Nilchian et al., 2020). In mesangial cells, high glucose can potently increase autocrine

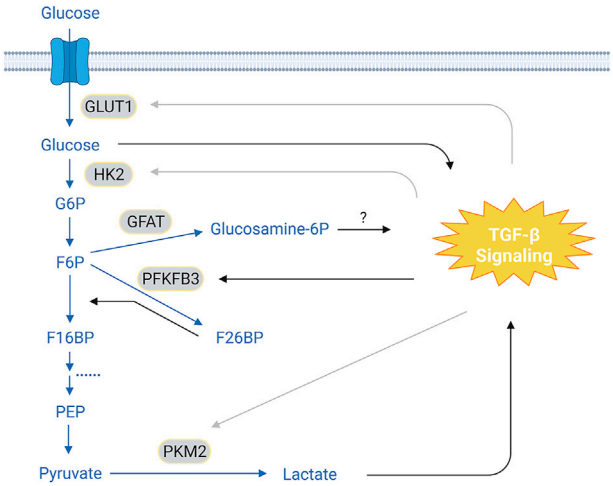

FIGURE 2 | Crosstalk Between TGF-β Signaling and Glycolysis. In the glycolytic pathway, glucose is converted to pyruvates via a cascade of enzymatic reactions. It has been reported that TGF-β signaling can either increase or decrease the expression of GLUT1 and HK2, depending on the cell types. This cell type-context effect is also seen on PKM2, an enzyme that catalyzes pyruvate into lactate. TGF-β signaling upregulates PFKFB3, leading to increased F26BP levels, which, in turn, accelerate conversion of F6P to F16BP. It has been suggested that glucose and lactate can promote TGF-β signaling; and inhibition of GFAT prevents glucose-induced expression of TGF-β ligands, implying a potential role of glucosamine-6-phosphotase in mediating this process. The conversion of F16BP to pyruvate has been omitted for clarity. Blue texts and arrows, glycolysis and its branches; gray arrows, cell-type dependent effect. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F16BP, fructose-1,6-biphosphate; F26BP, fructose-2,6-biphosphate; PEP, phosphoenolpyruvate; GLUT1, glucose transport 1; HK2, hexokinase 2; GFAT, glutamine:fructose-6-phosphate aminotransferase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PKM2, pyruvate kinase M2. Figure is created with BioRender.com.

secretion of TGF-β (Ziyadeh et al., 1994; Kolm et al., 1996; Kolm-Litty et al., 1998) (**Figure 2**). It seems that a positive feedback loop, in which elevated glucose levels stimulate TGF-β production and TGF-β, in turn, enhances glucose uptake, may pathologically contribute to the progression of diabetic nephropathy. Interestingly, production of TGF-β induced by high glucose is impaired by inhibition of Gfat (Glutamine: fructose-6-phosphate aminotransferase, the rate-limiting enzyme that converts fructose-6-phosphate into glucosamine-6-phosphate) (Kolm-Litty et al., 1998), suggesting a potential role of glucosamine-6-phosphotse in regulating TGF-β expression (**Figure 2**). In addition to promote TGF-β ligand production, high glucose is shown to increase cell membrane levels of both TβRI and TβRII and to induce latent-TGF-β activation by matrix metalloproteinases, leading to activation of the Akt-mTOR pathway and consequently causing cell hypertrophy in fibroblasts and epithelial cells (Wu and Derynck, 2009).

TGF-β signaling also regulates other components in the glycolytic pathway (**Figure 2**; **Table 1A**). For example, TGF-β treatment significantly decreases hexokinase 2 (HK2) expression in murine thymic-derived Tregs (Priyadharshini et al., 2018; Chen et al., 2020). However, HK2 levels are slightly increased

TABLE 1 | TGF-β-induced metabolic changes.

Target	Effect on metabolism	Implication	Cell type	Reference
A. Glucose metabolism				
GLUT1	Glucose uptake ↑	Proliferation	Fibroblasts	Kitagawa et al. (1991)
	Glucose uptake ↑	ECM production	Mesangial cells	Inoki et al. (1999)
	Glucose uptake ↓	Antiproliferation, EMT	Mammary epithelial cells	Nilchian et al. (2020)
HK2	Glycolysis ↓	NA	Thymic Treg cells	Priyadharshini et al. (2018); Chen et al. (2020)
	Glycolysis ↑	Osteoarthritis	Articular chondrocytes	Wang et al. (2018)
	Glycolysis ↑	Fibrosis	Lung fibroblasts	Yin et al. (2019)
PFKFB3	Glycolysis ↑	Invasion	Glioblastoma, pancreatic cancer cells	Rodriguez-Garcia et al. (2017); Yalcin et al. (2017)
PKM2	Aerobic glycolysis ↑	EMT	Colon cancer cells	Hamabe et al. (2014)
	Glycolysis ↓	Diabetic injury	Podocytes	Li et al. (2020)
PDC	OXPHOS ↓	Kidney injury	Fibroblasts	Smith and Hewitson, (2020)
Fumarase	Moonlighting effect	Cell cycle arrest	Lung cancer cells	Chen et al. (2019)
COX IV	OXPHOS ↓	Cell cycle arrest	Lung epithelial cells	Yoon et al. (2005)
COX I	OXPHOS & fatty acid oxidation ↑	EMT	Breast cancer cells	Liu et al. (2020)
ATP synthase	OXPHOS ↓	Impaired tumor immunity	Effector memory T cells	Dimeloe et al. (2019)
G6PC3	Gluconeogenesis ↑	HSC differentiation	Zebrafish embryonic cells	Zhang et al. (2018)
B. Lipid metabolism				
SCD	Unsaturated fatty acid synthesis ↑	NA	Epithelial cells and fibroblasts	Samuel et al. (2002)
FASN	Fatty acid synthesis ↓	EMT	Breast and lung cancer cells	Jiang et al. (2015); Yang et al. (2016); Liu et al. (2020)
SPHK1	Sphingosine-1P ↑	NA	Fibroblasts	Yamanaka et al. (2004)
	Sphingosine ↑	Dysfunctional placentae	Choriocarcinoma cells	Chauvin et al. (2015)
ASAH1	Sphingosine ↑	Dysfunctional placentae	Choriocarcinoma cells	Chauvin et al. (2015)
SHIP	$PI(3, 4, 5)P_3$ ↓	Apoptosis	Immune cells	Valderrama-Carvajal et al. (2002)
CYP24A1	$1,25(OH)_2D_3$ ↓	Impaired host defense	Airway epithelial cells	Schrumpf et al. (2020)
Ceramide	Ceramide ↑	ECM production	Fibroblasts	Sato et al. (2003)
	Ceramide ↓	Cell survival	Fibroblasts	Chen et al. (2003)
Lipid droplet	Fatty acids storage ↑	EMT, invasion	Acidosis-adapted cancer cells	Corbet et al. (2020)
	Fatty acids storage ↑	Impaired tumor immunity	Dendritic cells	Trempolec et al. (2020)
	Fatty acids storage ↑	Impaired tumor immunity	Macrophages	Bose et al. (2019)
C. Amino acid metabolism				
P4HA3	Reprogrammed amino acid metabolism	EMT	Lung cancer cells	Nakasuka et al. (2021)
GLS1	Glutamine anaplerosis ↑	NA	Hepatocellular carcinoma cells	Soukupova et al. (2017)
	Glutaminolysis ↑	EMT	Breast cancer cells	Lee et al. (2016)
	Glutaminolysis ↑	Fibrosis	Myofibroblasts	Bernard et al. (2018)
	Glutaminolysis ↑	Fibrosis	Fibroblasts	Choudhury et al. (2020)
ARG1	Polyamine synthesis ↑	Immunosuppression	Dendritic cells	Mondanelli et al. (2017)
	Polyamine synthesis ↑	Impaired tumor immunity	Macrophages	Boutard et al. (1995)
	Polyamine and proline synthesis ↑	ECM production	Vascular smooth muscle cells	Durante et al. (2001)
IDO1	Moonlighting effect	Immunosuppression	Dendritic cells	Mondanelli et al. (2017)
	Moonlighting effect	Self-tolerance	Dendritic cells	Pallotta et al. (2011)
	Tryptophan metabolism ↑	NA	Fibroblasts	Yuan et al. (1998)
ATF4	Serine-glycine synthetic pathway ↑	ECM production	Lung fibroblasts	Selvarajah et al. (2019)
SLC3A2	Leucine uptake ↓	Cell cycle arrest	Mammary epithelial cells	Loayza-Puch et al. (2017)
P5CS, PYCR1/2	Proline synthesis ↑	Fibrosis	Fibroblasts	Schworer et al. (2020)

(Continued on following page)

TABLE 1 | (*Continued*) TGF-β-induced metabolic changes.

Target	Effect on metabolism	Implication	Cell type	Reference
D. Redox, polyamine and other aspects of cell metabolism				
NOX4	ROS ↑	Fibrosis, cancer	Multiple tissues of origin	Cucoranu et al. (2005); Sturrock et al. (2006); Carmona-Cuenca et al. (2008); Michaeloudes et al. (2011); Boudreau et al. (2012)
Glutathione	Glutathione metabolism ↓	Fibrosis	Multiple tissues of origin	see Liu and Gaston Pravia, (2010) for review
	Glutathione metabolism ↑	Drug resistance	Squamous cell carcinoma cells	Oshimori et al. (2015)
ODC1	Polyamine synthesis ↓	NA	Leukemia cells	Motyl et al. (1993)
	Polyamine synthesis ↑	NA	Myofibroblasts	Blachowski et al. (1994)
AMD1	Polyamine synthesis ↓	NA	Leukemia cells	Motyl et al. (1993)
	Polyamine synthesis ↑	NA	Myofibroblasts	Blachowski et al. (1994)
Putrescine	Putrescine ↑	Impaired tumor immunity	Macrophages	Boutard et al. (1995)
	Putrescine ↑	ECM production	Vascular smooth muscle cells	Durante et al. (2001)
Spermidine	Spermidine ↑	Self-tolerance	Dendritic cells	Mondanelli et al. (2017)
PNPO	Vitamin B6 metabolism ↑	Cell proliferation	Ovarian cancer cells	Zhang et al. (2017b)

GLUT1, Glucose Transporter 1; HK2, Hexokinase 2; PFKFB3, Fructose-2,6-Biphosphatase 3; PKM2, Pyruvate Kinase M2; PDC, Pyruvate Dehydrogenase Complex; OXPHOS, oxidative phosphorylation; COX, Cytochrome c Oxidase; G6PC3, Glucose-6-Phosphatase Catalytic Subunit 3; SCD, Stearoyl-CoA Desaturase; FASN, Fatty Acid Synthase; SPHK1, Sphingosine Kinase 1; ASAH1, N-Acylsphingosine Amidohydrolase 1; SHIP, SH2 domain-containing 5′ Inositol Phosphatase; CYP24A1, Cytochrome P450 Family 24 Subfamily A Member 1; P4HA3, Prolyl 4-Hydroxylase Subunit Alpha 3; GLS, Glutaminase; ARG1, Arginase 1; IDO1, Indoleamine 2,3-Dioxygenase 1; ATF4, Activating Transcription Factor 4; SLC3A2, Solute Carrier Family 3 Member 2; P5CS, Delta-1-Pyrroline-5-Carboxylate Synthase; PYCR1/2, Pyrroline-5-Carboxylate Reductase 1/2; NOX4, NADPH Oxidase 4; ROS, reactive oxygen species; ODC1, Ornithine Decarboxylase 1; AMD1, Adenosylmethionine Decarboxylase 1; PNPO, Pyridoxamine 5′-Phosphate Oxidase; ↑, increase. ↓, decrease.

in TGF-β-treated articular chondrocytes from patients with osteoarthritis (Wang et al., 2018). TGF-β stimulation also specifically increases HK2 abundance in murine and human lung fibroblasts, which is required for profibrotic actions of TGF-β possibly through upregulating YAP/TAZ protein levels by an unknown mechanism (Yin et al., 2019). These results together suggest a cell-type dependent effect of TGF-β signaling on HK2 regulation. Phosphofructokinase 2 (PFK2), an enzyme that generates fructose-2,6-biphosphate that allosterically activates phosphofructokinases, is overexpressed in many cancer cells. TGF-β induces PFK2 expression in glioblastoma and pancreatic cancer cells (Rodriguez-Garcia et al., 2017; Yalcin et al., 2017), which is required for activation of SNAI1 transcription and promotes cell invasion (Yalcin et al., 2017). In SW480 colon cancer cells, increased pyruvate kinase M2 (PKM2) expression by TGF-β and EGF has been reported to be indispensable for EMT (Hamabe et al., 2014). In podocytes, the interaction of Smad4 with PKM2 interrupts the active PKM2 tetramer and reduces glycolysis activity (Li et al., 2020).

Lactate, the product of anaerobic glycolysis generated from pyruvate, appears to positively modulate TGF-β signaling (**Figure 2**; **Table 2A**). For instance, lactate induces TGF-β2 expression in glioma cells and knockdown of lactate dehydrogenase A (LDHA), an enzyme that catalyzes lactate production, downregulates TGF-β2 levels (Baumann et al., 2009). Lactate generated during exercising is associated with increased bioactive TGF-β concentration in rat cerebrospinal fluid (Yamada et al., 2012). Consistently, injection of lactate into mice results in elevated serum TGF-β2 levels, and incubation of adipocytes with lactate causes increased TGF-β2 concentrations in the media (Takahashi et al., 2019), though the underlying mechanism remains to be determined.

When oxygen is plentiful, pyruvate generally enters the TCA cycle, and most ATP is produced via oxidative phosphorylation (OXPHOS). TGF-β signaling has been shown to attenuate pyruvate dehydrogenase complex (PDC) activity in fibroblasts from injured kidneys and reduces free acetyl-CoA levels (Smith and Hewitson, 2020). TGF-β also causes phosphorylation of fumarase at T90 via the p38 pathway (Chen et al., 2019). Although the phosphorylated fumarase seems to retain normal catalytic activity, it gains non-metabolic functions and can shuttle into the nucleus to activate p21 expression through interaction with the CSL/RBPJ-p53 complex, thereby facilitating cell cycle arrest (Chen et al., 2019). TGF-β signaling also targets OXPHOS (**Table 1A**). In murine and human natural killer cells, TGF-β signaling dampens cell metabolism and represses OXPHOS (Viel et al., 2016; Zaiatz-Bittencourt et al., 2018; Slattery et al., 2021), in a mTOR signaling-dependent (Viel et al., 2016) or -independent manner (Zaiatz-Bittencourt et al., 2018). In addition, TGF-β suppresses the activity of ATP synthase in effector memory $CD4^+$ T cells and therefore reduces mitochondria respiratory capacity (Dimeloe et al., 2019). Since mitochondria are critical to many key immune functions (Mills et al., 2017), these inhibitory effects on OXPHOS in immune cells may underlie some negative effects of TGF-β in immunity. In mink lung epithelial Mv1Lu cells, TGF-β inhibits mitochondria complex IV activity and increases intracellular ROS accumulation, leading to senescence (Yoon et al., 2005). However, TGF-β has also been reported to enhance OXPHOS. For instance, in MCF-7 breast cancer cells, TGF-β increases the expression of OXPHOS-associated proteins, including NADH:ubiquinone oxidoreductase subunit B8 (NDUFB8), cytochrome c oxidase

TABLE 2 | Modulation of TGF-β signaling by metabolic changes.

Metabolic Event	Effect on TGF-β signaling	Outcome	Reference
A. Glucose metabolism			
High glucose	TGF-β production/ secretion ↑	ECM production	Ziyadeh et al. (1994); Kolm et al. (1996); Kolm-Litty et al. (1998)
		Cell hypertrophy	Wu and Derynck, (2009)
	TβRI/II membrane levels and TGF-β bioactivity ↑	Cell hypertrophy	Wu and Derynck, (2009)
Inhibition of GFAT	TGF-β production/ secretion ↓	ECM reduction	Kolm-Litty et al. (1998)
Increased lactate	TGF-β production/ secretion ↑	Cell migration	Baumann et al. (2009)
		Energy expenditure	Yamada et al. (2012); Takahashi et al. (2019)
B. Lipid metabolism			
Increased β-hydroxybutyrate	*TGFB* expression ↑	ECM production	Guh et al. (2003)
Overexpression of SGMS1	*TGFBRI* expression ↓	EMT inhibition	Liu et al. (2019)
Treatment of ceramide	TβRI/II membrane levels ↓	Inhibition of cell migration/invasion	Gencer et al. (2017)
Treatment of S1P	p-Smad2 levels ↑	NA	Yamanaka et al. (2004)
Loss of Nsdhl	*Tgfb1* expression and TGF-β production/ secretion ↑	EMT	Gabitova-Cornell et al. (2020)
Expression of NSDHL	TβRII levels ↑	Metastasis	Chen et al. (2021)
Treatment of RA with TGF-β	Smad3 and p-Smad3 levels ↑	Treg differentiation	Xiao et al. (2008)
Treatment of vitamin D	p-Smad2 levels ↓	Fibrosis inhibition	Halder et al. (2011); Beilfuss et al. (2015)
Activation of VDR	Smad3 binding to target DNA ↓	Fibrosis inhibition	Ding et al. (2013)
C. Redox, polyamine and other aspects of cell metabolism			
Depletion of intracellular PA	TβRI/II levels ↑ Total nuclear Smad3, 4 levels ↑	Cell cycle arrest	Patel et al. (1998); Rao et al. (2000) Liu et al. (2003)
Secretion of adenosine	p-Smad2/3 levels ↓	ECM reduction	Vasiukov et al. (2020)
Downregulation of XDH	TGF-β production/ secretion and p-Smad2/3 levels ↑	EMT	Chen et al. (2017)

GFAT, Glutamine:Fructose-6-Phosphate Aminotransferase; SGMS1, Sphingomyelin Synthase 1; S1P, Sphingosine-1-Phosphatase; NSDHL, NAD(P) Dependent Steroid Dehydrogenase-Like; RA, Retinoic Acid; VDR, Vitamin D Receptor; PA, Polyamine; XDH, xanthine dehydrogenase. ↑, increase; ↓, decrease.

subunit I (COX I) and mitochondrial transcription factor A (TFAM) during EMT, a cellular process that is thought to promote metastasis (Liu et al., 2020). In addition, TGF-β signaling in precursors of exhausted effector T cells promotes OXPHOS by repressing mTOR, enabling the preservation of mitochondrial metabolism that supports long-term T cell responses during chronic infection (Gabriel et al., 2021).

Aerobic glycolysis, or the Warburg effect, is widely adopted in many cancer cells (Hanahan and Weinberg, 2011), which is characterized by the preference of glycolysis over oxidative phosphorylation as a major source of energy production even when oxygen is abundant. Aerobic glycolysis can be induced in normal mammary fibroblasts by overexpression of constitutively active TβRI, powering the metabolically reprogrammed fibroblasts to fuel growth of cancer cells via energy transfer (Guido et al., 2012; Martinez-Outschoorn et al., 2012). In prostate cancer cells, overexpression of Smad2/3 enhances aerobic glycolysis independently of TGF-β stimulation but requires PKCε-mediated phosphorylation of the Smad3 linker region, which assists binding of Smad3 to the promoter of glycolytic genes (Xu et al., 2018). However, most of the studies were carried out in cell lines, and whether endogenous activation of TGF-β signaling promotes aerobic glycolysis in tumor cells awaits further investigation.

Compared to glucose catabolism, TGF-β1 has been documented to increase gluconeogenesis via the c-Jun/G6PC3 (glucose-6-phosphatase catalytic subunit 3) axis in zebrafish embryos, which fosters the nascent hematopoietic stem cells (Zhang et al., 2018). It would be worth exploring whether this mechanism can be applied to mammals or humans. Furthermore, there are many other metabolic pathways other than glycolysis that require glucose, including the pentose phosphate pathway, the hexosamine pathway, glycogenesis, the serine biosynthesis pathway and its many branches (Hay, 2016). Whether TGF-β signaling interacts with these pathways is unclear.

TGF-B SIGNALING AND LIPID METABOLISM

Lipids are a large group of water-insoluble molecules that, according to their diverse cellular functions, can be roughly divided into three categories represented by triglycerides that store energy; phosphoglycerides, sphingolipids and sterols that build the main structure of biological membrane; and many derivatives that actively engage in signal transduction and enzymatic reaction (Ridgway and McLeod, 2008).

Fatty acids can be released from triglycerides and provide the energetic needs through fatty acid oxidation (β-oxidation) in mitochondria. Blocking TGF-β signaling in mice via Smad3 ablation promotes brown adipogenesis within white adipose tissue and boosts mitochondria biogenesis in adipocytes, causing a significant elevation in fatty acid oxidation (Yadav

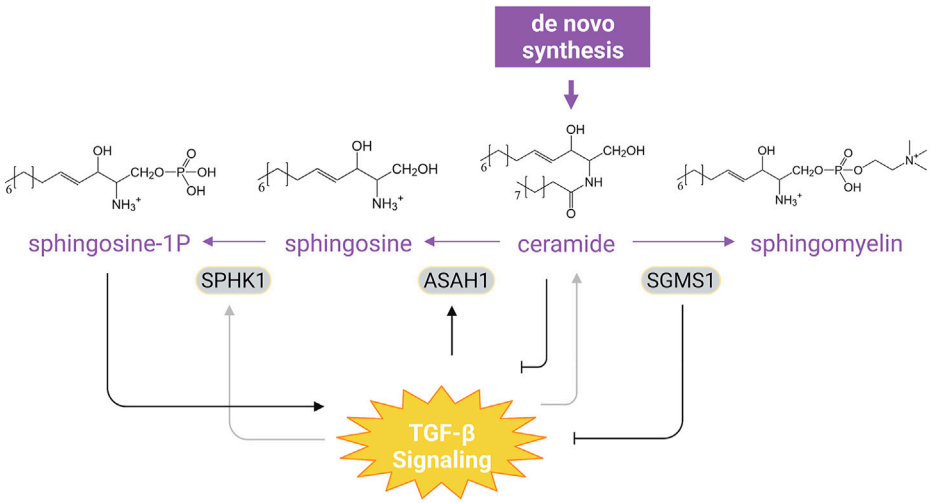

FIGURE 3 | Interaction of TGF-β Signaling with Sphingolipid Metabolism. TGF-β differentially regulates SPHK1 expression in a context-dependent manner and can upregulate ASAH1 to promote aberrant accumulation of sphingosine. Ceramide, the only sphingolipid that can be *de novo* synthesized, has been shown to constrain TGF-β signaling. Overexpression of SGMS1, which catalyzes synthesis of sphingomyelin from ceramide, also inhibits TGF-β signal transduction. Moreover, sphingosine-1P can evoke TGF-β-like responses in cells (see text). For simplicity, synthesis of other sphingolipid and the downstream catabolism of sphingosine-1P have been omitted. Purple texts and arrows, the sphingolipid metabolic pathway; gray arrows, cell-type dependent effect. Abbreviations: SPHK1, sphingosine kinase 1; ASAH1, N-acylsphingosine amidohydrolase 1; SGMS1, sphingomyelin synthase 1. Figure is created with BioRender.com.

et al., 2011). Conditional knockout of *Tgfbr2* in hepatocytes ameliorates CDAA (choline-deficient L-amino acid-defined) diet-induced steatohepatitis in mice, prevents CDAA-induced expression of genes related to lipogenesis, and enhances gene expression involved in β-oxidation (Yang et al., 2014). As inhibition of TGF-β signaling promotes fatty acid oxidation, it is plausible to postulate that TGF-β signaling activates the synthesis of fatty acids. Indeed, all three types of the TGF-β ligands, but not other members of the TGF-β superfamily, are shown to increase stearoyl-CoA desaturase expression in a Smad-dependent way in many human cell lines (Samuel et al., 2002). However, other studies demonstrate that the effect of TGF-β on fatty acid oxidation or synthesis is context-dependent (see **Table 1B** for details). Many studies report that TGF-β suppresses the expression of fatty acid synthase during the induction of EMT in cancer cells (Jiang et al., 2015; Yang et al., 2016; Liu et al., 2020). In Hep3B cells, TGF-β causes a significant reduction in carnitine-conjugated fatty acids, which coincides with upregulation of fatty acid transporter genes, implying increased carnitine-mediated entry of fatty acids into mitochondria that are destined for β-oxidation (Soukupova et al., 2021). In addition, TGF-β2 or TGF-β3, but not TGF-β1, is shown to reinforce fatty acid oxidation in myotubules and adipocytes (Takahashi et al., 2019). Ketone bodies are formed in the liver from acetyl-CoA produced by oxidation of fatty acids. As a major form of ketone body, β-hydroxybutyrate has been reported to increase TGF-β expression in HK-2 renal cells (Guh et al., 2003). However, the effect of TGF-β on the ketone bodies remains unknown.

TGF-β signaling also regulates the metabolism of some structural lipids that define the membrane architecture (**Table 1B**). Sphingolipids are a large class of membrane lipids, among which ceramide is the only one that can be *de novo* synthesized and serves as the structural precursor of higher sphingolipid members (**Figure 3**). Ceramide can be hydrolyzed by N-acylsphingosine amidohydrolase 1 (ASAH1) into sphingosine, which can be phosphorylated by sphingosine kinase 1/2 (SPHK1/2) into sphingosine-1-phosphate (S1P) to regulate a variety of physiological and pathological processes (Maceyka et al., 2012). In NMuMG cells and human normal bladder HCV29 cells, TGF-β can rewire glycosphingolipid composition to promote EMT by reducing intracellular levels of gangliotetraosylceramide or GM2 (Guan et al., 2009). TGF-β enhances the activity and expression of SPHK1 in human fibroblasts that are important for the expression of TIMP-1 (Yamanaka et al., 2004). In contrast, TGF-β1 and TGF-β3 downregulates SPHK1 expression but upregulates ASAH1 expression in the human choriocarcinoma JEG-3 cell line, leading to aberrant sphingosine accumulation characteristic of dysfunctional placentae in intrauterine growth restriction (Chauvin et al., 2015). TGF-β can also diminish ceramide production to inhibit apoptosis in NIH3T3 cells during serum starvation (Chen et al., 2003), while increasing ceramide levels in human dermal fibroblasts and Mv1Lu cells (Sato et al., 2003). The increased ceramide is shown to act as a positive regulator of TGF-β signaling by facilitating TGF-β-induced COL1A2 expression in foreskin fibroblasts (Sato et al., 2003). Ceramide has also been reported to inhibit TβRI/II trafficking to primary cilia by stabilizing the TβRI-Smad7 interaction, thereby attenuating cell migration and metastasis (Gencer et al., 2017). Consistent with the observation that TGF-β induces SPHK1 expression, exogenous sphingosine 1-phosphate can elevate phosphorylated Smad2 levels and increase TIMP-1 expression in rat renal mesangial cells (Yamanaka et al., 2004). Moreover,

overexpression of sphingomyelin synthase 1, a key enzyme that converts ceramides into sphingomyelins, downregulates TβRI expression and thus impairs TGF-β-induced EMT in breast cancer cell lines (Liu et al., 2019). Aside from sphingolipid metabolism, the metabolic pathway of cholesterol has been shown to regulate TGF-β signaling (**Table 2B**). Cholesterol is enriched in lipid rafts, a membrane microdomain which modulates TGF-β signaling. TGF-β receptors can be internalized via lipid raft-dependent endocytosis and transported to lysosome for degradation (Chen, 2009), while the location at lipid rafts of TGF-β receptors is required for TGF-β activation of MAP kinases (Zuo and Chen, 2009). Cholesterol depletion specifically inhibits TGF-β-induced activation of extracellular signal-regulated kinase (ERK) and p38 and therefore impairs EMT and cell migration (Zuo and Chen, 2009). In addition, loss of the rate limiting enzyme Nsdhl (NAD(P)-dependent steroid dehydrogenase-like) involved in cholesterol synthesis in mouse pancreatic ductal adenocarcinoma cells activates *Srebp1* (sterol regulatory element-binding protein 1), which enhances TGF-β1 expression and secretion and consequently facilitates EMT (Gabitova-Cornell et al., 2020). However, another study reported an opposite observation in human breast cancer cells: NSDHL expression augments TGF-β signaling by inhibiting TβRII degradation and therefore promotes cell migration (Chen et al., 2021). Hence, like in many other cases, this regulation is cell-specific.

The phosphorylated derivatives of phosphatidylinositol (PI) play a critical role in intracellular signal transduction. Phosphorylation of PI(4, 5)P_2 to PI(3, 4, 5)P_3 by PI-3-kinase triggers activation of Akt, inhibiting apoptosis and promoting cell survival (Manning and Toker, 2017). In hematopoietic cells, TGF-β signals are shown to counteract Akt signaling and promote apoptosis by upregulating the expression of the SH2-containing inositol phosphatase SHIP (Valderrama-Carvajal et al., 2002), which breaks down PI(3, 4, 5)P_3 to PI(3, 4)P_2. The interplay between TGF-β signaling and lipophilic hormones such as retinoic acid and vitamin D is well studied (**Tables 1B**, **2B**). Retinoic acid has been shown to synergistically increase the expression and phosphorylation of Smad3 in the presence of TGF-β during differentiation of $CD4^+$ T cells toward Treg (Xiao et al., 2008). The biologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), has been reported to revert TGF-β-increased OXPHOS and reactive oxygen species (ROS) in human bronchial epithelial cells (Ricca et al., 2019). 1,25$(OH)_2D_3$ has also been shown to antagonize TGF-β-mediated fibrogenesis. In the presence of the ligands, the vitamin D receptor (VDR) occupies Smad3-binding sites at profibrotic genes and reduces TGF-β-mediated hepatic fibrosis (Ding et al., 2013). Similarly, VDR ablation abolishes the antagonistic effect of 1,25$(OH)_2D_3$ on TGF-β-promoted hepatic fibrosis (Beilfuss et al., 2015). In human leiomyoma cells, 1,25$(OH)_2D_3$ can reduce Smad2 expression or activation by TGF-β and thus expression of profibrotic genes (Halder et al., 2011). In hepatic stellate cells, vitamin D supplementation also showed similar effects (Beilfuss et al., 2015). Reversely, TGF-β can cause vitamin D catabolism through upregulation of the vitamin D-24A-hydroxylase CYP24A1, resulting in undermined host defense in airway epithelium (Schrumpf et al., 2020). Interestingly, Smad3 can form a complex with VDR in a ligand-dependent manner and enhances its transactivation activity (Yanagisawa et al., 1999).

Lipid droplets (LD) are a type of organelle instrumental in lipid and energy homeostasis and also involved in diverse cellular activities other than lipid metabolism (Olzmann and Carvalho, 2019; Walther and Farese, 2012). TGF-β has been demonstrated to induce its formation in many cell types (**Table 1B**). TGF-β2 induces fatty acids storage and LD formation in acidosis-adapted cancer cells, which meets cellular energetic needs for EMT and cell invasion (Corbet et al., 2020). It also increases LD content in dendritic cells under acidic circumstances (Trempolec et al., 2020). In addition, treatment of murine macrophages with TGF-β causes LD accumulation, accompanied by a shift of macrophages from M1 phenotype to the pathological M2 phenotype (Bose et al., 2019). However, the mechanisms underlying TGF-β-induced LD formation are currently unclear.

TGF-β SIGNALING AND AMINO ACID METABOLISM

It has come to appreciate that amino acids, besides their fundamental role as substrates for protein synthesis, also perform multifarious cellular functions including energy homeostasis, cell growth and immune response (Wu, 2009). Taking advantage of metabolomics, Nakasuka and others have nicely demonstrated that TGF-β can change intracellular amino acid levels in non-small cell lung cancer cells (Nakasuka et al., 2021). Depletion of a particular amino acid (e.g., Phe, Thr, Leu, Ile, or Tyr), whose intracellular concentrations are significantly decreased by TGF-β, in culture media, induces EMT-like elongated morphology. They further showed that TGF-β induces the expression of prolyl 4-hydroxylase subunit alpha 3, an enzyme catalyzing proline to 4-hydroxylproline, whose knockdown abrogates TGF-β-induced amino acid changes and EMT (Nakasuka et al., 2021). It would be intriguing to know how altered expression of one gene involved in proline metabolism can cause global changes of amino acid levels.

In addition to its comprehensive effects on amino acid metabolism, TGF-β signaling also specifically mediates certain amino acid metabolic pathways (**Table 1C**). For instance, TGF-β modulates glutamine metabolism, which takes a key part in tumor development (Zhang et al., 2017a). In hepatocellular carcinoma cells, TGF-β augments glutamine metabolism by inducing the expression of glutamine transporter and glutaminase 1 (GLS1) and reduces oxidative metabolism, concomitant with enhanced EMT and cell migration (Soukupova et al., 2017). Interestingly, the way TGF-β induces *GLS1* expression seems to cell type-specific. In MCF-7 cells, TGF-β-induced *GLS1* expression is mediated by the transcription factor Dlx-2, leading to enhanced glutamine metabolism that contributes to EMT (Lee et al., 2016). In myofibroblasts, however, TGF-β upregulates *GLS1* expression via Smad3 and p38 and promotes myofibroblast differentiation (Bernard et al., 2018). Furthermore, TGF-β elevates GLS1 levels in

AKR-2B mouse fibroblasts by repressing the transcription factor SIRT7 and FOXO4, and the process requires Smad2/3 as well as mTOR (Choudhury et al., 2020).

Tryptophan metabolism, especially in immune cells, exemplifies another aspect of amino acid metabolism modulated by TGF-β signaling (**Table 1C**). At the core of tryptophan metabolism lies the kynurenine pathway, in which kynurenine is generated from tryptophan, serving as the common precursor for the synthesis of various downstream metabolites including NAD^+ (Kolodziej et al., 2011). Two serial enzymatic reactions convert tryptophan to kynurenine, and the first and rate-limiting step is catalyzed by three different enzymes: IDO-1 (indoleamine 2,3-dioxygenase-1), IDO-2 (indoleamine 2,3-dioxygenase-2) or TDO (tryptophan 2,3-dioxygenase). TGF-β prominently abolishes IFN-γ-induced IDO expression in human fibroblasts (Yuan et al., 1998). In contrast, IDO expression is upregulated by TGF-β in dendritic cells, which relies on the TGF-β-induced expression of arginase 1 and increased abundance of spermidine (Mondanelli et al., 2017). Importantly, TGF-β also confers IDO immunoregulatory function independently of its metabolic activity. By inducing phosphorylation of IDO at the putative immunoreceptor tyrosine-based inhibitory motifs (ITIMs) via the kinase Fyn, TGF-β promotes the complex formation of IDO with two tyrosine phosphatase SHP-1 and SHP-2, thereby activating a circuit of downstream signaling events required to maintain self-tolerance (Pallotta et al., 2011).

TGF-β also regulates other amino acid metabolic pathways (**Table 1C**). In human lung fibroblasts, TGF-β activates expression of ATF4, a master transcription factor of amino acid metabolism (Ameri and Harris, 2008; Kilberg et al., 2009), and leads to upregulation of *PHGDH*, *PSAT1*, *PSPH* and *SHMT2*, which are key players involved in glycine-serine synthesis (Nigdelioglu et al., 2016; Selvarajah et al., 2019). TGF-β inhibits leucine transporter SLC3A2 expression and therefore impairs leucine uptake, contributing to TGF-β-induced cell cycle arrest of mammary epithelial cells (Loayza-Puch et al., 2017). In NIH3T3 fibroblasts, the TGF-β/Smad signaling stimulates proline synthesis from glutamate by elevating protein levels of pyrroline-5-carboxylate synthase and pyrroline-5-carboxylate reductase 1/2 in the synthetic pathway to buffer mitochondrial redox stress (Schworer et al., 2020).

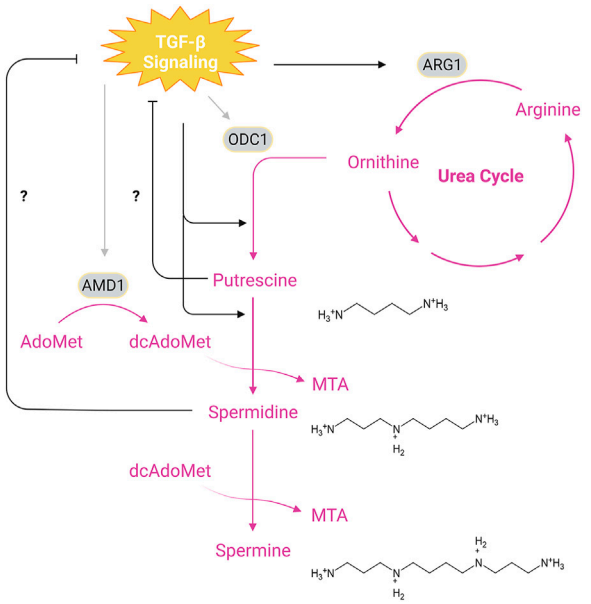

FIGURE 4 | Interplay Between TGF-β Signaling and Polyamine Synthesis. The main biosynthetic pathway of polyamines begins with ODC1-catalyzed formation of putrescine from ornithine, a product of the urea cycle, which is generated from arginine through hydrolysis by ARG1. Synthesis of spermidine and spermine requires transfer of aminopropyl groups from dcAdoMet, a decarboxylated product of AdoMet (S-adenosylmethionine) catalyzed by AMD1. Putrescine and spermidine may inhibit TGF-β signaling since depletion of cellular putrescine and spermidine has been shown to enhance TβRI/II levels and Smad nuclear translocation, while TGF-β signaling promotes synthesis of putrescine and spermidine through upregulation of ARG1. In addition, TGF-β signals can stimulate or dampen the activity of ODC1 and AMD1 depending on the cell types. Pink texts and arrows, the urea cycle and polyamine synthesis. Abbreviations: MTA, methylthioadenosine; ARG1, arginase 1; ODC1, ornithine decarboxylase 1; AMD1, adenosylmethionine decarboxylase 1. Figure is created with BioRender.com.

TGF-β SIGNALING AND OTHER ASPECTS OF CELL METABOLISM

Homeostasis of redox metabolism is crucial to an extensive range of cellular and physiological conditions. The redox imbalance, often arises from aberrant accumulation of ROS and is marked by oxidative stress, can promote progression of multiple diseases (Sies et al., 2017). The crosstalk between redox metabolism and TGF-β signaling during cancer and fibrosis is comprehensively reviewed elsewhere (Richter et al., 2015; Ramundo et al., 2021). Noteworthy, the enzyme NADPH oxidase 4 (NOX4) appears to play a main role in mediating TGF-β-induced ROS generation under many circumstances (Cucoranu et al., 2005; Sturrock et al., 2006; Carmona-Cuenca et al., 2008; Michaeloudes et al., 2011; Boudreau et al., 2012). TGF-β can also inhibit the key antioxidant systems by downregulating glutathione (GSH) metabolism (Liu and Gaston Pravia, 2010). However, increased GSH metabolism and alleviated ROS levels are also observed in TGF-β-mediated drug resistance of squamous cell carcinoma cells (Oshimori et al., 2015).

The polyamine metabolic pathway attracts great interests in the past decade due to their roles in cell biology beyond early described importance for cell proliferation (Miller-Fleming et al., 2015). Depending on the cell types, TGF-β signaling can differentially regulate the activity of the two rate-limiting enzymes ornithine decarboxylase (ODC1) and adenosylmethionine decarboxylase 1 (AMD1) in polyamine synthesis (**Figure 4**; **Table 1D**). TGF-β suppresses the enzymatic activity of ODC1 and AMD1 in leukemic cells (Motyl et al., 1993), while stimulating their activities in myofibroblasts (Blachowski et al., 1994). TGF-β can also indirectly regulates the polyamine synthesis in immune cells and vascular smooth muscle cells. By inducing the expression of arginase 1, an enzyme that converts arginine to ornithine that serves as the common precursor for polyamine synthesis, TGF-β is able to increase the arginine-dependent production of specific polyamines (Mondanelli et al., 2017; Boutard et al., 1995;

Durante et al., 2001). However, a recent study reported that the TGF-β blunt the increased influx of arginine to putrescine and spermidine during polarization of $CD4^+$ naïve T cells (Puleston et al., 2021). Polyamine metabolism can also modulate the TGF-β signal transduction (**Table 2C**). In mouse intestinal epithelial cells, depletion of intracellular polyamines by an ODC1 inhibitor DFMO leads to increased expression of TGF-β, TβRI, Smad3 and Smad4 as well as nuclear accumulation of these Smads, sensitizing cells to TGF-β-induced cytostasis (Patel et al., 1998; Rao et al., 2000; Liu et al., 2003).

In addition, TGF-β is able to upregulate the expression of *PNPO* (pyridoxamine 5′-phosphate oxidase), which encodes the rate-limiting enzyme in vitamin B6 metabolism, to produce active forms of vitamin B6 that may promote ovarian cancer progression (Zhang et al., 2017b). Adenosine secreted from myeloid cells is shown to modulate TGF-β signaling in proximal fibroblasts by reducing phosphorylation of Smad2/3 and to affect ECM deposition and therefore influence the tumor microenvironment of mammary carcinoma (Vasiukov et al., 2020). Furthermore, downregulation of the purine catabolism enzyme xanthine dehydrogenase increases TGF-β2/3 and phosphorylated Smad2/3 levels and contributes to EMT and cell migration in hepatocellular carcinoma cell lines (Chen et al., 2017). How these metabolic alterations convey their regulatory instructions to TGF-β signaling awaits further investigation.

CONCLUDING REMARKS

As summarized above, TGF-β signaling can exert its cellular and physiological effects through reprograming of cell metabolism. It controls the activity of many metabolic pathways as wells as the production of functional metabolites by regulating the expression of key metabolic proteins or enzymatic activities (Motyl et al., 1993; Blachowski et al., 1994; Yoon et al., 2005; Dimeloe et al., 2019; Hua et al., 2020; Li et al., 2020; Smith and Hewitson, 2020). In addition, TGF-β signaling is able to reprogram cell metabolism by conferring enzymes non-metabolic functions through post-translational modification (Pallotta et al., 2011; Chen et al., 2019). Of note, the metabolic outputs of TGF-β signaling in cells are context-dependent and highly specific to the cell type, which probably result from the different epigenetic landscapes of distinct cell types, or the different Smad-interacting transcriptional cofactors (Feng and Derynck, 2005; Massague, 2012; David and Massague, 2018). Importantly, rather than being passively regulated by TGF-β signaling, cell metabolism can also modulate TGF-β signaling. Intracellular metabolites and metabolic proteins affect the production or bioactivity of the TGF-β ligands, influence the expression and membrane levels of TGF-β receptors (Rao et al., 2000; Wu and Derynck, 2009; Gencer et al., 2017; Liu et al., 2019; Gabitova-Cornell et al., 2020; Chen et al., 2021), regulate phosphorylation or the abundance of Smad proteins (Inoki et al., 1999; Xiao et al., 2008; Halder et al., 2011; Beilfuss et al., 2015; Chen et al., 2017; Vasiukov et al., 2020), and impact translocation of Smad complex or their binding to TGF-β-target genes (Liu et al., 2003; Ding et al., 2013).

Despite reasonable knowledge have been acquired to date, many questions about the interconnection between TGF-β signaling and cell metabolism still remain. First, we lack a characterization of TGF-β-responsive metabolic gene signature across different cell types, and we do not know how many metabolites or metabolic enzymes can also function as signaling effectors in response to TGF-β. A combination of transcriptomics, untargeted metabolomics and phosphoproteomics will considerably aid in handling this problem. Second, the underlying mechanisms by which metabolites regulates TGF-β signaling remain poorly understood. Since control of gene expression appears to be a mainstay of metabolite-mediated regulation of TGF-β signaling, it would be worthy to investigate if epigenetic regulation by metabolites could account for their modulatory effects (Li et al., 2018). Last, the majority of experiments were conducted *in vitro* using cell lines and whether these findings could be reproduced at a physiological level are currently unknown. Hence, it is of great importance to develop mouse models to examine if the interactions between TGF-β and cell metabolism are indeed physiologically and pathologically relevant. These emerging problems at the interface between TGF-β signaling and cellular metabolism might offer new avenues for future research and bring therapeutic benefits to treat diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Extracellular Vesicles and Transforming Growth Factor β Signaling in Cancer

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Complexity in mechanisms that drive cancer development and progression is exemplified by the transforming growth factor β (TGF-β) signaling pathway, which suppresses early-stage hyperplasia, yet assists aggressive tumors to achieve metastasis. Of note, several molecules, including mRNAs, non-coding RNAs, and proteins known to be associated with the TGF-β pathway have been reported as constituents in the cargo of extracellular vesicles (EVs). EVs are secreted vesicles delimited by a lipid bilayer and play critical functions in intercellular communication, including regulation of the tumor microenvironment and cancer development. Thus, this review aims at summarizing the impact of EVs on TGF-β signaling by focusing on mechanisms by which EV cargo can influence tumorigenesis, metastatic spread, immune evasion and response to anti-cancer treatment. Moreover, we emphasize the potential of TGF-β-related molecules present in circulating EVs as useful biomarkers of prognosis, diagnosis, and prediction of response to treatment in cancer patients.

Keywords: cancer-associated fibroblast (CAF), epithelial – mesenchymal transition (EMT), extracellular vesicle (EV), metastasis, micro-RNA (miRNA), transforming growth factor β (TGF-β)

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INTRODUCTION

Extracellular vesicles (EVs) is an ISEV (International Society for Extracellular Vesicles)-endorsed, collective term for nanosized lipid membrane vesicles that are naturally released from cells (Théry et al., 2018). EVs are heterogeneous and are subtyped according to their biogenesis. Identifying or isolating the various EV subtypes is challenging as no definitive markers can discriminate between subtypes with high security. Accordingly, ISEV recommends the use of operational terms to differentiate various experimentally-obtained EV populations, such as small EVs (sEVs; EVs with a diameter <100 nm). The function of EVs is to mediate intercellular communication in physiological or pathological processes by trafficking biologically active molecules (proteins, nucleic acids, lipids and carbohydrates) from secreting to recipient cells, and even to remote sites *via* circulation through bodily fluids such as the blood (Raposo and Stoorvogel, 2013). EVs interact with recipient cells in several ways. They can be internalized by recipient cells *via* a membrane to membrane fusion process or *via* endocytosis that shuttles them to endocytic compartments where the cargo is released into the cytoplasm or shuttled to lysosomes for degradation (Raposo and Stoorvogel, 2013). Alternatively, EVs could exert their effects on cells without being internalized through various membrane proteins such as CD73, CD59 and others, as explained later. Certain EV

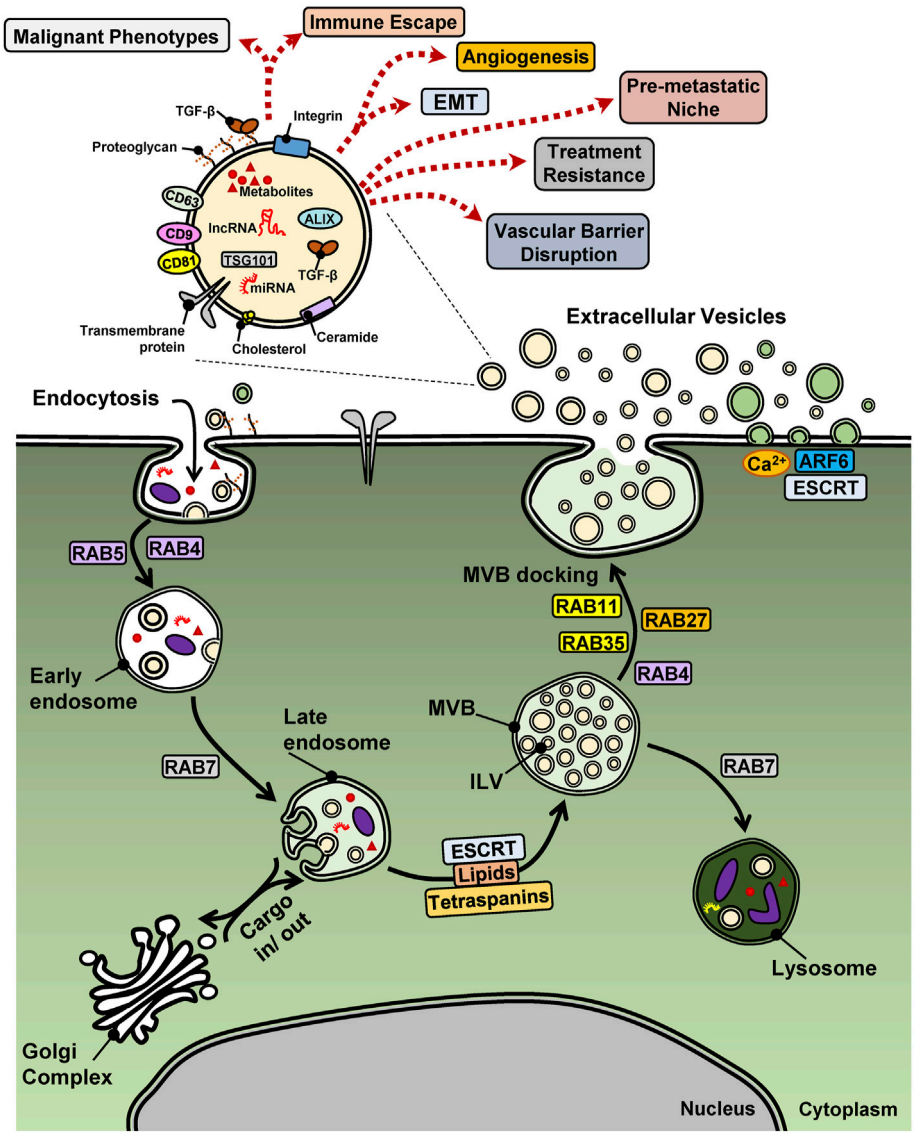

FIGURE 1 | EV biogenesis and biological functions in cancer. A cancer cell is shown with surface proteins and EVs undergoing endocytosis *via* the early and late endosome and the multi-vesicular body (MVB) that shuttles protein or vesicular (intra-luminal vesicles, ILV) cargo to lysosomes or the cell surface, thus releasing exosomes (beige EVs), meanwhile outward budding of the plasma membrane releases microvesicles (green EVs). The case of apoptotic bodies generated from cells undergoing cell death is not illustrated. Key regulatory proteins of endocytosis and EV biogenesis are shown with their names boxed. A single EV is magnified in order to highlight various cargo molecules. miRNAs and lncRNAs may be viewed with potential caution as to their functional importance as EV cargo. Dotted arrows indicate diverse cell biological functions of EVs that relate to the hallmarks of cancer.

membrane proteins could hydrolyze extracellular AMP to activate receptor-mediated signaling or inhibit complement complex formation and prevent cell lysis, respectively (Lai et al., 2012).

In the context of cancer, EVs are considered as important vehicles that assist the intercellular communication and the development of the microenvironment where tumors develop (**Figure 1**) (Liu et al., 2021; Schubert and Boutros, 2021). For example, EVs can mediate and maintain molecular gradients that lead to differential responses of the various cell types that populate the tumor microenvironment (TME), such as mesenchymal stem cells (MSCs), fibroblasts, adipocytes, neurons, immune cells and blood or lymphatic endothelial cells and pericytes (Oudin and Weaver, 2016). The preparation of metastatic sites, also known as "niches" by a process often called "seeding," has also been linked to tumor-derived EVs that generate a proper tissue microenvironment that fosters metastatic colonization (Peinado et al., 2017). Both at the primary tumor and at metastatic sites, EVs can mediate metabolic adaptations of the tumor cells and the cells of the TME, possibly assisting tumor cell survival during the interactions of these tumor cells with multiple other cell types along the metastatic

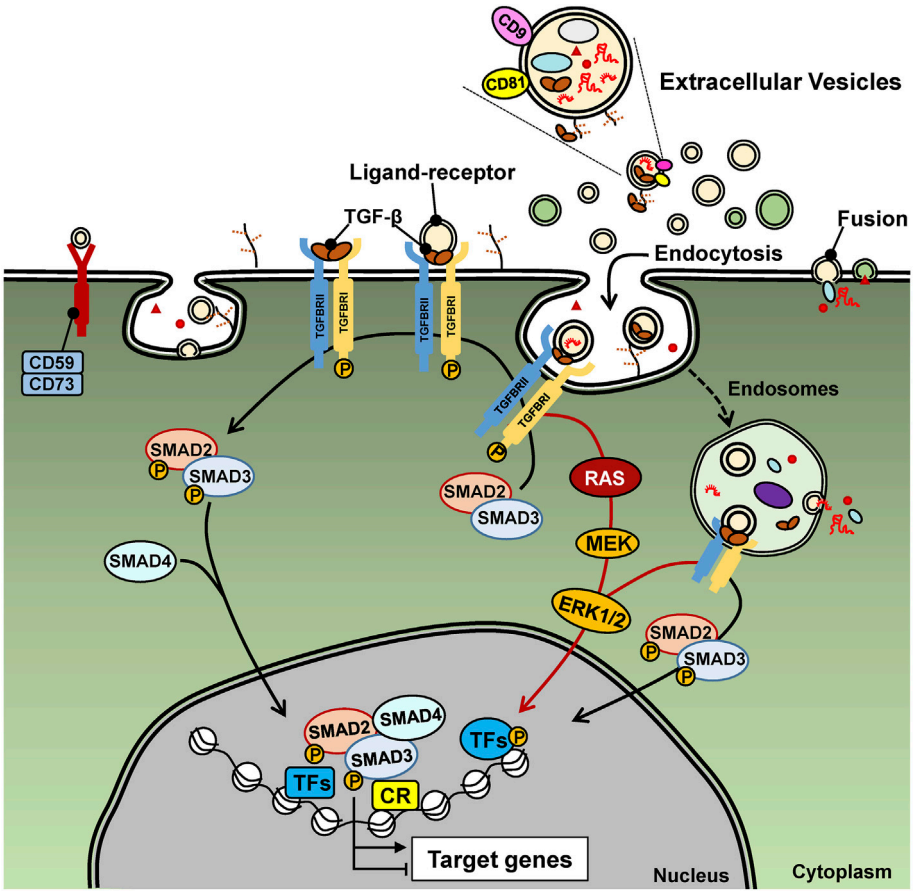

FIGURE 2 | TGF-β signaling. Extracellular TGF-β (usually deposited in the ECM) and here shown as free mature TGF-β, binds to the type II and type I receptors on the cell surface, which signal *via* inter-receptor trans-phosphorylation. The type I receptor phosphorylates SMAD2 and SMAD3 that results in their oligomerization with SMAD4. The ligand-bound receptors also activate RAS, MEK, ERK and other (not shown) protein kinase signaling pathways. EV-associated TGF-β signals in the same manner, yet the ligand is presented from the surface of EVs, as endocytosis of these EVs is in progress. The signaling proteins, SMADs and MAPKs regulate gene transcription *via* direct binding to DNA (SMADs) and *via* phosphorylation of transcription factors (TF) and association with chromatin regulatory protein (CR). MiRNAs and lncRNAs are illustrated as EV cargo and may be viewed with potential caution as to the ability of EVs to deliver functional RNAs to the recipient cells that can affect TGF-β signaling either in a positive or negative manner.

trajectory (Li and Simon, 2020; Bergers and Fendt, 2021). Special attention has recently been given to the communication mediated by EVs between tumor and immune cells, as this provides opportunities for the improvement of immunotherapy against cancer (Garner and de Visser, 2020). Whether EV-mediated processes may improve future treatment of cancer patients, remains to be evaluated (Witwer et al., 2019), yet multiple recent reports raise the utility of EVs and their molecular cargo as biomarkers with predictive and diagnostic potential that can improve cancer treatment by characterization and subclassification of tumors (Hoshino et al., 2020).

TGF-β Signaling in Cancer

Appreciating the potential of EVs in cancer biology, prompts the identification of specific molecular cargo carried in the lumen, the membrane or even bound to the surface of EVs. Among such cargo, the transforming growth factor β (TGF-β) has been steadily featured in cancer EV biology (Schubert and Boutros, 2021; Tan et al., 2021; Webber et al., 2010). This may not come with any surprise as TGF-β and other members of the TGF-β family exert versatile intercellular communication among all cell types and across metazoan evolution (Moustakas and Heldin, 2009; Tzavlaki and Moustakas, 2020). In a nutshell, the TGF-β family pathways are initiated by binding of ligands to type I and type II cell surface receptors (e.g. TGFβRI and TGFβRII in the case of TGF-β), causing their oligomerization and inducing activation of the protein kinase activity of the type I receptor (Heldin and Moustakas, 2016; Tzavlaki and Moustakas, 2020). Co-receptors also facilitate the recruitment of ligands to the signaling receptors (Heldin and Moustakas, 2016). The best studied co-receptor for TGF-β is the type III receptor (TGFβRIII, also known as betaglycan) (Heldin and Moustakas, 2016). TGFβRIII is a transmembrane proteoglycan that binds all three TGF-β ligands with high affinity and presents them to the TGFβRII and TGFβRI signaling complex. The activated TGFβRII and TGFβRI recruit and induce many signaling proteins such as

protein and lipid kinases, scaffolding proteins and small GTPases, whereas some of these proteins become directly phosphorylated by the TGFβ receptors. A well understood substrate of the TGFβRI is the family of SMAD proteins (e.g. SMAD2 and SMAD3 in the case of TGF-β), which, upon phosphorylation, oligomerize with SMAD4, accumulate in the nucleus and regulate gene transcription by binding to regulatory sequences in the genome together with other transcription factors and chromatin proteins (**Figure 2**) (Morikawa et al., 2013; Tzavlaki and Moustakas, 2020). Several of these proteins become post-translationally modified by the action of the signaling proteins initially activated by the TGF-β receptors (Tzavlaki and Moustakas, 2020). In addition to signaling protein mediators, the TGF-β family pathways are regulated by non-protein coding RNAs, including micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs) (Janakiraman et al., 2018; Lai et al., 2020; Papoutsoglou and Moustakas, 2020).

TGF-β was isolated as a tumor-derived growth factor with potential to induce oncogenic transformation in cell models in culture and has been proven to suppress benign and pre-malignant tissue hyperplasia, but also to facilitate the development of aggressive and metastasis-prone tumors (Drabsch and ten Dijke, 2012; Derynck et al., 2021). The effects of TGF-β signaling in cancer are multiple, complex and depend on the context of the intercellular interactions, making TGF-β pathways linked to every hallmark of cancer (Drabsch and ten Dijke, 2012; Derynck et al., 2021). Prominent and extensively studied functions of TGF-β in pre-malignant tissues include the arrest of the cell cycle at the G1 phase, which is cell type-independent, and the induction of apoptosis in specific cell types, e.g. liver or prostate epithelial cells (David and Massagué, 2018). In contrast, in tumors, TGF-β is known to induce epithelial-mesenchymal transition (EMT) that fosters cancer cell invasiveness and initiation of metastasis. Moreover, TGF-β mediates cancer-associated fibroblast (CAF) to myofibroblast differentiation and tissue fibrosis, and acts as a potent suppressor of anti-cancer immunity (Webber et al., 2010; Drabsch and ten Dijke, 2012; Moustakas and Heldin, 2012; Caja et al., 2018; Derynck et al., 2021). In the following sections, these important actions of TGF-β in cancer will be discussed from the point of view of the role of EVs in each of the specific processes that mediate cancer development.

Extracellular Vesicles and Their Biogenesis

One of the first reports about the relevance of EVs in tumor biology showed that tumor cells could secrete microparticles with pro-coagulant function (Dvorak et al., 1983). These particles were later renamed as microvesicles and their origins were linked to responses to stimuli that act on the cell membrane and result in vesicular shedding into the extracellular space (Hugel et al., 2005; Piccin et al., 2007). Other origins of EVs were linked to processes that regulate early endosome maturation into late endosomal compartments known as multi-vesicular bodies (MVBs). The term MVBs indicates that such vesicles can accumulate intraluminal vesicles (ILVs) in their lumen, due to a reverse budding mechanism of their membrane (Stoorvogel et al., 1991). These ILVs can later be released into the extracellular milieu as EVs/exosomes, through fusion of MVBs with the plasma membrane (**Figure 1**) (Johnstone et al., 1987).

Although the biogenesis of exosomes and microvesicles are different, both share membrane-trafficking processes that mediate membrane budding followed by a fission process, which generates EVs secreted inside the lumen of MVBs or in the extracellular milieu (van Niel et al., 2018). Thus, microvesicles are generated by the outward budding of the plasma membrane, a process incorporating changes in lipid bilayer composition, protein intercalation and Ca^{2+} levels, and regulated by the small GTPase ADP-ribosylation factor 6 (ARF6), which leads to the depolymerization of the actin cytoskeleton, and by TSG101 and ALIX, components of the endosomal sorting complex required for transport (ESCRT) (**Figure 1**) (Colombo et al., 2014). The ESCRT machinery, which comprises approximately 30 proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins (VPS4, VTA1, ALIX), is a major regulator of EV biogenesis, driving the sorting of cargo, the membrane shape and fission of ILVs present in MVBs (Hanson and Cashikar, 2012; Hurley, 2008). Furthermore, ESCRT-independent mechanisms involve specific lipids (cholesterol, ceramide and phosphatidic acid) and transmembrane proteins, the tetraspanins (CD9, CD63, CD81 and CD82), regulating the sorting of cargo and of membranous microdomains that will be incorporated into budding EVs (van Niel et al., 2018). Members of the Ras-related protein in brain (RAB) family also regulate vesicular trafficking along the endocytic pathway, either driving the MVB to lysosome route, or the MVB to plasma membrane fusion and secretion route (**Figure 1**) (Stenmark, 2009; Colombo et al., 2014). Additionally, apoptotic cells also release vesicles, denominated apoptotic bodies and formed by blebbing of the plasma membrane that surrounds among other constituents, nuclear fragments. Hence, the different processes that drive EV biogenesis and the physiological state of the cell (surviving or undergoing apoptosis) lead to heterogeneous EV subpopulations, with exosomes, microvesicles or apoptotic bodies coexisting simultaneously in a given cell or tissue microenvironment.

Extracellular Vesicles and Their Cargo

Several cargo molecules carried by EVs (proteins, nucleic acids, lipids and metabolites) (**Table 1**) seem to represent regulators of EV biogenesis. Moreover, the physiological cell state and extracellular stimuli modulate the molecular mechanisms inducing EV biogenesis, generating the context- and cell type-dependent shedding of EVs (van Niel et al., 2018). Among nucleic acids, non-protein coding RNAs (miRNAs and lncRNAs) have been reported to make up EV cargo (Valadi et al., 2007). However, it should be noted that RNAs in exosomes are usually in the range of 200–400 nt long, representing precursors to miRNAs or fragmented rRNAs, mRNAs and lncRNAs (Chen et al., 2010; Enderle et al., 2015; Jenjaroenpun et al., 2013; Kesimer et al., 2009). Quantitative analysis has also estimated that, on average, far less than one molecule of a given miRNA could be identified in the cargo of a single exosome (Chevillet et al., 2014). Furthermore, and in contrast to an extensive number of studies showing that EVs deliver

TABLE 1 | EV cargo molecules.

EV cargo				Molecular function	Process in cancer development	Recipient cell	Cancer type	Ref
Protein, metabolite, lipid	miRNA	lncRNA	EV source					
TGF-β1, TNFα, IL-6, MMPs	—	—	—	—	Tumorigenesis; Anchorage-independent growth	—	—	Redzic et al. (2013); Urciuoli et al. (2018)
Fibronectin	—	—	—	—	Tumorigenesis; Anchorage-independent growth	—	—	Antonyak et al. (2011)
—	*miR-200b*	—	—	*p27* mRNA downregulation	Tumorigenesis; Proliferation	—	CRC	Zhang et al. (2018)
—	*miR-19b; miR-92a*	—	—	*TGFBR1*, *TGFBR2* mRNA downregulation	Tumorigenesis; Proliferation	—	LUAD	Borzi et al. (2019)
—	*miR-142-3p*	—	—	*TGFBR1* mRNA downregulation	Tumorigenesis; Proliferation	—	ORCA	Dickman et al. (2017)
β-Catenin	*miR-23a*	—	—	—	EMT	—	A549, LUAD	Kim et al. (2016)
—	—	*HULC*	—	ZEB1 expression	EMT; Circulating cancer cells	—	PDAC	Takahashi et al. (2020)
—	*miR-21, miR-31, miR-145*	—	—	AR (androgen receptor)	EMT; Mesenchymal cells	—	PC	El-Sayed et al. (2017)
SLUG, SOX2	—	*MALAT1, linc-ROR*	—	—	EMT; CSCs	—	Thyroid cancer	Hardin et al. (2018)
—	*miR-424*	—	BMSC EVs	*TGFBR3* mRNA downregulation	Invasion; Metastasis	—	CRC	Zhang et al. (2021)
—	*miR-378b*	—	—	*TGFBR3* mRNA downregulation	Cancer aggressiveness; Angiogenesis	—	HCC	Chen et al. (2021a)
TGF-β	—	—	Breast milk EVs	—	EMT	Normal epithelial cell	—	Qin et al. (2016)
TGF-β	—	—	MSC EVs	—	EMT	Normal epithelial cell	—	Zhao et al. (2018)
TGF-β1	—	—	Mast cell EVs	—	EMT	—	Human LUAD cells	Yin et al. (2020)
Fibronectin	—	—	EVs	—	Migration; Metastasis	—	—	Sung et al. (2015); Sung et al. (2020)
MMPs	—	—	—	Invadopodia	Invasion	—	—	Clancy et al. (2015); Hoshino et al. (2015)
Integrin $\alpha_v\beta_6$	—	—	—	LAP-TGF-β1	Migration; Metastasis	—	PC	Fedele et al. (2015)
—	—	*lncMMP2-2*	—	MMP-2	Invasion; intravasation	—	LUAD	Wu et al. (2018)
ATP	—	—	—	P2X7 receptor activates JNK, ROCK1	Migration	—	LUAD	Takai et al. (2012)
—	—	*lncRNA-ATB*	—	*miR-204-3p* sponge and TGF-β upregulation	Migration; Invasion	—	Glioma	Bian et al. (2019)
TGF-β, IL-6, TNFα, MMPs	—	—	—	Hypoxia	Pre-metastatic niche	—	PC	Ramteke et al. (2015)
Chemokines	—	—	—	Integrins	Pre-metastatic niche (endothelium)	T effector and memory cells	—	Shulman et al. (2011)
TGF-β	—	—	—	IL-6 secretion	Pre-metastatic niche	MSCs	OS	Baglio et al. (2017)
—	—	*circPACRGL*	—	*miR-142-3p*, *miR-506-3p* inhibition and TGF-β upregulation	Pre-metastatic niche (N1-N2 neutrophils)	—	CRC	Shang et al. (2020)
MET	—	—	—	BMDCs, vasculogenesis	Pre-metastatic niche	—	—	Peinado et al. (2017)
—	*miR-21-5*	—	—	SMAD7, TGF-β signaling activation	Invasion; Angiogenesis	—	Gastric cancer	Li et al. (2018)
—	—	—	—	TGF-β upregulation	Pre-metastatic niche (liver)	Kupffer cells	PDAC	Costa-Silva et al. (2015)
—	*miR-92*	—	BMDC EVs	SMAD7, TGF-β signaling activation	Pre-metastatic niche (liver)	HSCs	Liver metastasis	Hsu et al. (2020)
Integrin $\alpha_v\beta_5$	—	—	—	—	Pre-metastatic niche	Kupffer cells	Liver metastasis	Hoshino et al. (2015)

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TABLE 1 | (*Continued*) EV cargo molecules.

EV cargo				Molecular function	Process in cancer development	Recipient cell	Cancer type	Ref
Protein, metabolite, lipid	miRNA	lncRNA	EV source					
Integrin $\alpha_6\beta_4$, $\alpha_6\beta_1$	—	—	—	—	Pre-metastatic niche	—	Lung metastasis	Hoshino et al. (2015)
ITGBL1	—	—	—	TGF-β,IL-6, IL-8	Liver metastasis	HSCs	CRC	Ji et al. (2020)
Integrin $\alpha_6\beta_4$, $\alpha_6\beta_1$ or $\alpha_v\beta_5$	—	—	—	—	Pre-metastatic niche; Biomarkers	—	CRC to lung or liver metastasis	Ji et al. (2020)
CEMIP	—	—	—	—	Pre-metastatic niche	—	Brain metastasis	Rodrigues et al. (2019)
TGF-β, VEGF	—	—	—	—	CAFs	ADSCs	BRCA, OVCA	Cho et al. (2011); Cho et al. (2012); Song et al. (2017)
TGF-β	—	—	—	—	CAFs	MSCs	Gastric cancer	Gu et al. (2012)
—	*miR-21*	—	—	—	CAFs	HSCs	HCC	Zhou et al. (2018b)
—	*miR-10b*	—	—	PI3K downregulation	CAFs	Fibroblasts	CRC	Dai et al. (2018)
TGF-β	—	—	—	—	CAFs	Fibroblasts	Bladder cancer	Ringuette Goulet et al. (2018)
TGF-β1	—	—	TAM EVs	—	CAFs	Fibroblasts	—	Umakoshi et al. (2019)
TGF-β, TGFβRIII	—	—	—	—	Myofibroblasts	Fibroblasts	BRCA, PC	Webber et al. (2010); Webber et al. (2015)
—	*miR-17-5p*	—	—	*RUNX3* mRNA downregulation, MYC upregulation, TGF-β1 upregulation	CAFs	—	CRC	Zhang et al. (2020)
—	*miR-192, miR-215*	—	—	*Caveolin-1* mRNA downregulation, TGF-β signaling activation	CAFs	—	HNSCC	Zhu et al. (2021)
Tumor antigenic peptides	—	—	—	MHC receptor activation	Immune escape	—	Diverse tumors	Robbins and Morelli, (2014); Seo et al. (2018); Whiteside, (2016)
TGF-β1	—	—	—	MDSC accumulation	Immune escape	—	Murine BRCA	Xiang et al. (2009)
TGF-β1	—	—	—	—	Immune escape	Antigen-presenting cells	Melanoma	Düchler et al. (2019)
TGF-β1	—	—	—	—	Immune escape	T_{reg}	CRC	Yamada et al. (2016)
TGF-β1, IL-12	—	—	—	—	Immune escape	—	CRC	Rossowska et al. (2019)
TGF-β1	—	—	—	—	Immune escape	NK cells	AML	Szczepanski et al. (2011)
TGF-β1	—	—	—	—	Immune escape	NK cells	CML (K562), Lung LCC	Berchem et al. (2016)
TGF-β1	—	—	—	—	Immune escape	DC, $CD4^+$ T, NK	Leukemia	Huang et al. (2017)
TGF-β1, PS	—	—	—	—	Immune escape	$CD8^+$ CTLs	EG7 lymphoma	Xie et al. (2009)
TGF-β1, PS	—	—	—	—	Immune escape	Macrophages	B16F10 murine melanoma	Lima et al. (2009)
Fibronectin, IL-6, MMP-10, MMP-12	—	—	—	Inflammasome	Immune escape	—	HNSCC	Bottino et al. (2021)
TIM-3?	—	—	—	—	Immune escape	M2 macrophages	MG63 OS	Cheng et al. (2021)
?	—	—	—	—	Immune escape	T_{reg}	NPC	Mrizak et al. (2015)
LAMP1, MMP-9	—	—	—	—	Immune escape	B cells	ECA	Li et al. (2015)
PD-L1, TGF-β1	—	—	—	—	Immune escape	—	—	Kang et al. (2020); Mathew et al. (2020)
—	*miR-93-5p*	—	—	*FOXA1* mRNA downregulation, TGF-β3 upregulation	CAFs; Treatment resistance	—	CRC	Chen et al. (2020)
TGF-β1, TGFβRII	—	—	—	—	Treatment resistance	Keratinocytes	ORCA	Languino et al. (2016)

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TABLE 1 | (*Continued*) EV cargo molecules.

EV cargo				Molecular function	Process in cancer development	Recipient cell	Cancer type	Ref
Protein, metabolite, lipid	miRNA	lncRNA	EV source					
TGF-β	—	*Linc-ROR*	—	—	Treatment resistance	—	HCC	Takahashi et al. (2014)
—	*miR-23-5p*	—	—	*GREM2* mRNA downregulation	Treatment resistance (taxanes)	—	PC	Shan et al. (2020)
TGF-β3	—	—	—	—	Treatment resistance (cisplatin)	—	HNSCC	Rodrigues-Junior et al. (2019)
TGFB1, *IL-8* mRNA	—	—	—	—	Drug response biomarkers	—	Glioma	Poggio et al. (2019)
THBS2, VCAN, TNC, FN1	—	—	—	—	Biomarkers	—	Diverse tumors	Hoshino et al. (2020)
TGF-β1	—	—	—	—	Biomarkers	—	PC	Signore et al. (2021)
—	*miR-122-5p*	—	—	—	Biomarkers	—	PDAC	Zhou et al. (2018a)

Table listing EV cargo in groups (proteins, metabolites, lipids, miRNAs, lncRNAs), EV source, function of the molecule, cancer process involvement, recipient cell, cancer type and the corresponding reference. Empty entries indicate lack of information or lack of relevance. The validity of these reported EV cargoes has been criticized in the text and caution is suggested to the careful reader of the table. EV cargoes are listed in the same order as they appear in the main text. Cancer type abbreviations: AML, acute myeloid leukemia; BRCA, breast cancer; CML, chronic myelogenous leukemia; CRC, colorectal cancer; ECA, esophageal cancer; HCC, hepatocellular carcinoma; HNSCC, head-and-neck squamous cell carcinoma; LUAD, lung adenocarcinoma; NPC, nasopharyngeal cancer; ORCA, oral cancer; OS, osteosarcoma; OVCA, ovarian cancer; PC, prostate cancer; PDAC, pancreatic adenocarcinoma.

functional RNAs to recipient cells (**Table 1**), it was recently reported that miRNAs are minor constituents of EVs that are rarely delivered to target cells (Albanese et al., 2021). Yet, another recent study points to a specific molecular mechanism, *via* which RNA sequences within the central body of the precursors of miRNAs are recognized by RNA-binding proteins and thus specify miRNA retention or enrichment within EVs or within cells (Garcia-Martin et al., 2022). In agreement with a more prominent role of the protein cargo carried by EVs, it was demonstrated that the main cargo attribute that drives MSC-derived exosome function is likely to be made of proteins and not RNAs (Toh et al., 2018). In summary, publications reporting the function of bioactive molecules, including proteins or RNAs, carried by tumor-derived EVs, are often based on gain or loss of function of a given cargo molecule; it thus remains possible that such genetic perturbations performed on the secreting cells, may induce changes in the amount of secreted EVs and/or in the overall molecular content of the EVs, leading to indirect effects of the perturbed molecule and not true effects of the analyzed cargo (Théry et al., 2018). Hence, ISEV has raised concern about the validation of such functional studies of EVs carrying bioactive molecules, requiring a complete characterization of the "engineered EVs," considering at least a small-scale analysis of EV amount or common EV-associated proteins in comparison to the unperturbed EVs (Théry et al., 2018). In view of this critical evaluation of the EV cargo, all cases presented in this article (**Table 1**), should be considered with the appropriate caution.

For instance, *TGFBR2* deficiency that inactivates most if not all aspects of TGF-β signaling, affects the miRNA and protein content of EVs derived by colorectal cancer (CRC) cells, indicating that TGF-β signaling may regulate EV biogenesis or secretion (Fricke et al., 2019a; Fricke et al., 2019b). It is well known that the TGF-β pathway regulates the transcriptional and post-transcriptional expression of different genes, but to date, the impact of TGF-β signaling on the content of EV cargo is yet poorly studied. Hence, the findings by Fricke et al. raise new perspectives about how TGF-β signaling could be implicated in the cell-to-cell communication mediated by vesicles (Fricke et al., 2019a; Fricke et al., 2019b).

To understand how the different molecules carried by EVs can affect the recipient cells, it is important to mention the different interaction routes of cell-to-cell communication mediated by vesicles. These can represent interactions of ligands present on the surface of EVs (e.g. TGF-β1) with cell surface receptors, inducing the activation of intracellular signaling (**Figure 2**). Alternatively, EVs could fuse with the cell membrane of the recipient cells and the EV content released into the cytoplasm, affecting downstream signaling (**Figure 1**) (Del Conde et al., 2005; Valadi et al., 2007). Nevertheless, EV uptake also occurs *via* energy-dependent, receptor-mediated endocytosis, in which EVs remain intact during and after cell entry, until specific cargo molecules interact with molecular pathways that initiate at the endocytic or phagocytic compartments (Svensson et al., 2013). In the latter context, we here discuss how molecules carried by tumor-derived EVs can affect TGF-β signaling positively or negatively in different cell types, inducing responses in recipient tumor cells, as well as in other cells in the TME.

Extracellular Vesicles, Tumorigenesis and Epithelial-Mesenchymal Transition

Tumor-derived EVs (TDEs) can directly alter the physiology of surrounding and distant normal cells to promote cancer growth. For instance, TDEs can influence endothelial cells by inducing

neo-angiogenesis and vascular leakiness, causing fibroblast differentiation into CAFs and suppressing immune cells allowing for generation of pro-tumorigenic and pro-metastatic phenotypes that lead to cancer progression and dissemination (Liu D. et al., 2016; Nabet et al., 2017; Nazarenko et al., 2010; Webber et al., 2010), aspects that are discussed later. Interestingly, disruption of EV biogenesis by inhibition of the small GTPase RAB27a, which regulates EV secretion, hinders primary tumor growth as well as metastasis of malignant cells (Bobrie et al., 2012). Increasing evidence suggests that TDEs perform these cell phenotypic changes by horizontal transfer of functional molecules/oncoproteins and activation of downstream signaling pathways in the recipient cells. For instance, EVs derived from various cancer cell types, in addition to TGF-β, transfer tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and matrix metalloproteinases (MMPs) to normal recipient cells, promoting their proliferation, migration and anchorage-independent growth (**Table 1**) (Redzic et al., 2013; Urciuoli et al., 2018). Numerous studies have also indicated the role of EVs in the generation of a microenvironment permissive to tumor growth (Liu et al., 2021; Schubert and Boutros, 2021). TDEs carry fibronectin, which, once bound to integrin receptors of normal fibroblasts, promotes their anchorage-independent growth (Antonyak et al., 2011). Similarly, prostate cancer (PC) cell-derived EVs enriched in TGF-β1, induced SMAD3 and α smooth muscle actin (αSMA) expression in normal fibroblasts, promoting their differentiation to myofibroblasts (**Table 1**) (Webber et al., 2010).

EVs have also been reported to carry miRNAs and lncRNAs (**Table 1**), resulting in altered expression of tumor-suppressing or tumor-promoting genes in the recipient cells (Wei et al., 2014; Qu et al., 2016; Zheng et al., 2016; Hsu et al., 2017; Hardin et al., 2018; Ota et al., 2018; Bian et al., 2019; Borzi et al., 2019; Bier et al., 2020). As explained in detail in the previous section, these findings should be considered with caution.

In colorectal carcinoma, TGF-β1 was found to upregulate *miR-200b* levels; transfer of *miR-200b* to recipient cells *via* EVs directly targeted the 3′- UTR of the *p27* mRNA, suppressing expression of this cell cycle inhibitor, and leading to increased cancer cell proliferation (Zhang et al., 2018). In addition, EVs derived from lung adenocarcinoma cell lines increased proliferation of pre-neoplastic bronchial epithelial cells, favoring tumor growth, due to the transfer of *miR-19b* and *miR-92a*, which attenuated the expression of TGFBRI and TGFBRII in the recipient epithelial cells, where the anti-proliferative role of TGF-β is well established (Borzi et al., 2019). Furthermore, tumor-suppressive miRNAs can be selectively packaged into EVs, thus eliminating their anti-tumor function from the donor cancer cell. To this end, selective elimination of the *miR-142-3p via* EVs led to enhanced proliferation of the donor oral cancer cells as well as to induced pro-angiogenic activity in the recipient stromal cells, *via* altered expression of TGFBRI (Dickman et al., 2017).

An integral process involved in cancer progression, first proposed more than a 100 years ago by Santiago Ramon y Cajal, is the EMT (Nieto et al., 2016). EMT is linked to the modification of the primary TME in order to facilitate invasion. This is achieved by reassembly of epithelial cell-cell adhesions, modification of cell-extracellular matrix (ECM) interactions, reorganization of cytoskeleton and remodelling of the secreted extracellular proteins (Derynck and Weinberg, 2019; Moustakas and Heldin, 2012; Nieto et al., 2016). Currently, the EMT status in a primary tumor is validated by the expression of a combination of epithelial and mesenchymal genes, as was established in a breast cancer (BRCA) microarray (Sarrio et al., 2008). As already introduced, the TGF-β pathway prominently induces EMT (Moustakas and Heldin, 2012). TGF-β signaling is directly coupled to the transcriptional induction of a cohort of transcription factors that initiate the EMT (EMT-TFs), such as SNAI1, SNAI2, ZEB1, ZEB2, TWIST1 and TWIST2, many of which also receive signals from TGF-β that control their activity (de Herreros et al., 2010; Moustakas and Heldin, 2012; Nieto et al., 2016). Induction of EMT in A549 human lung adenocarcinoma cells upon TGF-β1 treatment altered the protein and miRNA cargo of EVs, which reflected the phenotypic condition of the cells they derived from (Kim et al., 2016). Although the EVs were not properly characterized in this study, the data showed an increment of β-catenin and *miR-23a* levels in A549-derived EVs treated with TGF-β1 (Kim et al., 2016). In the same cell model, β-catenin mediates signaling that promotes EMT (Tian and Phillips, 2002), whereas *miR-23a* downregulates the adherens junction protein E-cadherin (Cao et al., 2012). Moreover, stimulation of human pancreatic ductal adenocarcinoma cells by TGF-β induced the lncRNA *HULC*, which caused ZEB1 upregulation and promoted EMT (Takahashi et al., 2020). *HULC*-containing EVs caused increased *HULC* levels in recipient human pancreatic ductal adenocarcinoma cells (**Table 1**), which resulted in further induction of the EMT program (Takahashi et al., 2020). In addition, *HULC* encapsulated in EVs was upregulated in the serum of pancreatic ductal adenocarcinoma patients, suggesting that the EVs and their *HULC* cargo might serve as diagnostic biomarkers (Takahashi et al., 2020).

Mesenchymal cell TDEs can transform normal or pre-malignant epithelial cells in experimental systems. For instance, EVs derived by mesenchymal PC cells triggered phenotypic changes in the recipient androgen-dependent epithelial PC cells by direct inhibition of androgen receptor signaling and activation of TGF-β signaling (El-Sayed et al., 2017). The miRNAs *miR-21*, *miR-31* and *miR-145* directly regulate androgen receptor levels and appeared elevated in both mesenchymal-derived TDEs and in recipient carcinoma cells (**Table 1**), suggesting a horizontal transfer of cargo, which promoted the survival of a more plastic (EMT) state and generation of an aggressive PC cell subpopulation (El-Sayed et al., 2017). In addition, EVs isolated from cancer stem cells upon culture with normal thyroid cells upregulated the lncRNA *MALAT1* and the *long intergenic non-protein coding RNA, regulator of reprogramming* (*linc-ROR*) levels (**Table 1**), as well as the EMT transcription factor SNAI2 and the stem cell transcription factor SOX2 (Hardin et al., 2018). The cells receiving these EVs presented increased proliferative and invasive ability when compared to control cells (Hardin et al., 2018).

Disruptions of TGF-β signaling in CRC drives tumor progression (Itatani et al., 2019). In this context, EVs derived from bone marrow-derived MSCs (BMSCs) carrying *miR-424* induced an aggressive phenotype in CRC cells *in vivo* by targeting *TGFBR3* (TGFβRIII) transcripts (Zhang et al., 2021). Similarly, downregulation of *TGFBR3* expression in hepatocellular carcinoma (HCC) was driven by HCC cell-derived EVs carrying *miR-378b*, which increased progression and angiogenesis (Chen W. et al., 2021). In these two examples, downregulation of TGFβRIII is thought to inhibit physiological TGF-β signaling, which acts homeostatically, and thus, the loss of the co-receptor TGFβRIII might be equivalent to the loss-of-function mutations in TGFβRII that sometimes are required for the progression of CRC. Remarkably, in addition to malignant cells, EVs derived from normal cells contain high TGF-β levels, i.e. EVs secreted by breast epithelial cells of healthy lactating women in their milk and EVs secreted from human umbilical cord MSCs, were reported to induce EMT and malignant transformation of both cancer and benign epithelial cells (**Table 1**), as observed by activation of the canonical and non-canonical TGF-β signaling pathways, and altered expression or assembly of EMT related proteins (E-cadherin, vimentin, αSMA, filamentous actin) in the recipient cells (Qin et al., 2016; Zhao et al., 2018). Furthermore, mast cell-derived EVs rich in TGF-β1 on their surface, induced EMT when taken up by epithelial human lung adenocarcinoma cells as observed by increased mRNA and protein levels of EMT-TFs (TWIST1, SNAI1, SNAI2) and of induced phosphorylation of cellular proteins involved in EMT (TGM2, annexin-A1, VACM1, Chrombox3), cell-cell junctions (E-cadherin, N-cadherin), cell-ECM interactions (MMP-2, MMP-9) and cell proliferation (c-KIT) (Yin et al., 2020). Altogether these findings suggest that key regulators of TGF-β signaling can be shed extracellularly as content of EVs and thus, they can induce signaling that modulates the adjacent and distant TME in order to culminate tumor progression and metastasis initiation.

Extracellular Vesicles and TGF-β as Mediators of Cancer Metastasis

Metastatic outgrowth of the primary tumor to distant organs is the major cause of death due to cancer. This requires the invasion of neoplastic cells from the primary tumor through the basement membrane and dissemination *via* the circulation. Accumulating evidence suggests that TDEs may promote metastases at secondary sites, and that the EV-mediated pro-metastatic signal transmission can take place either within the primary tumor or at distant organs and/or tissues contributing to premetastatic niche formation.

In the primary tumor, EVs show a prominent role in modifying the motility of cancer cells and their invasive abilities. Secretion of EVs is required for *in vivo* movement and cell migration of cancer cells by promotion and stabilization of leading-edge adhesive protrusions (Sung et al., 2015). Development of a pH-sensitive EV reporter (pHluo_M153R-CD63) that allowed the monitoring of cellular interactions with EVs, demonstrated that the EVs are secreted at the front edge of the migrating cells and can be used by other cell types as migrating tracks in 2D and 3D tissue culture environments (Sung et al., 2020). These cells also leave behind exosome trails and cancer cells migrating towards the leading cells in the migratory trail can actively endocytose the layered EVs, which fuel the transmission of migratory behavior (Sung et al., 2020).

EVs also act as carriers of ECM components promoting adhesion and altered cell-ECM interactions. For example, fibronectin is sorted into the cargo of EVs after integrin receptor binding and promotes cell motility (Sung et al., 2015). Furthermore, it has been shown that EVs contribute to the formation of invadopodia or acquisition of an amoeboid mode of migration *via* MVB-dependent transfer of MMPs (Hoshino et al., 2013; Clancy et al., 2015). Another study demonstrated that PC cell-secreted EVs that carry high levels of integrin $\alpha_v\beta_6$, after delivery to $\alpha_v\beta_6$-negative PC cells allowed binding of the inactive latency-associated peptide (LAP)-TGF-β form, promoting its activation and the release of mature TGF-β, whose signaling induces alterations of the ECM and mediates cell migration (Fedele et al., 2015). In the same line, TGF-β-treated human lung adenocarcinoma cells secrete EVs that are enriched in *lnc-MMP2-2*, which promotes the expression of MMP-2, regulating migration and invasion of lung cancer cells and intravasation into the vasculature (Wu et al., 2018). Alternatively, TGF-β1 stimulation of human lung adenocarcinoma cells can promote exocytosis of ATP *via* vesicles, which in turn activates the ionotropic P2X7 receptor that promotes actin remodeling through activation of the Jun N-terminal kinase or Rho kinase leading to increased lung cancer cell migration (Takai et al., 2012).

By sequestering certain miRNAs, specific lncRNAs are involved in the epigenetic regulation of gene expression in several diseases including cancer (Su et al., 2021). As an example, TGF-β signaling downregulates *miR-622*, which normally targets the lncRNA *HULC* and attenuates cell invasion and migration by suppression of EMT signaling *via* EV transfer (Takahashi et al., 2020). Moreover, glioma cell-derived exosomal *lncRNA activated by TGF-β* (*lncRNA-ATB*) suppresses *miR-204-3p* in an argonaute 2-dependent manner in recipient astrocytes, causing activation of migration and invasion of glioma cells by induction of TGF-β signaling (Bian et al., 2019).

Development of hypoxia is a common pathophysiological condition observed during tumor growth and is characterized by limited supply of oxygen and nutrients to the cells of the core of the tumor mass. This condition induces angiogenesis and activates altered metabolic pathways leading to increased migration of the invasive tumor front. Thus, EVs derived from hypoxic PC cells contain elevated numbers of proteins implicated in EMT and pre-metastatic niche formation (TGF-β2, IL-6, TNF1α, MMPs, **Table 1**) (Ramteke et al., 2015). The cargo proteins in these EVs regulate adherens junctions, by downregulation of E-cadherin and accumulation of nuclear β-catenin, and by remodeling of the actin cytoskeleton, thus enhancing the motility and invasiveness of the PC cells (Ramteke et al., 2015). In another case, hypoxia enhanced TGF-β signaling in cancer cells, which promoted alternative splicing of hMENA, a cytoskeletal remodeller during EMT that supports fast actin polymerization, promoted cell migration and invasiveness (Ahuja et al., 2020).

Extracellular Vesicles and the Metastatic Niche

The contribution of EVs in malignant progression by aiding in the formation of premetastatic niches can be related to the increased number of tumor-derived EVs present in the blood circulation of cancer patients (Logozzi et al., 2009; Baran et al., 2010; Galindo-Hernandez et al., 2013; Baglio et al., 2017) and to the fact that elevated levels of several EV cargoes have been associated with poor prognosis of cancer patients with metastatic progression (Peinado et al., 2012). Interestingly, injections of metastatic cell-derived EVs into the mouse blood circulation, induced formation of a pre-metastatic niche even in the complete absence of tumor cells (Grange et al., 2011; Peinado et al., 2012; Costa-Silva et al., 2015; Hoshino et al., 2015; Liu Y. et al., 2016). After generation of the niche, incoming tumor cells communicate with surrounding fibroblasts, endothelial and immune cells by receptor-mediated cell-cell interactions, *via* paracrine secretion of growth factors, chemokines and cytokines and *via* EVs. Interestingly, vascular endothelial cell-derived chemokines have been shown to be stored in vesicles which docked on actin fibers beneath the endothelial plasma membrane (Shulman et al., 2011). These chemokines were released at lymphocyte-endothelial synapses, allowing for establishment of contacts between adhesive integrins and T effector and memory cells within the inflamed endothelia (Shulman et al., 2011). Furthermore, using a zebrafish model, it was assessed how exogenous melanoma MemBright-labeled EVs circulate in the blood flow and how they are internalized by normal cells during formation of a pre-metastatic niche (Hyenne et al., 2019). Endothelial cells and macrophages were the major circulating cells that received EVs, and these cell types presented increased uptake efficiency of the tumor-derived EVs for degradation in lysosomes (Hyenne et al., 2019).

Plethora of studies suggest that molecular cargo released from EVs can "educate" cells to activate signaling cues favoring metastatic development mainly by induction of inflammation, immune suppression, vascular leakiness and stromal cell activation (Liu Y. et al., 2016; Nabet et al., 2017; Nazarenko et al., 2010). EV-transduced TGF-β signaling has been shown to underlie many such processes (**Table 1**). For example, EVs from metastatic osteosarcoma cells have been shown to carry elevated levels of a membrane-associated form of TGF-β that interacts with its receptor on the surface of MSCs and "educates" them to produce IL-6 and thus trigger a proinflammatory loop favoring metastatic seed and progression (Baglio et al., 2017). Potential mechanisms of action of such EVs may relate to a special conformation that EV-bound TGF-β takes, making this TGF-β capable of activating not only TGF-β receptors but also alternative signaling receptors that could act as co-receptors in this case. Such a mechanism remains to be experimentally confirmed. Alternatively, specific cargoes in EVs, such as inflammatory miRNAs may cooperate with TGF-β and activate Toll-like receptor signaling in the MSCs, as is the case for *miR-21* and *miR-29a* action on Toll-like receptors of immune cells during metastasis (Fabbri et al., 2012). Another plausible mechanism is that EV surface cargo signals together with TGF-β, thus providing a combinatorial message that mediates the MSC response in terms of IL-6 secretion, as is the case of fibroblast to myofibroblast differentiation by a combinatorial action of EV-carried TGF-β together with heparan sulphate, also carried on the EV surface (Webber et al., 2015). Irrespective of the specific mechanism, when injected into a preclinical mouse model, the "educated" MSCs promoted osteosarcoma growth and formation of lung metastasis, supporting the establishment of a tissue microenvironment favoring tumor growth and metastasis formation through the induction of the pro-inflammatory IL-6/STAT3 pathway (Baglio et al., 2017). Colorectal carcinomas secrete EVs carrying the non-coding RNA *circPACRGL* that regulates the expression of TGF-β by binding to *miR-142-3p* and *miR-506-3p*, promoting cancer cell proliferation and metastasis, mediated by the transformation of N1 neutrophils to N2 in the TME (Shang et al., 2020). Furthermore, the contribution of bone marrow derived cells (BMDCs) in metastasis has been well established (Kaplan et al., 2005; Hara et al., 2017; Xu et al., 2017). In this context, it was demonstrated that TDEs first recruit BMDCs through upregulation of pro-inflammatory molecules at premetastatic sites, and second, educate BMDCs to support tumor vasculogenesis, invasion and metastasis by horizontal transfer of the MET oncoprotein to bone marrow progenitors (Peinado et al., 2012). Alternatively, gastric cancer-derived exosomal *miR-21-5p* that targets SMAD7, an inhibitor of TGF-β signaling, has been shown to promote mesenchymal transition of peritoneal mesothelial cells, a process that promotes invasion through matrix remodeling and angiogenesis of the peritoneum (Li et al., 2018).

Organ-specific metastasis, the tendency of primary tumors to develop secondary malignancies in specific organs, has only recently started being understood. Several studies suggest that TDEs prepare the microenvironment at future metastatic sites and mediate non-random patterns of metastasis. For example, EVs secreted from pancreatic ductal adenocarcinoma (PDAC) promoted the formation of a pre-metastatic niche in the liver and thus increased the liver metastatic burden (Costa-Silva et al., 2015). Uptake of EVs by Kupffer cells in the liver induced the TGF-β signaling pathway *via* activation of the macrophage migration inhibitory factor (MIF), a known mediator of liver inflammation and fibrosis. This in turn promoted fibronectin production that arrested bone marrow-derived macrophages in the liver, establishing alterations of the ECM that supported metastasis (Costa-Silva et al., 2015). EVs derived from BMDCs of mouse lung tumors, contain *miR-92*, which promotes metastasis to the liver (Hsu et al., 2020). This was achieved by enhancement of TGF-β signaling in hepatic stellate cells (HSCs) (**Table 1**) through *1*) direct pairing of *miR-92* to the 3' UTR of *SMAD7*, causing SMAD7 protein suppression and TGF-β pathway de-repression, *2*) accumulation of immunosuppressive cells, and *3*) upregulation of collagen type I, supporting cancer cell and myeloid-derived suppressor cell (MDSC) attachment (Hsu et al., 2020).

The targeting of EVs to specific recipient cells and internalization is proposed to depend on the presence of ECM-related proteins that promote EV adhesion to certain organs thereby initiating pre-metastatic niche formation. For example, EVs expressing the integrins $\alpha_v\beta_5$ mediated liver tropism by binding to Kupffer cells, whereas EVs expressing the integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ mediated lung tropism by binding to lung fibroblasts and epithelial cells (**Table 1**) (Hoshino et al., 2015). Thus, integrins transported by EVs, apart

from inducing adhesion to specific organs, "educate" these organs, creating an environment where metastatic cells could grow and form secondary neoplasia (Hoshino et al., 2015). In the same line, CRC cells secrete EVs carrying integrin β-like 1 (ITGBL1) to the circulation, activating fibroblasts and stellate cells in the lung and the liver, which in turn secrete growth factors and cytokines, including TGF-β, IL-6 and IL-8 that promote metastatic cancer growth and invasiveness (Ji et al., 2020). Interestingly, the content of circulating TDEs secreted from CRC cells predicted the metastatic site; integrins α6, β1 and β4 were high in plasma EVs of colorectal patients showing metastasis to the lung, while high EV integrin α5 and β5 correlated with liver metastasis of different colorectal patients (Ji et al., 2020). It is likely that there are more mechanisms through which EVs promote organ-specific metastasis. Bone-tropic EVs were reported to induce vascular leakiness in the lung instead of expressing a specific repertoire of integrins (Hoshino et al., 2015). In addition, cell migration-inducing and hyaluronan-binding protein (CEMIP) levels were elevated in EVs from metastatic cells of the brain but not in EVs from lung or bone metastasis (Rodrigues et al., 2019). CEMIP triggered vasculogenesis and promoted a pro-inflammatory state in the brain which supported metastatic colonization (Rodrigues et al., 2019). Altogether these findings provide examples on how EVs can "educate" a plethora of cell types in order to generate a metastatic microenvironment permissive of successful engraftment of incoming tumor cells. Of significance is the fact that EVs, based on their cargo, display specific preference for target organs/tissues and thus support non-random patterns of metastasis.

Extracellular Vesicles and Cancer-Associated Fibroblasts

We briefly referred to CAFs in the EMT section. CAFs are important cell types of the TME. They are derived from resident, tissue fibroblasts, from infiltrating MSCs originating from the bone marrow, occasionally from adipocytes and possibly some other sources, and reside within the TME, where they secrete ECM proteins and enzymes that remodel the ECM (Östman and Augsten, 2009; Sahai et al., 2020; Chen Y. et al., 2021). CAFs are often defined negatively, being non-epithelial, non-endothelial, non-immune and non-hematopoietic elongated cells that are non-cancerous, in other words they lack the genetic mutations that define a cancer, and consequently, CAFs cannot be confused with mesenchymal cells generated *via* EMT (Sahai et al., 2020; Chen Y. et al., 2021). TGF-β "activates" CAFs, in other words induces their differentiation to a myofibroblastic phenotype that is highly secretory and contractile. The high secretory function of CAFs is connected to the production of EVs. We will here discuss two main actions of EVs in CAF biology: the role of EVs in CAF generation and the role of EVs in myofibroblast differentiation in various tumor types.

EVs derived from ovarian or BRCA cells cultured *in vitro*, induced a myofibroblast phenotype to adipose tissue-derived MSCs (ADSCs) (Cho et al., 2011; Cho et al., 2012; Song et al., 2017). TGF-β1 carried by the EVs was a key mediator of the myofibroblast phenotype, although the EVs carried additional growth factors, such as vascular endothelial growth factor (**Table 1**). Upon incubation of the EVs with the ADSCs, the differentiating cells upregulated expression of their TGF-β receptors and activated TGF-β signaling, which explains why inhibition of TGF-β receptor kinase activity blocked the differentiation process (Cho et al., 2011; Cho et al., 2012; Song et al., 2017). Unexpectedly, the ovarian cancer EVs induced SMAD2 or AKT signaling in the differentiating ADSCs, depending on the cancer cell line where the EVs were isolated from, an observation that deserves further investigation and explanation (Cho et al., 2011). Similarly, EVs produced by gastric cancer cells induced CAF generation from human umbilical cord MSCs *via* activation of TGF-β/SMAD2 signaling (Gu et al., 2012). EVs produced by HCC cells acted on isolated HSCs causing generation of CAFs that secreted TGF-β1 (Zhou Y. et al., 2018). In addition to MSCs, incubation of resident vesical fibroblasts or primary fibroblasts with EVs derived either from bladder cancer cells or from CRC cells, generated CAFs (Dai et al., 2018; Ringuette Goulet et al., 2018). The CRC EVs induced secretion of TGF-β1 and expression of αSMA, a hallmark cytoskeletal protein of myofibroblasts, when incubated with primary fibroblasts, and the resulting CAFs enhanced CRC cell proliferation *in vitro* and CRC in recipient mice where the latter cells were xenografted together with the CAFs (Dai et al., 2018). On the other hand, bladder cancer EVs carried a substantial proportion of secreted TGF-β1, induced TGF-β/SMAD2 signaling in vesical fibroblasts and CAF differentiation was inhibited by a TGF-β receptor kinase inhibitor (Ringuette Goulet et al., 2018). Induction of high expression of fibronectin in response to TGF-β carried by EVs promotes invasion of fibroblasts mediated by binding of fibronectin to integrin $\alpha_5\beta_1$ on their surface (Chanda et al., 2019). A final example of EV-mediated CAF generation stems from studies of tumor-associated macrophages (TAMs), which secrete EVs carrying TGF-β1, and which upon interaction with fibroblasts induced CAFs (**Table 1**), but also acted on peritoneal mesothelial cells that responded with EMT, and on endothelial cells, in a gastric epithelium invasive model (Umakoshi et al., 2019). This model is relevant to the generation of a pro-metastatic niche, as discussed earlier, and provides evidence for a strong ability of the EVs to penetrate the gastric parenchyma.

Observations of myofibroblast differentiation of resident fibroblasts or CAFs are also abundant. Comparative analysis of EVs secreted by salivary adenoid cystic carcinoma cells relative to EVs secreted by CAFs from the same tumor type, showed that the CAF EVs carried a stronger potential of generating the pre-metastatic niche to the lung; the EVs acted on lung fibroblasts that generated CAFs in the metastatic colony area (Kong et al., 2019). As discussed in the tumorigenesis section, EVs from breast or prostate carcinoma cells that carry TGF-β and TGFβRIII co-receptor on their surface, induced stromal fibroblast to myofibroblast differentiation with strong αSMA expression (Webber et al., 2010; Webber et al., 2015). An important finding made in these studies was that the TGF-β carried by EVs was more potent than recombinant human TGF-β1, which was provided to the same responding fibroblasts, in terms of pro-tumorigenic and pro-angiogenic activity (Webber et al., 2010; Webber et al., 2015). The generation of the EVs required intact RAB27a GTPase activity and the response of the fibroblasts to TGF-β depended on the heparan sulphate content of the EVs, possibly reflecting the function

of the co-receptor function of TGFβRIII (Webber et al., 2015). A possible reason why TGF-β carried by EVs is superior to TGF-β secreted by cancer cells, is the presence of receptors in the same EVs. Thus, EVs secreted from stromal fibroblasts of oral squamous cell carcinoma carry TGF-β1 and TGFβRII (**Table 1**) and mediate drug resistance to recipient oral squamous cell carcinoma keratinocytes (Languino et al., 2016). Fibroblast-derived EVs transferred fully competent TGF-β responses in patient-derived keratinocytes carrying mutations in *TGFβRII* and thus lacking active receptor signaling (Languino et al., 2016). This mechanism whereby tumor cells mutate components of the TGF-β pathway, and surrounding stromal cells complement this defect by utilizing their EVs as a vehicle for communication, highlight the complexity of signaling events taking place during cancer development.

Extracellular Vesicles Carry miRNAs To Regulate CAF Biology

Based on the possibility that miRNAs are cargo of EVs secreted by cancer cells, it is appropriate to discuss a few molecular mechanisms controlled by such miRNAs and which center around the TGF-β pathway, although the validity of these mechanisms should be viewed critically. In the example of HCC EVs that generate CAFs out of HSCs, the EV cargo of relevance is *miR-21* (**Table 1**), which directly downregulates the PTEN phosphatase, thus promoting phosphoinositide 3′ kinase (PI3K) activity and further releasing the activity of the AKT protein kinase pathway, which is required for initiation of CAF differentiation (Zhou Y. et al., 2018). Based on its mechanistic role, *miR-21* levels measured in EVs isolated from the serum of HCC patients correlated with CAF to myofibroblast differentiation and the vascularity of the tumors (Zhou Y. et al., 2018). Focusing on the same signaling pathway, the CRC EV mechanism that generates CAFs involved the cargo *miR-10b* (**Table 1**), whose target was the catalytic subunit of PI3K (PIK3CA) (Dai et al., 2018). This example contrasts the previous data on *miR-21*, considering that PIK3CA was downregulated by EVs carrying the *miR-10b*. Consequently, PI3K/AKT signaling activity was reduced and the recipient fibroblasts exhibited reduced proliferation, yet they turned on expression of TGF-β1 and αSMA, which mark the differentiation of CAFs (Dai et al., 2018). Thus, CAF differentiation is linked to the inhibition of progenitor fibroblast proliferation. Two more examples from CRC exemplify the complexity of the molecular networks involved in CAF function during cancer development. Pro-metastatic actions of EVs secreted by CAFs in CRC are associated with cargo *miR-17-5p* (**Table 1**), which downregulates the transcription factor RUNX3 (Zhang et al., 2020). RUNX3 and c-MYC form complexes on the regulatory sequences of the *TGFB1* gene and RUNX3 suppresses the transcriptional activity of c-MYC, which positively induces expression of TGF-β1, the latter promoting CRC cell invasiveness; thus, when CAFs secrete their EVs, they provide *miR-17-5p* to CRC cells, negatively regulating RUNX3 and consequently de-repressing the *TGFB1* gene *via* c-MYC. The newly synthesized TGF-β1 from CRC cells feeds back to the CAFs and further enhances CAF differentiation, enforcing higher secretion of EVs enriched in *miR-17-5p*. It is worth noting that *miR-17-5p*, in addition to RUNX3, negatively regulates many components of the TGF-β signaling pathway, yet, whether TGF-β regulates expression of *miR-17-5p* remains to be examined. CRC CAFs can also generate EVs carrying *miR-93-5p*, whose target is the *FOXA1* mRNA (**Table 1**), encoding a transcriptional repressor of the *TGFB3* gene (Chen et al., 2020). This mechanism has functionally been linked to escape from radiation-mediated cell death of CRC cells *in vitro* in xenografted mice (Chen et al., 2020). Thus, EVs secreted from CAFs act on CRC cells, causing de-repression and expression of TGF-β3, *via* the action of *miR-93-5p* on FOXA1; the increased amounts of TGF-β3 contributed to the proliferation after escape from apoptosis that was induced by radiation (Chen et al., 2020). However, the proposed anti-apoptotic or pro-survival mechanism of TGF-β3 in this model requires further analysis. Lastly, EVs secreted from hypoxic head and neck squamous cell carcinoma (HNSCC) cells carry TGF-β1 as cargo that promotes CAF differentiation in co-culture experiments (**Table 1**); the EVs also carry *miR-192* and *miR-215*, which downregulate the caveolar protein caveolin-1, an established negative regulator of TGF-β signaling (Zhu et al., 2021). In this manner, the EVs generate a positive feedback loop whereby the EV-carried TGF-β is permitted to signal on recipient cells, and thus generate more TGF-β, which enriches the HNSCC with more CAFs. These examples highlight mechanisms whereby CAFs influence carcinoma cell invasiveness but also induction of CAF differentiation by engaging carcinoma cell-derived EVs carrying specific miRNAs that directly or indirectly impact on expression and signaling activation of the TGF-β pathway. As repeatedly noted in this article, such miRNA-based mechanisms should be viewed with appropriate caution.

Regulation of Cancer Immunity by Extracellular Vesicles

The content of EVs secreted by cancer and stromal cells in the TME have been proposed to stimulate or suppress the activity of immune cells, including their progenitor cells. In this context, EVs can directly expose antigens in their surface, which are recognized by major histocompatibility receptors, or indirectly the EVs induce antigen presentation by transferring tumor antigenic peptides to antigen-presenting cells (Robbins and Morelli, 2014; Whiteside, 2016; Seo et al., 2018). Strong evidence supports induction of local immunosuppressive responses by tumor-derived EVs, through the generation of MDSCs (comprising myeloid progenitor cells, immature macrophages, granulocytes, and dendritic cells - DCs), which drive the function of regulatory T (T_{Reg}) cells, inhibiting anti-tumoral responses (Huber et al., 2005; Robbins and Morelli, 2014). Hence, since TGF-β signaling can modulate immunosuppressive activities in several innate and adaptive immune cells (Batlle and Massagué, 2019; Derynck et al., 2021), in this section we discuss how different tumor-derived EVs promote immune evasion through TGF-β signaling.

In light of this, TDEs isolated from murine mammary adenocarcinoma carried functional TGF-β (**Table 1**), which effectively induced the accumulation of MDSCs, a process that could be blocked by pre-incubating the TDEs with an anti-TGF-β antibody (Xiang et al., 2009). Moreover, TGF-β transported by melanoma-derived EVs contributed to the promotion of a suppressive phenotype by antigen-presenting cells (Düchler et al.,

2019), while the EVs released by CRC loaded with TGF-β1 induced phenotypic alteration of T to T_{Reg}-like cells through activating TGF-β/SMAD and inactivating MAPK signaling (Yamada et al., 2016). Additionally, EVs purified from MC38 colon carcinoma cells overexpressing IL-12 and deprived of TGF-β1 by transfecting shRNA molecules targeting *TGFB1*, efficiently inhibited tumor growth and induced anti-tumor immunity, together with DC-based vaccines (Rossowska et al., 2019).

In a different cancer context, EVs isolated from the sera of a small cohort of newly diagnosed acute myeloid leukemia patients carried TGF-β1 (**Table 1**), apart from being positive for expression of classical EV and myeloid blast markers (CD34, CD33, and CD177) (Szczepanski et al., 2011). These circulating patient-derived EVs decreased natural killer group 2D (NKG2D) receptor levels and reduced the cytotoxicity of natural killer (NK) cells. In addition, an anti-TGF-β1 antibody blocked efficiently the EV-mediated suppression of NK cell function and the concomitant NKG2D downregulation (Szczepanski et al., 2011). Similarly, EVs purified from K562 (chronic myelogenous leukemia) cells and IGR-Heu (lung large cell carcinoma) under hypoxic conditions carried TGF-β1 (**Table 1**) and decreased NK cell suppressive activity by reducing NKG2D expression (Berchem et al., 2016). Moreover, EVs derived from *TGFB1*-silenced leukemic cells, promoted DC activation, facilitated $CD4^+$ T-cell proliferation and Th1 cytokine secretion, and further stimulated cytotoxic responses in lymphocytes and NK cells when compared to the control leukemia-EVs (Huang et al., 2017). Thus, by treating mice with leukemia-derived EVs carrying lower TGF-β1 levels prolonged animal survival, suggesting that such EVs were more effective in both protective and therapeutic antitumor tests than non-modified EVs carrying a higher load of TGF-β1 (Huang et al., 2017).

Of note, molecules constitutively present on EV surfaces such as milk fat globule EGF and factor V/VIII domain-containing gene (MFGE8), tetraspanins, and externalized phosphatidylserine (PS) can mediate the interaction of tumor-derived EVs with immune cells (Hao et al., 2007; Robbins and Morelli, 2014; Thery et al., 1999). Tolerogenic EVs isolated from EG7-lymphoblasts undergoing irradiation-induced cell death were enriched in PS and histone H3 and suppressed DC_{OVA}-stimulated $CD8^+$ cytotoxic T lymphocyte (CTL) responses, *via* the induction of $CD8^+$ T-cell anergy and type 1 regulatory $CD4^+$ T-cell responses (Xie et al., 2009). Furthermore, the irradiation-induced apoptosis led to an increase in both TGF-β1 levels on cells and in their secreted EVs due to the activation of nuclear factor of activated T-cells (NF-AT), a transcription factor positively regulating the *TGFB1* promoter (Xie et al., 2009). Thus, an anti-TGF-β1 antibody was able to block the EV-mediated immune suppression through $CD8^+$ CTL responses and anti-tumor immunity *in vivo* (Xie et al., 2009). Furthermore, PS presented on EVs isolated from B16F10 malignant murine melanoma cells, elicited anti-inflammatory responses on macrophages by inducing TGF-β1 secretion and enhancing the metastatic potential of B16F10 cells in C57BL/6 mice, while these effects were abrogated when the PS on EVs was blocked with annexin V (Lima et al., 2009). In addition to inflammatory responses mediated by macrophages, the inhibition of inflammasomes is another mechanism used by tumor cells to escape from the immune system (Ghiringhelli et al., 2009). Based on this, the NLRP3 (nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeat-containing receptors (NLRs) family pyrin domain containing 3) is one of the best-described inflammasome proteins, and EVs isolated from HNSCC patients, which were enriched in TGF-β signaling molecules, were able to inhibit the induction of pro-IL-1β and pro-caspase-1 proteins, in addition to the downregulation of *NLRP3* expression during the priming phase of inflammasome activation (Bottino et al., 2021). Moreover, MG63 osteosarcoma cell-derived EVs induced M2 macrophage differentiation and also enhanced expression of cytokine transcripts, such as *IL10*, *VEGF* and *TGFB1 in vivo* (Cheng et al., 2021). Nonetheless, although this study claimed that T cell immunoglobulin and mucin domain containing-3 (TIM-3) was the mediator of such effect transferred by osteosarcoma-derived EVs (**Table 1**), the mechanisms by which this protein drives immunosuppression and *TGFB1* expression were not fully investigated. In the context of TDEs inducing TGF-β1 expression in T cells, nasopharyngeal cancer (NPC)-derived EVs recruited T_{reg} cells, inducing TGF-β1 release, and converting $CD4^+CD25^-$ T cells to $CD4^+CD25^{high}$ T cells (Mrizak et al., 2015). Moreover, EVs isolated from esophageal cancer (ECA) cells carrying lysosomal associated membrane protein 1 (LAMP1) and MMP-9 induced naive B cells to differentiate into TGF-β-producing regulatory B cells (**Table 1**), which led to immunosuppressive effects on $CD8^+$ T-cells (Li et al., 2015).

Together, all these cases show that EVs derived from different types of tumors are often loaded with TGF-β1, educating the immune system to act in favor of tumorigenesis. Thus, the use of approaches to avoid the delivery of EVs carrying TGF-β1 (e.g. anti-TGF-β1 antibodies or hybrid anti-PD-L1 and TGFβRII biomolecules, such as bintrafusp-α (Gulley et al., 2021)) is a promising tool to avoid the immune evasion promoted by EVs and TGF-β signaling. Nevertheless, it is important to mention that TGF-β signaling is not the only pathway regulated by tumor-derived EVs and acting on immune cells. Hence, to inhibit the immunomodulatory mechanism promoted by EVs, their multi-factorial mode of action remains to be targeted.

Extracellular Vesicles and Their Cargo as Tumor Biomarkers and Drug Resistance

The management and prognosis of different cancer patients have improved over the last decades. Yet, a significant proportion of patients still fail the treatment protocols incorporating radiotherapy, chemotherapy, targeted therapy and immunotherapy. Biomarker-directed therapeutic decisions remain the cornerstone for precision oncology, most widely practiced when protocols of targeted therapy are utilized. The mechanisms driving treatment resistance in cancer cells are multi-factorial. TGF-β has been implicated as a key player for treatment resistance in several tumors, since TGF-β signaling induces EMT, maintains stem-like cell populations in tumors and modulates the TME (Colak and ten Dijke, 2017). Furthermore, EVs can also mediate drug resistance by *1*) transferring functional proteins and possibly even functional RNAs from resistant donor cells to sensitive recipient cells, *2*) sequestering drugs from the target sites, causing reduction to the local cytotoxic concentration (Samuel et al., 2017), and *3*) by carrying membrane proteins that can capture

therapeutic monoclonal antibodies which aimed at blocking target receptors at the tumor cell surface (Whiteside, 2016). Therefore, assuming that the role of TGF-β signaling in controlling the content of EVs as previously suggested (Fricke et al., 2019b) can be strengthened, one can consider that EVs carrying molecules linked to TGF-β signaling can provide new mechanisms to understand not only how resistance to treatment rises in tumors, but also how the resistance can spread through tumor cells that exhibit rather heterogeneous phenotypes.

Thus, one of the first reports connecting TGF-β signaling molecules carried by EVs to resistance to cancer treatment involved HCC (Takahashi et al., 2014). TGF-β significantly induced the expression of several lncRNAs, including the *linc-RoR* (**Table 1**), which was also enriched in HCC-derived EVs (Takahashi et al., 2014). Mechanistically, *linc-RoR* induced chemoresistance to the protein kinase receptor inhibitor sorafenib, due to an increase in the number of $CD133^+$ tumor-initiating cells (Takahashi et al., 2014). However, whether *linc-RoR* could be functionally delivered to the recipient cells by EVs, or whether the chemoresistance phenotype transferred by EVs to HCC cells was due to other EV cargo molecules, affected by the action of *linc-RoR* in the donor cells, remains unresolved. Moreover, PC-associated CAFs release EVs carrying *miR-423-5p*, which targets *gremlin-2* (*GREM2*) (Shan et al., 2020). The downregulation of *GREM2* by *miR-423-5p* increased PC resistance to taxanes (**Table 1**). GREM2 is a known extracellular ligand-binding inhibitor of bone morphogenetic proteins (BMP), and thus, the proposed mechanism by which GREM2 could impact TGF-β signaling awaits further studies. CRC CAF-derived EVs can deliver the *miR-93-5p* to CRC cells (Chen et al., 2020). Mechanistically, *miR-93-5p* targeted *FOXA1* transcripts (**Table 1**), inducing their downregulation, and due to the lack of FOXA1, the *TGFB3* gene promoter is de-repressed (Chen et al., 2020). Hence, by increasing TGF-β3 levels, EVs containing *miR-93-5p* and secreted from CRC CAFs, increased CRC tumor growth and rescued these cells from radiation-induced apoptosis (Chen et al., 2020). HNSCC cell lines that exhibit resistance to the widely used chemotherapeutic cisplatin, released EVs carrying TGF-β3 and were capable of transferring the drug-resistant phenotype to sensitive cells through activation of TGF-β signaling (Rodrigues-Junior et al., 2019). Furthermore, this study also evaluated TGF-β3 levels in specific EV fractions circulating in the plasma of HNSCC patients treated with chemo-radiation (Rodrigues-Junior et al., 2019). In this screen, TGF-β3 was significantly more abundant in the plasma EVs of HNSCC patients that did not respond to chemo-radiation treatment and the high levels of TGF-β3 in plasma EVs was associated with poor progression-free survival, highlighting the relevance of the use of EV-based biomarkers in oncology. Moreover, EVs derived from BRCA cells mediated intercellular transfer of TGF-β1 in addition to inducing EMT and increasing resistance to the cytotoxic drug adriamycin (Tan et al., 2021). Hence, since the EV content can reflect features of the tumor that secretes them, studies on EVs may generate important insights into the tumor milieu, with potential to identify reliable markers not only for prognosis but also for cancer detection and subtype segregation (Rodrigues-Junior et al., 2019; Hoshino et al., 2020).

Anti-cancer immunotherapies aiming at reviving tumor-reactive CTLs are promising in a large group of metastatic patients, but immune evasion can develop. Hence, by understanding the molecular mechanisms of resistance to immunotherapy, better strategies can be adopted to improve the clinical outcome for cancer patients (Sharma et al., 2017). Thus, immunosuppressive molecules, including programmed death-ligand 1 (PD-L1) and TGF-β1 (**Table 1**), which facilitate tumor immune evasion, are carried by tumor-derived EVs (Mathew et al., 2020). Beyond PD-L1 and TGF-β, cancer-derived EVs may act as immune system suppressors based on the mechanism of NKG2D downregulation and subsequent natural killer cell cytotoxicity suppression described above (Szczepanski et al., 2011). Alternatively, cargoes such as miRNAs or enzymatically active arginase-1, can impact on macrophage differentiation or T cell activation respectively (reviewed in (Zhou et al., 2020; Marar et al., 2021)). Of note, when human lung fibroblasts are stimulated with TGF-β1, activate SMAD2/3 and YAP/TAZ signaling, which enhance the deposition of PD-L1 into EVs (Kang et al., 2020). Similarly, TGF-β present in the TME can induce BRCA cells to release EVs loaded with PD-L1, while the blockage of TGF-β signaling by a chemical inhibitor (SB431542) reduced the PD-L1 levels of these EVs (Chatterjee et al., 2021). Furthermore, when PD-L1 is present on the surface of EVs that circulate systemically, this ligand can bind to its receptor PD-1 on effector T cells, eliciting the immune checkpoint response (Chen et al., 2018; Poggio et al., 2019; Kang et al., 2020). Therefore, the levels of PD-L1 on EVs cannot only stratify clinically tumor patients treated with anti-PD-1 antibodies as responders and non-responders, but also represent a new therapeutic target, that may possibly overcome resistance to current anti-PD-1 immunotherapies (Chen et al., 2018; Theodoraki et al., 2018; Poggio et al., 2019). Furthermore, a phase I/II trial for clinical markers in glioma patients receiving anti-tumor vaccines evaluated the mRNA content of circulating EVs in the plasma of patients pre- and post-vaccine treatment (Muller et al., 2015). In this trial, *TGFB1* and *IL-8* mRNA positively correlated with the immunologic responses to glioma antigens in the EVs isolated from the post-vaccine group, suggesting the potential of mRNAs carried by EVs to assess the response of vaccination therapy in glioma patients (Muller et al., 2015). It is worth noting that such studies aimed at biomarker discovery can be valid even in the case of monitoring fragmented RNAs in the EV cargo.

One of the largest screens to identify TDE proteins as new biomarkers for early-stage cancer detection and identification of primary tumor types in patients of many different cancers was recently conducted (Hoshino et al., 2020). The analysis of TDEs in this study identified the ECM or transmembrane proteins thrombospondin 2 (THBS2), versican (VCAN), and tenascin C (TNC) as genes whose expression analysis could distinguish tumors from normal patients with 90% sensitivity and 94% specificity (Hoshino et al., 2020). Both VCAN and TNC are upregulated by TGF-β signaling (Jinnin et al., 2004; Yeung et al., 2013), whereas thrombospondins regulate latent TGF-β activation in the ECM (Munger et al., 1997). Of note, fibronectin, another protein upregulated by TGF-β signaling, was found highly expressed in all (426 human tissue explants, plasma and other bodily fluids) TDEs evaluated in this study (Hoshino et al., 2020). In another screen of

EV proteins aiming at revealing new biomarkers for PC patients, using reverse-phase protein microarrays, a protein signature with prognostic significance was identified, with TGF-β1 being among the proteins presented by EVs with statistical significance in recurrent PC patients (Signore et al., 2021).

Screening for miRNAs circulating in the plasma of pancreatic cancer patients, a signature of six miRNAs that was able to distinguish non-tumor from cancer tissue was reported (Zhou X. et al., 2018). One of the six miRNAs, the *miR-122-5p*, was also enriched in EVs circulating in the plasma of the patients in comparison to non-cancer patients (Zhou X. et al., 2018). Moreover, *in silico* data showed that *miR-122-5p* could have a relevant role in negatively regulating TGF-β signaling by targeting *TGFBR2* transcripts (Ding et al., 2020). A screen for lncRNAs upregulated by TGF-β in pancreatic cancer that could enhance cargo levels loaded into PDAC-derived EVs, identified 21 such lncRNAs including *HULC* (Takahashi et al., 2020). The level of *HULC* in PDAC-derived EVs was further validated by digital PCR as significantly increased in PDAC patients compared to healthy individuals or intraductal papillary mucinous neoplasm patients, suggesting that non-coding RNAs regulated by TGF-β can contribute to the diagnosis for human PDAC. Hence, the evidence suggests that the molecular content of EVs is a new promising tool that can allow oncologists to improve early tumor detection and offer treatment decisions to cancer patients (Rodrigues-Junior et al., 2019; Hoshino et al., 2020; Signore et al., 2021). Furthermore, large-scale production of EVs from healthy cells, such as MSCs, may be an alternative avenue to deliver promising molecules that may facilitate multiple translational approaches, including the fight against resistance to cancer therapy (Witwer et al., 2019).

CONCLUSION

This article aims primarily at providing a comprehensive view of the relationship between EVs and TGF-β in the context of cancer (**Figure 2**). This relationship can be summarized as follows: *1*) TGF-β signaling regulates the enrichment of specific cargo molecules (proteins, RNAs, metabolites) in EVs, thus offering a qualitative input for downstream functions mediated by the EVs. The large number of examples presented in the article fall into this category, and in this manner, TGF-β signaling joins many other growth factor and cytokine pathways that regulate the content of EVs. Yet, the functional significance of RNA cargo molecules must be viewed with caution. *2*) EVs carry as cargo ligands of the TGF-β family or other key components of the TGF-β signaling machinery, possibly including their respective mRNAs, as evidenced in a variety of cancers. Thus, recipient cells in various tissues, including the TME and the pre-metastatic niche respond to TGF-β, usually mediating pro-tumorigenic actions. *3*) EVs carry indirect regulators of TGF-β signaling, possibly including miRNAs that target key signaling components (e.g. the receptors) or lncRNAs that sponge complementary miRNAs and thus relieve TGF-β signaling from negative regulators. These three scenarios provide an elaborate series of mechanisms by which TGF-β as a component of EVs, and EVs, as cell-to-cell communication vehicles, coordinate processes critical for cancer development. Since TGF-β, like many other cytokines, is known to be secreted and deposited in the ECM of tumors, its presence as EV cargo and that of its various regulators leaves the interested investigator with the central question of what might the purpose of using such alternative routes of TGF-β delivery to recipient cells in the TME be. Is TGF-β cargo in EVs biologically different from TGF-β deposited in the ECM? Is the distance between tumor cells that produce TGF-β and tumor or stromal cells that respond to TGF-β a decisive factor that necessitates transport *via* EVs instead of local ECM deposition? These central questions remain open for investigation as, at the same time, the link between TGF-β and EV biology promises important contributions to biomarker and novel treatment development in several, if not all, cancer types.

AUTHOR CONTRIBUTIONS

DR-J, CT, and AM designed and wrote the first draft. All five authors considerably revised and contributed to the generation of the final submitted article.

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REVIEW
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Bone Morphogenetic Protein Signaling in Cancer; Some Topics in the Recent 10 Years

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Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) family, are multifunctional cytokines. BMPs have a broad range of functions, and abnormalities in BMP signaling pathways are involved in cancer progression. BMPs activate the proliferation of certain cancer cells. Malignant phenotypes of cancer cells, such as increased motility, invasiveness, and stemness, are enhanced by BMPs. Simultaneously, BMPs act on various cellular components and regulate angiogenesis in the tumor microenvironment. Thus, BMPs function as pro-tumorigenic factors in various types of cancer. However, similar to TGF-β, which shows both positive and negative effects on tumorigenesis, BMPs also act as tumor suppressors in other types of cancers. In this article, we review important findings published in the recent decade and summarize the pro-oncogenic functions of BMPs and their underlying mechanisms. The current status of BMP-targeted therapies for cancers is also discussed.

Keywords: BMPs, ALKs, Smads, cancer, metastasis, angiogeneis

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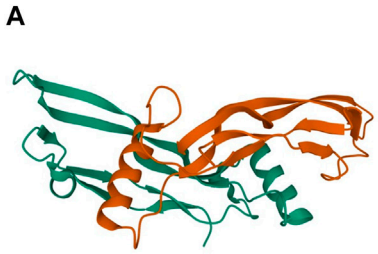
INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally identified as bone- and cartilage-inducing factors in the bone matrix (Wozney et al., 1988; reviewed in; Katagiri & Watabe, 2016). Subsequent studies revealed that BMPs exert a wide range of biological effects. Since then, a variety of BMP roles have been shown in cancer progression. BMPs induce the proliferation of several types of cancer cells, suggesting that BMPs act as pro-tumorigenic factors. BMPs enhance malignant phenotypes of cancer cells, such as cell motility and invasiveness. BMPs also act on various cellular components in the tumor microenvironment, regulating angiogenesis and the immune landscape. In contrast, BMPs serve as tumor suppressors in certain types of cancers. These divergent roles of BMPs have been discussed in many other review articles, including our previous review articles (Ehata, et al., 2013; Davis et al., 2016). However, the precise mechanisms for the pro-oncogenic or tumor-suppressive functions of BMPs remain to be elucidated.

BONE MORPHOGENETIC PROTEIN SIGNALING PATHWAY

BMPs, which are members of the transforming growth factor-β (TGF-β) family, are multifunctional cytokines. More than a dozen BMPs have been identified in vertebrates (**Figures 1A,B**) (Miyazono et al., 2010; Morikawa et al., 2016). Several BMPs are also known as osteogenic proteins (OPs) or growth differentiation factors (GDFs). BMPs bind to two different groups of cognitive kinase



B

Groups	BMP ligands	Type II receptors	Type I receptors
BMP-2/4 group	BMP-2, BMP-4	BMPR-II, ActR-II, ActR-IIB	ALK3(BMPR-IA), ALK6(BMPR-IB)
OP-1 group	BMP-5, BMP-6, BMP-7, BMP-8A, BMP-8B	BMPR-II, ActR-II, ActR-IIB	ALK2(ActR-I), ALK6(BMPR-IB)
BMP-9/10 group	BMP-9, BMP-10	BMPR-II, ActR-II, ActR-IIB	ALK1, ALK2(ActR-I)
GDF-5/6/7 group	GDF-5(BMP-12), GDF-6(BMP-13), GDF-7(BMP14)	BMPR-II, ActR-II, ActR-IIB	ALK6(BMPR-IB)

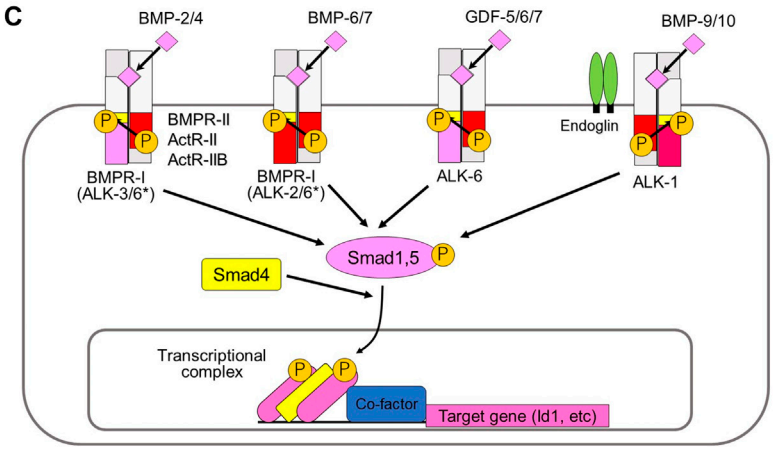


FIGURE 1 | Activation of BMP signaling pathways by various BMP ligands and type II and type I receptors. **(A)** Three-dimensional structure of human BMP-6 homodimer. This figure is created based on the PDB information deposited by Juo and Seeherman (https://www.rcsb.org/structure/6OMO). **(B)** Classification of BMPs based on their binding affinities to receptors. **(C)** BMPR-II, ActR-II, and ActR-IIB are type II receptors, while ALK1, ALK2, ALK3, and ALK6 are type I receptors for BMPs. The binding profiles of BMP-2/4 group, the BMP-5/6/7/8 group, the GDF-5/6/7 group, and the BMP-9/10 group to type I receptors are shown. *BMPs of BMP-2/4 group preferentially bind to ALK3 and ALK6, while those of OP-1 group mainly bind to ALK2 and ALK6. However, BMP-2/4 may transduce signals through ALK2, while OP-1 group ligands may transduce signals through ALK3 under certain conditions. Type II and type I receptors form a heteromeric complex and transduce intracellular signals by phosphorylating Smad1/5, as well as through non-Smad signaling pathways. The phosphorylated Smads form complexes with Smad4 and associate with various transcription factors and transcriptional coregulators in the nucleus, thereby regulating transcription of the target genes, including ID1.

receptors, namely type I and type II TGF-β family receptors, both of which are required for signal transduction. Unlike TGF-β, certain BMPs can bind to type I receptors in the absence of type II receptors. However, the binding affinities of BMPs to type I receptors are facilitated by the presence of type II receptors. Among the five different type II receptors in mammals, BMPs bind to BMP type II receptor (BMPR-II), activin type II receptor (ActR-II), and activin type IIB receptor (ActR-IIB). Among the seven type I receptors, BMPs bind to activin receptor-like kinase (ALK) 1, 2, 3, and 6 (**Figures 1B,C**).

BMPs can be classified into four subgroups according to their structural similarities and ability to bind to type I receptors (Miyazono et al., 2010; Sanchez-Duffhues et al., 2020). The BMP-2/4 group preferentially binds to ALK3 and ALK6, whereas the BMP-5/6/7/8 group mainly binds to ALK2 and ALK6. The GDF-5/6/7 (also known as BMP-12/13/14) group binds to ALK6, but does not bind to other type I receptors. The BMP-9/10 group binds to ALK1 and binds weakly to ALK2. BMP signaling is modified by membrane proteins, as well as by diverse secreted proteins (Brazil et al., 2015; Correns et al., 2021). A co-receptor, endoglin (also known as CD105) upregulates BMP-9/10-ALK1 signaling in endothelial cells and downregulates TGF-β-ALK5 signaling (Dallas et al., 2008). BMP and activin membrane bound inhibitor (BAMBI) acts as a pseudoreceptor and negatively regulates BMP signaling. Structurally diverse secreted proteins, such as noggin, chordin, and gremlin1, bind directly to BMPs and regulate their availability as antagonists (Chang et al., 2016).

Upon binding to type I and type II receptors, BMPs form heterotetrameric receptor complexes (Miyazono et al., 2010). Type II receptor serine/threonine kinases are constitutively active, and they activate type I receptor kinases through phosphorylation of the Gly-Ser-rich (GS) domain of type I receptors. Type I receptors activate receptor-regulated Smads (R-Smads: Smad1 and Smad5). Although Smad8 (also known as Smad9) is structurally similar to Smad1 and Smad5, its function has not been fully elucidated. Recent findings have suggested that Smad8 acts as an antagonist of Smad1/5 (Tsukamoto et al., 2014). Some mutations affect residues in the GS domain or the ATP-binding pocket of the kinase domain of ALK2, leading to increased phosphorylation of R-Smads (Wu et al., 2014; Sanchez-Duffhues et al., 2020). Phosphorylated R-Smads induce heteromeric assembly with the common partner Smad (Co-Smad; Smad4). R-Smad/Co-Smad complexes bind to various transcription factors and transcriptional co-activators [p300,

cAMP response element-binding protein-binding protein (CBP), and general control non-depressible 5 (GCN5)] or co-repressors (c-Ski and SnoN) in the nucleus, leading to regulation of the transcription of target genes, including inhibitor of DNA binding (*ID*)*1*. Inhibitory Smads (I-Smads; Smad6, and Smad7) repress TGF-β family signaling, mainly through interaction with type I receptors (Miyazawa & Miyazono, 2017). While both TGF-β signaling and BMP signaling are inhibited by Smad7, Smad6 preferentially inhibits BMP signaling through ALK3 and ALK6 (Goto et al., 2007). In addition to these Smad-dependent signaling pathways, BMPs activate non-Smad signaling pathways, including the extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (Jnk), p38 mitogen-activated protein (MAP) kinase, phosphoinositide 3 (PI3) kinase-Akt, and small GTPase pathways. Non-Smad signaling pathways cooperate with Smad signaling pathways to regulate various cellular responses.

ALTERED EXPRESSION OF BONE MORPHOGENETIC PROTEINS AND THEIR SIGNALING COMPONENTS IN CANCERS

Altered expression of BMPs or related signaling components has been observed in many cancers. In addition to conventional histopathological analyses using clinical specimens, analyses of public databases have indicated the clinical significance of BMPs in cancer progression. Here, we introduce recent findings for each type of cancer.

Hepatocellular Carcinoma

Numerous reports have suggested that BMP signaling is activated in hepatocellular carcinoma (HCC) tissues. The expression levels of BMP-2/4 and ALK3 were upregulated in tumor tissues. Many of these are correlated with various clinical or biological parameters, such as unfavorable prognosis, tumor grade, TNM stage, vascular invasion, and the expression of the stem cell marker CD133 (Guo D et al., 2012; Zhang et al., 2012; Ma et al., 2017; Feng et al., 2019; Li et al., 2020; Guo et al., 2021). Increased BMP-9 expression is also observed in HCCs and significantly associated with poor outcomes and the T stage of HCC (Herrera et al., 2013; Li et al., 2013; Chen et al., 2021). Of note, high levels of BMP-9 were detected, especially at the tumor borders, in samples from an HCC mouse model (Li et al., 2013). Although conflicting data have also been reported (Wang et al., 2018), the pro-oncogenic function of BMP signaling in HCC has been supported by many reports.

Colorectal Cancer

It is well known that germline mutations in *MADH4* or *BMPR1A* (encoding Smad4 or ALK3, respectively) are present in a large number of patients with juvenile polyposis syndrome (Ehata et al., 2013). Phosphorylation of Smad1/5 has been reported to be absent in most colorectal cancers (CRCs). A recent study estimated that methylation of *BMP2* occurs in 60.2% of sporadic CRCs (Miura et al., 2020). Based on these findings, many reports have indicated that BMPs act as tumor suppressors in CRC. On the contrary, BMP signaling also acts as a pro-tumorigenic factor by promoting tumor invasion, epithelial-mesenchymal transition (EMT), and tumor proliferation (Davis et al., 2016). Fan et al. revealed that BMP-9 expression gradually increased during the transition from normal mucosa to adenoma and subsequent adenocarcinoma in the colon (Fan et al., 2020). Our analysis showed that BMP-4 was highly expressed in CRC tissues compared to normal tissues, and that BMP-4 is involved in CRC progression as an autocrine factor (Yokoyama et al., 2017). One possible explanation for the different roles of BMPs in CRC might be the BMP-induced non-Smad signaling pathway. Based on the expression of the BMP receptor and Smad4, Voorneveld. et al. subdivided CRC cases and found that the expression of normal BMP receptors in Smad4-negative tumors was associated with poor prognosis, suggesting that Smad4-independent BMP signaling may accelerate the progression of CRC (Voorneveld et al., 2014). They also reported that patients with a combination of high BMP-2 expression in the stroma and loss of Smad4 in tumors showed a significantly poorer overall survival (Ouahoud et al., 2020).

Lung Cancer

In non-small cell lung cancers, elevated serum BMP-2 levels are observed in many patients, and they serve as a marker for effective treatment (Choi et al., 2012; Fei et al., 2013). In contrast, Liu et al. revealed that in small cell lung cancers, BMP-7 expression was not detected in cancer tissues, and that BMP-7-positive tumors were correlated to the absence of bone metastasis (Liu et al., 2012).

Breast Cancer

There are still conflicting data regarding the role of BMPs in the progression of breast cancer. The activation of Smad-dependent BMP signaling has been observed in primary and metastatic bone tumors in breast cancer (Katsuno et al., 2008; Owens et al., 2015). BMP-2/5/6/7 are observed in breast cancers and are correlated with the expression of the stem cell marker CD44 (Owens et al., 2015; Huang et al., 2017). The expression of *BMPR1A* (encoding ALK3) is correlated with poor relapse-free survival (Pickup et al., 2015). However, an inverse correlation was observed. BMP-5 expression is decreased in invasive breast cancer, and is associated with cancer recurrence (Romagnoli et al., 2012). The expression of BMP-9 is significantly decreased in the breast cancer tissues, compared with paracancerous tissues (Li et al., 2018). The expression of BMP antagonists, such as noggin or follistatin, is correlated to bone metastasis, but not to metastasis to other organs (Tarragona et al., 2012; Mock et al., 2015).

Renal Cell Carcinoma

Although BMP-9 expression is found over 80% of RCC (Wang et al., 2016), recent studies mainly support the tumor-suppressive role of BMPs in renal cell carcinoma (RCC). BMP-2 is downregulated by the promoter CpG methylation of *BMP2* in RCC cells. The resultant loss of BMP-2 is correlated to poor prognosis in RCC (Mitsui et al., 2015). Consistent with these findings, we observed increased expression of the transcriptional co-repressor c-Ski in cancer cells in RCC tissues (Taguchi et al.,

2019). However, it should be mentioned that c-Ski may accelerate cancer progression mainly through the suppression of TGF-β-dependent Smad signaling, and not through the suppression of BMP-dependent Smad signaling in an experimental setting. Our latest data also indicated that endoglin expression was heterogeneously upregulated in highly malignant derivatives obtained after serial transplantation of human RCC cells (Momoi et al., 2021).

Ovarian Cancer

The expressions of BMPs and their signaling components, BMP-2/7, ALK2/3, BMPR-II, and phosphorylated Smad5, are elevated in ovarian cancers (Hover et al., 2015; Peng et al., 2016; Guan et al., 2019; Fukuda et al., 2020). These findings consistently suggest pro-oncogenic roles of BMPs. Moreover, Fukuda et al. observed high BMP-2 expression after chemotherapy for ovarian cancer (Fukuda et al., 2020). The role of anti-Müllerian hormone (AMH), a member of the TGF-β family, has also been investigated in ovarian cancers. AMH activates the Smad1/5-mediated signaling pathway through the binding to AMH type II receptors (AMHRII) and type I receptors (ALK2/3/6). AMH is produced by granulosa cells in females, and it acts as a key factor in sexual differentiation. Interestingly, AMH has been shown to inhibit the proliferation of ovarian granulosa cell tumor cells (Anttonen et al., 2011) and epithelial ovarian cancer cells (Zhang et al., 2018). Further analyses are needed to elucidate the role of BMP signaling in ovarian cancer.

Endometrial Cancer

High expression of *BMP2* and *BMP7* mRNA is associated with poorer survival in patients with endometrial cancer, albeit not significantly for *BMP2* (Fukuda et al., 2021). In addition, mutations in *ACVR1,* the gene encoding ALK2, are more frequently observed in endometrial cancer (>6%) than in most other cancers. Mutations in *ACVR1* were originally found in patients with fibrodysplasia ossificans progressiva (FOP) (Shore et al., 2006), and have also been found in pediatric brain tumors (see below). The mutant ALK2 in the GS domain (R206H) shows hyperactivation of the kinase domain, and it acquires the ability to bind activins and BMPs (Hatsell et al., 2015). Concerning the expression of BMP-10, a controversial implication has also been reported in endometrial cancer (Hu et al., 2021).

Prostate Cancer

In prostate cancer, decreased BMP-2 expression in cancer tissue is correlated to recurrence and the Gleason score, which represents histological patterns and prognosis (Tae et al., 2018). We have also reported that BMP-7 inhibits the proliferation of prostate cancer cells in cell culture and a xenograft model (Miyazaki et al., 2004). However, BMP-7 expression in metastatic prostate cancer tissues is associated with shorter patient survival, suggesting a context-dependent contribution of BMPs in prostate cancer (Liu et al., 2015).

Squamous Cell Carcinoma in the Esophagus, Head, and Neck

Two independent groups revealed that high BMP-7 was observed in primary esophageal cancer tissues, with a correlation to prognosis and metastasis-related parameters (Megumi et al., 2012; Xu et al., 2013). Interestingly, the level of phosphorylated Smad1/5 is elevated in the tumor tissues of patients with cetuximab-resistant oral squamous cell carcinoma with poor prognosis (Yin et al., 2018).

Nasopharyngeal Carcinoma

High BMP2 expression is significantly associated with clinical stage, distant metastasis, and shorter survival in patients with nasopharyngeal carcinoma (Wang et al., 2017).

Glioma

BMPs suppress the tumorigenic function of human glioma-initiating cells by inducing cell differentiation, cell cycle arrest, and apoptosis (see below). Accordingly, several reports have shown that BMP-4 is expressed in low-grade gliomas, and that it serves as a favorable prognostic marker in gliomas (Bao et al., 2013; Nayak et al., 2020). BMP-4 is overexpressed in gliomas harboring *IDH1* mutations, which are a hallmark of better prognosis (Zhou et al., 2020).

In contrast, in diffuse intrinsic pontine glioma (DIPG), a glial tumor in the brainstem with highly infiltrative properties in children, mutations in *ACVR1* may exhibit pro-oncogenic functions. Four independent research groups reported that *ACVR1* mutations were found in approximately 20–30% of DIPG (Buczkowicz et al., 2014; Fontebasso et al., 2014; Taylor et al., 2014; Wu et al., 2014). Notably, many somatic *ACVR1* mutations in DIPG are identical to germline mutations found in FOP. However, given that FOP is not accompanied by a tumor predisposition, other oncogenic mechanism(s) are required for the pathogenesis of DIPG. The increased activation of *ACVR1* may contribute to the pathogenesis of DIPG in the presence of several histone modifications. Co-expression of *ACVR1* G328 and histone H3.3K27M additively increased the expression of *ID1* and *ID2* (Buczkowicz et al., 2014). Evolutionary analysis showed specific associations between H3K27M and mutations in *TP53*, *PPM1D*, *ACVR1*, and *PIK3R1* (Nikbakht et al., 2016). Hoeman et al. recently demonstrated that *ACVR1* R206H or G328V with H3.1K27M activates signal transducer and activator of transcription 3 (STAT3) signaling (Hoeman et al., 2019). Inhibition of BMP signaling may suppress the progression of *ACVR1* mutation-positive DIPG (Carvalho et al., 2019). However, how BMP signaling affects the progression of *ACVR1* mutation-negative DIPG cells remains unknown. It should also be noted that mutations in the *ACVR1* gene are observed in other types of cancers, including endometrial cancer, although the frequencies of the *ACVR1* mutations are lower than those in DIPG (Fukuda et al., 2021).

EFFECT OF BONE MORPHOGENETIC PROTEINS ON THE PROLIFERATION AND SURVIVAL OF CANCER CELLS

BMPs promote the progression of many types of cancers through the activation of proliferation and survival of cancer cells (Ehata

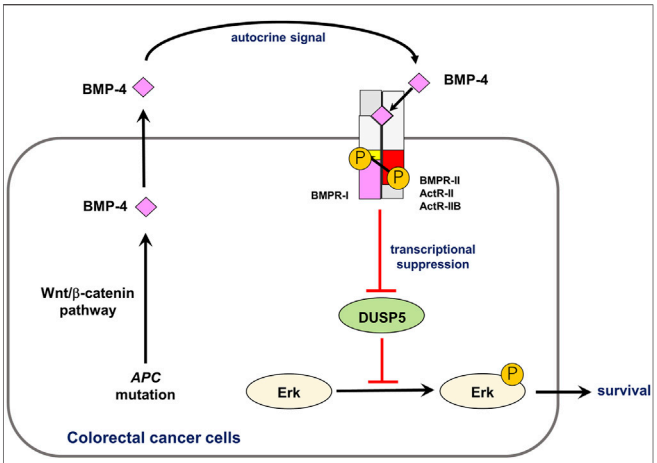


FIGURE 2 | Role of autocrine BMP-4 signaling in CRC. Aberrant activation of the Wnt/β-catenin pathway induces expression of *BMP4* mRNA, activating endogenous BMP signaling. This endogenous signaling promotes phosphorylation of Erk MAP kinase *via* DUSP5 suppression, which results in survival of CRC cells. Modified from Yokoyama et al. (2017).

et al., 2013). The detailed mechanisms of these pro-oncogenic roles of BMPs have recently been uncovered, especially in HCC. BMP-4 upregulates the expression of cyclin-dependent kinase (CDK) 1 and cyclin B1 in HCC cells and accelerates cell cycle progression by activating Erk MAP kinase (Chiu et al., 2012). Ma et al. reported that exogenous BMP-4 increases the expression of cyclin A and CDK2 and promotes the G1-S phase transition in several HCC cells. The BMP-4-mediated cell proliferation was attenuated by silencing ID2 (Ma et al., 2017). The human HCC cell line HepG2 produces BMP-9 in an autocrine fashion, which triggers cell cycle progression and abolishes apoptosis induced by serum starvation. Notably, BMP-9 promotes the growth of HCC cells, but not of immortalized human hepatocytes, suggesting that other oncogenic signaling pathways may modulate the effect of BMP-9 (Herrera et al., 2013). BMP-4 has also been shown to promote the proliferation of HCC cells *via* autophagy activation through Jnk1/Bcl-2 signaling (Deng et al., 2018). In addition to HCC, the pro-survival effect of BMP signaling is indicated in other cancers. We have revealed that aberrant activation of the Wnt/β-catenin pathway induces *BMP4* mRNA expression, activating endogenous BMP signaling in CRC cells (**Figure 2**). This endogenous signaling enhances the phosphorylation of Erk MAP kinase through the downregulation of dual-specificity phosphatase 5 (*DUSP5*), thereby promoting the survival of CRC cells (Yokoyama et al., 2017). Stress-induced phosphoprotein 1 (STIP1) is secreted by ovarian cancer cells. The binding of STIP1 to ALK2 activates the Smad signaling pathway, leading to the transcriptional activation of ID3, promoting cell proliferation (Tsai et al., 2012). Most of *ACVR1* mutations in DIPG cause constitutive activation of ALK2, which increases the expression of the downstream targets *ID1*, *ID2*, and *SNAI1* (encoding Snail), and also increases cell proliferation (Buczkowicz et al., 2014; Fontebasso et al., 2014). Treatment of tumor cells with a small-molecule ALK2 inhibitor, LDN-193189, attenuated their viability (Taylor et al., 2014).

In contrast, BMPs have been shown to negatively regulate cell cycle progression in other types of cancer cells, including gastric and prostate cancer cells (Ehata et al., 2013). We previously found that BMP-4 induces G1 arrest in diffuse-type gastric carcinoma cells *via* induction of p21 through the Smad pathway, inhibiting cell proliferation (Shirai et al., 2011). Similarly, BMP-2 inhibits esophageal cancer cell growth by inducing p21 through the Smad pathway and activating the Hippo signaling pathway (Kim et al., 2016). BMP-7 also inhibits prostate cancer cell proliferation by inducing p21 and suppressing CDK2 activity (Miyazaki et al., 2004). BMP-10 suppresses the proliferation of HCC cells by inhibiting the signal transducer and activator of transcription 3 (STAT3) signaling. Mechanistically, cytoplasmic BMP-10 interacts with both protein tyrosine phosphatase sigma (PTPRS) and STAT3, thus facilitating the dephosphorylation of STAT3 by PTPRS (Yuan et al., 2019). Olsen et al. reported that BMP-9 induces apoptosis in multiple myeloma cells through the ALK2-mediated signaling pathway (Olsen et al., 2014). As different types of cancers cannot account for the diverse effects of BMPs, they are thought to regulate the proliferation or survival of each cancer cell in a context-dependent manner.

EFFECT OF BONE MORPHOGENETIC PROTEINS ON CANCER STEM CELLS

Cancer stem cells (CSCs) or cancer-initiating cells may be responsible for cancer recurrence. As BMPs act as differentiation factors in several organs, the activity of CSCs is diminished by BMPs. BMPs induce differentiation of glioma-initiating cells, leading to cell cycle arrest or apoptosis (Piccirillo et al., 2006; Lee et al., 2008). Various mechanisms have been determined for the BMP-mediated differentiation of glioma-initiating cells. BMPs induce the EMT-associated transcription factor Snail *via* Smad-dependent pathways, which results in the deletion of tumorigenic potential (Savary et al., 2013). As a mechanism, Snail transcriptionally represses *TGFB1* through interaction with Smads, thereby regulating astrocytic differentiation (Caja et al., 2018). Our research group has also shown that distal-less homeobox 2 (DLX2), an essential transcription factor induced by BMPs, is important for neural differentiation and apoptosis of glioma-initiating cells (Raja et al., 2017). In addition to these transcription factors, BMPs affect glioma-initiating cells *via* epigenetic mechanisms. We found that the expression of paired related homeobox 1 (PRRX1) is induced by BMPs in glioma-initiating cells. The longer isoform of PRRX1, pmx-1b, interacts with DNA methyltransferase 3A (DNMT3A) and induces promoter methylation of the *PROM1* gene encoding CD133, thereby attenuating stem cell-like properties (Tanabe et al., 2022) (**Figure 3**). We have also reported that the tyrosine kinase Eph receptor A6 (EPHA6) promotes apoptosis in BMP-2-sensitive glioma-initiating cells (Raja et al., 2019). BMPs thus play tumor-suppressive roles in the progression of glioma, acting on glioma-initiating cells. However, some glioma cells acquire resistance to the action of BMP-Smad signaling pathways, and several extracellular proteins are associated with the sensitivity of some glioma cells to BMPs. Increased expression of the

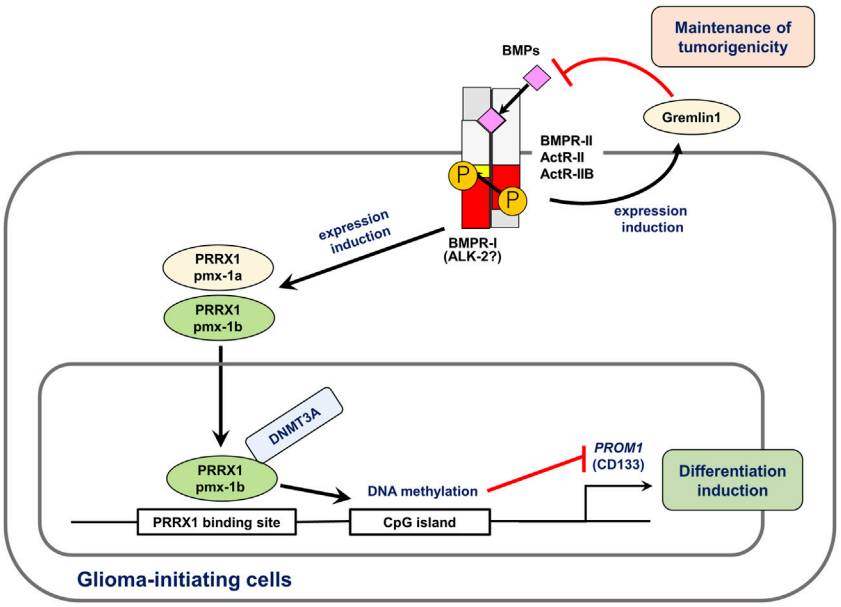


FIGURE 3 | BMPs induce differentiation and apoptosis of glioma-initiating cells. BMPs regulate expression of various target genes in glioma-initiating cells. In this figure, the roles of PRRX1 (Tanabe et al., 2022) and gremlin1 (Yan et al., 2014) are shown. Of the two splice isoforms of PRRX1, only pmx-1b induces differentiation of glioma-initiating cells. Through interaction with DNMT3A, pmx-1b induces the methylation of the *PROM1* gene promoter and suppressed the $CD133^+$ glioma-initiating cell population. BMPs induce the expression of gremlin1, an antagonist of BMP, which is involved in the maintenance of pro-tumorigenic functions and stem cell properties of glioma-initiating cells. Modified from Tanabe et al. (2022).

extracellular antagonists gremlin1 or follistatin-like 1 promotes the maintenance of glioma-initiating cells through the attenuation of BMP signaling (Yan et al., 2014; Jin et al., 2017).

Likewise, the effect of BMPs on the differentiation of CSCs has been investigated in cancers other than glioma. Although TGF-β-induced EMT may render breast cancer cells with stem cell properties, BMPs, particularly heterodimeric BMP-2/7, antagonize TGF-β-induced EMT and reduce the aldehyde dehydrogenase $(ALDH)^h$ $CD44^h$ $CD24^{l-}$ CSC population (Buijs et al., 2012). In normal mammary epithelial cells, BMP-4 acts as a pro-differentiation factor and promotes acinar formation. However, in triple-negative breast cancer (TNBC) cells, TGF-β inhibits the expression of BMP-4 through the Smad pathway and cyclin D1. Thus, TGF-β enhances tumor formation and increases the highly tumorigenic $CD44^{high}$ $CD24^{low}$ CSC population (Yan et al., 2021). Whissel et al. found that *BMP4* transcription is regulated by GATA-binding protein 6 (GATA6), which is related to the self-renewal of adenoma stem cells in the colon (Whissell et al., 2014). In RCC cell lines, BMP-2 downregulates the expression of stem cell markers in $ALDH^+$ cells and potently inhibits their growth (Wang et al., 2015). CSC-like cells in head and neck cancer display decreased levels of phosphorylated Smad1/5 and BMP target gene *ID1*, whereas Smurf1, a negative regulator of BMP signaling, is highly expressed compared to that in non-CSC populations. When BMP signaling pathways are reactivated by Smurf1 knockdown in CSC-like cells, adipogenic differentiation and loss of tumorigenic capacity are observed (Khammanivong et al., 2014). BMP-2 is thought to act as a potent inducer of tumor cell transdifferentiation in osteosarcoma (Geng et al., 2014).

These observations indicate that BMPs promote the differentiation of CSCs and attenuate the tumor-forming capability of several cancer cells. Thus, BMP administration is thought to be suitable for these cancers (Reguera-Nuñez et al., 2014; Nayak et al., 2020). For example, exogenous BMP-4 induces the differentiation of CSCs within HCC through the activation of the Erk1/2 MAP kinase signaling pathway, inhibiting the self-renewal and tumorigenic capacities of $CD133^+$ CSCs. Simultaneously, the expression of one of the ABC transporters, ATP-binding cassette subfamily G member 2 (ABCG2), is decreased in hepatic cancer stem cells after BMP-4 treatment, which enhances sensitivity to chemotherapeutic agents (Zhang et al., 2012). González-Gómez et al. employed BMP-7-loaded microspheres in a xenograft model and demonstrated that controlled release of BMP-7 potently inhibited the growth of glioblastoma and reduced CSC markers, including CD133, Olig2, and glial fibrillary acidic protein (GFAP)δ (González-Gómez et al., 2015).

However, BMPs are also important for the maintenance of CSCs in HCC. Zhang et al. revealed that high-dose exogenous BMP-4 promotes the differentiation of $CD133^+$ CSCs in HCC, whereas low-dose exogenous BMP-4 upregulates CD133 expression, suggesting a concentration-dependent effect of BMPs on CSC maintenance (Zhang et al., 2012). Silencing of BMP-2 in HCC suppresses Erk MAP kinase and inhibits sphere formation and the expression of stemness-related and EMT markers in CSCs from HCC cell lines (Guo et al., 2021).

BMP-9 promotes CSC properties in epithelial cell adhesion molecule (EpCAM)-positive HCC subtypes by enhancing ID1 expression (Chen et al., 2021).

EFFECT OF BONE MORPHOGENETIC PROTEINS ON CANCER CELL MIGRATION, INVASION, AND METASTASIS

Similar to TGF-β, BMPs enhance the migration and invasion of several types of cancer cells, thereby potentiating their metastatic ability. This pro-metastatic function of BMPs has been supported by numerous recent studies (Guo X et al., 2012; Maegdefrau & Bosserhoff, 2012; Xu et al., 2013; Leinhäuser et al., 2015; Liu et al., 2015; Guan et al., 2019; Huang et al., 2020). The BMP-Smad pathway plays a critical role in the induction of EMT in many cancer cells. For example, BMP-4 induces the expression of the EMT-associated transcription factors *SNAI1* and *SNAI2*, which encode Snail and Slug, respectively, in a Smad4-dependent manner in ovarian and pancreatic cancer cells. This regulatory mechanism is critically dependent on matrix rigidity and Yes-associated protein 1 (YAP1) (Serrao et al., 2018). BMP-4 enhances EMT and stem cell properties *via* the Smad-dependent pathway in both mammary epithelial and breast cancer cells, which is accompanied by the activation of Notch signaling (Choi et al., 2019). In HCC cell lines, autocrine BMP-9 signaling induces Snail expression *via* the ALK1- and ALK2-Smad1 pathways (Li et al., 2013).

Non-Smad pathways, including PI3 kinase-Akt, mTOR, and Rock, are involved in BMP-mediated EMT. BMP-2 induces EMT and stemness of breast cancer cells through the Rb and CD44 signaling pathways, which act not only *via* Smad-dependent pathways but also *via* the PI3 kinase-Akt signaling pathway (Huang et al., 2017). The mTORC1 inhibitor rapamycin blocks BMP-2-induced EMT in nasopharyngeal carcinoma cells (Wang et al., 2017). In CRC, Smad4-independent BMP signaling induces EMT and invasion *via* the Rock pathway (Voorneveld et al., 2014). In chondrosarcoma cells, BMP signaling regulates the expression of matrix metalloproteinases (MMPs) and promotes invasiveness *via* non-Smad signaling pathways, including p38 MAP kinase and Akt pathways (Chen et al., 2014; Yahiro et al., 2019).

Despite these findings, exogenous BMP-2 does not affect the metastatic phenotype of osteosarcoma cells (Gill et al., 2017). Moreover, the suppressive effects of BMPs on metastasis have been documented in breast cancer and osteosarcoma (Ren et al., 2014a; Ren et al., 2014b; Xiong et al., 2018). Overexpression of some BMP antagonists, such as gremlin1 or noggin, promotes metastasis of breast cancer cells (Tarragona et al., 2012; Sung et al., 2020). Although the reason for these conflicting effects remains unclear, the experimental methods employed in each report might have affected the function of BMPs.

EFFECT OF BONE MORPHOGENETIC PROTEINS ON TUMOR ANGIOGENESIS

ALK1 is predominantly expressed in proliferating vascular endothelial cells. As BMP-9/10 have been identified as ligands for ALK1, the role of BMP-9/10-ALK1 signaling in vascular angiogenesis has been well established (see **Figure 1**) (Ehata et al., 2013). The BMP-9/10-ALK-1 signaling pathway activates the proliferation of endothelial cells under certain conditions, and plays an important role in the maintenance of vascular homeostasis. We previously showed that BMP-9 induces tumor angiogenesis in a mouse xenograft model of human pancreatic cancer (Suzuki et al., 2010). However, the effect of BMP-9/10-ALK1 signaling on lymphatic endothelial cells may differ from that on vascular endothelial cells. Yoshimatsu et al. revealed that BMP-9 directly downregulates prospero homeobox 1 (Prox1) expression *via* ALK1 in human dermal lymphatic endothelial cells (HDLECs) and reduces their proliferation. BMP-9 was shown to inhibit tumor lymphangiogenesis in a mouse breast cancer allograft model (Yoshimatsu et al., 2013).

In contrast to BMP-9/10-ALK1 signaling, the role of ALK2/3/6-mediated BMP signaling in angiogenesis remains controversial. Elevated expression of BMP-2 is found in HCC and is positively correlated to angiogenesis in tumor tissues. A loss-of-function assay using shRNAs revealed that BMP-2 secreted from HCC cells activates the p38 MAP kinase signaling pathway in endothelial cells, thus enhancing the proliferation, migration, and angiogenic abilities of endothelial cells (Feng et al., 2019). BMP-7 exhibits a pro-angiogenic effect through the expression of repulsive guidance molecule family member B (RGMb), a co-receptor for BMPs, in endothelial cells (Sanders et al., 2014). Taken together, the impairment of tumor angiogenesis may be achieved by blocking BMP signaling pathways other than BMP-9/10-ALK1 (Jia et al., 2016). However, under certain conditions, the direct administration of BMPs to tumor-bearing mice exerts an anti-angiogenic effect. Intraperitoneal treatment with BMP-4 suppressed tumor angiogenesis and reduced tumor formation in xenograft and allograft models of some cancer cells. As its mechanism, BMP-4 reduces vascular endothelial growth factor (VEGF) expression *in vivo* in a thrombospondin 1 (TSP1)-dependent manner (Tsuchida et al., 2014). BMP-7v, a modified BMP-7, is capable of reducing the number of microvessels in CSC-based avatars, resulting in the sensitization of CRC cells to chemotherapeutic reagents (Veschi et al., 2020).

EFFECT OF BONE MORPHOGENETIC PROTEINS ON OTHER CELLULAR COMPONENTS IN THE TUMOR MICROENVIRONMENT

BMPs mediate the interactions between cancer cells and various cellular components in the tumor microenvironment. BMP-4 expression is upregulated in cancer-associated fibroblasts (CAFs) in HCC tissues compared to non-cancerous liver fibroblasts. BMP-4 overexpression in normal fibroblasts activates these cells to a CAF-like phenotype. Simultaneously, BMP-4 enhances the production of interleukin (IL)-6, IL-8, and chemokine (C-C motif) ligand (CCL)2 and promotes cancer cell invasion (Mano et al., 2019). BMP-2 is upregulated in

fibroblasts upon stimulation with conditioned medium from Smad4-deficient CRC cells, which in turn increases the liver metastasis of Smad4-deficient CRC cells, but not that of Smad4-proficient CRC cells. BMP-2 expression in fibroblasts appears to be regulated by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) derived from Smad4-deficient CRC cells. Thus, a reciprocal loop in which TRAIL from Smad4-deficient CRC cells induces BMP-2 in fibroblasts plays a critical role in cancer progression (Ouahoud et al., 2020). BMPs and hedgehogs mediate the interactions between cancer cells and the tumor microenvironment. We previously reported that BMP-4 induces the production of sonic hedgehog in prostate cancer cells, thereby enhancing osteoblastic differentiation of stromal cells and may account for osteoblastic metastasis of prostate cancer (Nishimori et al., 2012). Hedgehogs secreted from ovarian tumor cells induce BMP-4 expression in carcinoma-associated mesenchymal stem cells. BMP-4 reciprocally increases hedgehog expression in ovarian tumor cells, indicating a positive feedback loop. The interruption of this loop with a hedgehog pathway inhibitor or BMP-4-blocking antibody prevents the enrichment of CSCs and reverses chemotherapy resistance (Coffman et al., 2016).

BMPs suppress anti-tumor immunity (reviewed in Sconocchia & Sconocchia, 2021). Recent studies revealed that BMP signaling is shown to be involved in the activation of macrophages or dendritic cells during cancer progression. Bladder cancer cells produce BMP-4, which enhances the macrophage polarization toward anti-inflammatory M2 phenotype (Martinez et al., 2017). BMP-4 derived from acute lymphoblastic leukemia cells promotes the generation of dendritic cells with immunosuppressive features and polarizes macrophages towards a less pro-inflammatory phenotype (Valencia et al., 2019). Ihle et al. utilized a LysMCre-mediated myeloid-specific *Bmpr1a* conditional knockout mouse model along with a syngeneic prostate cancer model and demonstrated the pro-tumorigenic role of ALK3 in myeloid cells. They also confirmed that macrophage polarization is altered by ALK3 inhibition in this setting (Ihle et al., 2020). BMP-7 is upregulated in tumors in a mouse model that does not respond to treatment with immune checkpoint inhibitors (Cortez et al., 2020). Mechanistically, tumor cell-derived BMP-7 downregulates MAP kinase (MAPK) 14, which regulates some cytokines and chemokines, including *IL1A*, *IL1B*, *TNF*, and *CCL5* *via* Smad1 activation in macrophages.

The role of BMPs on the lymphocyte functions has recently been investigated. Kuczma et al. extracted *Bmpr1a* as a gene that regulate the immune suppressive function of regulatory T cells (Treg) (Kuczma et al., 2014). Inactivation of *Bmpr1a* in T cells resulted in impaired generation of Treg, leading to the reduced tumor growth in mice bearing B16 melanoma cells. BMP-7 decreases $CD4^+$ T-cell activation by downregulating interferon γ and IL-2 expression *via* Smad/MAPK14 signaling, resulting in resistance to immunotherapy (Cortez et al., 2020). These findings support the idea that inhibition of BMP signaling may be beneficial for cancer treatment.

However, other reports have shown that BMPs suppress the function of tumor-promoting immune cells (Sconocchia & Sconocchia, 2021). BMP-4 reduces the secretion of granulocyte colony-stimulating factor (G-CSF) from mammary tumors, which is likely a critical factor for the expansion of myeloid-derived suppressor cells (MDSCs) and progression of metastasis (Cao et al., 2014). Although the anti-inflammatory effects of TGF-β are well established, the precise role of BMP signaling in the immune system remains fully uncovered.

APPLICATIONS OF SMALL-MOLECULE BONE MORPHOGENETIC PROTEIN INHIBITORS IN CANCER TREATMENT

Many researchers have attempted to use BMP type I receptor inhibitors to treat various types of cancers. As mutations in the *ACVR1* gene are responsible for the pathogenesis of FOP, and such mutations are also found in patients with DIPG, the development of ALK2-specific inhibitors is expected. The current computational approach reveals small changes in the binding site residue type or side-chain orientation in ALKs, as well as subtle structural modifications of the inhibitors, which can be used to improve the specificity of BMP inhibitors (Alsamarah et al., 2015). Dorsomorphin is a prototype BMP type I receptor inhibitor containing a pyrazolo [1,5-a]-pyrimidine scaffold. Subsequently, more ALK2-selective and metabolically stable inhibitors, LDN-193189 and LDN-212854, were developed (Rooney & Jones, 2021). In addition to pyrazolo [1,5-a]-pyrimidine, other scaffolds have also been identified, such as pyridines, quinazolinones, and pyrazoles. M4K2163, a pyridine-based compound, has been shown to have good permeability in the brain (Rooney & Jones, 2021). Our group recently developed the pyrazole-based compounds RK-59638 and RK-71807 (Sato et al., 2020).

These bioavailable inhibitors have allowed examination of their beneficial effects in various types of mouse tumor models. The therapeutic effects of these inhibitors have been documented for DIPG. Treatment of mice bearing *ACVR1* R206H mutation-harboring DIPG cells with LDN-193189 or LDN-214117 extended the survival of host mice compared with vehicle controls (Carvalho et al., 2019). Many reports have also shown that ALK2 inhibitors potently inhibit the formation and progression of other cancers. In breast cancer, treatment of a mouse cancer model with LDN-193189 suppressed tumor-initiating capacity and EMT induction, as well as prolonged tumor latency (Balboni et al., 2013). When MMTV.PyVmT-expressing mice were treated with an osmotic pump containing the BMP type I receptor inhibitor, DMH1, the tumors were less proliferative and more apoptotic, reducing lung metastasis. Simultaneously, DMH1 affects fibroblasts, lymphatic vessels, and macrophages, reducing their tumor-promoting effects (Owens et al., 2015). Our group revealed that intraperitoneal administration of LDN-193189 reduced the Erk MAP kinase signaling pathway in CRC cells, attenuating primary tumor formation in mice-bearing CRC cells (Yokoyama et al., 2017). In HCC, treatment with LDN-212854 repressed ID1 and EpCAM expression in cancer cells *in vivo*, suggesting that the repression of the BMP-9-induced CSC phenotype was attenuated

by the inhibitor (Chen et al., 2021). In brain tumors, ovarian cancer, lung cancer, oral squamous cell carcinoma, endometrial cancer, and melanoma, the therapeutic benefits of ALK2 inhibitors are suggested (Langenfeld et al., 2013; Hao et al., 2014; Hover et al., 2015; Peng et al., 2016; Newman et al., 2018; Yin et al., 2018; Mihajlović et al., 2019; Zhou et al., 2020; Fukuda et al., 2021; Kalal et al., 2021). Our research group recently reported that a BMP type I receptor inhibitor, RK-783, inhibited the growth of ovarian cancer cells *in vivo* (Fukuda et al., 2020).

As the survival of cancer cells is augmented by BMP signaling, BMPs determine the sensitivity to chemotherapeutic drugs (Camara-Clayette et al., 2013; Bach et al., 2018). ALK2 inhibitors are expected to be used in combination with cytotoxic agents to treat cancers. DMH1 enhances the sensitivity of ovarian cancer cells to cisplatin treatment (Hover et al., 2015). DMH1 reduces the growth of cetuximab-resistant oral squamous cell carcinoma (Yin et al., 2018). LDN-193189 augmented the growth-inhibitory effects of carboplatin (Fukuda et al., 2021).

However, these inhibitors are not entirely specific to ALK2. Among the pyrazolo [1,5-a]-pyrimidine-containing inhibitors, dorsomorphin typically induces autophagy in ovarian cancer cells, whereas LDN-193189 induces ROS-mediated apoptosis in the same cell lines. As this discrepancy is thought to be caused by the off-target effects of each inhibitor, the development of more specific ALK2 inhibitors is required (Ali et al., 2015). In addition, as mentioned above, because BMPs have tumor-suppressive functions under certain conditions, tumor formation or metastasis might be promoted through the inhibition of BMP signaling (Vollaire et al., 2019; Sharma et al., 2021). We should clarify which types of cancer ALK2 inhibitors are effective against.

TARGETING ANGIOGENESIS *VIA* INHIBITION OF ALK1 SIGNALING

To suppress tumor angiogenesis, various strategies have been utilized to inhibit BMP-9/10-ALK1 signaling, such as soluble form receptors, neutralizing antibodies, and small-molecule receptor inhibitors. Dalantercept (ACE-041), a soluble form of ALK1, acts as a ligand trap for BMP-9/10, inhibiting the interactions between BMP-9/10 and ALK1. The therapeutic potential of dalantercept has been demonstrated in various mouse tumor models (Mitchell et al., 2010; Cunha et al., 2015; Hawinkel et al., 2016). Notably, dalantercept inhibits tumor angiogenesis by regulating signaling pathways other than VEGF signaling. Thus, dalantercept is considered a promising inhibitor for treating RCC, in which escape from VEGF-mediated tumor angiogenesis is critical (Philips & Atkins, 2014; Wang et al., 2016). Initial studies have shown that dalantercept is well tolerated in humans. However, clinical trials with patients with RCC have failed to show therapeutic benefits (Bendell et al., 2014; Voss et al., 2017; Voss et al., 2019). Clinical studies on patients with other cancers have revealed that dalantercept has insufficient efficacy in HCC, ovarian cancer, endometrial cancer, and head and neck cancer (Makker et al., 2015; Jimeno et al., 2016; Burger et al., 2018; Abou-Alfa et al., 2019). PF-03446962 is a fully humanized monoclonal antibody that targets and neutralizes human ALK1 (van Meeteren et al., 2012). Its pharmacokinetics and therapeutic benefits were evaluated in a preclinical model, followed by evaluations in humans (Hu-Lowe et al., 2011; Luu et al., 2012; Li et al., 2014). Although the results of a phase I study of PF-0334962 in patients with cancer initially supported further evaluation (Doi et al., 2016; Goff et al., 2016; Simonelli et al., 2016), phase II studies have demonstrated insufficient efficacy or unacceptable toxicity in the treatment of CRC, urothelial cancer, and mesothelioma (Necchi et al., 2014; Wheatley et al., 2016; Clarke et al., 2019). The small-molecule kinase inhibitor K02288 inhibits BMP-9-induced phosphorylation of Smad1/5 in human umbilical vein endothelial cells to reduce both Smad- and Notch-dependent transcriptional responses. K02288 caused dysfunctional vessel formation in a chick chorioallantoic membrane angiogenesis assay (Kerr et al., 2015). It is currently unknown why the inhibition of ALK-1 signaling failed to show clinical effects, and further studies on the *in vivo* effects of ALK-1 inhibitors on tumor angiogenesis are needed. Additional information will be forthcoming.

CONCLUSION

In the present article, the diverse effects of BMPs are reviewed based on the latest literatures. As BMPs are considered potentially important therapeutic targets in some cancers, treatments with BMP inhibitors have been attempted. However, favorable results have not always been obtained. BMPs act in a context-dependent manner and become tumor suppressors under certain conditions. It is difficult to understand the fact that conflicting data have been obtained for the same cancers, even in the analyses using same cancer cell lines. The key factor(s) that switch the pro-oncogenic or tumor-suppressive functions of BMPs remains unknown. Different BMPs may act differently on each receptor. In addition, BMPs may act not only on many cancer cells but also on cellular components in the tumor microenvironment, leading to different results in different experimental settings. Thus, discovery of biomarkers which can discriminate the responses of certain cancers to BMPs is required.

Finally, the amounts of different BMPs biologically available in the cancer microenvironment should be considered. In the past, to observe cellular responses, many cancer cells were stimulated with ligands or overexpressed with BMPs. Although these evaluations have greatly contributed to our understanding of the diverse effects of BMPs, they do not always reproduce the roles of BMPs under physiological conditions. Notably, some biological effects of BMPs occur in a concentration-dependent or biphasic manner. In addition, crosstalk between BMPs and other signaling pathways may be important. In particular, crosstalk between BMP signaling and TGF-β signaling, which is a strong determinant of the metastatic phenotype of cancer cells, may be important. BMPs often exert their biological functions by enhancing or counteracting the effects of TGF-β. Therefore, evaluating not only the intensity of BMP signaling but also the intensity of TGF-β signaling may be needed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Analysis of the Effect of SNAI Family in Breast Cancer and Immune Cell

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SNAI family members are transcriptional repressors that induce epithelial-mesenchymal transition during biological development. SNAIs both have tumor-promoting and tumor-inhibiting effect. There are key regulatory effects on tumor onset and development, and patient prognosis in infiltrations of immune cell and tumor microenvironmental changes. However, the relationships between SNAIs and immune cell infiltration remain unclear. We comprehensively analyzed the roles of SNAIs in cancer. We used Oncomine and TCGA data to analyze pan-cancer *SNAI* transcript levels. By analyzing UALCAN data, we found correlations between *SNAI* transcript levels and breast cancer patient characteristics. Kaplan–Meier plotter analysis revealed that SNAI1 and SNAI2 have a bad prognosis, whereas SNAI3 is the opposite. Analysis using the cBio Cancer Genomics Portal revealed alterations in SNAIs in breast cancer subtypes. Gene Ontology analysis and gene set enrichment analysis were used to analyze differentially expressed genes related to SNAI proteins in breast cancer. We used TIMER to analyze the effects of *SNAI* transcript levels, mutations, methylation levels, and gene copy number in the infiltration of immune cell. Further, we found the relationships between immune cell infiltration, SNAI expression levels, and patient outcomes. To explore how SNAI proteins affect immune cell, we further studied the correlations between immunomodulator expression, chemokine expression, and SNAI expression. The results showed that SNAI protein levels were correlated with the expression of several immunomodulators and chemokines. Through analysis of PharmacoDB data, we identified antitumor drugs related to SNAI family members and analyzed their IC50 effects on various breast cancer cell lines. In summary, our study revealed that SNAI family members regulate different immune cells infiltrations by gene copy number, mutation, methylation, and expression level. SNAI3 and SNIA1/2 have opposite regulatory effects. They all play a key role in tumor development and immune cell infiltration, and can provide a potential target for drug therapy.

Keywords: SNAI, breast cancer, immune cell, methylation, immunomodulator, drug

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INTRODUCTION

Breast cancer is the most common tumor and has a high fatality rate worldwide (Siegel et al., 2021). The new cases of breast cancer worldwide surpassed that of lung cancer, and breast cancer became the cancer with the highest number of diagnoses in 2020. In the past year, there were approximately 19.3 million new cancer cases globally. Among these, there were approximately 2.3 million newly female breast cancer cases, accounting for 11.7% (Sung et al., 2021). The onset of breast cancer is

related to numerous factors. Through whole-genome sequencing of breast cancer patients, numerous breast cancer-related genes, including genes of the *SNAI* family, have been identified.

The SNAIL family of zinc finger transcription factors consists of three members in vertebrates: SNAIL1 (encoded by *SNAI1*), SNAIL2 (encoded by *SNAI2*, also named SLUG), and SNAIL3 (encoded by *SNAI3*, also named SMUC) (de Herreros et al., 2010). All three SNAI proteins are expressed in the mammary glands and function as transcriptional repressors in physiological and pathological states (Peinado et al., 2007). SNAI1 is involved in the induction of epithelial-to-mesenchymal transition (EMT) and embryonic mesoderm formation and maintenance (Puisieux et al., 2014; Baulida et al., 2019). Mechanistically, it transcriptionally represses CDH1, which leads to loss of E-cadherin expression, EMT induction, and tumor cell invasion (Cano et al., 2000). EMT is important in malignant transformation and tumor progression (Larue and Bellacosa, 2005). SNAI2 is also involved in EMT induction (Bolos et al., 2003). It plays an essential role in TWIST-induced EMT by repressing BRCA2 expression and E-cadherin/CDH1 gene transcription (Tripathi et al., 2005; Caramel et al., 2013). SNAI1/2 are related to the malignant biological properties of tumor cells. Clinical data indicate that these two proteins are positively correlated with a high rate of metastasis and poor prognosis in various malignant tumors (Shin et al., 2012). Compared to SNAI1 and SNAI2, SNAI3 is less well studied. The function of SNAI3 in tumor progression is unknown, but it has been reported that it may be related to survival (Madden et al., 2014). SNAI3 has five zinc finger domains and is structurally similar to SNAI1 and SNAI2.

Several studies have demonstrated that SNAI family members are associated with patient prognosis in various tumor malignancies. We used different databases to comprehensively analyze the correlation between SNAIs and breast cancer to identify its complexity. In this study, we analyzed *SNAI* transcript levels, promoter methylation levels, and gene alterations in breast cancer, and their relationships with immune cell infiltration and patient prognosis. In addition, we analyzed the tumor killing effects of small-molecule drugs related to SNAI proteins. Our research shows that the SNAI family members play important roles in breast cancer and immune cell infiltration, and may provide some help for the early diagnosis of breast cancer and the development of new drugs.

METHODS

Pan-Cancer Analysis

By using Oncomine database to compare the expression levels of SNAI members among various cancer types. The *p*-value was set at 1E-4, and the fold change was set at 2.

Interactive Gene Expression Profiling Analysis

Using the Gene Expression Profiling Interactive Analysis (GEPIA) tool to profile and compare SNAI expression in various breast cancer stages, which was developed based on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases (Tang et al., 2017).

UALCAN Database Analysis

Using UALCAN portal (Chandrashekar et al., 2017) to analyze the clinical characteristics of breast cancer patients correlate with SNAI expression profiles and SNAI promoter methylation status.

Kaplan–Meier Plotter Analysis

The relationships between SNAI expression levels and the prognosis of breast cancer patients, was analyzed using the Kaplan–Meier plotter (Gyorffy et al., 2010). In total, 2032 patients were divided in two groups according to the median SNAI expression level. The two groups were compared for overall survival (OS), distant metastasis-free survival (DMFS), relapse-free survival (RFS), and progression-free survival (PFS).

cBio Cancer Genomics Portal (cBioPortal) Analysis

SNAI alterations in breast cancer subtypes were analyzed using the breast invasive carcinoma dataset (TCGA, PanCancer Atlas) (Cerami et al., 2012), which includes data from 1,084 samples.

LinkedOmics Database Analysis

LinkedOmics is available portal containing data on 32 cancer types and 10 clinical proteomics tumor analysis consortium (CPTIC) cancer cohort (Vasaikar et al., 2018). It was used to analysis Kyoto Encyclopedia Genes and Genomes (KEGG) pathway and Gene Ontology (GO) according to biological process, cellular component and molecular function.

TIMER Analysis

Using the TIMER web server (Li et al., 2017) analyze different immune cell infiltrations, including B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells, in breast cancer. The correlations between gene expression, mutation, copy number alterations and immune cell infiltrations were analyzed.

Gene Set Cancer Analysis

Using the Gene Set Cancer Analysis database (Liu et al., 2018) analyze the relationships between *SNAI* methylation levels and immune cell infiltrations in breast cancer.

PharmacoDB Analysis

The PharmacoDB database was used to analyze the IC50 effects of different drugs on various cancer cells. The cutoffs used were an absolute value of correlation >0.1 and $p < 0.05$.

TCGA Pan-Cancer Data Analysis

The Xena browser was used to analyze RNA-seq data, clinical information, and stemness scores based on mRNA and immune subtype data for various cancer types. These samples were used to compare gene expression profiles and any-omics data within a gene or transcript between tumors and normal tissues.

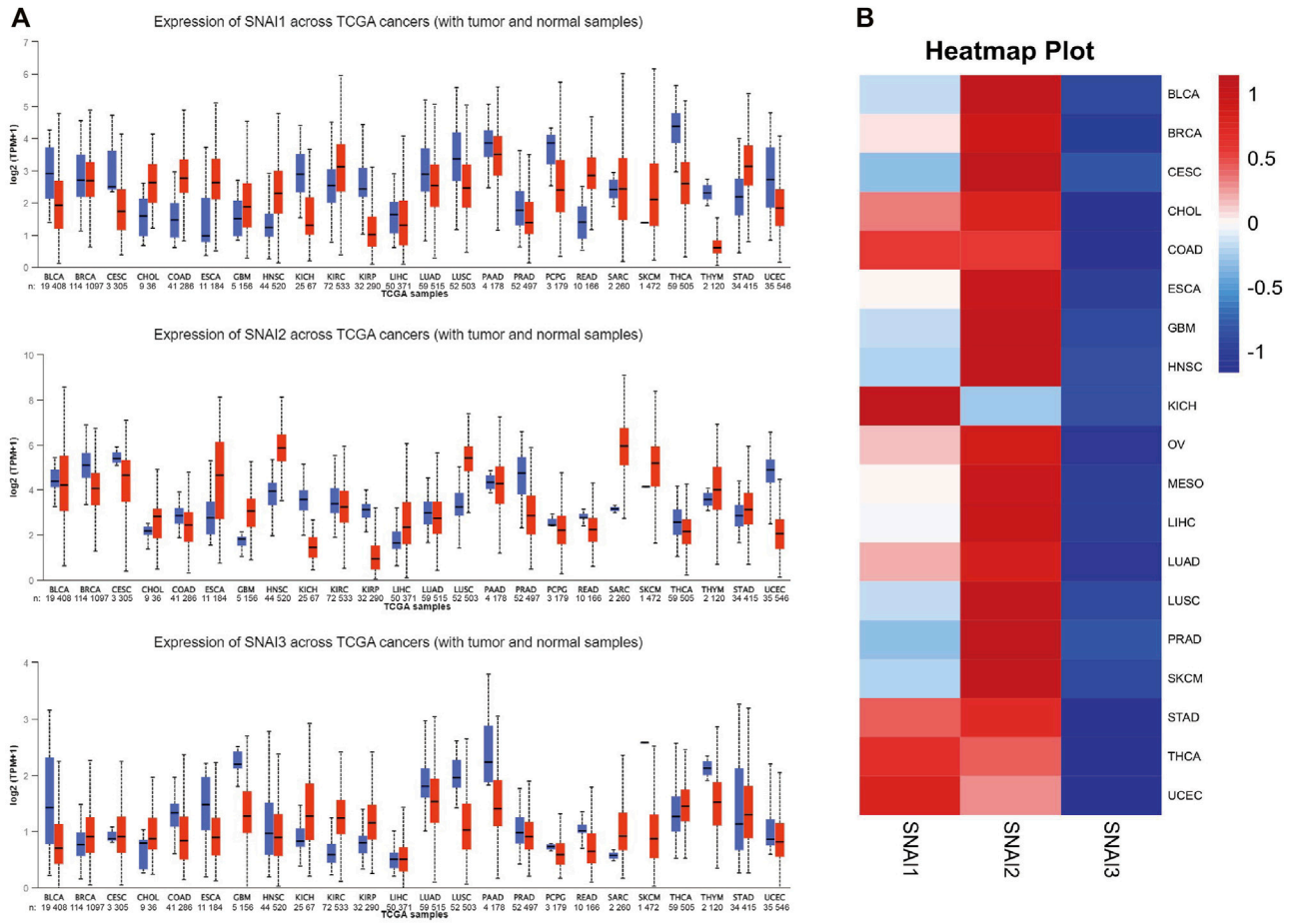

FIGURE 1 | Transcript levels of SNAI zinc finger genes in various types of cancers **(A)** Transcript levels of *SNAI1*, *SNAI2*, and *SNAI3* in tumor tissues compared with normal tissues for various cancers **(B)** Comparison of transcript levels of *SNAI1*, *SNAI2*, and *SNAI3* among various cancer types based on The Cancer Genome Atlas data.

TISIDB Analysis

TISIDB is used for interactions between tumor and immune system that integrates multiple heterogeneous data types (Ru et al., 2019). The database was used to analyze correlations between SNAI expression levels and immune cell infiltration as well as immunomodulator and chemokine levels in breast cancer.

RESULTS

Pan-Cancer Analysis of SNAI Expression

To study the roles of SNAIs expression levels in tumors, we retrieved clinical data and RNA-seq data of 33 tumors and non-cancer tissues. The data showed that SNAI expression differs among different types of cancer (**Figure 1A**). Transcript levels of SNAI1 and SNAI2 were increased in most types of tumors when compared with non-cancer tissues. SNAI3 expression was decreased in various tumor types. In breast cancer, SNAI1 and SNAI2 expression was high, whereas SNAI3 expression was low (**Figure 1B**).

SNAI Transcript Levels in Breast Cancer

We used UALCAN to study the SNAIs expression in breast cancer cells. *SNAI1* expression level was no significantly different in primary cancer cells and non-cancer tissues (**Figure 2A**). However, *SNAI2* and *SNAI3* expression were downregulated and upregulated, respectively, in primary cancer cells (**Figures 2B,C**). These results were not consistent with previous findings. Due to the use of different algorithms, the resulting results would also be different direction. These results may indicate that SNAI proteins are more relevant in the breast cancer metastasis. Further, we found the SNAI proteins expression levels did not significantly differ according to the stage of breast cancer (**Figures 2D–F**).

The relationships between SNAI expression levels and breast cancer patient characteristics were evaluated using TCGA data. We discovered the expression level of SNAI1 was lower in men than in women and tended to be higher in Asians than in Americans and Africans (**Supplementary Figures S1A, B**). We found no obvious difference in SNAI1 levels between histological types (**Supplementary Figure S1C**). *SNAI1* transcript levels and *TP53* mutation status correlation found through study; patients

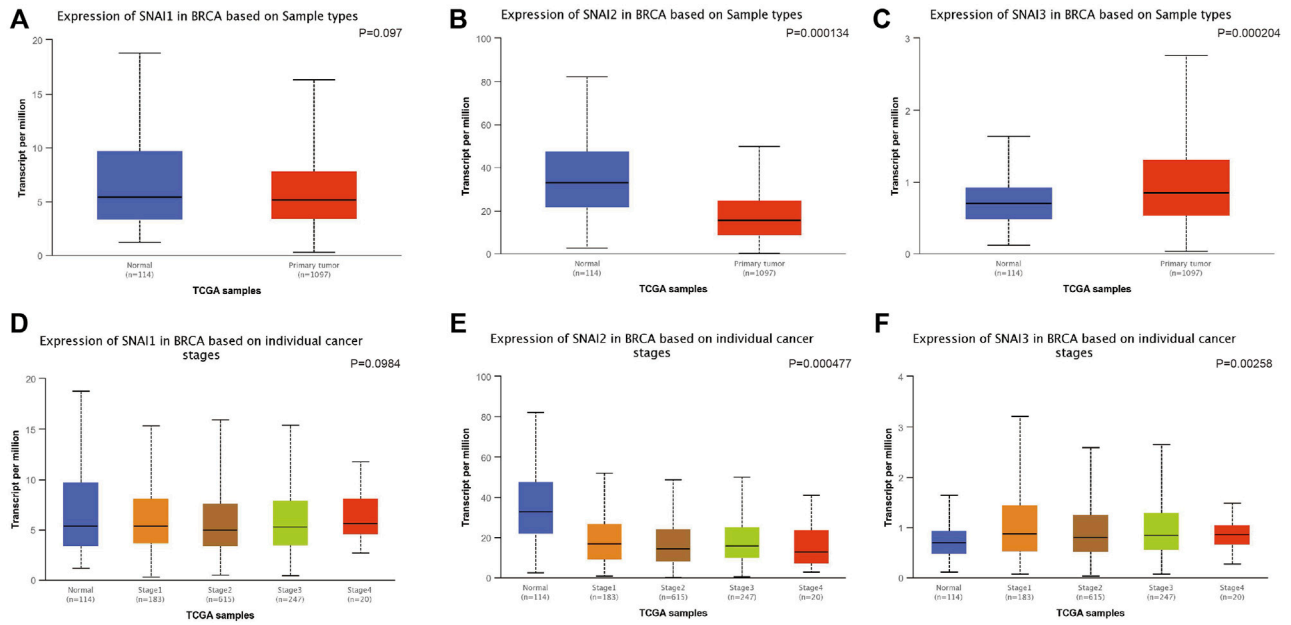

FIGURE 2 | Correlations between SNAI expression and breast cancer. Transcript levels of *SNAI1* **(A)**, *SNAI2* **(B)**, and *SNAI3* **(C)** in breast cancer tissues compared with normal tissues. Transcript levels of *SNAI1* **(D)**, *SNAI2* **(E)**, and *SNAI3* **(F)** in various stages of breast cancer.

with *TP53* mutation have higher *SNAI1* transcript levels than healthy people (**Supplementary Figure S1D**). At the same time, we also further analyzed the expression levels of SNAI2 and SNAI3 in breast cancer. These results indicated that SNAI transcript levels are related to the *TP53* mutation in patients with breast cancer.

Survival Analysis

Using Kaplan–Meyer plotter analysis analyzed the relationships between SNAI expression levels and patient prognosis. Results showed higher SNAI1 expression levels in patients had shorter OS, DMFS, and RFS. Higher SNAI2 expression had shorter DMFS and RFS, whereas higher SNAI3 expression had longer OS and RFS (**Figure 3**). These results indicated that SNAI1 and SNAI2 are associated with a poor prognosis and may play key effects in the DMFS and RFS, whereas SNAI3 seems to have an opposite effect and is related with a good prognosis.

SNAI Alterations in Various Cancers

We used cBioPortal to analyze SNAI alterations, including fusions, amplifications, mutations, structural variants, deep deletions, and multiple alterations, in various cancer subtypes. SNAI1 and SNAI2 showed similar alteration tendencies in different subtypes, whereas SNAI3 showed different. The highest frequencies of SNAI alteration types among tumors were colorectal adenocarcinoma, invasive breast carcinoma, endometrial carcinoma, and epithelial ovarian carcinoma (**Supplementary Figure S2A**). For SNAI1, amplification ranked first among all alteration types in colorectal adenocarcinoma (6.73%), esophageal adenocarcinoma (4.4%), and invasive breast carcinoma (3.41%). For SNAI2, amplification was the most frequent alteration, with 4.43% on breast invasive carcinoma, and of 3.06% in endometrial carcinoma. In contrast, for SNAI3, deep deletion was the most frequent alteration, with a percentage of 2.4% in epithelial ovarian carcinoma and of 2.49% in invasive breast carcinoma. SNAI2 showed the highest number of alterations among the three SNAIs (**Supplementary Figure S2B**). When we analyzed the mutation sites in the SNAI genes, we found that the major mutation sites are located near the C2H2 domain (**Supplementary Figure S3A**). When we simulated the mutation sites of 3D protein structure, we found that the major mutations have quite a large impact on the protein structure (**Supplementary Figure S3B**).

Analysis of DEGs Correlated With *SNAI* Genes in Breast Cancer

LinkedOmics was used to analyze DEGs related with *SNAI1* in breast cancer (**Figure 4A**). We showed the 50 genes that most positively and negatively related to *SNAI1* in the form of heatmaps (**Figures 4B,C**). Significant DEGs were subjected to KEGG pathway enrichment analysis by GSEA (**Figure 4D**). We found that the TNF signaling pathway was significantly upregulated, whereas nitrogen metabolism, propanoate metabolism, and aminoacyl-tRNA biosynthesis were significantly downregulated in breast tumors. We next classified the DEGs using GO analysis (**Figure 4E**). The three most enriched biological process terms were biological regulation, metabolic process, and response to stimulus. The three most enriched cellular component terms were membrane, nucleus, and cytosol. The most enriched molecular function terms was protein binding. Classification and enrichment for DEGs correlated with

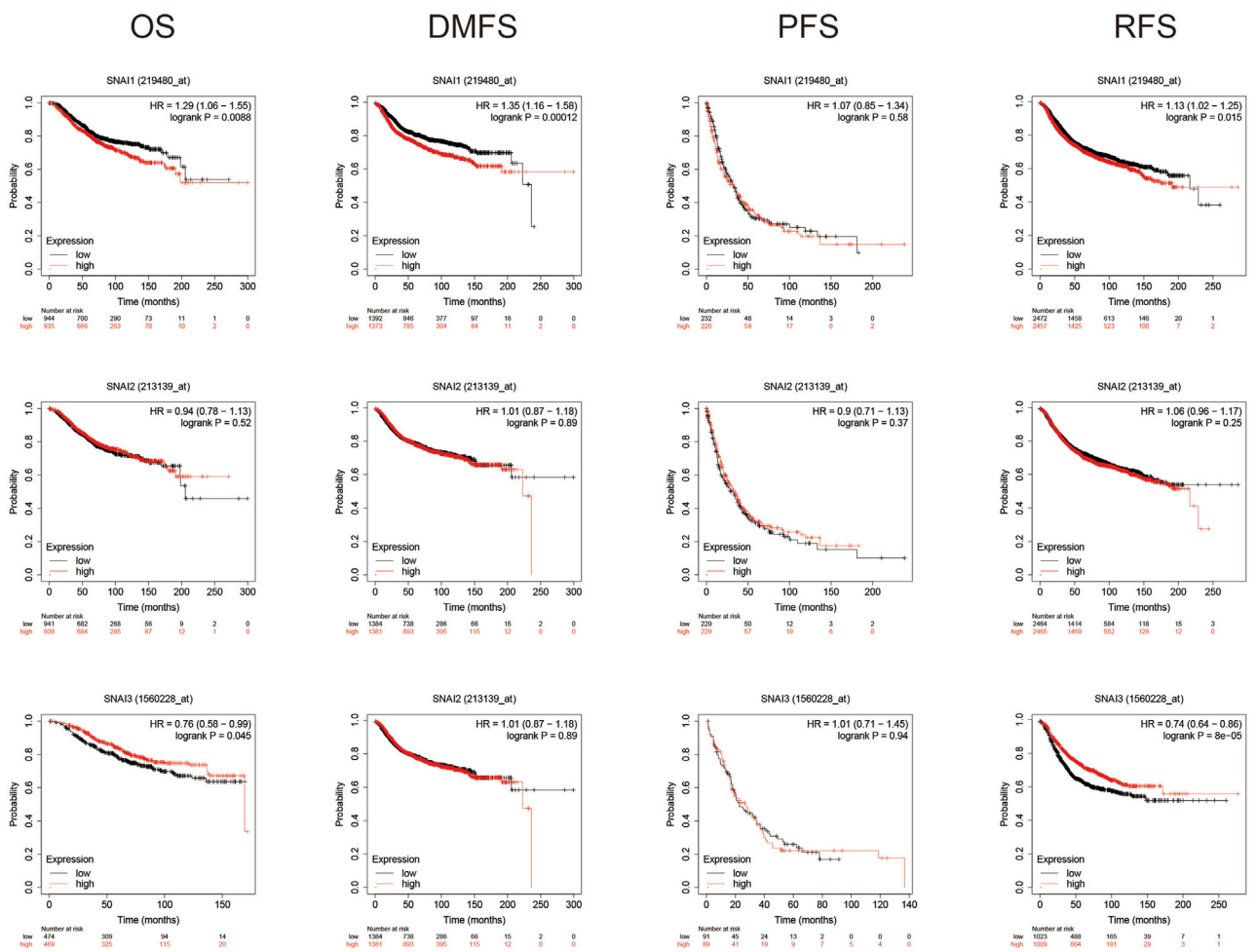

FIGURE 3 | Prognostic value of *SNAI* transcript levels in breast cancer patients. Breast cancer patients were classified into two groups based on the median expression levels of *SNAI1*, *SNAI2*, or *SNAI3*. Overall survival (OS), distant metastasis-free survival (DMFS), progression-free survival (PFS), and relapse-free survival (RFS) were compared between patients with high SNAI expression and those with low SNAI expression.

SNAI2 and SNAI3 are shown in **Supplementary Figures S4, S5**. GSEA showed that genes correlated with autophagy, JAK-STAT signaling, TNF signaling, and VEGF signaling were significantly enriched in SNAI1-high patients. In SNAI2-high patients, genes correlated with autophagy, hippo signaling, EGFR tyrosine kinase inhibitor resistance, and TGF-β signaling were significantly enriched. In SNAI3-high patients, genes correlated with natural killer cell-mediated cytotoxicity, NF-κB signaling, T cell receptor signaling, and chemokine signaling were significantly enriched. These results show that the SNAI family is related to variety of signaling pathways, and may change the fate of cells by regulating different signal pathways.

SNAI Promoter Methylation in Breast Cancer

Gene promoter methylation plays an important role in the regulation of gene expression. We conducted UALCAN analysis to study SNAI1 promoter methylation and their correlation with patient characteristics. In all clinical stages of breast cancer, we found the level of SNAI1 promoter methylation was lower in tumor tissues than in non-cancer tissues, and similar findings were made for subclass, TP53 mutation, and histological type (**Supplementary Figure S6**). MEXPRESS data was used to analyze the SNAI promoter methylation levels in breast cancer, and got similar results. These results indicated that methylation levels of the SNAI promoters may account for the different effects of SNAI proteins on outcomes.

Correlation Between SNAI Expression Levels and Immune Infiltrate Abundance in Breast Cancer

SNAI1 proteins regulate the immune cells infiltration; however, the relationships between SNAI expression levels and immune cell infiltration in breast cancer are unclear. TIMER data was used to study the correlations between SNAI expression levels and the infiltration of six types of immune cells (i.e., B cells, $CD8^+$ T cells,

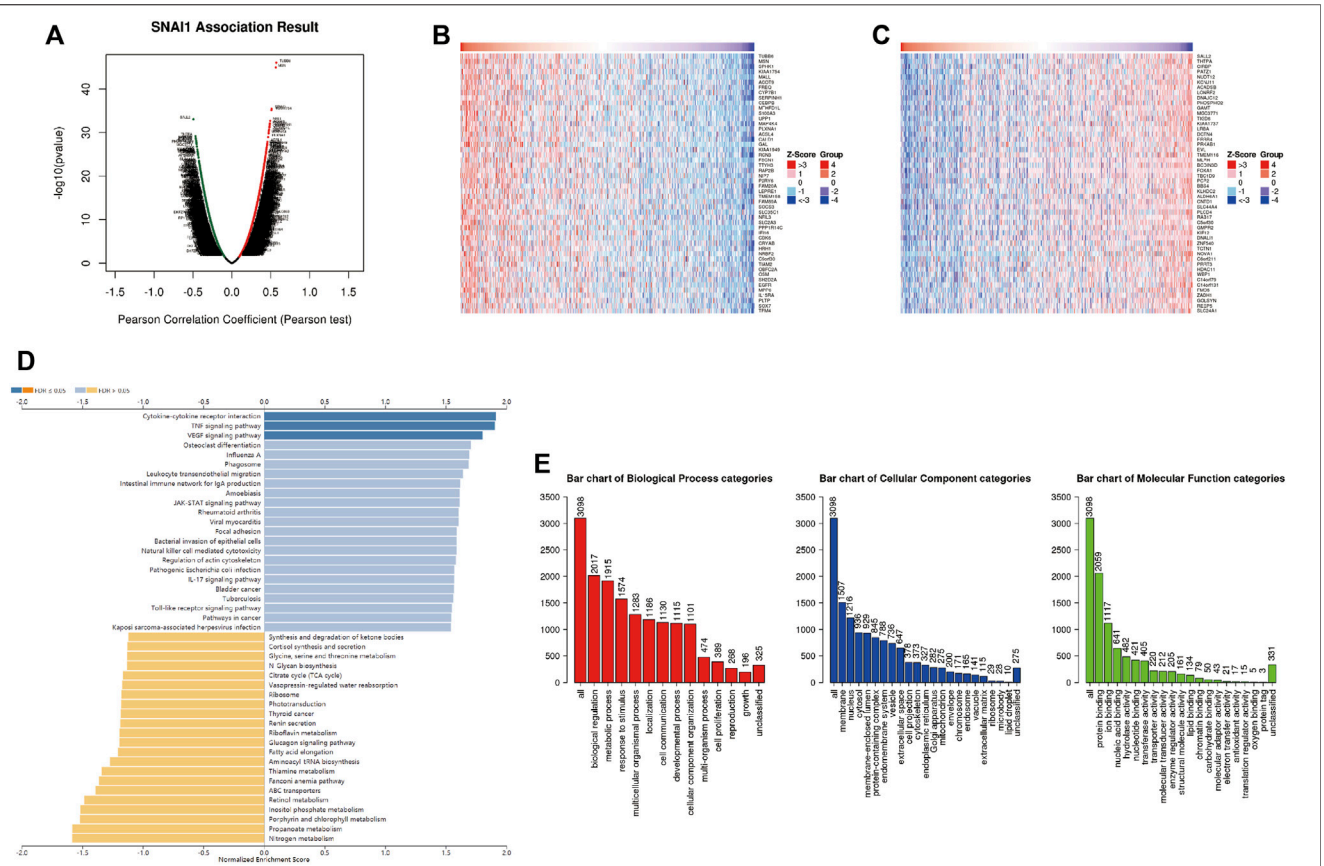

FIGURE 4 | Analysis of differentially expressed genes (DEGs) in correlation with SNAI1 in breast cancer **(A)** Volcano plot showing the up- and downregulated genes correlated with SNAI1 expression (Pearson test). Significantly positively correlated **(B)** and negatively correlated **(C)** genes are shown in heatmaps **(D)** Kyoto Encyclopedia Genes and Genomes (KEGG) pathway analysis of the significant DEGs in correlation with SNAI1 **(E)** Gene ontology (GO) analysis of the significant DEGs in correlation with SNAI1.

$CD4^+$ T cells, macrophages, neutrophils, and dendritic cells) in breast cancer. We found the SNAI1 expression level was positively correlated with the infiltration of $CD8^+$ T cells (R = 0.093, p = 3.50E-03), $CD4^+$ T cells (R = 0.043, p = 1.77E-01), macrophages (R = 0.094, p = 3.03E-03), neutrophils (R = 0.396, p = 1.43E-38), and dendritic cells (R = 0.312, p = 7.34E-24), whereas it was negatively correlated with B cell infiltration (R = –0.178, p = 1.70E-08) (**Figure 5A**). SNAI2 was positively related with the infiltration of $CD8^+$ T cells (R = 0.36, p = 7.79E-32), macrophages (R = 0.455, p = 7.72E-52), neutrophils (R = 0.326, p = 5.01E-26), and dendritic cells (R = 0.208, p = 3.50E-11), whereas it was negatively correlated with B cell infiltration (R = –0.253, p = 5.04E-16) and $CD4^+$ T cells infiltration (R = –0.127, p = 5.99E-05) (**Figure 5B**). SNAI3 was positively correlated with the infiltration of B cells (R = 0.206, p = 5.43E-11), $CD4^+$ T cells (R = 0.387, p = 8.01E-37), neutrophils (R = 0.255, p = 2.92E-16), and dendritic cells (R = 0.488, p = 1.54E-60), whereas it was negatively correlated with the infiltration of $CD8^+$ T cells (R = –0.028, p = 3.75E-01) and macrophages (R = –0.066, p = 3.68E-02) (**Figure 5C**).

The correlations between SNAI1 expression and the immune cells infiltration in breast cancer subtypes were also analyzed. The SNAI1 expression level was positively related with the infiltration of $CD8^+$ T cells (R = 0.1, p = 1.88E-01), $CD4^+$ T cells (R = 0.139, p = 6.78E-02), macrophages (R = 0.121, p = 1.11E-01), neutrophils (R = 0.199, p = 8.50E-03), and dendritic cells (R = 0.1, p = 1.91E-01) in the BRCA-basal type. In the BRCA-HER2 subtype, the SNAI1 expression level was positively related with the infiltration of $CD8^+$ T cells (R = 0.173, p = 1.47E-01), macrophages (R = 0.332, p = 4.33E-03), neutrophils (R = 0.052, p = 6.63E-01), and dendritic cells (R = 0.247, p = 3.67E-02). In the BRCA-luminal subtype, the SNAI1 expression level was positively related with the infiltration of $CD8^+$ T cells (R = 0.235, p = 6.76E-08), macrophages (R = 0.245, p = 1.76E-08), neutrophils (R = 0.362, p = 1.75E-17), and dendritic cells (R = 0.279, p = 1.00E-10) (**Supplementary Figure S7**).

Relationships Between *SNAI* Gene Copy Numbers and Immune Cell Infiltration in Breast Cancer

We conducted TISIDB analysis to unravel the relationships between *SNAI* gene copy numbers and immune cell

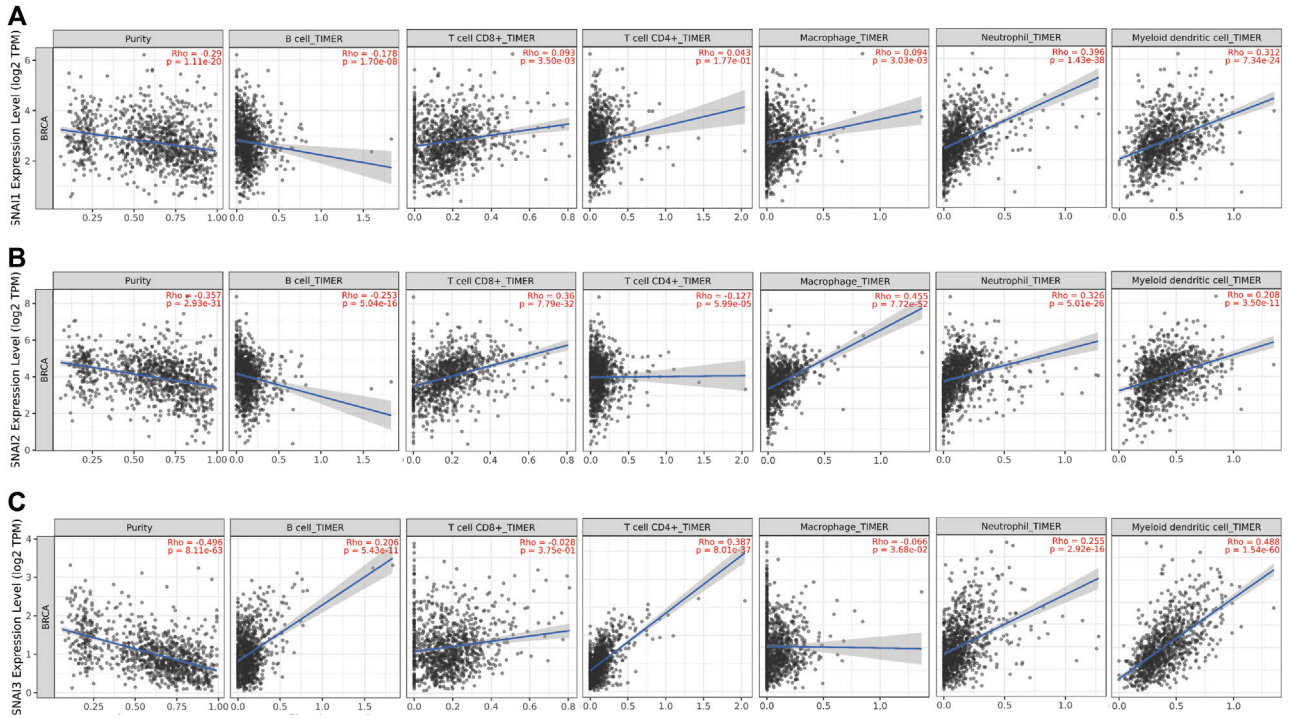

FIGURE 5 | Correlations between differentially expressed SNAIs and immune cell infiltration in breast cancer. Correlations between transcript levels of **(A)** *SNAI1* **(B)** *SNAI2*, and **(C)** *SNAI3* and the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in breast cancer are shown.

infiltration. Correlations between *SNAI1* copy numbers and different immune cells and tumor types are visualized in heatmaps in **Supplementary Figure S8A**. A high *SNAI1* copy number was positively correlated with the infiltration of $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells, whereas it was negatively correlated with the infiltration of B cells, neutrophils and macrophage (**Supplementary Figure S8B**). *SNAI2* and *SNAI3* copy numbers among different immune cell types and tumor types are shown in **Supplementary Figures S8C, E**. The *SNAI2* copy number was positively correlated with the infiltration of $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells, as also found for *SNAI1* (**Supplementary Figure S8D**). The *SNAI3* copy number was significantly correlated with the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (**Supplementary Figure S8F**). These results suggested that changes in the copy numbers of *SNAI* genes reflect the infiltration of various types of immune cells in breast cancer.

Next, we analyzed SNAI alterations in multiple immune cell types. For SNAI1, arm-level deletion ranked first in B cells, $CD8^+$ T cells, and neutrophils. The most frequent SNAI1 alterations in immune cells were arm-level deletion and arm-level gain (**Supplementary Figure S9A**). For SNAI2, arm-level gain ranked first among all six immune cells analyzed (**Supplementary Figure S9B**). For SNAI3, arm-level deletion ranked first in B cells, $CD8^+$ T cells, macrophages, neutrophils, and dendritic cells. The most frequent SNAI2 and SNAI3 alterations in different types of immune cells were arm-level deletion and arm-level gain (**Supplementary Figure S9C**). These results suggested that the main alteration types in SNAI genes in various immune cell types in breast cancer are arm-level deletion and arm-level gain.

Relationships Between *SNAI* Gene Methylation and Immune Cell Infiltration

The above analyses revealed changes in the methylation levels of SNAI genes in breast cancer as well as the relationships between SNAI expression levels and immune cell infiltration in breast cancer. However, it remained unclear whether changes in SNAI gene methylation and immune cell infiltration are related. Therefore, we analyzed the relationships between SNAI methylation levels and the infiltration of six types of immune cells in breast cancer using TISIDB data. SNAI1 methylation levels among different immune cell types and tumor types are visualized in heatmaps (**Figure 6A**). SNAI1 methylation was weak positively correlated with the infiltration levels of B cells, macrophages, and neutrophils, and weak negatively correlated with the infiltration levels of $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells (**Figures 6B–G**). SNAI2 and SNAI3 methylation levels among different immune cell types and tumor types are shown in **Supplementary Figures S10A, C**. The SNAI2 methylation level was weak positively correlated with the infiltration levels of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, and neutrophils, whereas it was weak negatively correlated with the infiltration levels of B cells and dendritic cells (**Supplementary Figure S10B**). The methylation level of

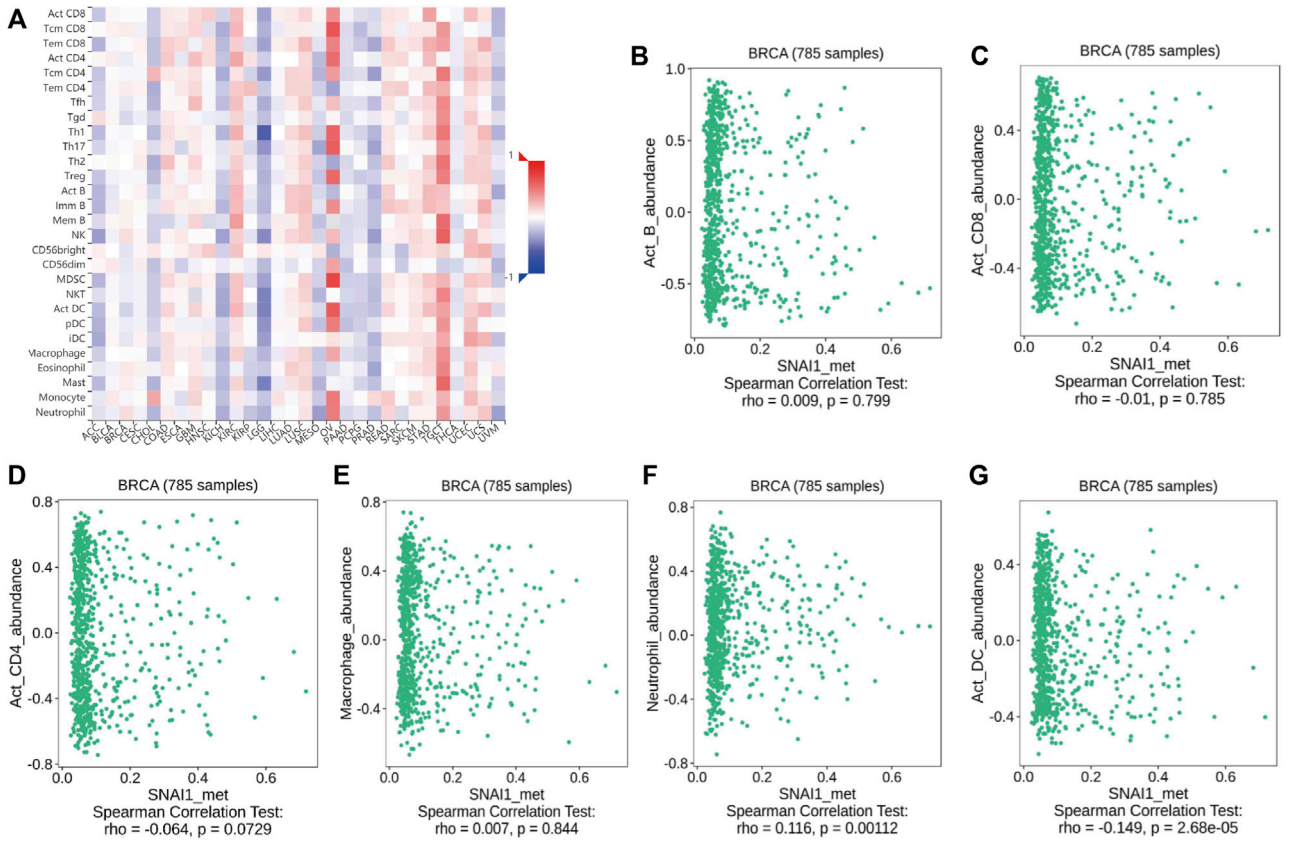

FIGURE 6 | Correlations between SNAI1 methylation levels and immune cell infiltration in breast cancer **(A)** *SNAI1* methylation levels in various cancer types and immune cell types are shown in heatmaps. Correlations between changes in the *SNAI1* methylation level and the infiltration of **(B)** B cells **(C)** $CD8^+$ T cells **(D)** $CD4^+$ T cells **(E)** macrophages **(F)** neutrophils, and **(G)** dendritic cells in breast cancer are shown.

SNAI3 was weak positively correlated with the infiltration levels of B cells, macrophages, and neutrophils, whereas it was weak negatively correlated with the infiltration levels of $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells (**Supplementary Figure S10D**). These results suggested that SNAI methylation levels are related to immune cell infiltration and may have opposite functions in different immune cell types. But SNAI methylation may be not a major factor in immune cell infiltration.

Relationships Between SNAI Protein Mutations and Immune Cell Infiltration

Next, we analyzed the relationships between SNAI protein mutations and immune cell infiltration using TIMER data. The results showed SNAI1 mutation was positively correlated only with the infiltration level of neutrophils, whereas it was negatively correlated with the infiltration levels of B cells, $CD8^+$ T cells, macrophages, and dendritic cells (**Supplementary Figure S11A**). SNAI2 mutation was positively correlated with the infiltration of macrophages, whereas it was negatively correlated with the infiltration of B cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (**Supplementary Figure S11B**). In contrast, SNAI3 mutation was positively correlated with the infiltration of $CD8^+$ T cells, macrophages, neutrophils, and dendritic cells, whereas it was negatively correlated with the infiltration of B cells and $CD4^+$ T cells (**Supplementary Figure S11C**). These results suggested that SNAI protein mutation affects immune cell infiltration and that the three SNAI proteins play different roles in immune cell infiltration.

Immune Cell-related Survival Analysis

We used TIMER to analyze the correlations between SNAI expression levels, immune cell infiltration, and the survival rate of breast cancer patients. The results showed that patients with high SNAI1 expression and low B cell infiltration levels had a lower life expectancy than those with high SNAI1 expression and high B cell infiltration levels. Similarly, patients with high SNAI1 expression and low neutrophil infiltration levels had a poor prognosis. However, patients with high SNAI1 expression and low $CD8^+$ T cell, $CD4^+$ T cell, macrophage, or dendritic cell infiltration levels had a longer life expectancy than those with high SNAI1 expression and high immune cell infiltration levels (**Supplementary Figure S12A**). Patients with high SNAI2 expression and high $CD8^+$ T cell, macrophage, neutrophil, or dendritic cell infiltration levels had a lower life expectancy than those with high SNAI2 expression and high immune infiltration levels, whereas patients with high SNAI2 expression and high B cell or $CD4^+$ T cell infiltration levels had a

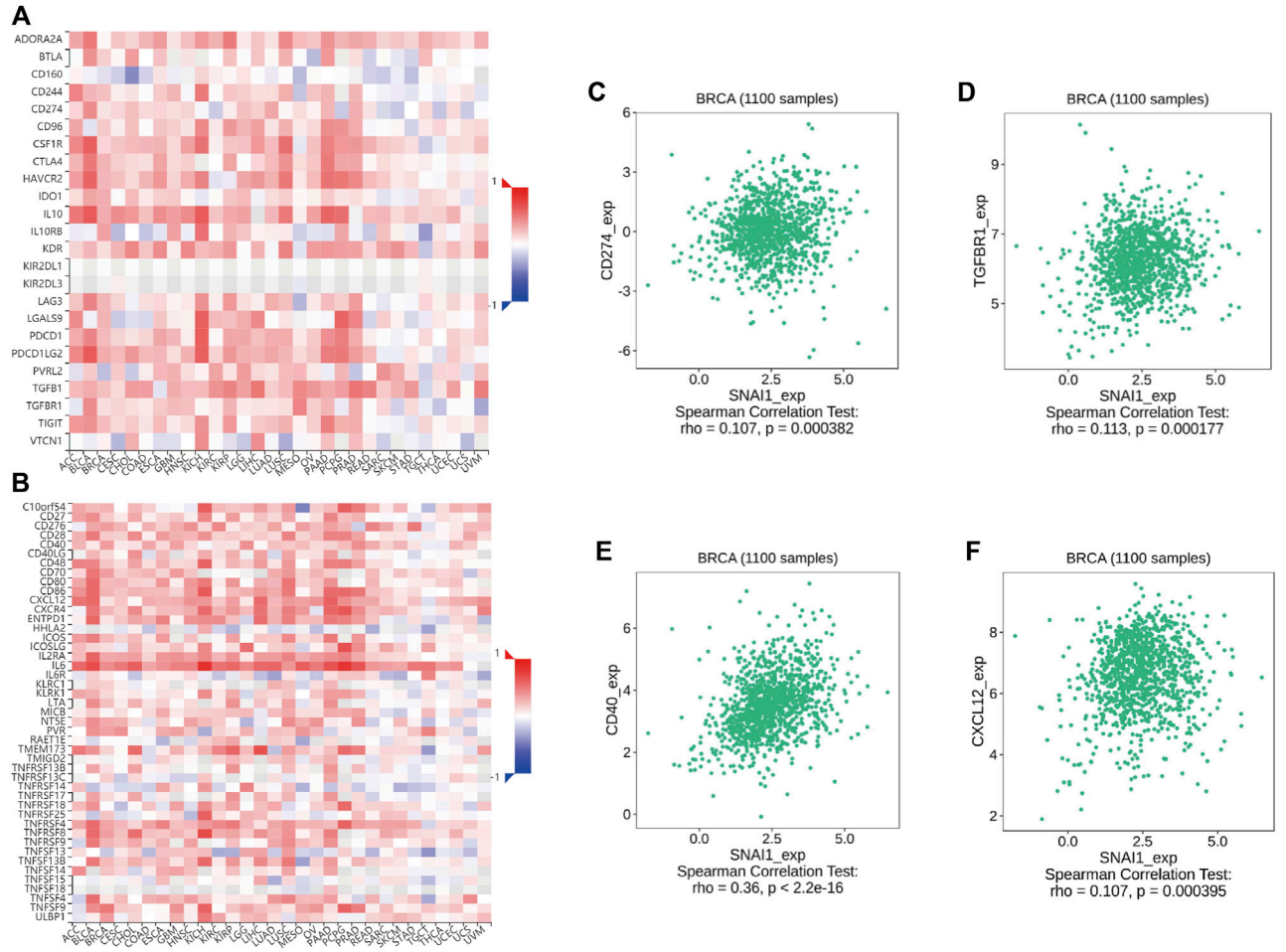

FIGURE 7 | Correlations between differentially expressed SNAI1 and the expression levels of immuno-inhibitors and immunostimulators in breast cancer **(A)** Correlations between SNAI1 expression levels in various cancer types and various immuno-inhibitors are shown in heatmaps. Correlations between SNAI1 expression levels and the expression levels of **(B)** CD274 and **(C)** TGFBR1 in breast cancer are shown **(D)** Correlations between SNAI1 expression levels in various cancer types and immunostimulators are shown in heatmaps. Correlations between SNAI1 expression levels and the expression levels of **(E)** CD40 and **(F)** CXCL12 in breast cancer are shown.

longer life expectancy than those with high SNAI2 expression and high immune cell infiltration levels (**Supplementary Figure S12B**). Patients with low SNAI3 expression and high B cell, $CD8^+$ T cell, macrophage, neutrophil, or dendritic cell infiltration levels had a lower life expectancy than those with low SNAI3 expression and low immune infiltration levels (**Supplementary Figure S12C**). These results suggested that high immune cell infiltration levels in patients with high SNAI1 and SNAI2 expression are associated with a bad prognosis, whereas SNAI3 in relation with high immune cell infiltration has the opposite effect and is related with a good prognosis.

Correlations Between SNAI Expression Level and Immunomodulator Expression in Breast Cancer

Immunomodulators are divided into immuno-inhibitors and immunostimulators. It is currently unclear whether immunomodulators are related to SNAI expression. Using TISIDB data, we found the correlations between SNAI expression levels and immunomodulator expression in breast cancer. The correlations between SNAI1 expression levels and immuno-inhibitors and immunostimulators are visualized in heatmaps (**Figures 7A,B**). The results showed that the expression of CD274 and TGFBR1, which are immuno-inhibitors, was correlated with the SNAI1 expression level (**Figures 7C,D**). The expression of CD40 and CXCL12, which are immunostimulators, were also correlated with the SNAI1 expression level (**Figures 7E,F**). The correlations between SNAI2 and SNAI3 expression levels and immuno-inhibitors and immunostimulators are shown in **Supplementary Figures S13A,B, G,H**, respectively. CD274, TGFBR1, CD40, and CXCL12 expression was correlated with the SNAI2 expression level (**Supplementary Figures S13C–F**). CD274, CD40, and CXCL12 expression was positively correlated with the SNAI3 expression level, whereas that of

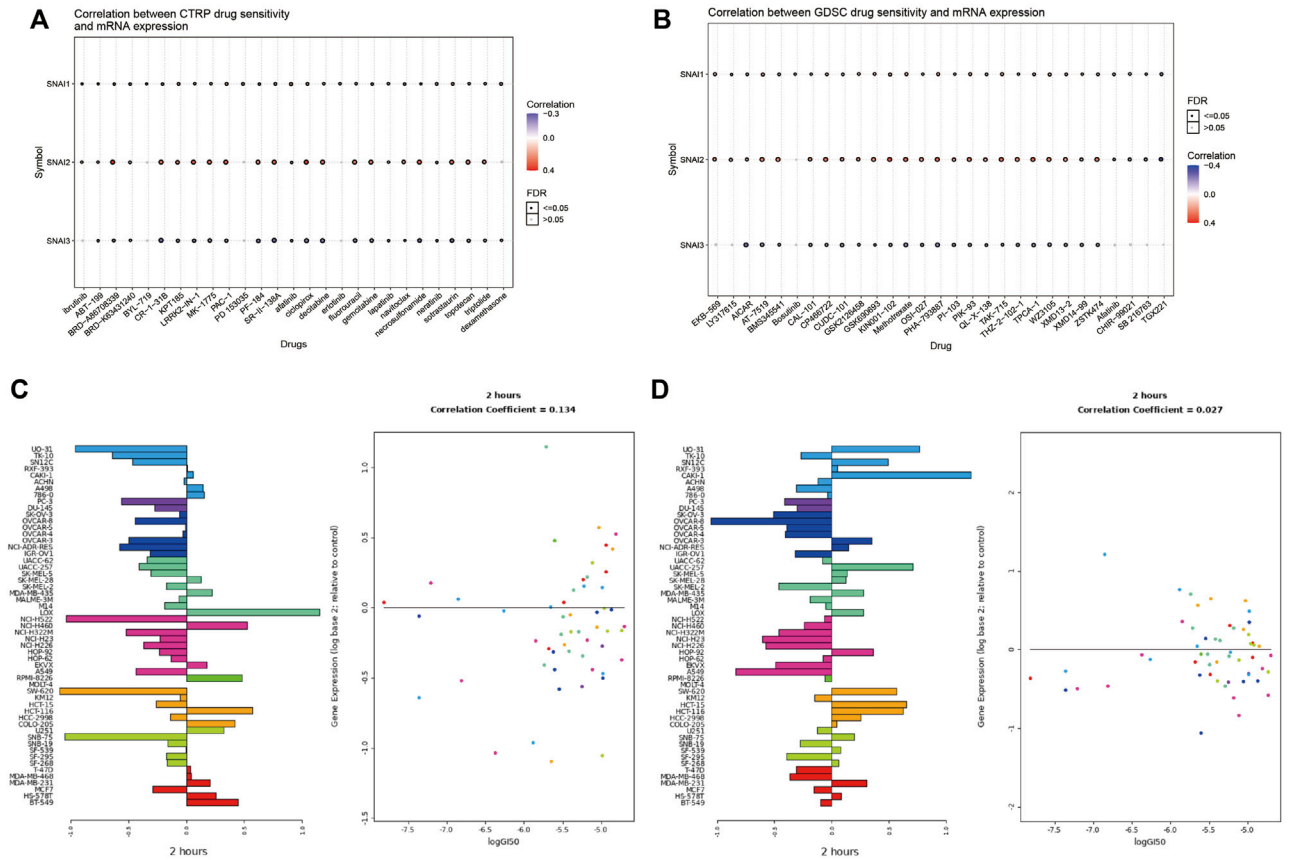

FIGURE 8 | Correlations between drug sensitivity and SNAI expression levels **(A)** Correlations between drug sensitivity and *SNAI* mRNA expression based on CTRP data **(B)** Correlations between drug sensitivity and *SNAI* mRNA expression based on GDSC data **(C)** SNAI1 expression levels after lapatinib treatment for 2 h in various cancer cell lines **(D)** SNAI2 expression levels after lapatinib treatment for 2 h in various cancer cell lines.

TGFBR1 was negatively correlated with the SNAI3 expression level (**Supplementary Figures S13I–L**). These results suggested that SNAI expression affects the expression of immunomodulators, which may influence immune system function in breast cancer.

Correlations Between SNAI Expression Levels and Chemokine Expression in Breast Cancer

The chemokine family plays a vital role in controlling the migration and residence of immune cells through receptor recognition. However, it remains unclear whether chemokines and receptors are related to SNAI expression levels. Using TISIDB data, we analyzed the correlations between SNAI expression levels and chemokine expression levels. The correlations between SNAI1 expression and chemokine and receptor levels are visualized in heatmaps (**Supplementary Figures S14A,B**). The expression of the chemokines CCL2 and CCL5 was positively correlated with the SNAI1 expression level (**Supplementary Figures S14C,D**). The expression of the receptors CCR5 and CCR7 was also positively correlated with the SNAI1 expression level (**Supplementary Figures S14E,F**). The correlations between SNAI2 and SNAI3 expression level and chemokines and receptors are shown in **Supplementary Figures S14G,H, M,N**, respectively. The expression of CCL2, CCL5, CCR5, and CCR7 was positively correlated with the SNAI2 expression level (**Supplementary Figures S14I–L**). The expression of CCL2, CCL5, CCR5, and CCR7 was positively correlated with the SNAI3 expression level (**Supplementary Figures S14O–R**). These results suggested that SNAI expression affects the expression of chemokines and their receptors, which may influence immune cell activation and killing power in breast cancer.

Correlations Between Drug Sensitivity and SNAI Expression Levels

The above analyses revealed the relationships between SNAI family members and tumors. Finally, we analyzed whether SNAI family members may serve as therapeutic targets for inhibiting tumor development. We used drug databases to

analyze drugs related to *SNAI* expression levels. The 30 drugs for which we found the strongest correlations between *SNAI* gene expression and drug sensitivity in pan-cancer in the CTRP and GDSC databases are shown in bubble charts in **Figures 8A,B**. For most drugs, drug sensitivity increased with increasing *SNAI2* expression. Next, we analyzed the effect of lapatinib, a commonly used antitumor drug, on SNAI expression in different types of cancer cells. The results showed that after treatment with lapatinib for 2 h, the *SNAI1* and *SNAI2* expression levels in most tumor cell types were decreased, as was their survival ability (**Figures 8C,D**). These results suggested that SNAI family members have a strong effect on tumors and may serve as therapeutic targets for the development of new antitumor drugs.

DISCUSSION

The onset and development of breast cancer is complex and involves numerous genetic and environmental factors (Polyak, 2007). An increasing number of studies show immune cell infiltration varies widely and affects patient prognosis. Infiltrating immune cells affect tumor development and metastasis through different signaling pathways. Further, numerous gene alterations have been identified by comparing tumor and normal tissues, and *SNAI* family members show a high mutation frequency. In mammals, the SNAI family comprises SNAI1–3. However, the clinical relevance of immune cells and related SNAI expression in breast cancer remains unclear.

In the current study, we analyzed the effects of the SNAI family members on tumors and their relationships with survival outcome by probing various public databases. The results indicated that SNAI1 and SNAI2 are highly expressed in breast cancer and are positively correlated with poor prognosis. However, the SNAI3 expression had an opposite effect on the prognosis of breast cancer patients. These results suggest that SNAI3 has an opposite role to SNAI1 and SNAI2 in the onset and development of breast cancer. SNAI1 is one of the key proteins of EMT and is closely related to E-cadherin (Cano et al., 2000). Thus, it may serve as a prognostic factor in various tumors. Studies have demonstrated that SNAI1 is a tumor-promoting factor. Our data corroborated that SNAI may serve as a prognostic biomarker in breast cancer. SNAI3 has been less well studied, but we found that it is positively correlated with good prognosis in breast cancer. A clinical study showed that the decrease of SNAI1 expression level will lead to the increase of CDH1 expression level, thereby promoting cell migration, invasion, and poor prognosis (Chen et al., 2018). The finding is consistent with our results and corroborates that SNAI3 can inhibit tumor onset and development. Our results provide a clinical basis for follow-up research. Epigenetics plays an important role in the onset and development of breast cancer (Wu et al., 2015). It has been reported that gene promoter methylation is frequent in breast cancer. Consistent herewith, *SNAI3* promoter methylation was increased, resulting in decreased *SNAI3* expression. We comprehensively analyzed methylation changes in *SNAI* genes. *SNAI* methylation changes may serve as biomarkers in breast cancer from these findings. Loss or mutation of *TP53* is the most common genetic lesion in cancer (Petitjean et al., 2007; Hainaut and Pfeifer, 2016). In the current study, we found that patients with *TP53* mutation tended to have high SNAI1 expression (Lee et al., 2009). This result suggests that *SNAI1* and *TP53* mutation are positively correlated, which indicates that *SNAI1* is correlated with the degree of malignancy of tumors.

SNAIs have critical roles in regulating immune cell. For example, SNAIs induce regulatory T cells or tumor-associated macrophages which belong to immunosuppressive cells (Kudo-Saito et al., 2009; Hsu et al., 2014). In ovarian cancer, they induce intratumoral trafficking of myeloid-derived suppressor cells by upregulating CXCR2 ligands (Taki et al., 2018). It is important to analyze the correlations between SNAI expression levels and the infiltration of different immune cell types. TIMER data was used to analyze the correlations between SNAI expression levels and the infiltration of immune cells. We found that high SNAI1 expression was significantly associated with increased in infiltration of $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells. Further, we analyzed the relationships between SNAI mutations and immune cell infiltration. To explore how SNAI affects the infiltration of immune cells, we analyzed the relationships between SNAI family members and immunomodulator and chemokine expression. The chemokine CXCL5 enhances the activation of Snail causing EMT to induce invasion of colorectal cancer (Zhao et al., 2017). IL-25 is negatively correlated with patient prognosis by inducing alternative activation of macrophages promoted tumorigenesis by increasing SNAIL expression (Li et al., 2019). We found that numerous immunomodulators and chemokines are positively correlated with SNAI expression in breast cancer. This finding provides a clinical basis for further experimental verification.

Finally, we found that certain drugs affect the expression of SNAI family members and thus may affect targeted tumor therapies. In future, we can screen small-molecule compounds that affect SNAI expression to develop new drugs for tumor treatment.

In conclusion, the current study deepened our understanding of the roles of SNAI family members and their relationships with immune cells in breast cancer. This study provided a basis for elucidating the molecular mechanisms of SNAIs in the onset and development of breast cancer and provided a potential therapeutic target for treating breast cancer.

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 in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

LZ and KS constructed the study and revised this manuscript. YT and PF performed the data analysis and wrote the manuscript. Authors read and approved the final manuscript. YT and PF contributed equally to this work.

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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.2022.906885/full#supplementary-material

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