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Review

Review

TGF-β signaling in cancer metastasis

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Abstract

The transforming growth factor (TGF)-β signaling events are well known to control diverse processes and numerous responses, such as cell proliferation, differentiation, apoptosis, and migration. TGF-β signaling plays context-dependent roles in cancer: in pre-malignant cells TGF-β primarily functions as a tumor suppressor, while in the later stages of cancer TGF-β signaling promotes invasion and metastasis. Recent studies have also suggested that the cross-talk between TGF-β signaling and other signaling pathways, such as Hippo, Wnt, EGFR/RAS, and PI3K/AKT pathways, may substantially contribute to our current understanding of TGF-β signaling and cancer. As a result of the wide-ranging effects of TGF-β, blockade of TGF-β and its downstream signaling components provides multiple therapeutic opportunities. Therefore, the outlook for anti-TGF-β signaling therapy for numerous diseases appears bright and will provide valuable information and thinking on the drug molecular design. In this review, we focus on recent insights into the regulation of TGF-β signaling in cancer metastasis which may contribute to the development of novel cancer-targeting therapies.

Key words: TGF-β, cancer, metastasis, therapies

Introduction

The transforming growth factor-β (TGF-β) superfamily of cytokines comprises TGF-βs, activins, inhibins, nodal, growth and differentiation factors (GDFs), and bone morphogenetic proteins (BMPs) [1,2]. Each family member plays crucial roles in many cellular processes, including immune-suppression, growth inhibition, epithelial–mesenchymal transition (EMT), cell migration, invasion, and extracellular matrix (ECM) remodeling [2,3]. During the early phases of tumor development, TGF-β frequently acts as a tumor suppressor, whereas in later phases, tumor cells can become resistant to its antimitogenic effects and TGF-β can switch into a tumor promoter [4–6]. In the later phases of cancer progression, TGF-β signaling can promote EMT by increasing the expression of mesenchymal markers, such as N-cadherin and vimentin, and reducing expression of epithelial markers, such as E-cadherin [7,8]. EMT is essential for normal embryonic development, but its exploitation during cancer progression is thought to contribute to tumor invasion and metastasis. In addition to EMT, TGF-β signaling can stimulate metastatic dissemination, for instance during metastasis of breast and prostate tumor cells to bone and lung. This review will focus on the different levels of regulation that determine the levels, persistence, and functional effects of TGF-β signaling that are related to tumor metastasis.

TGF-β SMAD Signaling and Regulation by Ubiquitination

TGF-β cytokines signal through a transmembrane receptor complex that comprises the Type I and Type II receptor serine-threonine kinases (**Fig. 1A**). During activation TGF-β first appears to bind to the constitutively active Type II receptor (TβRII), which triggers recruitment of the TGF-β Type I receptor (TβRI). Subsequently, the

Type I receptor is activated by TGF-β Type II receptor-mediated phosphorylation in its 30-amino acid regulatory segment called the GS region [9]. Zhang *et al.* have demonstrated that in the absence of TGF-β, TβRI and TβRII can be present on the cell surface as monomers rather than homodimers [10], while dimerization of both receptors occurs upon TGF-β stimulation [11]. The activated receptor complexes initiate the so-called canonical TGF-β signaling through C-terminal phosphorylation of the receptor-activated SMADs (R-SMADs), SMAD2 and SMAD3, also called receptor-regulated or associated SMADs. These R-SMADs then interact with SMAD4 (Co-SMAD, common mediator SMAD) and the SMAD4–SMAD2/3 complex shuttles to the nucleus where it can associate with other transcription cofactors at DNA elements of target genes, thereby activating or repressing TGF-β target gene expression [12,13].

TGF-β signaling is regulated at all levels from the ligands outside of the cell to DNA-bound SMADs. Ubiquitination of TGF-β signaling components is emerging as a key mechanism of TGF-β pathway control [14–16]. SMAD6 and SMAD7, so-called inhibitory SMADs (I-SMADs), play crucial roles in the repression of TGF-β signaling via multiple mechanisms. First of all, SMAD6/7 can compete with the recruitment of R-SMADs to the Type I receptors and prevent R-SMAD activation by phosphorylation. Secondly, SMAD7 can promote Type I receptor ubiquitylation and degradation by recruiting the E3 ligases SMURF1 and SMURF2 [17,18]. In the receptor–SMAD7–SMURF1/2 complex, SMAD7 recruits the ubiquitin-conjugating E2 enzyme UbcH7 to stimulate the activity of SMURF1/2 [19]. In addition, SMAD7 can inactivate TβRI by recruiting two HECT-type E3 ligases, WWP1/Tiul1 and NEDD4-2, leading to TβRI ubiquitylation and degradation [20]. SMAD7 is regulated at the transcriptional level by TGF-β signaling, and it participates in a negative feedback loop regulating the amplitude and duration of TGF-β/SMAD signaling.

The E3 ubiquitin ligase RLIM/RNF12 has been identified as a key regulator of SMAD7 at the protein level [21]. RNF12 was originally identified as a protein that binds to the homeodomain protein LIM, and targets the LIM cofactor CLIM for degradation [22]. Recent studies have shown that mouse RNF12 is an X-encoded, dose-dependent inducer of X chromosome inactivation (XCI) in mouse embryonic stem cells (ESCs) [23,24].

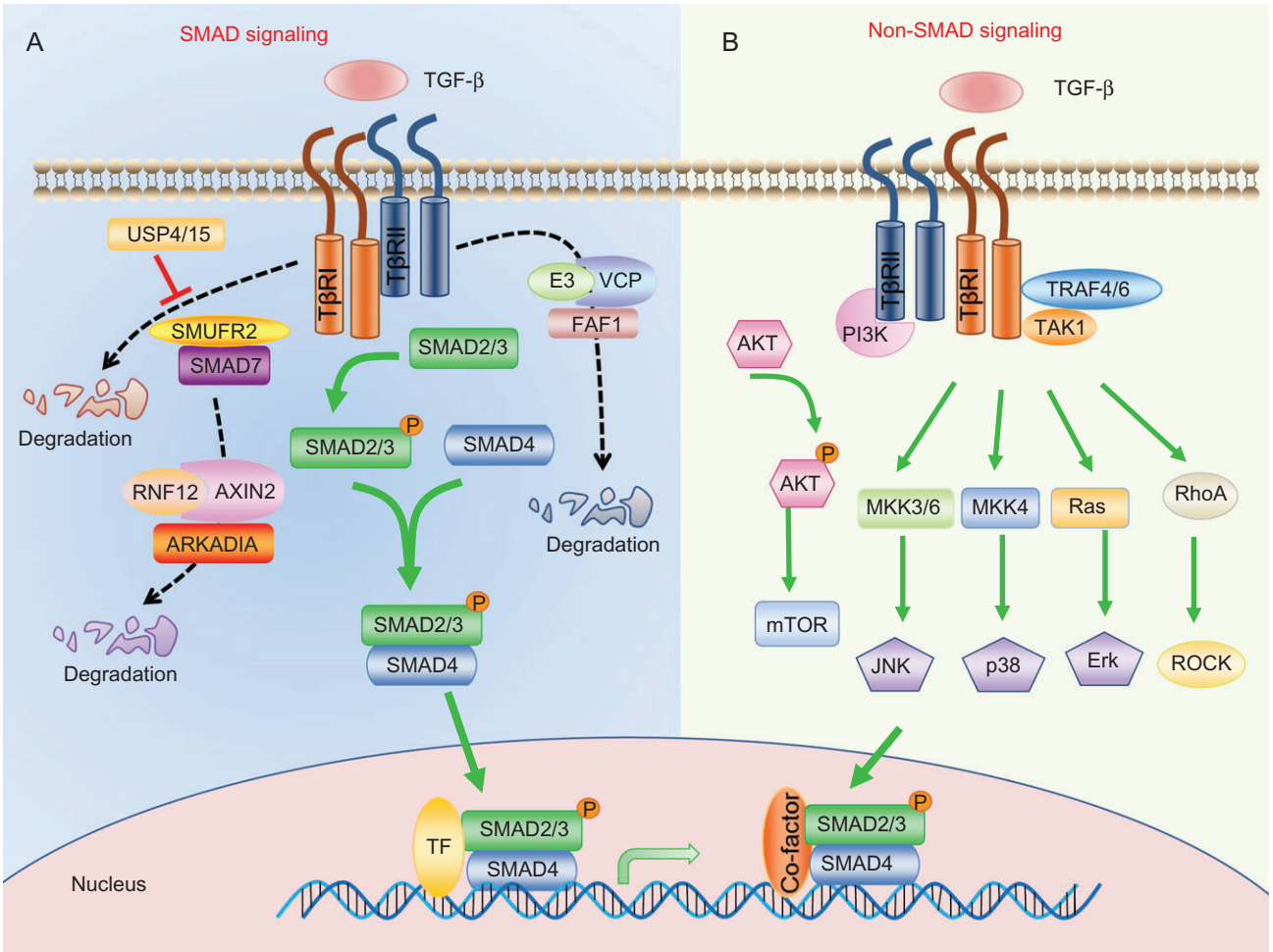

Figure 1. Regulation of SMAD signaling and non-SMAD signaling TGF-β signals via specific complexes of TβRI and TβRII Ser/Thr kinase receptors. The activated TβRI induces SMAD2/3 phosphorylation; phosphorylated SMAD2/3 forms hetero-oligomers with SMAD4, which accumulate in the nucleus to regulate the expression of target genes. SMAD7 functions as an inhibitory SMAD by recruiting the E3 ligase SMURF1/2 to TβRI. ARKADIA–RNF12–AXIN2 potentiates TGF-β signaling by targeting SMAD7 for polyubiquitination and degradation. Ubiquitin-specific protease (USP) 4/15 deubiquitinases can remove ubiquitin chains from TβRI, and thus stabilize the TβRI receptor. FAF1 destabilizes TβRII on the cell surface by recruiting the VCP/E3 ligase complex, thereby limiting excessive TGF-β response. Non-SMAD signaling pathways: the phosphatidylinositol kinase (PI3K)-AKT-TOR pathway, Erk, p38 and JNK mitogen-activated protein kinases (MAPKs) and Rho-like GTPase signaling.

The E3 ligase ARKADIA can enhance TGF-β signaling by ubiquitin-mediated degradation of SMAD7 as well [25]. However, ARKADIA also directly interacts with receptor-phosphorylated SMAD2/3 and ubiquitinates these proteins, resulting in highly transient SMAD2/3 activation and degradation in embryonic cells [26]. The effects of ARKADIA on TGF-β signaling might be distinct from the effects of RNF12; whereas ARKADIA inhibits, RNF12 potentiates TGF-β signaling in ESCs.

The nuclear receptor NR4A1 was identified as a potent activator of TGF-β signaling via its effects on SMAD7. NR4A1 was found to facilitate AXIN2–RNF12/ARKADIA-induced SMAD7 degradation [27]. Importantly, inflammatory cytokines potently induce NR4A1 expression, and potentiate TGF-β-mediated breast cancer cell migration, invasion and metastasis both *in vitro* and *in vivo*. In line with this, more and more evidence indicates that immune cells, including inflammatory cells, have tumor-promoting activity. And the role of NR4A1 has revealed a new mechanism by which the microenvironment can stimulate breast cancer cell invasion and metastasis. However, another group has reported NR4A1 as an endogenous inhibitor of TGF-β signaling by recruiting a repressor complex comprising SP1, SIN3A, CoREST, LSD1, and HDAC1 to TGF-β target genes, thereby limiting pro-fibrotic effects of TGF-β [28].

The conjugating function of E3 ligases is opposed by deubiquitylating enzymes (DUBs) [29]. Two studies have identified ubiquitin-specific peptidase-4 (USP4) and −15 (USP15) as DUBs for the TβRI [30,31]. USP4 and USP15 were found to activate the Type I receptors by counteracting receptor-ubiquitinating complexes through different mechanisms: USP15 influences TβRI-signaling indirectly, by acting on the SMAD7–SMURF2 complex, whereas USP4 acts directly on TβRI. Moreover, USP15 interacts with USP4 and does not affect TβRI ubiquitylation in USP4-deficient cells. USP15, highly expressed in glioblastoma, is associated with poor prognosis and promotes TGF-β-dependent oncogenesis [32]. USP15 has also been shown to act as a DUB of monoubiquitylated R-SMADs, thereby counteracting the inhibition of DNA binding [30]. Depletion of USP4 can mitigate TGF-β-induced epithelial to mesenchymal transition and metastasis. AKT activation by growth factors or TGF-β triggers USP4 phosphorylation and relocalization to the cell membrane. USP4 thereby functions as an important mediator of the cross-talk between the TGF-β and AKT signaling pathways. Interestingly, activated AKT and TGF-β cooperate to antagonize the inhibitory effect of FAS-associated factor 1 (FAF1) on TGF-β signaling [33].

TGF-β Non-SMAD Signaling

In addition to SMAD-mediated canonical TGF-β signaling, the TGF-β receptors can also activate other intracellular pathways, either through phosphorylation of, or through direct interaction with critical signaling intermediates. These so-called non-SMAD signaling pathways include several branches: the phosphatidylinositol kinase (PI3K)–AKT–TOR pathway, the Erk, p38 and JNK mitogen-activated protein kinase (MAPK) cascades, and pathways downstream of Rho-like GTPase signaling intermediates (**Fig. 1B**) [34–36]. To understand the complexity of the TGF-β response, non-SMAD signaling mechanisms have more recently been the subjects of intense studies. Both TβRII and TβRI appear to be directly involved in the activation of the PI3K/AKT pathway by interacting with the p85 subunit of PI3K [37]. AKT then activates its downstream effector mammalian target of rapamycin (mTOR), which controls translational responses. JNK and p38 MAPK pathway activation by TGF-β is induced via TAK1, a MAPK kinase kinase (MAPKKK) family member [38]. It has been reported that TGF-β activates TAK1 through catalytic activation of the ubiquitin ligase tumor necrosis factor (TNF) receptor-associated factors 4 and 6 (TRAF4/6) [39–41]. TRAF4 and TRAF6 were originally known as adapter proteins that signal downstream of Toll-like receptors and activate the transcription factor nuclear factor kappa B (NFκB) [42–44]. Upon TRAF4 interaction with TβRI, TGF-β-induced K63-polyubiquitination of TRAF4 contributes to the activation of TAK1 and, as a consequence, p38 MAPK. TRAF6 also associates with TβRI, which is required for its autoubiquitination upon TGF-β stimulation and subsequent Lys63-linked polyubiquitination of TAK1 [39]. Recent studies have revealed that TGF-β, via TRAF6, also can induce Lys63-linked polyubiquitination of TβRI, which promotes cleavage of TβRI by TNF-α converting enzyme (TACE) in a PKCζ-dependent manner [45]. In addition, a recent paper has reported that TGF-β can activate PI3K via TRAF6, although this result has not yet been widely proved [46]. TRAF6 was found to polyubiquinate the PI3K regulatory subunit p85α and to promote complex formation between TβRI and p85α, leading to the activation of PI3K and AKT [46]. Concomitant activation of TAK1 results in activation of mitogen-activated protein kinase kinases 3, 4, and 6 (MKK3/4/6), leading to JNK and p38 activation. Intriguingly, TGF-β can also induce phosphorylation of tyrosine residues on TβRI and TβRII, thereby recruiting Grb2 and SOS which activate Ras and the Raf–MEK–Erk MAPK cascade [47]. TGF-β also activates GTPase RhoA and its downstream target p160 (ROCK), inducing actin stress fiber formation and mesenchymal characteristics [48,49].

Insights in the Switch of TGF-β's Function in Cancer Progression

In many types of cells, TGF-β-induced growth inhibition is mediated through SMAD-dependent inhibition of the expression of the c-MYC oncogene, cyclin-dependent kinases (CDKs) and CDC25A [5,6]. In addition to causing cell cycle arrest, TGF-β signaling can also induce apoptosis or differentiation of epithelial cells. The most aggressive tumors preferentially acquire oncogenic mutations, such as mutations in RAS/RAF, PI3K/AKT, or p53. These oncogenic mutations can confer selective insensitivity to, for example, TGF-β/SMAD-dependent $p15^{INK4B}$/$p21^{CIP1}$ induction or c-MYC reduction, and tumor cells with such a signature therefore fail to execute TGF-β/SMAD-mediated growth arrest. The non-affected SMAD-dependent gene responses in these cells usually facilitate cancer cell migration and metastasis. Tumor cells can undergo partial EMT in which cells lose cell polarity and cell–cell contacts, become more motile, and/or acquire fibroblast-like properties [50,51]. It is poorly understood in many cases how TGF-β switches from a tumor suppressor to a tumor promoter. Recently, several groups have provided some clues that explain how TGF-β can be converted to a metastasis promoter. They showed that the phosphoserine/phosphothreonine-binding protein 14-3-3ζ can serve as a molecular switch, by turning off TGF-β-mediated tumor suppression in mammary epithelial cells and promoting TGF-β-induced bone metastasis in breast cancer (**Fig. 2**) [52]. 14-3-3ζ, which belongs to a family of evolutionary conserved proteins in eukaryotes with seven mammalian isoforms, is expressed in more than 40% of breast cancers. In breast and other cancers, the 14-3-3ζ gene is found to be amplified, which is associated with cancer recurrence and increased incidence of metastasis. Previously, Lu *et al.* [53] demonstrated that 14-3-3ζ can potentiate TGF-β signaling during EMT and breast tumor progression. They found that 14-3-3ζ can cooperate

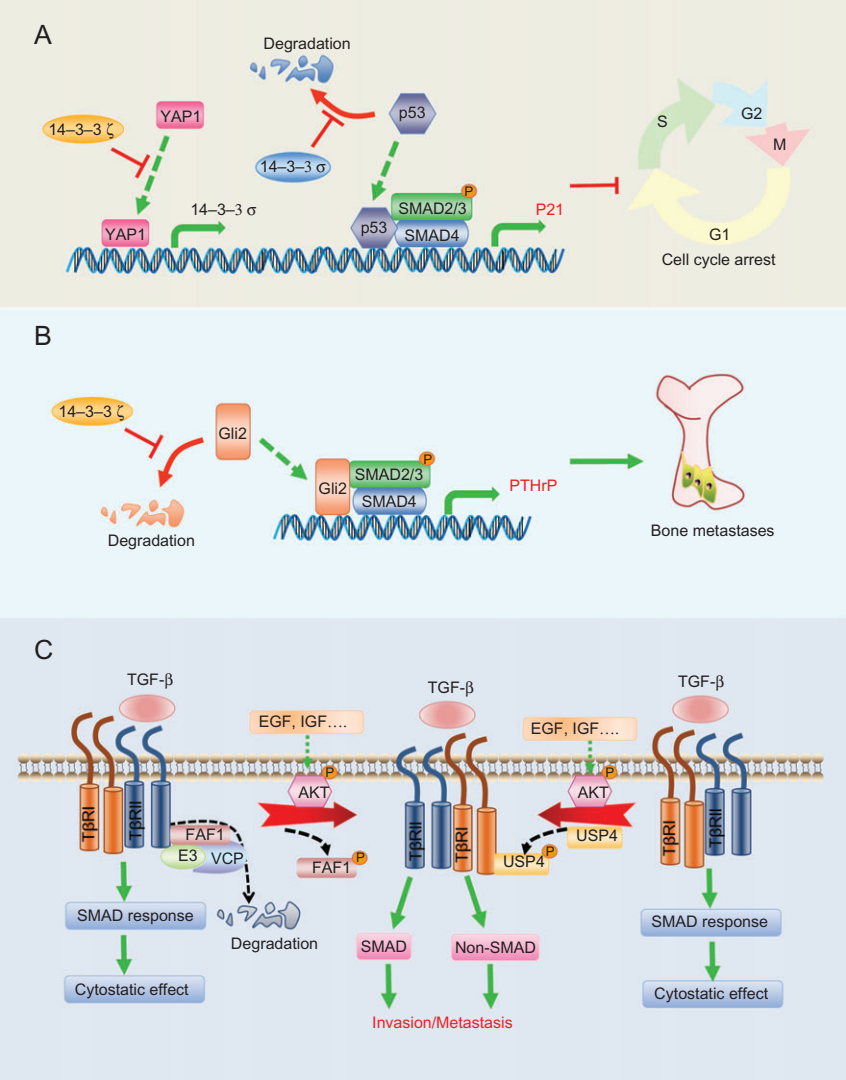

Figure 2. The dual role of TGF-β in cancer is switched by 14-3-3ζ and FAF1 (A) In pre-malignant cells, 14-3-3ζ inhibits TGF-β/SMAD-induced growth inhibition by repressing Yes-associated protein (YAP)-induced 14-3-3σ expression. The nuclear YAP1 transactivates the 14-3-3σ tumor suppressor gene. 14-3-3σ stabilizes p53 by inhibiting the E3 ubiquitin ligase MDM2. p53 can partner with SMAD2/3/4 to activate p21 to inhibit cell cycle progression. (B) In breast cancer cells, 14-3-3ζ turns on TGF-β's metastasis promoter function by stabilizing GLI2. 14-3-3ζ can displace βTrCP from GLI2. GLI2 interacts with TGF-β-induced SMAD complexes to promote the expression of PTHrP. PTHrP mediates TGF-β-induced bone metastasis of breast cancer. (C) The non-SMAD signals downstream of TβRII incorporates AKT, leading to the positive amplification loop of AKT–TGF-β signaling. FAF1 destabilizes TβRII on the cell surface by recruiting the VCP/E3 ligase complex, thereby limiting excessive TGF-β response as tumor suppressor. Activated AKT by oncogenic mutations directly phosphorylates FAF1, disrupts the FAF1–VCP complex and reduces FAF1 at the plasma membrane, resulting in an increase in TβRII at the cell surface and promoting both TGF-β-induced SMAD and non-SMAD signaling as tumor promoter.

with the tyrosine kinase receptor (ErbB2) in driving the progression of non-invasive ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC). Moreover, they also found that 14-3-3ζ can promote cancer cell proliferation by downregulating the cell cycle inhibitor p27 and activating PI3K/AKT [54]. Recently, Xu *et al.* [52] reported that 14-3-3ζ can inhibit TGF-β/SMAD-induced growth inhibition in pre-malignant cells by repressing Yes-associated protein (YAP)-induced 14-3-3σ expression (**Fig. 2A**). YAP1, which is downregulated in breast cancers, has been reported as a tumor suppressor in multiple types of cancer [55,56], whereas 14-3-3σ stabilizes tumor suppressor p53, a SMAD partner for transactivation of p21 [57], one of the key executors of TGF-β's cytostatic function. In breast cancer cells, 14-3-3ζ was found to turn on TGF-β's metastasis promoting function by stabilizing glioma-associated oncogene homolog 2 (GLI2) (**Fig. 2B**). GLI2 is a SMAD partner in TGF-β-induced parathyroid hormone-related protein (PTHrP) expression, a previously identified key determinant of TGF-β-induced bone metastasis [58].

USP4 was identified as an important mediator of the cross-talk between the TGF-β and AKT signaling pathways [31] and activated AKT cooperates with TGF-β to antagonize the inhibitory effect of FAS-associated factor 1 (FAF1) on TGF-β signaling [33]. These mechanisms, depicted in **Fig. 2C**, are important for TGF-β-induced pro-invasive and pro-metastatic responses in advanced tumors. As the first target of the TGF-β ligand, cell surface TβRII is thought to be required for all TGF-β signaling responses. A low dose of TβRII appears to be sufficient for cytostatic SMAD activation, while an increase in TβRII levels contributes to cancer and drug resistance [59]. Moreover, TβRII expression in CAFs has been found to

support cancer growth and survival [60]. FAF1 can destabilize TβRII on the cell surface by recruiting the VCP/E3 ligase complex, thereby limiting excessive TGF-β responses. When AKT is aberrantly activated in cells (e.g. by oncogenic mutations or excessive amounts of growth factors), it can phosphorylate FAF1 at Ser582, which disrupts the FAF1–VCP complex and reduces the levels of FAF1 at the plasma membrane. It results in an increase in TβRII on the cell surface, enhancing both TGF-β/SMAD and non-SMAD signaling. Blocking PI3K/AKT activity in metastatic breast cancer cells reduces TβRII expression to a very low level, demonstrating that TβRII-mediated oncogenic TGF-β signaling is supported by AKT activity. AKT activation by growth factor or TGF-β also induces phosphorylated USP4 to relocate and stabilize TβRI in the plasma membrane, thereby enforcing TGF-β-induced pro-tumorigenic responses in breast cancer cell (**Fig. 2C**) [31]. Targeting of PI3K/AKT pathway for the treatment of cancer patients holds significant therapeutic promise [61].

TGF-β in Cancer Metastasis

Breast cancer starts as a local disease, but it can metastasize to the lymph nodes and distant organs, such as lung, bones, brain, and liver. Certain tumors produce metastasis to specific organs independent of vascular anatomy, rate of blood flow, and the number of tumor cells delivered to each organ, and this organotropic metastasis depends on the extracellular vesicles such as exosomal integrins α6β4 and α6β1 [62]. As a result of their uncontrolled proliferation, cancer cells usually first produce a primary tumor. Multiple cells in this primary tumor can eventually acquire a malignant phenotype. This enables these cells to escape from the primary tumor and invade into the bloodstream or lymphatic system. Subsequently, they can settle and form secondary tumors at a distant site. In breast cancer patients, it is not the primary tumor, but its metastases in other tissues that are the main cause of death. The molecular mechanisms underlying metastasis are still poorly understood and most studies have focused on features intrinsic to tumor cells. However, the tumor microenvironment also plays an important role in metastasis [63].

Prostate cancer (PCa) is one of the most frequently occurring malignant cancers in men, associated with a high risk of bone metastasis. A recent study showed that TGF-β supports the development of PCa bone metastases [64]. Moreover, TGF-β signaling is activated in PCa bone metastatic patients because TGF-β is one of the most abundant growth factors in bone and is released during osteoclastic bone resorption. In breast cancer bone metastases, TGF-β increased the expression of genes previously shown to be associated with bone metastases, including *PTHRP*, *IL11*, *CTGF*, *CXCR4*, *MMP1*, and *JAG1* [65–67]. To develop therapeutic treatments, it is critical to understand the mechanisms of breast cancer bone metastases. Recent research has shown that PMEPA1 (also known as TMEPAI, STAG1, and N4wwBP4) interacts with R-SMADs and ubiquitin ligases and inhibits TGF-β signaling by a nonproteasomal mechanism (**Fig. 3A**) [68]. The membrane-bound PMEPA1 was found to interact with R-SMADs via its C terminus and to interact with HECT E3 ubiquitin ligase via its PPxY domains. In PCa patients, the expression of PMEPA1 is increased by a high level of TGF-β in the primary tumor [69]. High level of PMEPA1 in turn regulates negative feedback loops in TGF-β signaling [70]. Interestingly, the E3 ubiquitin ligase AIP4 can also inhibit TGF-β signaling independent of ubiquitination via SMAD7 and TβRI [71]. In the early stages of cancer, induction of PMEPA1 expression by environmental factors can inhibit TGF-β signaling and thereby its tumor-suppressing function. However, compared with the primary tumor, expression of PMEPA1 was decreased in distant metastases of PCa patients due to accumulated methylation in the *PMEPA1* promoter [72], suggesting that methylation of the *PMEPA1* promoter may predict patient outcome, and that low methylation of the *PMEPA1* is associated with bad patient prognosis. According to this model, lower level of PMEPA1 increases TGF-β signaling and TGF-β-regulated pro-metastatic genes resulting in an increase in metastases and a decrease in patient survival. PMEPA1 may thus be a prognostic biomarker in patients suffering from breast or prostate cancer.

It has been reported that the adipokine angiopoietin-like 4 (ANGPTL4) can enable human breast cancer cells to form lung metastasis by disrupting capillary vascular endothelial cell junctions [73]. As described above, cancer cells in advanced tumors usually become unresponsive to the growth inhibitory functions of TGF-β, but still can be induced to invade and undergo EMT. TGF-β produced abundantly by stromal cells in the tumor microenvironment stimulates the expression of ANGPTL4 by activating SMAD transcription factors (**Fig. 3B**). Secretion of ANGPTL4 was found to enable estrogen receptor (ER)-negative breast cancer cells to extravasate into lung tissue and to seed micrometastases [73].

Emerging Roles of LncRNAs in TGF-β Signaling

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides. LncRNAs can regulate many important cancer-associated phenotypes, including proliferation, apoptosis, or cell migration, through their interactions with DNA, chromatin, signaling and regulatory proteins, and a variety of cellular RNA species [74,75]. Multiple cancer-associated lncRNAs have been identified as a new class of players regulating cancer invasion and metastases [76,77]. LncRNA BRAF (V600E) regulates melanoma cell migration and lncRNA GAPLINC regulates CD44-dependent cell invasiveness [78,79]. Recently, Yuan *et al.* [80] reported that lncRNA-ATB is activated by TGF-β in hepatocellular carcinoma (HCC) cells and promotes epithelial to mesenchymal transition, cellular invasion, and organ colonization by HCC cells via two distinct RNA–RNA interactions (**Fig. 4**). It has been reported that many RNA transcripts function as competing endogenous RNAs (ceRNAs), by competitively binding common microRNAs (miRNAs) [81–83]. The miR-200 family has been identified to repress EMT and tumor invasion by targeting the 3′ UTRs of the zinc-finger E-box binding factors 1 and 2 (ZEB1/2) [84,85]. The TGF-β-induced lncRNA-ATB functions as a ceRNA for miR-200s and thereby upregulates ZEB1 and ZEB2 during EMT. On the other hand, interaction of lncRNA-ATB with interleukin-11 (IL-11) mRNA enhances signal transducer and activator of transcription3 (STAT3) signaling to promote metastasis [80]. The breast cancer-associated lncRNA BCAR4 also stimulates cell migration and metastasis [86]. LncRNA BCAR4 binds to SMAD nuclear-interacting protein 1 (SNIP1) and serine/threonine-protein phosphatase 1 regulatory subunit 10 (PPP1R10, also known as PNUTS) in response to the chemokine CCL21, leading to GLI2-dependent gene activation. LncRNAs also mediate cancer metastasis through chromatin deregulation. The HOX-associated lncRNA HOTAIR can reprogram the chromatin landscape genome-wide via recruitment of polycomb repressive complex 2 (PRC2), leading to increased breast cancer invasiveness and metastasis [87]. By a high-density Affymetrix Genechip platform from 1008 prostate cancer patients, the prostate cancer lncRNA second chromosome locus associated with prostate-1 (SChLAP1) was identified as the highest-ranked

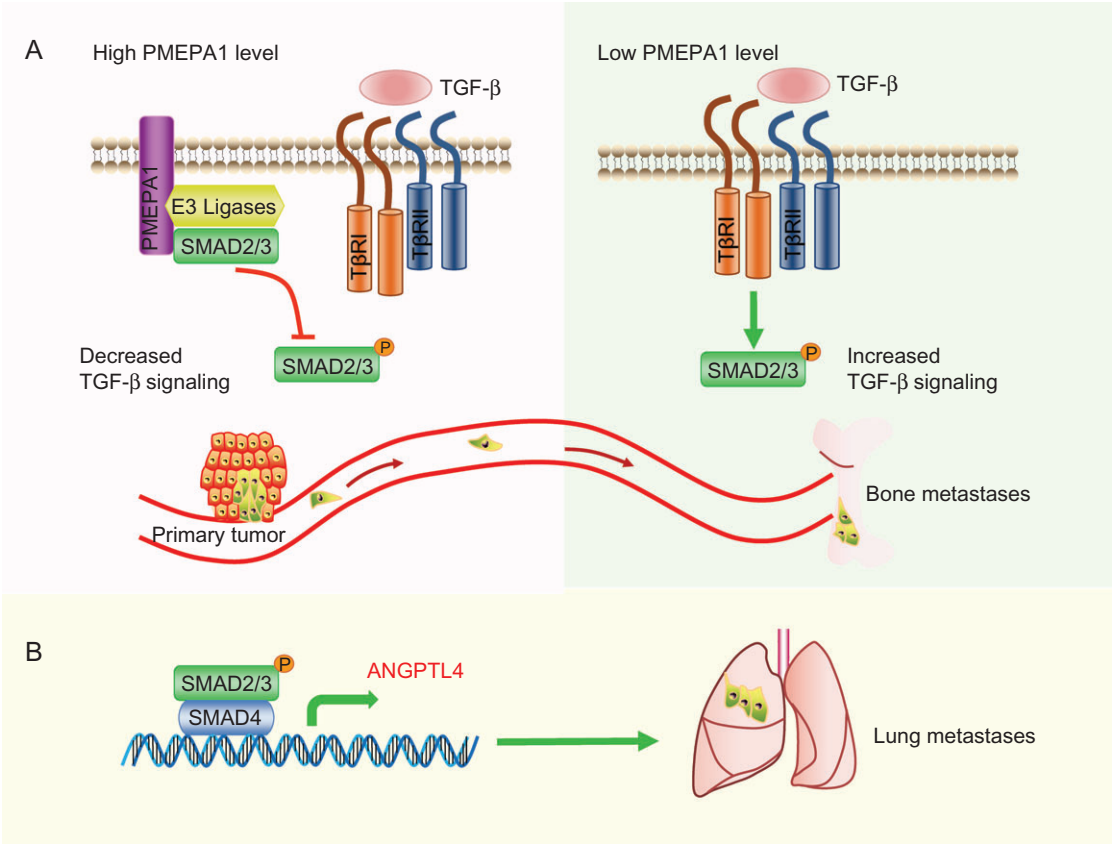

Figure 3. Regulation of TGF-β signaling in cancer metastasis (A) High expression of PMEPA1 in primary tumor decreases TGF-β signaling. PMEPA1 interacts with R-SMADs and HECT E3 ubiquitin ligase and prevents their phosphorylation and downregulates TGF-β signaling by a mechanism independent of the proteasome. Lower levels of PMEPA1 increase TGF-β signaling and TGF-β-regulated pro-metastatic genes, resulting in an increase in bone metastases. (B) TGF-β stimulates expression of adipokine angiopoietin-like 4 (ANGPTL4) by activating SMAD transcription factors. ANGPTL4 drives human tumor cells for lung metastasis.

overexpressed gene, associated with poor prognosis and metastatic progression [88]. Mechanistically, SChLAP1 was found to promote prostate cancer invasion and metastasis by antagonizing the tumor-suppressive functions of the SWI/SNF complex [89].

In addition to lncRNAs, miRNAs also play critical roles in regulation of TGF-β signaling, EMT, and cancer metastasis [90,91]. MiRNAs are small non-coding RNAs of 19–24 nucleotides in length and exhibit their regulatory functions by mRNA degradation or translational inhibition. MiR-182 has been found to antagonize the response of SMAD7 to TGF-β and promote cancer cell EMT, invasion, and metastasis (**Fig. 4**) [92]. TGF-β signaling and/or its target genes can also be regulated by miR-145, miR-203, miR-206, miR-181a, miR-155, and miR-1269 (**Fig. 4**) [93–97]. LncRNAs, miRNAs, and ceRNA may also modulate the cancer epigenome. In summary, these RNAs could be new cancer diagnostic and therapeutic targets.

Therapeutic Targeting of the Tumor-promoting Arm of TGF-β

Because of the broad panel of functions of TGF-β during tumor metastasis and other diseases, multiple therapeutic agents have been developed to block TGF-β ligand and/or receptor activity as well as SMAD functions. The four major classes of TGF-β pathway inhibitors include ligand traps, antisense oligonucleotides (ASOs), small molecule receptor kinase inhibitors, and peptide aptamers.

A pan-neutralizing anti-mouse TGF-β monoclonal antibody, 1D11, has been developed to bind all three TGF-β isoforms and reduce their biological activities (**Fig. 5**) [98]. 1D11 has been shown to reduce the expression of both Gli2 and PTHrP and to significantly inhibit bone tumor metastases induced by NMuMG cells [52]. Furthermore, fully humanized pan-TGF-β neutralizing monoclonal antibodies were developed by Genzyme for use in patients, including Fresolimumab (GC1008) [99], Metelimumab (CAT192) [100], and Lerdelimumab (CAT-152) [101,102] (**Fig. 5**). In another therapeutic strategy, ASOs are used to reduce TGF-β synthesis. ASOs can hybridize to their complementary RNA sequence and induce mRNA degradation. Trabedersen (AP 12009) has been developed as an ASO specifically targeting human TGF-β2 RNA and inhibiting TGF-β mediated metastasis [103].

Targeting of receptor kinase activity has been another successful therapeutic strategy. SB-431542, a small molecule inhibitor of TβRI developed by GlaxoSmithKline has been widely used in basic research studies. Similar TβRI inhibitors, including Ki26894, LY364937, and SD-208, also have been shown to block TGF-β signaling and reduce invasion and motility of breast cancer cells (**Fig. 5**) [104–106]. In addition, recombinant Fc-fusion proteins containing the soluble ectodomain of TβRII (TβRII-Fc) have been shown to inhibit TGF-β signaling and to reduce breast tumor metastasis in transgenic mice [107].

Another class of therapeutics is formed by peptides, which have a high specificity to bind targets *in vivo* and have relatively few

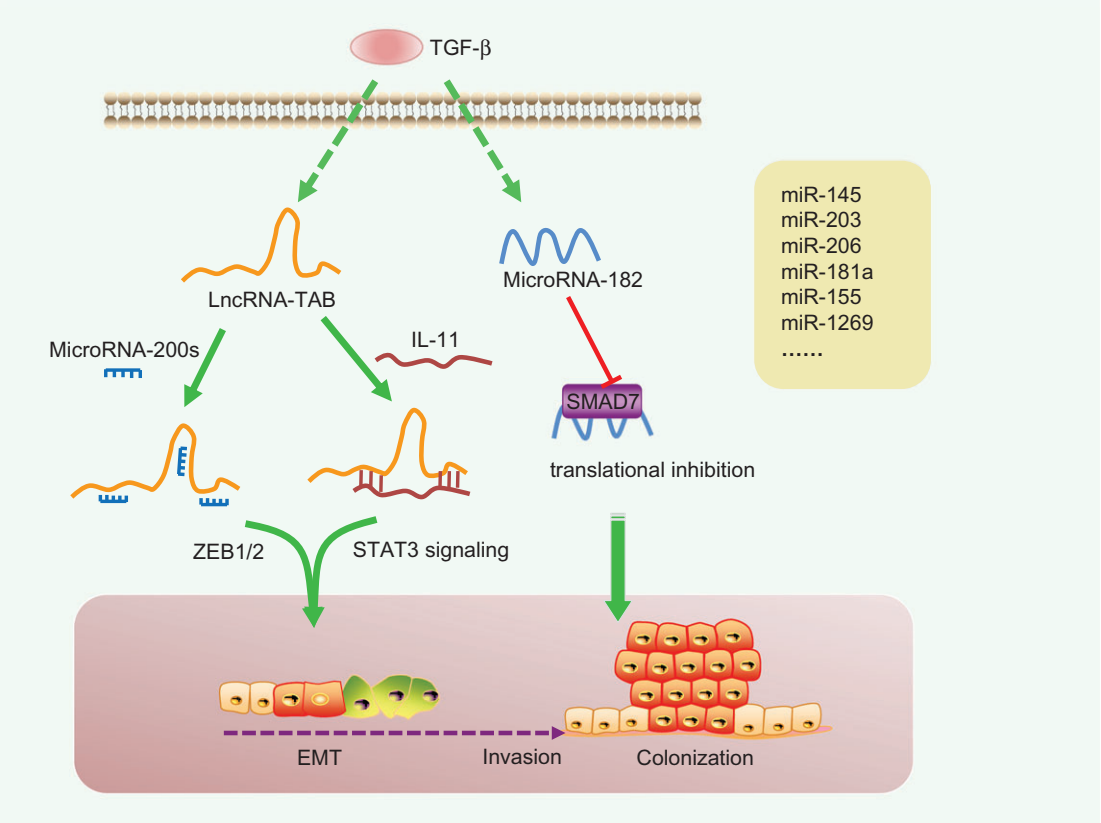

Figure 4. The role of lncRNAs, miRNAs, and ceRNAs in TGF-β signaling On one hand, lncRNA-ATB promotes EMT and invasion. LncRNA-ATB, upregulated by TGF-β, functions as a ceRNA for miR-200 family to upregulate ZEB1/2. On the other hand, lncRNA-ATB promotes HCC cell colonization at the site of metastases. The interaction of lncRNA-ATB with interleukin-11 (IL-11) mRNA enhances signal transducer and activator of transcription3 (STAT3) signaling. MiR-182 antagonizes the response of SMAD7 to TGF-β and promotes cancer cell EMT, invasion and metastasis.

off-target side effects compared with other small molecules [108,109]. A very recent paper described a TAT-SNX9 peptide that inhibits pro-fibrotic effects of TGF-β in murine cells and human lung fibroblasts [110]. Sorting nexin 9 (SNX9) belongs to the PX/BAR subfamily of intracellular trafficking proteins [111–113]. SNX9 binds to phosphorylated SMAD3 (pSMAD3) and promotes pSMAD3 (but not pSMAD2) nuclear import together with importin8 and importinβ (**Fig. 5**) [114]. Trx-SARA aptamer represents another TGF-β-pathway inhibitory peptide, designed to bind SMAD2 and SMAD3 and thereby disrupt their interaction with SMAD4 (**Fig. 5**) [115]. Trx-SARA treatment has been shown to inhibit TGF-β-induced EMT.

In addition, Ying *et al.* [116] recently identified chaperonin containing TCP1 subunit 6 A (CCT6A) as a new inhibitor which can directly bind with SMAD2 to suppress SMAD2 function and promote TGF-β-induced metastasis in NSCLC. This study indicated that targeting SMAD3 or the specific SMAD2 inhibitor CCT6A may represent a novel anti-metastasis strategy for specifically targeting the tumor-promoting branch of TGF-β signaling in NSCLC (**Fig. 5**). Combining anti-CCT6A or anti-SMAD3 agents with TGF-β inhibitors may achieve more efficient suppression of metastasis.

Conclusion

TGF-β signaling plays complex roles in tumor progression. Much still needs to be learned about the TGF-β signaling-related processes that initiate and enforce tumor metastasis. Recent advances have further exposed the mechanisms of TGF-β signaling involved in cancer and provided deeper understanding of the cell type, tissue-specific and context-dependent functions of TGF-β, as well as of its downstream components and modulating factors. However, development of treatments that can specifically stop or slow down the growth of primary tumors and the ever-emerging problem of metastasis is urgently needed. There are three potential strategies: (i) inhibiting TGF-β itself or downstream signaling; (ii) blocking the interplay between TGF-β signaling and other signaling pathways in cancer; (iii) switching TGF-β's tumor-promoting function back to its tumor-suppressive function.

Given the experimental evidence showing TGF-β's role in cancer metastasis, TGF-β and components of TGF-β signaling have indeed been regarded as candidates for anti-metastasis therapies, and multiple therapeutic agents have been developed to block TGF-β and its signaling. Clinical trials with several of these agents are currently in progress. Some drugs have reached Phase III clinical trials for a number of disease applications, particularly cancer progression and metastasis.

Further investigation should focus on the cross-talk between TGF-β signaling and other signaling pathways. For instance, SMAD2/3 interaction with YAP/TAZ, effectors of the Hippo pathway, plays an important role in SMAD nuclear import. A recent study reported that Hippo pathway activation by RAS effector and Hippo kinase scaffold RASSF1A negatively regulates the nuclear YAP1/SMAD2 fraction, leading to limited TGF-β transcriptional activation [117]. In the absence of YAP/TAZ, R-SMADs fail to

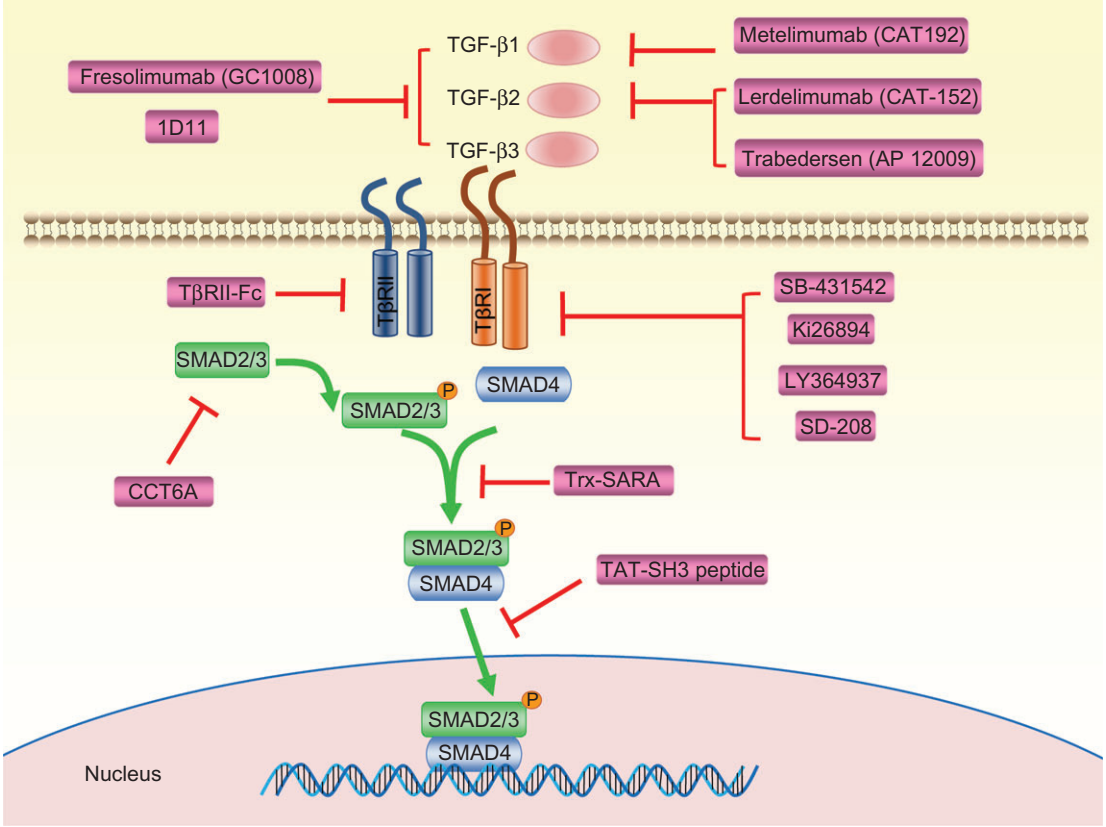

Figure 5. Therapeutic targeting of TGF-β signaling A range of anti-TGF-β-signaling drugs have been developed for cancer therapy. The four major classes of TGF-β inhibitors include ligand traps such as 1D11 or Fresolimumab (GC1008), antisense oligonucleotides (ASOs) like Trabedersen (AP 12009), small molecule receptor kinase inhibitors (e.g. SB-431542, Ki26894, and LY364937), and peptide aptamers (e.g. Trx-SARA and TAT-SNX9).

accumulate in the nucleus, which blocks TGF-β signaling. Recent studies also expand our understanding of the interplay between the TGF-β/SMAD and oncogenic activation of the PI3K/AKT pathway in cancer progression [33,118], which explains how TGF-β can be switched from a tumor suppressor to a tumor promoter. Disrupting the cross-talk of TGF-β with other oncogenic signaling pathways might therefore be one of the most promising approaches for therapeutic intervention.

Importantly, both the TGF-β pathway and other signaling cascades including Wnt and EGFR/RAS can, often cooperatively, induce EMT during cancer metastasis. EMT is driven by an interactive network of transcriptional repressors including SNAIL1, SNAIL2 (also known as SLUG), ZEB1, ZEB2, and KLF4 [119–121]. In cancer cells, TGF-β-induced EMT promotes invasiveness and stem cell-like features [122]. However, a recent study provided a novel concept in which TGF-β can suppress tumorigenesis by inducing a lethal EMT program, involving conversion of transcription factor Sox4 from pro-tumorigenic to pro-apoptotic [123]. Previously, EMT and apoptosis were viewed as separate fates for TGF-β-stimulated cancer cells, and opposite poles of the duality of TGF-β in cancer. However, in mutant Ras expressing pancreatic ductal adenocarcinoma (PDA) cells, TGF-β was found to induce a SMAD4-dependent EMT by promoting remodeling of the transcription factor landscape, including repression of the gastrointestinal lineage-master regulator Klf5. Klf5 cooperates with Sox4 in oncogenesis and prevents Sox4-induced apoptosis. This provides a new potential strategy for cancer therapy, i.e., inducing cancer cell apoptosis by targeting appropriate transcription factors.

Finally, a large number of context-dependent factors contribute to the phenotypic outcomes of TGF-β signaling. For instance, the interplay between TGF-β and inflammatory signaling in both tumor cells and the stromal cells also offer key clues for the development of new anticancer drugs. Obviously, possible side effects of these strategies in normal tissues need to be carefully considered. With the current advances in DNA sequencing and transcriptional and proteomic profiling, it is likely that genomic, transcriptional and proteomic profiles will be determined within clinical trials in the future. Blood-based tumor cell-derived material, such as circulating tumor cells (CTCs), cell-free tumor DNA, and tumor-derived secreted micro-vesicles (termed exosomes), can be isolated and analyzed from patients before and during clinic treatment [124–126]. Pre-clinical and clinical trials are therefore expected to result in higher efficiency and less off-target effects in the future.

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