

# The involvement of circular RNAs in breast cancer metastasis: Insight into EMT-related signaling pathways

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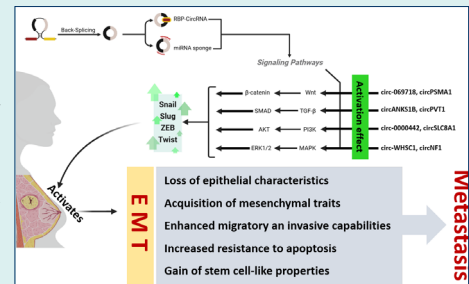
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## Abstract

Breast cancer, a complex and heterogeneous disease marked by uncontrolled cell growth in breast tissue, presents a significant global health challenge due to its high mortality rate. Metastasis, a major driver of cancer progression and mortality, poses a significant challenge in clinical oncology. This complex process involves profound changes in cytoskeletal dynamics and interactions with the extracellular matrix, including integrin-mediated adhesion and the activity of matrix metalloproteinases (MMPs). Circular RNAs (circRNAs) are a type of endogenous non-coding RNA with a covalently closed-loop structure. They serve as critical regulators of gene expression and modulate essential cellular processes, including proliferation, cell cycle control, and invasion. Through intricate interactions with key signaling pathways, including Wnt/ $\beta$ -catenin, PI3K/AKT, TGF- $\beta$ /SMAD, and the MEK-MAPK pathway, circRNAs modulate these processes, particularly epithelial-to-mesenchymal transition (EMT), which is crucial in breast cancer metastasis. Mechanistically, circRNAs activate transcription factors associated with EMT, such as Slug, Snail, Twist, and Zeb. Moreover, circRNAs have emerged as promising clinical biomarkers in breast cancer, offering new opportunities for early diagnosis, prognostic assessment, and the development of targeted therapeutic strategies. In this article, the role of circRNAs in modulating EMT-regulating signaling pathways in the context of breast cancer metastasis will be reviewed.



## Introduction

Worldwide, breast cancer is the 5th leading cause of cancer-related death and the most prominent cause of cancer-related death in women. As a highly heterogeneous disease, understanding its tissue origin and classification is critical for effective diagnosis and treatment. The breast is anatomically composed of glandular, stromal, and lymphatic tissues.<sup>1,2</sup> The glandular tissue is primarily responsible for the synthesis and secretion of milk. The cells most commonly involved in breast cancer originate from the glandular tissue, which consists of ducts (facilitating milk transport) and lobules (functioning in milk production).<sup>2</sup> To guide clinical management and research, breast cancer is classified based on both histopathological and molecular features. Histologically, lobular carcinoma in situ (LCIS) and ductal carcinoma in

situ (DCIS) are the two main types of non-invasive breast cancer, and infiltrating (or invasive) ductal carcinoma (IDC) and infiltrative lobular carcinoma (ILC) constitute the two main invasive types of breast cancer. It should be noted that IDC is responsible for about 80% of breast cancer's total incidence, while ILC is responsible for 10-

## Significance

The importance of studying circRNAs lies in the fact that they are in close contact with the regulation of various biological processes such as proliferation, gene expression, metastasis, and can also be indicators of pathological conditions. Investigating the circRNA profile in patients with metastatic breast cancer is seen as the initial crucial step towards leveraging circRNAs to combat breast cancer and its metastatic progression, ultimately enhancing prognosis through targeted therapeutic interventions.



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## Review Highlights

### What is the current knowledge?

- Circular RNAs act as miRNA sponges and regulate gene expression in cancers.
- EMT drives breast cancer metastasis via TGF- $\beta$ , Wnt/ $\beta$ -catenin, PI3K/AKT, and MAPK pathways.
- circRNA-miRNA axes modulate signaling components controlling EMT and metastatic traits.
- circRNAs influence post-EMT processes: ECM remodeling, invasion, angiogenesis, and immune evasion.
- Many circRNA-EMT interactions are characterized in other cancers but underexplored in breast cancer.

### What is new here?

- This review synthesizes circRNA-mediated regulation of EMT specifically in breast cancer metastasis.
- We highlight circANKS1B, circ-0000043 and others that promote EMT via miRNA/Smad pathways.
- Evidence shows circRNAs both activate and inhibit Wnt, PI3K/AKT, and MAPK signaling affecting EMT.
- We emphasize circRNAs' roles in post-EMT events and potential therapeutic targeting opportunities.
- We propose targeted research directions to validate circRNA-based biomarkers and anti-metastatic strategies.

15% of it. Molecular subtyping, which is determined by the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2, further stratifies tumors into luminal A, luminal B, HER2-positive, and triple-negative subtypes (Table 1).<sup>3-5</sup>

Studies have reported the risk factors involved in breast cancer in the form of two categories. The first category is assigned to non-manipulable conditions such as genetic background, mutation, aging, and race. On the contrary, the second category refers to conditions that a person has the ability to improve or eliminate, such as alcohol consumption, smoking, obesity, drugs, and chemicals.<sup>14,15</sup> By integrating anatomical, histological, molecular, and epidemiological perspectives, we set the stage to explore how dysregulated regulatory networks drive breast cancer metastasis.

The metastatic cascade in cancer involves multiple sequential stages, beginning with cellular changes that promote increased motility and detachment from the primary tumor. Subsequent phases include molecular adaptations that enable cancer cells to intravasate into, survive within, and extravasate from the circulatory system, thereby facilitating colonization of distant organs.<sup>16,17</sup> In breast cancer, epithelial-mesenchymal transition (EMT) is recognized as a critical early event in

metastasis, characterized by the loss of epithelial polarity and intercellular adhesion, coupled with the acquisition of migratory and invasive mesenchymal traits.<sup>16</sup> Multiple signaling axes, including TGF- $\beta$ /SMAD, Wnt/ $\beta$ -catenin, PI3K/AKT, and MEK/MAPK, converge on EMT-associated transcription factors such as Snail, Slug, and Twist.<sup>18</sup> Elucidating the signaling-based molecular regulators that orchestrate these critical metastatic processes is essential for the development of novel and effective therapeutic strategies.

Circular RNAs (circRNAs) are a large class of long non-coding RNAs characterized by a covalently closed loop structure, lacking the typical 5' cap and 3' poly(A) tail found in linear RNAs. The covalently closed structure resulting from back-splicing endows these molecules with remarkable stability.<sup>19</sup> Functionally, many circRNAs act as molecular sponges for microRNAs (miRNAs), thereby modulating gene expression post-transcriptionally. In addition, they can interact with RNA-binding proteins, serve as scaffolds for protein complexes, or, in some cases, be translated into peptides, expanding their functional repertoire. Accordingly, their involvement in key processes like proliferation, gene expression, and metastasis makes circRNAs potential indicators of pathological conditions.<sup>20</sup> Among these pathological processes, metastasis in breast cancer remains the leading cause of mortality, making it a critical focus for exploring the regulatory roles of circRNAs. Given EMT's pivotal role in cancer cell dissemination, understanding its circRNA-mediated control offers valuable insight into breast cancer metastatic progression. In this review, relevant studies were gathered from major databases without language restrictions to clarify how circRNAs contribute to breast cancer metastasis, particularly through their involvement in EMT and associated signaling pathways.

### Breast cancer pathogenesis

The development and progression of breast cancer are influenced by three major factors: hormonal, genetic, and environmental. These factors collectively contribute to breast tumorigenesis by promoting cellular proliferation and suppressing apoptosis.

(i) Hormonal factors: Estrogen and progesterone are the physiological triggers of the development of the female reproductive system through their receptors, ER and PR, respectively.<sup>21</sup> Interestingly, ER and PR expression is restricted to a subset of terminally differentiated breast epithelial cells, which are non-proliferative. Therefore, most of the breast cells are not sensitive to these hormones and divide independently of ER and

**Table 1.** Molecular subtypes of breast cancer

Molecular Subtype	Receptor Status	Prognosis	Incidence (%)	Treatment	Common Metastatic Site	Ref.
Triple-negative (basal-like)	ER-, PR-, HER2-	Poor	15-20	Targeted therapy	Lung, Brain, Bone	6, 7
HER2 +	ER-, PR-, HER2 +	Variable	10-15	Targeted therapy	Liver, Brain	8, 9
Luminal A	ER +, PR +, HER2-	Good	40	Endocrine and Targeted therapy	Bone, Liver	10, 11
Luminal B	ER +, PR +, HER2 +/-	Variable	20	Endocrine and Targeted therapy	Bone, Lung, Liver	12, 13

PR. This small population, in response to estrogen and progesterone, by secreting mediators such as Wnt and insulin-like growth factor 2, triggers the development and proliferation of adjacent cells. Unlike normal breast cells, the majority of breast cancer cells are ER and PR positive. Consequently, estrogen and progesterone play a pivotal role in promoting breast cancer cell growth.<sup>22,23</sup> In addition to the oncogenic role, some studies have partially revealed a tumor-suppressive role for certain estrogen receptor isoforms, which can be valuable in the handling of breast cancer.<sup>24</sup>

(ii) Environmental factors: Metalloestrogens are divalent metals, such as cadmium, copper, zinc, iron, nickel, aluminum, arsenic, cobalt, and tin, that can mimic estrogenic activity by binding to the hormone-binding domain of estrogen receptor alpha (ER $\alpha$ ). Therefore, by stimulating the expression of the target genes of estrogen, they can stimulate the proliferation and development of breast cancer.<sup>25,26</sup> A large body of studies has acknowledged organochlorine pesticides, solar ultraviolet radiation, and ionizing radiation as environmental factors associated with breast cancer. Even though the escalation in the potential of DNA damage and the molecular level of reactive oxygen species (ROS), because of the mentioned factors, are the first-hand candidates for the connection with breast cancer, in cases such as organochlorine pesticides, the mechanism that increases the risk of breast cancer is still unknown.<sup>27-29</sup>

(iii) Genetic factors: Permanent genetic alterations, including deletions and mutations, increase the risk of breast cancer. The studies conducted to determine the involvement of genetic changes in the overall incidence of breast cancer show that the cause of 5-10% of breast cancer is due to hereditary abnormalities, and 90% is because of acquired changes in genetics.<sup>30</sup> BRCA1 and BRCA2 genes, which are implicated in various processes such as DNA repair, are known as the most likely breast cancer predisposing genes that increase the risk of breast cancer by 11.4-fold and 11.7-fold, respectively.<sup>31</sup> Studies have shown that mutations in BRCA1 and BRCA2 account for nearly 50% of hereditary breast cancer cases.<sup>32</sup> In addition, TP53, PTEN, STK11, CDH1, PALB2, CHEK2, and CHEK2 are also among the most known predisposing genes.<sup>33</sup>

### **Molecular mechanisms of breast cancer metastasis; from EMT to colonization**

The final stage in the evolutionary process of many malignancies is the metastasis-inducing phase, which is directly associated with poor prognosis. In breast cancer, this phase involves the formation of micrometastases at secondary sites, primarily including bone (up to 60%), lung (up to 34%), liver (up to 20%), and brain (up to 16%). However, with advances in early detection, effective screening methods such as mammography have gained clinical significance, leading to a significant reduction in breast cancer-related mortality in recent years.<sup>34-37</sup>

From a molecular perspective, the metastatic process

can be categorized into three phases. The first phase involves the triggering of molecular alterations and the enhancement of dissemination capacity into the circulatory system, primarily characterized by EMT.<sup>16</sup>

EMT is a multistep dedifferentiation process, during which cells acquire a hybrid state expressing both epithelial and mesenchymal markers. This process can be divided into three types: the first primarily functions in embryonic development, the second contributes to wound healing and tissue regeneration, and the third is associated with cancer progression by enhancing metastatic potential.<sup>38</sup>

The activation of EMT-associated transcription factors (e.g., Slug, Snail, ZEB, and Twist) drives a cascade of cellular alterations, the cumulative effect of which culminates in enhanced motility and invasiveness. In more detail, the downregulation of adhesion molecules such as E-cadherin, occludin, claudins, desmoplakin, EpCAM, and plakophilin, alongside the upregulation of mesenchymal markers associated with cellular motility, including N-cadherin, vimentin, fibronectin, integrin  $\beta$ 6, and  $\alpha$ -SMA, represents a prominent cellular strategy for detachment from the surrounding matrix.<sup>39,40</sup> In parallel, the induction of cytoskeletal remodeling and the activation of matrix metalloproteinases (MMPs) such as MMP15, MMP2, and MMP9 further potentiate this migratory and invasive phenotype.<sup>39,41</sup> It is noteworthy that integrin, as an adhesion receptor implicated in tissue integrity, activates MMP.<sup>42</sup>

However, the primary challenge associated with cellular detachment during EMT is the induction of programmed cell death triggered by loss of contact with the extracellular matrix, known as anoikis.<sup>43</sup> Circumventing anoikis involves multiple mechanisms, among which the upregulation of anti-apoptotic components such as survivin, Bcl-2, and Bcl-xL, along with the activation of Focal Adhesion Kinase (FAK)-dependent survival pathways, are the most well-established.<sup>16</sup> Nevertheless, studies have also documented the roles of Twist and ZEB1 in enhancing cell survival potential during EMT through mechanisms involving the suppression of p14ARF and p73, respectively.<sup>44</sup>

The second phase pertains to the plasticity of circulatory tumor cells (CTCs). Phenotypic plasticity in CTCs allows them to dynamically oscillate between epithelial and mesenchymal states. This plasticity is controlled by signaling pathways such as TGF- $\beta$ , hypoxia-induced HIF-1 $\alpha$ , Wnt/ $\beta$ -catenin, Notch, and Hedgehog, which in turn activate EMT transcription factors including Snail, Slug, Twist, and ZEB1/2.<sup>16</sup> As a result, CTCs often acquire a hybrid epithelial/mesenchymal phenotype that confers enhanced survival and motility, as well as stem-like traits such as self-renewal and unlimited proliferative capacity.<sup>45</sup>

A cancer cell that enters the bloodstream implements tactics to escape from life-threatening factors, such as the immune system and apoptosis, induced by the lack of attachment to the matrix. One of them is activation and attachment to platelets, respectively, through the

augmented expression of tissue factor and P-selectin ligands.<sup>46</sup> Furthermore, platelets secrete TGF- $\beta$ , which promotes the epithelial-mesenchymal transition (EMT). They also physically shield tumor cells from NK cell-mediated lysis. In this regard, studies have shown that increased platelet count is associated with poor prognosis in malignancies.<sup>47</sup>

The third phase involves the extravasation of CTCs to colonize and establish microscopic tumors at secondary sites. This phase hinges on a finely tuned interplay among CTCs, endothelial cells, and extracellular matrix (ECM) components, in which adhesion molecules (selectins, ICAM, and VCAM) initiate vascular arrest and MMPs orchestrate basement membrane degradation and tissue invasion.<sup>48,49</sup> Effective colonization then demands tumor cell adaptation to the new microenvironment, immune evasion, and neangiogenesis. At this stage, phenotypic plasticity marked by a partial return to epithelial traits is important for successful engraftment.<sup>50</sup> Notably, some disseminated cells may enter dormancy, retaining the capacity for later reactivation and secondary lesion formation.<sup>51</sup>

A concise overview of this cascade clearly highlights the critical importance of the EMT process; therefore, focusing on this aspect could represent a significant step forward in overcoming metastasis in breast cancer.

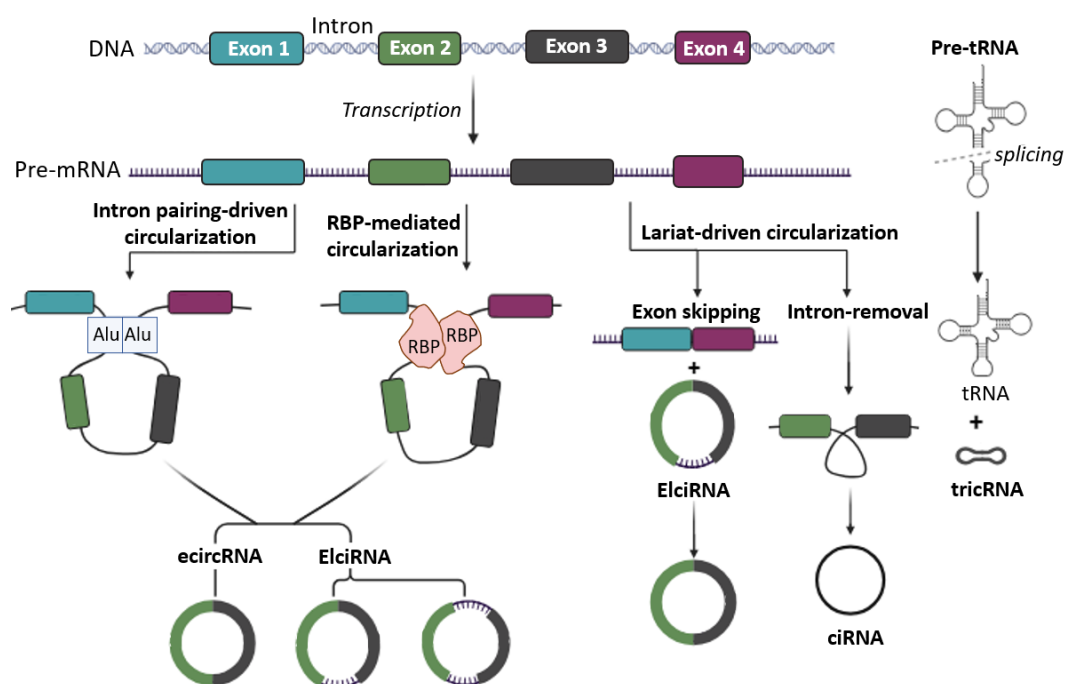
### Circular RNAs: biogenesis and methodological landscape

Formation of circRNAs from pre-mRNAs predominately occurs by back splicing or exon skipping. The closed-loop form of this class of non-coding RNAs provides durability against exonucleases due to the absence of free ends. This means that the 3' and 5' ends are linked via covalent bonds.<sup>20</sup>

Currently, there is significant interest in three models that elucidate the formation process of circRNAs. Model 1: RNA binding protein (RBP)-mediated circularization, which is based on the regulatory effect of various RBPs, such as DHX9, QKI, and MBL, on adjoining splicing sites. Model2: Circularization based on intron pairing, which is based on the pairing of reverse complementary sequences, especially Alu elements, in adjacent introns. Model3: Exon skipping or lariat-based circularization, which is based on the formation of a lariat with RNA splicing. This process can be explained by joining the 3' end of one exon with the 5' end of another exon and separating the intron and exon between these two exons in a circular form (Fig. 1).<sup>52,53</sup>

The categorization of circRNAs into four distinct groups based on their constituent parts has opened avenues for deeper investigation. In addition, the names of the four groups are clearly related to their components, including (i) exonic circular RNA or ecircRNA, (ii) circular intronic RNA or ciRNA, (iii) exon-intron circular RNA or EICIcRNA, and (iv) tRNA-intronic circular RNA or tricRNA. For instance, ecircRNAs consist solely of exonic sequences, whereas ciRNAs are composed of intronic sequences. EICIcRNAs contain both exonic and intronic regions, while tricRNAs contain tRNA sequences within their structure. This clear categorization facilitates targeted investigations into the roles and regulatory mechanisms of circRNAs in various biological processes and diseases (Fig. 1).<sup>54-56</sup>

Noteworthy, circRNAs contribute to cellular stress responses by modulating key signaling pathways such as MAPK, p53, and NF- $\kappa$ B.<sup>57,58</sup> They are also detected in stress granules, membrane-less ribonucleoprotein structures that help cells adapt to conditions like nutrient deprivation, oxidative stress, heat shock, and viral



**Fig. 1.** Schematic illustration of the core models presented in the construction of circRNA. Circularization based on intron pairing, RNA binding protein (RBP) mediated circularization and Exon skipping or lariat-based circularization. The circRNA family encompasses four distinct structural groups: exonic circular RNA (ecircRNA), circular intronic RNA (ciRNA), exon-intron circular RNA (EICIcRNA), and tRNA-intronic circular RNA (tricRNA).

infection.<sup>59</sup> Stress granules temporarily halt translation and protect mRNAs. While the presence of circRNAs in stress granules suggests their involvement in stress responses, much about their specific role remains unclear.<sup>60</sup>

Studies in this area typically start with whole-transcriptome RNA sequencing (RNA-seq) paired with specialized bioinformatic pipelines to detect backsplice junctions characteristic of circRNAs.<sup>61</sup> Because circRNAs constitute only a minor fraction of total RNA, samples are usually enriched by depleting ribosomal RNA and selecting against polyadenylated transcripts, often combining both approaches to maximize circRNA recovery.<sup>62,63</sup> In some workflows, Rnase R treatment is also applied to selectively degrade linear RNAs, further enhancing the relative abundance of circular species.<sup>61</sup> Definitive validation hinges on PCR amplification of the backsplice junction using divergent primers: each primer anneals just outside the junction and faces away from its partner, yielding a product only if the template is truly circular.<sup>64</sup> Ultimately, northern blotting remains the gold standard for directly visualizing circRNA size and topology.<sup>65</sup>

Several methodological challenges complicate circRNA detection. For instance, backsplice junction reads from RNA-seq may result from repeated exonic regions or overlapping transcripts rather than true circular RNAs. Moreover, reverse transcriptase can sometimes switch templates during cDNA synthesis, creating chimeric artifacts that resemble circular junctions in PCR. It should be noted that circRNA prediction algorithms vary considerably in their stringency and detection criteria. Studies have shown that between 10% and 45% of predicted circRNAs may represent false positives, and approximately 40% of identified candidates are reported by only a single detection tool.<sup>62,66</sup>

For future circRNA research, it is essential to integrate multiple detection algorithms with biochemical verification methods and to employ unique molecular identifiers to minimize artefacts.<sup>67-69</sup> Moreover, adopting three-dimensional culture systems and single-cell RNA sequencing, together with comprehensive integration of TCGA datasets and nanopore sequencing data, will

facilitate the direct translation of experimental findings into clinical applications.<sup>70-72</sup>

### Role of circRNAs in metastatic cascade

A large body of studies has demonstrated the multifaceted involvement of circRNAs in the metastatic cascade of breast cancer. In light of the critical role of EMT, we delineate these interactions into two main domains: circRNA-mediated regulation of EMT and circRNA-driven control of other metastatic phases.

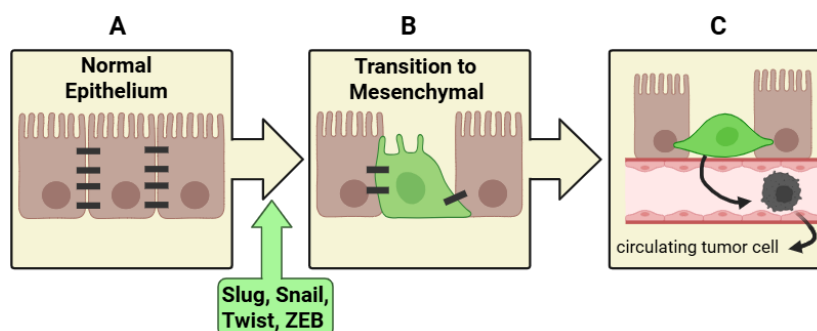
### Circular RNAs in the regulation of early-stage EMT

As previously stated, EMT is a multistep dedifferentiation process that converts polarized epithelial cells into non-polarized mesenchymal cells. Four general steps make up the EMT process in cancer cell metastasis: 1) initiation, 2) loss of epithelial properties, 3) gain of mesenchymal characteristics, and 4) remodeling of cytoskeleton and cell morphology (Fig. 2).

Transcription factors directly implicated in inhibiting CDH1 expression include the Snail family (Snail, Slug, Smuc), ZEB family (Zeb1/2), and b-HLH family (Twist1/2). Additionally, there are indirect repressors of CDH1, such as FoxC2, Goosecoid, TCF4, and PRRX1. These regulatory elements collectively drive the progression of EMT, facilitating the phenotype transformation.<sup>16</sup>

In the subsequent phase, crucial proteins responsible for maintaining cell-to-cell connections and apical-basal cell polarity, including those involved in tight junctions, adherens junctions, and gap junctions, are downregulated. In other words, proteins such as E-cadherin, cytokeratin, connexins, desmoplakin, zonula occludens, beta-catenin, and occludin experience systematic reduction during this phase of cellular transformation.<sup>16</sup> The cell develops mesenchymal traits in the following stage. A closer look reveals that the cell exhibits a notable resistance to apoptosis and that its motility/invasive capacity is increased by the elevated expression of mesenchymal markers such as vimentin, fibronectin, N-cadherin, and MMPs.<sup>16,73</sup>

The three main components of the cytoskeleton are intermediate filaments, microtubules, and actin.<sup>74,75</sup> Intermediate filaments, mainly composed of keratins and vimentin, provide structural support and flexibility



**Fig. 2.** Schematic illustration of the EMT process. (A) Normal epithelium: Epithelial cells maintain robust intercellular adhesions through well-organized junctional complexes, preserving tissue architecture and polarity. (B) EMT induction: Upregulation of EMT-associated transcription factors, including Snail, Slug, Twist, and ZEB, leads to the repression of epithelial markers and activation of mesenchymal genes, resulting in morphological changes and enhanced cellular motility. (C) Invasion and intravasation: Mesenchymal-like tumor cells detach from the primary epithelium and enter the bloodstream, enabling them to infiltrate distant sites and establish metastatic colonies.

to cells.<sup>76</sup> Notably, EMT involves a switch from epithelial-specific keratins to mesenchymal-specific vimentin, a hallmark of this transition.<sup>77,78</sup> Actin stress fibers, formed during EMT, offer contractile strength essential for cell movement and invasion.<sup>79,80</sup> Meanwhile, the disorganized microtubule network in mesenchymal cells promotes the creation of membrane protrusions like filopodia and lamellipodia, aiding cell movement.<sup>81,82</sup> These fundamental alterations in the cytoskeleton induce morphological changes, transitioning cells from their typical cuboidal epithelial shape to an elongated, spindle-like mesenchymal morphology.<sup>83</sup> Overall, a cell gains the ability to separate from the primary site, enter and exit blood and lymphatic vessels, colonize the secondary site, and form microscopic metastatic tumors after passing through the transformational process of EMT.<sup>16</sup> The intimate connection between signaling pathways and the molecular alterations driving EMT progression is readily apparent. Therefore, by focusing investigations on the circRNA-signaling effector axis, researchers can uncover novel regulatory mechanisms and identify potential therapeutic targets to modulate EMT.

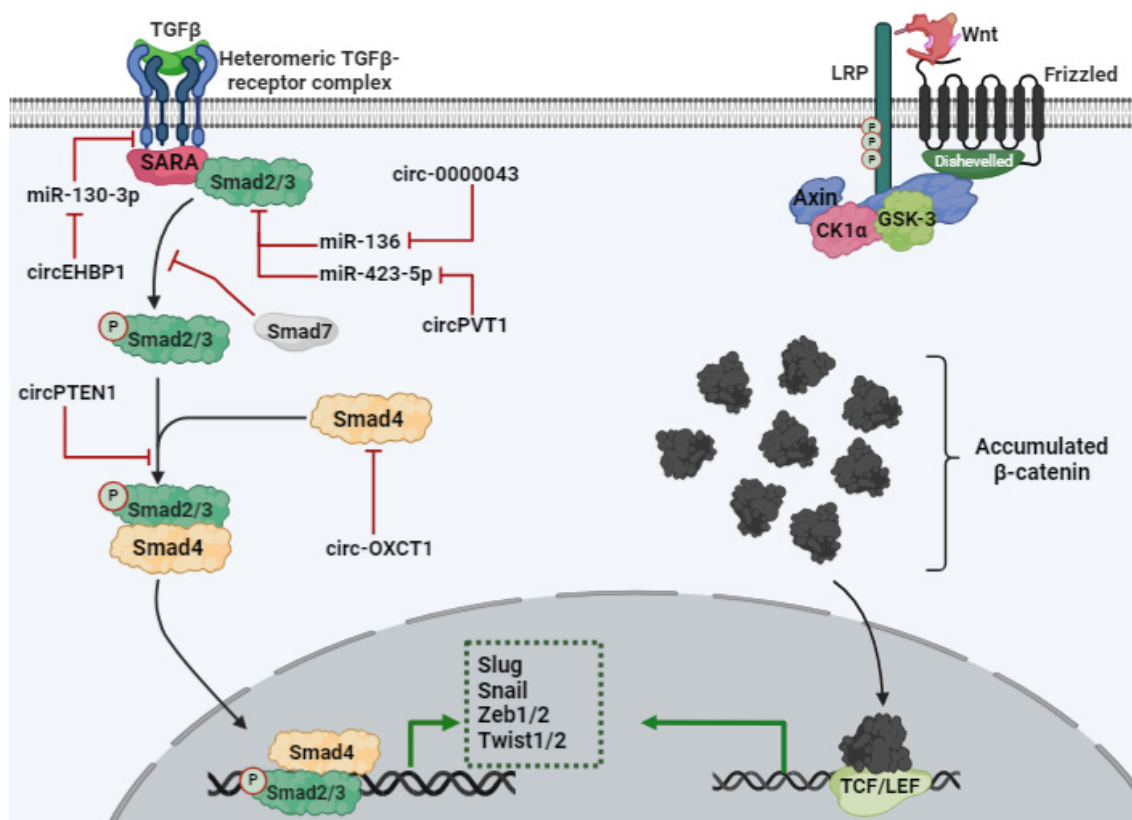
#### TGF $\beta$ /SMAD mediated regulation of EMT

Both TGF- $\beta$ RI and TGF- $\beta$ RII possess serine/threonine kinase activity; however, TGF- $\beta$ RII is constitutively active.<sup>84</sup> Upon ligand (in dimer form) binding, TGF- $\beta$ RI and TGF- $\beta$ RII form a heteromeric receptor complex, leading to the activation of TGF- $\beta$ RI through phosphorylation of its cytoplasmic domain by TGF- $\beta$ RII. This phosphorylation event triggers the initiation of

downstream signaling pathways. TGF- $\beta$ RI phosphorylates the SSXS motif of receptor-regulated Smads (R-Smads), such as Smad2 and Smad3, in a process facilitated by SARA. This leads to the formation of R-Smad/Smad4 complexes. Subsequently, these complexes translocate into the nucleus to regulate gene expression. It's worth noting that Smads are mediators of the TGF- $\beta$  family, and TGF- $\beta$ RI phosphorylates both Smad2 and Smad3, members of the R-Smad family, in response to TGF- $\beta$  signaling. After translocation into the nucleus, the R-Smad/Smad4 complex regulates gene expression. However, Smad7 (an inhibitory Smad) can suppress this pathway by blocking the activation of TGF- $\beta$ RI.<sup>85-92</sup> Finally, Smad can regulate the expression of EMT acceleration factors such as Slug, Zeb, Snail, and Twist (Fig. 3).<sup>93</sup>

The research findings demonstrate a correlation between EMT and the TGF- $\beta$  signaling pathway. Elevated levels of TGF- $\beta$ , induced by the activation of the upstream stimulatory family 1 (USF1), have been associated with increased EMT. Notably, studies suggest that miR-148a-3p and miR-152-3p act as inhibitors of USF1, thereby regulating EMT. CircANKS1B, identified for its role in promoting malignant characteristics in breast cancer cells and exacerbating prognosis, indirectly boosts TGF- $\beta$ -induced EMT by suppressing these two miRNAs.<sup>94</sup> Consistent with these observations, circ-0000043, recognized for its oncogenic properties in breast cancer, exacerbates EMT by suppressing miR-136, through a Smad3-dependent mechanism.<sup>96</sup>

Although studies in this area remain limited in breast cancer, the circRNA/miRNA/TGF $\beta$ /Smad/EMT axis has



**Fig. 3.** Schematic illustration of TGF- $\beta$ /Smad and Wnt/ $\beta$ -catenin signaling pathways. Both pathways are related to EMT, mainly by the expression of transcription factors related to EMT (Slug, Zeb1/2, Snail, and Twist1/2).

been well characterized in other malignancies. Targeted investigations based on these findings may accelerate progress and enhance our understanding of its role in breast cancer metastasis. Several examples stand out clearly in this regard. In gastric cancer, the circRNAs circPVT1 and circOXCT1 exhibit opposing roles in EMT regulation through their interaction with the TGF $\beta$  signaling pathway. CircPVT1 acts as a sponge for miR-423-5p, which leads to the upregulation of Smad3, promoting EMT. Conversely, circ-OXCT1 inhibits EMT by attenuating the TGF- $\beta$  pathway through suppressing Smad4.<sup>97,98</sup> Similarly, research has highlighted the roles of circPTK2 (hsa\_circ\_0008305) and circEHBP1 in modulating EMT. CircPTK2 inhibits EMT in lung cancer by up-regulating TIF1 $\gamma$  through the inhibition of miR-429/miR-200b-3p, thus attenuating the TGF- $\beta$ -induced EMT process. On the other hand, circEHBP1 enhances EMT in bladder cancer by up-regulating TGF- $\beta$ R1 via the inhibition of miR-130-3p.<sup>99,100</sup> Furthermore, in colorectal cancer, circPTEN1 inhibits TGF- $\beta$ -induced EMT by preventing the binding of Smad4 to Smad2/3.<sup>101</sup> Overall, despite the noticeable scarcity of related data, the critical role of circRNAs in the fine-tuned regulation of TGF $\beta$ /SMAD-driven EMT remains a reliable and compelling aspect of current evidence.

#### *Wnt/ $\beta$ -catenin mediated regulation of EMT*

In the Wnt/ $\beta$ -catenin signaling pathway, the control center revolves around the buildup of a crucial cytoplasmic co-activator called  $\beta$ -catenin. When Wnt signaling is inactive, a specialized degradation complex remains highly active, tasked with preventing the accumulation of  $\beta$ -catenin.<sup>102</sup> This complex comprises key components: Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK-3, a Ser/Thr kinase), and casein kinase 1 $\alpha$  (CK1 $\alpha$ ). Axin acts as a central scaffold protein within this complex, facilitating interactions between GSK-3, CK1 $\alpha$ , and  $\beta$ -catenin. Through these interactions,  $\beta$ -catenin undergoes phosphorylation by GSK-3 and CK1 $\alpha$ , marking it for subsequent degradation.<sup>103</sup>

$\beta$ -catenin is ubiquitinated and degraded by the proteasome system after receiving four phosphates (CK1 $\alpha$  adds one phosphate, while GSK-3 adds the three phosphates). CK1 $\alpha$  and GSK-3 also phosphorylate Axin and APC in addition to phosphorylating  $\beta$ -catenin in order to promote  $\beta$ -catenin binding and structural stability of the complex.<sup>104-106</sup>

Wnt binds to the Frizzled receptor (Fz), a seven-pass transmembrane protein, along with its co-receptor LRP5/6. This interaction triggers a series of key events that regulate the Wnt/ $\beta$ -catenin signaling pathway. In other words, Wnt binding causes CK1 $\alpha$  and GSK-3 to phosphorylate LRP5/6. This phosphorylation recruits these kinases to the plasma membrane and enhances their binding to LRP5/6.<sup>107,108</sup> It should be noted that Dishevelled, activated by Frizzled, is crucial for facilitating the interaction of Axin and GSK-3 with LRP5/6. Cytoplasmic accumulation of  $\beta$ -catenin induces

translocation to the nucleus to activate transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family (Fig. 3).<sup>103</sup>

Investigating the impact of the Wnt signaling pathway on facilitating EMT can be approached from three key perspectives: (i) upregulation of EMT-associated transcription factors (such as Zeb and Snail), (ii) disruption of cell-to-cell junctions via preventing  $\beta$ -catenin phosphorylation, and (iii) promotion of stem cell-like characteristics. In triple-negative breast cancer (TNBC), miR-506-3p functions as a tumor suppressor; however, its activity is reduced by circ-0008784, which results in increased activation of the Wnt/ $\beta$ -catenin pathway. Furthermore, circ-0008784 promotes oncogenic properties and upregulates mesenchymal markers.<sup>109</sup> Conversely, circ-ITCH, which suppresses miR-214 and miR-17, counteracts TNBC's oncogenic properties by overwhelming the Wnt/ $\beta$ -catenin pathway.<sup>110</sup> Recent research has shed light on the pivotal role of CircABCC4 in the progression of breast cancer. Its regulatory role in promoting the activation of NF- $\kappa$ B and Wnt/ $\beta$ -catenin signaling pathways through miR-154-5p inhibition, coupled with the suppression of apoptosis and enhancement of metastatic potential in cancer cells, has been comprehensively elucidated.<sup>111</sup> On the other hand, investigations have confirmed an elevated expression pattern of circRNA-069718 in patients with TNBC. CircRNA-069718 knockdown has demonstrated a notable reduction in the levels of key components of the Wnt/ $\beta$ -catenin pathway.<sup>112</sup> CircPSMA1, by inhibiting miR-637, is implicated in promoting the proliferation and metastasis of TNBC. This is expected to occur through upregulating  $\beta$ -catenin expression via Akt1, which is a target of miR-637.<sup>113</sup> CircRNA seldom yields peptides, but instances like the production of EIF6-224aa from hsa\_circ-0060055 (circ-EIF6) demonstrate its possibility. EIF6-224aa enhances MYH9 stability, thereby bolstering the activity of the Wnt/ $\beta$ -catenin pathway. While the circ-EIF6 oncogenic role in breast cancer is determined, its association with EMT warrants further investigation.<sup>114</sup>

Interactions between circRNAs and the EMT pathway have been observed in various malignancies, including colorectal cancer. In this context, circPDSS1 and circ-0026628 have been found to promote Wnt/ $\beta$ -catenin-induced EMT by suppressing miR-320c and miR-346, respectively.<sup>115,116</sup> Conversely, increasing levels of circ-0026344 have been shown to inhibit EMT by targeting the miR-183-dependent Wnt/ $\beta$ -catenin signaling pathway in colorectal cancer. Additionally, the inhibition of EMT in the case of increasing circ-0026344 by hindering the miR-183-dependent Wnt/ $\beta$ -catenin signaling has been reported in colorectal cancer.<sup>117</sup>

Similar to the previous signaling pathway, targeted research in this area can also be proposed. For example, in colorectal cancer, the upregulation of circ-0038718 leads to increased expression of Axin2 by inhibiting miR-195-5p. This observation further highlights the intricate connection between circRNAs and the EMT pathway.<sup>118</sup>

Hsa-circ-000984 and hsa-circ-0007059 have opposite effects on lung cancer cell's potential to metastasis, as evidenced by their relative inhibitory and acceleratory effects on Wnt/ $\beta$ -catenin-induced EMT, respectively.<sup>119,120</sup> These findings, which summarized in Fig. 3, demonstrate that circRNAs critically modulate Wnt/ $\beta$ -catenin signaling to drive EMT, tumor cell invasion, and metastatic spread.

#### PI3K/AKT mediated regulation of EMT

The initiation of PI3K activity is primarily triggered by ligand binding to receptors such as receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs), which serves as the fundamental step for downstream biological processes.<sup>121</sup> This cascade entails the conversion of PIP2 (phosphatidylinositol 4,5-bisphosphate) into PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) via PI3K-mediated phosphorylation. This event crucially localizes and activates AKT (protein kinase B) within the plasma membrane.<sup>103</sup>

Activation of AKT via phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) triggers its full activation, unleashing a cascade of downstream effectors. These include mTORC1, GSK-3, Bad, and FOXO, regulating processes that are implicated in growth, cell survival, homeostasis, and gene expression.<sup>122</sup>

The primary impact of AKT on the EMT process lies in its kinase function in transcription factor activation like, Snail, Slug, Twist1/2, and Zeb1/2.<sup>123</sup> Furthermore, AKT with GSK-3 phosphorylation ensures stability and cytoplasmic accumulation of  $\beta$ -catenin.<sup>124</sup> On the other hand, AKT also serves a central role in modulating key signaling pathways involved in EMT, including the Wnt/ $\beta$ -catenin and TGF- $\beta$  pathways.

Considering the oncogenic nature of the PI3K/AKT signaling pathway and the positive link to EMT, exploring

how the circRNA/miRNA axis affects EMT via this pathway is promising. Reduced levels of hsa-circ-001569 in breast cancer result in diminished malignancy-associated traits, including decreased activity of the PI3K/AKT pathway, lower expression levels of EMT markers at the molecular level, and reduced proliferation. Consequently, it presents a promising therapeutic target for enhancing prognosis in breast cancer cases.<sup>125</sup>

In breast cancer, circSEMA4B functions oppositely by impeding AKT phosphorylation, thereby exerting inhibitory effects on the EMT process.<sup>126</sup> Research has elucidated that miR-338-3p exerts a suppressive function on EMT by targeting Zeb2 in breast cancer.<sup>127</sup> In parallel, circKIF4A is a suppressor of metastasis in TNBC by inhibiting miR-375.<sup>128</sup> Interestingly, PDK1 was mentioned as a target of miR-375 in pancreatic cancer.<sup>129</sup> However, while the inhibitory effect of circ-0000326 on miR-338 has been established in bladder cancer, this relationship remains unexplored in breast cancer, highlighting a notable gap in current understanding.<sup>130</sup> Furthermore, research exploring the relationship between hsa-circ-0003288 and N-cadherin expression levels in hepatocellular carcinoma has demonstrated a supportive role in EMT (Fig. 4).<sup>131</sup> Overall, these studies reveal that circRNAs critically orchestrate PI3K/AKT pathway activity, either by sponging miRNAs or modulating key kinases, to regulate EMT and metastatic behavior. Table 2 presents a summary of additional relevant studies within this field.

#### MAPK-MEK mediated regulation of EMT

One of the most important members of the cellular signaling network is the classical MAP kinase pathway, which is also known as the Ras/Raf/MEK/ERK cascade. It is involved in biological processes including apoptosis, autophagy, differentiation, growth, and metastasis. Stimulation of receptors, mostly GPCRs and RTKs, leads

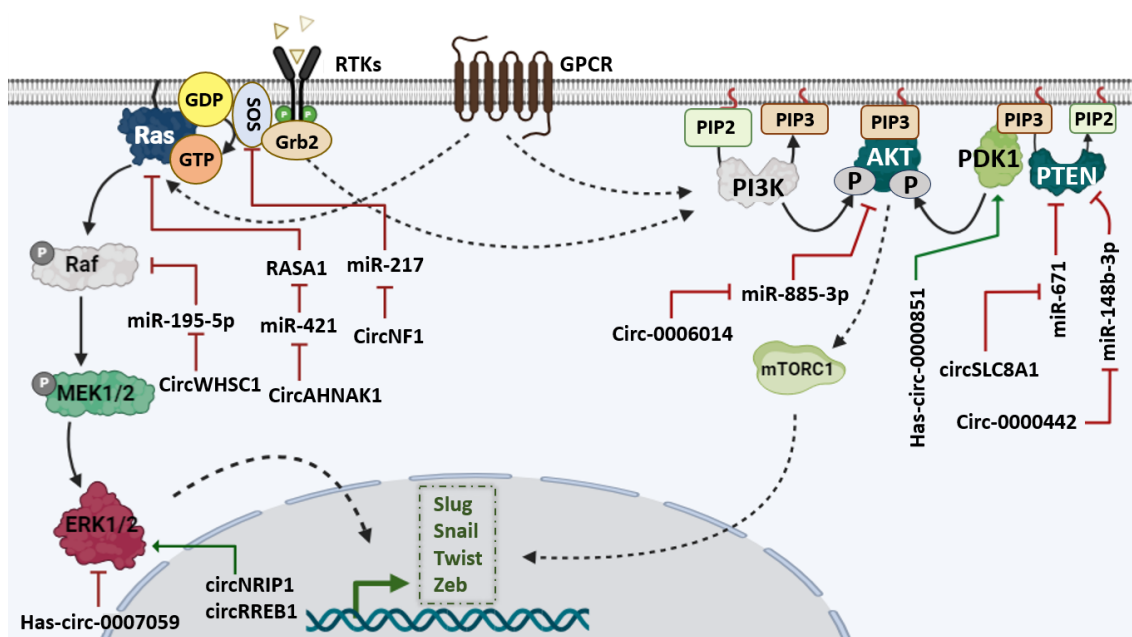


Fig. 4. Schematic picture of the interaction of circRNAs with MAPK-MEK and PI3K/AKT signaling pathways.

to the activation of RAS, a monomeric G-protein, which is mediated by GRB2 and SOS. Active Ras activates a serine-threonine kinase called Raf.<sup>138</sup> In turn, Raf phosphorylates and activates a dual-specificity kinase called MAPK/ERK kinase or mitogen-activated protein kinase (MEK). By phosphorylating ERK, MEK enables the phosphorylation of a wide range of cytoplasmic/nuclear mediators by ERK.<sup>139</sup>

Induction of transcription factors related to EMT, regulation of cytoskeleton and cell-to-cell connection, increase of cell resistance to apoptosis, and regulation of tumor environment to facilitate metastasis (for example, by increasing MMPs) are among the most important effects of the MAPK pathway on progression of EMT in breast cancer. Besides this, the interaction with other pathways facilitating EMT, such as TGF- $\beta$ , Wnt/ $\beta$ -catenin, and Notch, shows another dimension of the effect of the map kinase pathway on EMT.<sup>140-142</sup>

Research into the connection between circRNAs and EMT in breast cancer has highlighted the significant roles of circAHNAK1 and circRREB1.<sup>143</sup> CircRREB1 promotes breast cancer progression by enhancing tumor cell migration and proliferation while inhibiting apoptosis, with activation of ERK1/2 identified as a key underlying mechanism. Conversely, circAHNAK1 is downregulated in triple-negative breast cancer, which abolishes its tumor-suppressive effect. By failing to inhibit miR-421, reduced circAHNAK1 expression leads to decreased RASA1 activity, thereby enhancing Ras-mediated signaling and promoting cancerous traits such as proliferation and metastasis.<sup>144,145</sup>

Other circRNAs implicated in the MAPK-EMT network are summarized in Table 3. Collectively, these findings underscore the crucial regulatory roles of circRNAs in breast cancer metastasis and highlight the circRNA-

MAPK axis as a potential biomarker for assessing tumor progression.

#### *Other pathways-mediated regulation of EMT*

The binding of STAT3 to an active receptor leads to STAT3 phosphorylation and dimerization. After then, dimerized STAT3 is translocated to the nucleus, where it attaches to STAT-binding elements (SBEs) to start the transcription of different genes.<sup>152</sup> Studies have found that elevated levels of circNOLC1 in breast cancer are correlated with decreased E-cadherin and increased vimentin expression. The mechanism by which circNOLC1 enhances the oncogenic characteristics and metastatic potential of breast cancer involves acting as a sponge for miR-365a-3p, thereby sustaining STAT3 activity.<sup>153</sup> Increased expression of circSEPT9 in TNBC is also associated with poor prognosis. The mechanism of circSEPT9 in increasing the occurrence of oncogenic properties, including metastasis, is by inhibiting miR-637 in order to sustain the activity of the Leukemia Inhibitory Factor (LIF)/STAT3 axis.<sup>154</sup> Other malignancies have reported an increase in STAT3 activity associated with circ-0068871, acting as a sponge for miR-124; ciRS-7, acting as a sponge for miR-7; circFCHO2, acting as a sponge for miR-194-5p; and hsa-circ-0000043, acting as a sponge for miR-4492.<sup>155-159</sup>

The notch signaling pathway initiates by binding the notch receptor (Notch-1, -2, -3, and -4) in a cell to the ligand in the adjacent cell. This connection triggers proteolytic reactions that release the intracellular part of the notch receptor (NICD). In turn, NICD transfers to the nucleus and eventually increases the expression of the notch target genes.<sup>103</sup> Although studies have not presented a clear connection between the Notch signaling pathway and circRNA in mediating EMT in breast cancer, recent studies have shed light on the role of hsa-

**Table 2.** summary of circRNAs implication in the PI3K-EMT network

circRNA	Role	Cancer	Target miRNA	Pathway-related miRNA Target	Ref.
circSLC8A1	Anti-tumor	Breast cancer	miR-671	PTEN	132
circ-0000442	Anti-tumor	Breast cancer	miR-148b-3p	PTEN	133
circ-0006014	pro-tumor	Breast cancer	miR-885-3p	NTRK2/PIK3	134
hsa-circ-0000851	pro-tumor	triple-negative breast cancer	miR-1183	PDK1/p-AKT	135
circWAC	pro-tumor	triple-negative breast cancer	miR-142	WWP1/PI3K	136
circ-100395	Anti-tumor	gastric cancer	miR-142-3p	PTEN	137

**Table 3.** summary of circRNAs implication in the MAPK-EMT network

circRNA	Role	Cancer	Target miRNA	Pathway-related miRNA Target	Ref.
circWHSC1	Pro-tumor	breast cancer	miR-195-5p	FASN/AMPK	146
circ-0042881 (circNF1)	Pro-tumor	breast cancer	miR-217	SOS1	147
circAHNAK1	Anti-tumor	triple-negative breast cancer	miR-421	RASA1/Ras	145
hsa-circ-0007059	Anti-tumor	lung cancer	miR-378	Wnt/ $\beta$ -catenin and ERK1/2	120
circ-ZKSCAN1	Pro-tumor	non-small cell lung cancer	miR-330-5p	FAM83A	148
circNRIP1	Pro-tumor	gastric cancer	miR-149-5p	AKT1	149
	Pro-tumor	cervical cancer	miR-629-3p	ERK1/2	150
circ-0087558	Pro-tumor	breast cancer	miR-643	MAP2K6	151

circRNA-102051, circKIF4A, circAPLP2, and circPDK1 in the activation of the Notch pathway by inhibiting miR-203a, miR-375/1231, miR-101-3p, and miR-377-3p, respectively. Conversely, circ-0000390 inhibits the notch as a tumor suppressor. However, these findings require further validation through additional investigations in the field of breast cancer.<sup>160-164</sup>

The Hedgehog signaling pathway starts with connecting secretory ligands (i.e., Hedgehog proteins) to a membrane receptor called a Patched. This connection removes the Patched inhibition effect from the Smoothed. This signaling cascade eventually leads to the activation of transcription factors of the Glioma-associated oncogene (Gli) family, which can increase the molecular level of transcription factors associated with EMT.<sup>103</sup> CircZNF609 in hepatocellular carcinoma promotes Hedgehog pathway activation by suppressing miR-15a-5p/15b-5p, while Circ-0000034 in retinoblastoma enhances this pathway through upregulation of ADAM19 via sponging miR-361-3p.<sup>165,166</sup> Although a substantial portion of circRNA interactions with other signaling pathways remains uncharacterized, the limited evidence available highlights the urgent need to direct research efforts towards this domain.

### **Circular RNAs in the regulation of late-stage; post-EMT events**

Following the occurrence of EMT, events such as invasion, angiogenesis, immune evasion, and colonization at distant sites play a pivotal role in the success of metastasis. In this context, circRNAs act as critical regulators orchestrating these post-EMT processes.<sup>16</sup>

The ECM, composed of both fibrous and non-fibrous components, acts as a protective barrier within tissues, preventing the spread and invasion of cancer cells. ECM remodeling is essential for enhancing the ability of cancer cells to infiltrate the circulatory system and evade immune surveillance during metastasis, as it facilitates the transmission of oncogenic signals. A prominent example is circACAP2, an oncogenic circRNA that is upregulated in breast cancer and promotes the expression of the COL5A1 gene by inhibiting miR-29a-3p and miR-29b-3p.<sup>167</sup> Qu et al demonstrated that the upregulated activity of circRNA-CER causes the high expression of MMP13 through a mechanism dependent on the inhibition of miR-136, consequently accelerating ECM remodeling.<sup>168</sup> Invasion involves specialized actin-rich protrusions known as invadopodium, which cancer cells utilize to degrade the ECM and infiltrate surrounding tissues. A study by Du WW et al. demonstrated that circSKA3 promotes invadopodium formation by complexing with Tks5 and integrin  $\beta$ 1.<sup>169</sup> It is noteworthy that alongside circRNAs that activate invasiveness, such as hsa\_circ\_0052112 and circ\_0103552, some antitumor circRNAs like circASS1 attenuate the invasive phenotype.<sup>170-172</sup>

The interaction of circRNAs with the immune system in order to facilitate the escape of cancer cells from the immune system or to inhibit this system for the development of cancer is a very noteworthy area in cancer

studies. In breast cancer research, the investigation into the role of circ-002172 serves as a striking illustration of circRNA's interaction with the immune system. This upregulated circRNA not only suppresses the infiltration of cytotoxic T cells but also counteracts the inhibitory effect of miR-296-5p, consequently enhancing the expression of CXCL12. While CXCL12 impacts both the innate and adaptive components of the immune system, its precise role in cancer development remains uncertain.<sup>173</sup> Another example in this regard is circWWC3, which raises the possibility of breast cancer metastasizing by raising the molecular level of interleukin-4.<sup>174</sup> In breast cancer, upregulated hsa\_circ\_0067842 promotes the stabilization of CMTM6, thereby impeding the ubiquitination-mediated degradation of PD-L1. The subsequent binding of PD-L1 to PD-1 on T cells attenuates antitumor immunity by driving T cell exhaustion.<sup>175</sup> Another group of circRNAs, including circ\_0001598, circ\_0000512, circATAD2, and circGSK3 $\beta$ , has been shown to elevate PD-L1 expression levels, thereby suppressing CD8<sup>+</sup> T cell-mediated antitumor immune surveillance in breast cancer. However, current studies do not specifically attribute this immune evasion to the metastatic process alone.<sup>176-179</sup>

Angiogenesis is essential in the metastatic process, providing cancer cells with the necessary resources to survive.<sup>180</sup> According to reports, hsa-circRNA-002178 in breast cancer increases angiogenesis and worsens prognosis by suppressing the inhibitory effect on COL1A1 through a sponge-like function for miR-328-3p.<sup>181</sup> On the other hand, exosomal lncRNASNHG12 has been shown to promote angiogenesis in breast cancer through the PBRM1/MMP10 axis.<sup>182</sup>

CircRNAs play a multifaceted role in creating favorable conditions for metastatic cell colonization. Their involvement in regulating various processes such as angiogenesis, ECM remodeling, EMT, and modulation of immune response in the tumor microenvironment (TME) collectively promotes colonization potential in an indirect manner.<sup>183,184</sup>

It is important to note that although the role of circRNAs in individual post-EMT processes such as invasion, immune evasion, ECM remodeling, and angiogenesis is well-documented, these mechanisms have largely been studied in isolation. A more integrated investigation that considers their coordinated contribution within the full metastatic cascade may yield deeper insights into the functional relevance of circRNAs in breast cancer progression.

### **Translational and clinical relevance of circular RNA in breast cancer**

The remarkable stability and abundance of circRNAs in physiological fluids such as blood and urine have highlighted their clinical significance as candidates for liquid biopsy-based diagnostics. Aberrant expression patterns of circRNAs in breast cancer tissues, along with their tissue-specific characteristics, underscore their potential as reliable biomarkers for diagnosis and

prognosis, therapeutic targets, and mediators of treatment resistance.<sup>185</sup> In addition, the differentiation of subtypes of breast cancer is possible based on the expression of circRNAs. For instance, circ-0044234 expression is notably lower in TNBC compared to other breast cancer subtypes, suggesting its utility in subtype differentiation.<sup>186</sup>

Although no circRNA-based panel has yet been implemented in routine clinical practice for breast cancer diagnosis, accumulating evidence underscores their substantial diagnostic potential. In support of this, Libin et al showed that in 136 breast cancer patients, hsa\_circ\_0006969 achieved AUCs of 0.889 in tumor tissue and 0.823 in blood; 0.672 and 0.675 for grades 1 and 3; 0.648 in ER-positive cases; 0.687–0.757 across TNM stages I–III (0.751 for stages I–II); and outperformed CA15-3 in detecting metastasis (0.742 vs. 0.712).<sup>187</sup> Similarly, the ROC analysis of hsa\_circ\_0000615 demonstrated an AUC of 0.904 (95% CI: 0.863–0.944), with a sensitivity of 76.8% and specificity of 88.4% in distinguishing non-metastatic breast cancer cases.<sup>188</sup> Moreover, hsa\_circ\_0001785 in plasma showed a diagnostic AUC of 0.784.<sup>189</sup> Consistently, several studies have identified circRNAs as promising diagnostic and prognostic biomarkers for TNBC. For instance, hsa\_circ\_0072309 is downregulated in TNBC and discriminated patients from healthy controls with AUCs of 0.78 (FFPE) and 0.81 (frozen), and its activity appears linked to interactions with RNA-binding proteins affecting PTEN and RUNX1 signaling.<sup>190</sup> circZCCHC2 and circCAPG are upregulated in breast tumors including TNBC, with diagnostic AUCs of 0.787 and 0.8723, respectively; circCAPG is furthermore associated with poorer overall survival.<sup>191</sup> From a prognostic perspective, elevated circWHSC1 (AUC 0.968) correlates with shorter overall survival in TNBC, and serum circPRMT5 (AUC 0.9323) is associated with metastasis and treatment resistance, highlighting their potential for noninvasive monitoring.<sup>191</sup> These candidates warrant validation in larger cohorts and mechanistic studies.

Several circRNA profiles identified in breast cancer have been proposed as prognostic markers in prior research. Elevated levels of oncogenic circRNAs like circEPSTI1 and circKIF4A are associated with a worse prognosis, whereas increased expression of tumor-suppressive circRNAs such as circ-VRK1 correlates with improved outcomes for breast cancer patients.<sup>192–194</sup> Specifically, patients with high circEPSTI1 expression showed significantly shorter mean disease-free survival and overall survival compared to those with low expression levels.<sup>192</sup> It should be noted that, according to ClinicalTrials.gov, an observational study (NCT05771337) is currently recruiting to evaluate the diagnostic and prognostic value of hsa\_circ\_0001785 and hsa\_circ\_100219 in serum samples from breast cancer patients, with estimated completion in 2025.<sup>195</sup>

On the other hand, circRNAs contribute pointedly to drug resistance and restrict treatment options in breast cancer by regulating multiple cellular processes such as autophagy, apoptosis, cell cycle progression, DNA repair, and drug efflux. This sustains the survival of cancer

cells, contributing to increased mortality rates in breast cancer patients. Research in this field has elucidated the involvement of specific circRNAs in drug resistance mechanisms: circ-BGN in resistance to trastuzumab, circ-MMP11 in resistance to lapatinib, hsa-circ-0092276 in promoting autophagy and resistance to doxorubicin, circ-CDR1as in enhancing resistance to chemotherapy, and circPVT1 in augmenting drug resistance specifically in the ER+ subtype. Consequently, circRNA expression profiling in breast tumors holds significant promise for predicting drug resistance patterns and informing the selection of tailored therapeutic strategies. Such precision-guided approaches may not only improve treatment outcomes but also accelerate the integration of personalized medicine into breast cancer care.<sup>196–200</sup>

As previously mentioned, circRNAs regulate breast cancer progression primarily by functioning as competing endogenous RNAs, which sponge tumor-suppressor or oncogenic miRNAs. This, in turn, modulates key signaling cascades such as PI3K/AKT, Wnt/ $\beta$ -catenin, and MAPK/ERK. In this regard, oncogenic Hsa\_circ\_001569 and CircCHIPK3 promote proliferation and metastasis via PI3K/AKT activation, while circRNA-SFMBT2 and Circ-RNF111 drive therapy resistance by sequestering miRNAs that would otherwise sensitize tumors to chemotherapy. Targeted inhibition of these circRNAs in preclinical models has restored drug sensitivity and suppressed tumor characteristics, underscoring their potential as both biomarkers of resistance and actionable targets for precision therapies in breast cancer.<sup>201–204</sup> Another example in this area is the silencing of circAGFG1 using antisense oligonucleotides, which significantly inhibited proliferation and EMT *in vitro*, while also restoring sensitivity to chemotherapy.<sup>205</sup> Despite advances in engineering exosomes for targeted delivery of antitumor compounds such as miRNAs, this field remains largely unexplored for tumor-suppressive circRNAs.

Despite growing interest in the role of circRNAs in cancer-related EMT, much of the available evidence remains confined to *in vitro* studies using cancer cell lines or limited tissue-level analyses. In many cases, investigations have focused solely on altered circRNA expression and their impact on migration or proliferation, without extending these observations to *in vivo* models or patient-derived data. While notable exceptions exist, such as circANKS1B,<sup>206</sup> which has been functionally validated in tumor xenograft models and shown to promote *in vivo* tumorigenesis, most circRNAs proposed to regulate EMT lack such mechanistic or translational validation. As a result of these preclinical gaps, the clinical translation of circRNA biomarkers remains delayed by the absence of standardized and integrated analytical frameworks. Without precise data-quality metrics, targeted enrichment strategies and ultra-sensitive platforms (e.g., ddPCR), unified SOPs for isolation and quantification, and consensus-driven multicenter validation, reproducibility suffers and diagnostic adoption is hindered.

### Interpretation of findings: functional discrepancy

CircRNAs frequently behave paradoxically: the same circular transcript can act as a tumor promoter in one context and as a suppressor in another. Early studies largely reduced circRNAs to “miRNA sponges,” but mounting evidence indicates that protein interactions, translation of circRNA-derived peptides, and other post-transcriptional mechanisms often play equal or greater roles. Take the TGF $\beta$ /SMAD axis as an example. In breast cancer models, circANKS1B and circ-0000043 have been reported to drive EMT by sequestering miR-148a-3p/miR-152-3p and miR-136, thereby amplifying Smad-dependent mesenchymal programs.<sup>94,96</sup> Yet other tumor types tell a different story: circPVT1 upregulates Smad3 and promotes EMT in gastric cancer,<sup>97</sup> whereas circ-OXCT1 suppresses EMT through Smad4 inhibition.<sup>98</sup> Such cross-tissue contrasts are likely rooted in differences in epigenetic state and baseline pathway activity (for instance, tissue-specific TGF $\beta$  levels) which make straightforward extrapolation hazardous. For meaningful interpretation of circRNA function in breast cancer, we therefore need experiments conducted *in situ* and in models that preserve tumor subtype identity and microenvironmental context. Only then can we distinguish context-specific biology from artifacts of simplified systems.

A comparable functional divergence emerges within the Wnt/ $\beta$ -catenin pathway. For example, circ-0008784 and circABCC4 have been reported to promote EMT by sequestering miR-506-3p and miR-154-5p, respectively; whereas circ-ITCH appears to counteract EMT through inhibition of miR-214 and miR-17.<sup>109-111</sup> Importantly, most evidence supporting circ-ITCH comes from *in vitro* systems. Without *in vivo* corroboration, its perceived effect size may be inflated because complex TME interactions are not captured. Studies in non-breast malignancies echo this heterogeneity. CircPDSS1 and circ-0026628 drive Wnt-dependent EMT in colorectal and lung cancers, while circ-0026344 exerts an inhibitory influence.<sup>115-117</sup> Assertions that these opposing roles are evolutionarily conserved remain premature in the absence of phylogenetic analyses and may reflect publication bias. Adding another layer of complexity, some circRNAs encode peptides (for instance, EIF6-224aa from circ-EIF6) which can alter regulatory outcomes depending on isoform abundance and subcellular targeting.<sup>114</sup> Because bona fide translation of circRNAs appears infrequent under physiological conditions, rigorous biochemical and functional validation is essential to discriminate genuine biology from experimental artifact.

Parallel contradictions appear in PI3K/AKT and MAPK/MEK signaling. For example, circ-0006014 and hsa-circ-0000851 have been reported to activate PI3K/AKT and thereby promote EMT.<sup>134,135</sup> By contrast, circSEMA4B reduces AKT phosphorylation and suppresses the same program.<sup>126</sup> Crucially, most of these claims rely on knockdown or overexpression systems. Without quantification of endogenous circRNA abundance, the physiological relevance of such perturbations remains

ambiguous.

The picture is further muddled by indirect inferences. CircKIF4A in TNBC has been proposed to restrain PDK1 through miR-375 inhibition,<sup>161</sup> yet this link has not been validated in breast tumors. Such extrapolations risk creating a domino effect of untested assumptions and mask potential pathway crosstalk. To move beyond speculation, we need loss- and gain-of-function experiments that precisely target backsplice junctions, for example, CRISPR-based editing.

In MAPK/MEK signaling similar mixed signals emerge. CircWHSC1 appears to enhance Raf1-dependent EMT via miR-195-5p sponging,<sup>146</sup> whereas circ-ZKSCAN1 and circNRIP1 act inhibitory in other malignancies.<sup>148, 150</sup> Do circRNAs operate as finely tunable rheostats, subtly modulating pathway flux? Or do they behave as binary switches that flip cellular states? Framing the question this way is misleadingly simple. A more realistic model places circRNAs inside stochastic, noisy regulatory networks where genetic variation and environmental context shape net output. In practice, resolving these conflicts will require three things: accurate, absolute quantification of endogenous circRNAs; CRISPR-based causal tests; and integrative network modeling that accommodates stochastic behavior and context dependency.

The absence of standardized benchmarks for circRNA-miRNA interactions (for example, agreed-upon binding-affinity or free-energy cutoffs) undermines reproducibility across studies. *In silico* predictions frequently diverge from biochemical assays, producing spurious target lists. Widely used algorithms (e.g., miRanda, TargetScan) have a well-documented tendency to overcall interactions when used in isolation.<sup>207</sup> To avoid false positives, computational screens must be coupled with orthogonal experimental evidence. Integrating transcriptome-wide CLIP datasets, AGO-pull-downs, or direct reporter assays into a unified validation pipeline will greatly improve confidence in functional assignments.

The functional ambiguity of circRNAs extends well beyond EMT. Some circular RNAs, such as circACAP2 and circRNA-CER, drive ECM remodeling and invasion by upregulating COL5A1 and MMP13.<sup>167, 168</sup> Others, for example circASS1, act in opposition and blunt invasive behavior.<sup>172</sup> These opposing effects indicate that circRNAs can exert bidirectional control over metastatic fitness. Critically, investigations that focus narrowly on ECM remodeling frequently overlook immune interactions. CircRNAs may reshape the tumor stroma indirectly via cancer-associated fibroblasts, or by altering chemokine and checkpoint axes that modulate immune surveillance. For example, circ-002172 and hsa\_circ\_0067842 have been linked to T-cell exhaustion through CXCL12/PD-L1 signaling.<sup>173,175</sup> However, whether these immune effects are specific to metastatic niches or also operate in primary tumors is unresolved.

A major translational limitation is model choice. Many immune studies use immunocompromised hosts, which cannot recapitulate human antitumor immunity.

Humanized mouse models and complementary ex vivo systems, are therefore essential to validate immune-related claims.

Angiogenesis adds another layer of uncertainty. Reports implicate hsa-circRNA-002178 in neovascularization, yet direct, subtype-stratified comparisons are lacking.<sup>181</sup> It remains unclear if pro-angiogenic circRNA activity is universal across breast cancer subtypes or enriched in subsets such as TNBC.

Finally, exosomal circRNAs are an attractive mechanism for intercellular signaling, but their functional role is under-validated. Demonstrating bona fide transfer and biological effect requires rigorous in vivo tracing and quantitative uptake assays. In short, resolving these contradictions demands integrated, physiologically relevant models that couple stromal, immune, and vascular readouts with rigorous in vivo validation.

Taken together, these context-dependent and often contradictory effects underscore a core challenge in circRNA research. Many studies rely heavily on gain- and loss-of-function experiments in conventional cell lines. Such approaches can artificially amplify circRNA functions due to overexpression, while ignoring the natural stoichiometry and compensatory mechanisms present in vivo. As a result, the generalizability of these findings is limited. Relying on simplified models further constrains progress. To address this, more physiologically relevant systems are essential. Organoids, for example, preserve tissue architecture and cellular diversity. Single-cell RNA sequencing can capture heterogeneity across cell types and reveal microenvironmental influences. Integrating these advanced platforms will allow a more accurate and nuanced understanding of circRNA functions in cancer biology.

### Conclusion and Future Perspectives

CircRNAs regulate EMT in breast cancer through several distinct mechanisms. These include functioning as competing endogenous RNAs (ceRNAs) to sponge miRNAs, interacting with RNA-binding proteins, modulating transcription, influencing alternative splicing, and affecting post-transcriptional gene regulation. Collectively, these categories illustrate the diverse ways in which circRNAs can impact EMT and contribute to breast cancer progression.

In parallel, circRNAs have also emerged as pivotal regulators of breast cancer metastasis-related signaling pathways. Comprehensive profiling of circRNAs in metastatic breast cancer patients is a critical first step towards developing circRNA-directed therapies. Promising strategies include silencing oncogenic circRNAs and delivering tumor-suppressive circRNAs via bioengineered exosomes as adjuncts to existing treatments. However, several challenges must be addressed before clinical translation can occur. These include incomplete functional characterization of many circRNAs, a lack of standardized analytical protocols, hurdles in achieving targeted tissue delivery, the inherent complexity of

circRNA-regulatory networks, limited clinical validation, and regulatory and manufacturing barriers.

Looking ahead, integrating circRNA research with artificial intelligence and high-throughput computational platforms promises to uncover novel regulatory circuits through machine-learning-driven network analysis. Such integration can enhance metastasis-prediction models by incorporating circRNA signatures into prognostic algorithms and enable earlier detection of aggressive tumor subtypes via multi-omic biomarker panels. Establishing robust, interoperable circRNA databases will be instrumental in advancing these efforts.

Ultimately, circRNAs represent a cutting-edge frontier in precision oncology, offering new avenues to overcome drug resistance, inhibit metastatic spread, and improve clinical outcomes for patients battling advanced breast cancer.

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The authors declare that ChatGPT (OpenAI, San Francisco, CA, USA) was used solely for language editing and improving the readability of the manuscript. The tool was not involved in the generation of scientific content, data analysis, or interpretation of results.

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