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## A Review of the Signal Transduction Pathways involved in Epithelial-Mesenchymal Transition induced in Breast Cancer Metastasis and Their Cross-talks

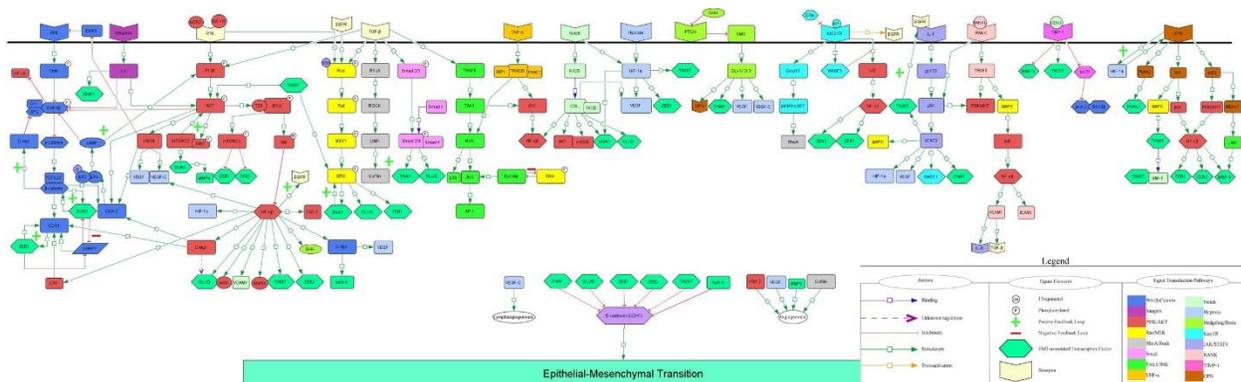
### **Introduction**

Epithelial-Mesenchymal Transition (EMT) is a biological process utilized by epithelial cells to transform into motile mesenchymal cells, initiating metastasis in cancer. EMT is also utilized during development and wound healing [10]. This process allows for cancerous cells to detach themselves from their primary tumor and invade normal tissue in preferred organ sites, forming secondary tumors called metastases. Metastasis is very important in the progression of cancer in patients as it is the process responsible for the mortality of patients through the collection of metastases that affect vital organs like the brain, lung, or immune system. The most common metastases for malignant breast tumors are the brain, lungs, lymph nodes, liver, and bone [1]. As metastasis increases the mortality rate of patients, it is vital to understand then how this process is initiated by EMT. EMT occurs due to epithelial cells losing their cell-cell adhesion and polarity. This is due to downstream targets of signal transduction pathways causing an integral step of EMT, the loss of epithelial cadherin (E-cadherin) expression. These downstream targets are zinc finger protein SNAI1 (Snail), zinc finger protein SNAI2 (Slug), zinc finger e-box binding homeobox 1 (ZEB1, TCF8), zinc finger e-box binding homeobox 2 (ZEB2, SIP1), and Twist-related protein 1 (Twist), that are known as EMT-associated transcription factors.

The second requirement for tumors to metastasize is the process of angiogenesis or lymphangiogenesis, which is the formation of new blood vessels or lymphatic vessels, respectively. Both processes allow for the primary tumor to connect to the blood stream or lymphatic system, allowing for mesenchymal cells to run through to invade healthy tissues. Both

of these processes are initiated by the hypoxic conditions that the malignant tumor stimulates via its own metabolism and proliferation [148].

Despite the general understanding of how metastasis occurs, there are several signal transduction pathways involved in this process that are complicated. This paper aims to discuss an up-to-date understanding of these signal transduction pathways, their cross-talks, and role in metastasis, specifically in breast cancer metastasis. Figure 1 provides a visual of all of these signal transduction pathways that are discussed in this paper. To download figure 1, please click on the additional file above labeled as “Signal Transduction Pathways involved in Epithelial-Mesenchymal Transition.pdf” or click here: [PDF image of Figure 1:](#)



**Figure 1.** Shown above are the signal transduction pathways involved in Epithelial-Mesenchymal Transition (EMT), induced in breast cancer metastasis, by promoting an EMT-associated transcription factor (Slug (SNAI2), SNAI1 (Snail), Zeb1 (TCF8), Zeb2, and Twist) caused by downstream targets. EMT is the biological process of which epithelial cells lose their cell-cell adhesion and polarity, allowing for the cells to transition into a motile mesenchymal state triggering metastasis. A hallmark of EMT is the repression of E-cadherin, which occurs when an EMT-associated transcription factor is activated. EMT is involved in the initiation of metastasis, wound healing, and development.

Second, angiogenesis or lymphangiogenesis must occur for metastasis to occur, which is the formation of new blood vessels or lymphatic vessels. Cancer stem cells utilize the blood stream or lymphatic stream in order to metastasize to different parts in the body. The process of angiogenesis is initiated by vascular endothelial growth factor (VEGF), which binds to VEGFR on the interior of blood vessel walls. This causes endothelial cells to begin to proliferate, to sprout out of the normal blood vessel to form a new blood vessel connected to the tumor. Activated matrix metalloproteinase (MMP-3/9) aids in this process by degrading the proteins that keep the vessel walls solid.

## WNT/ $\beta$ -catenin Signaling Pathway

The WNT signaling pathway plays a role in cell proliferation [2], cell polarity [4], cell fate determination [5], and cell adhesion [3, 4]. WNT is a key signaling pathway that is used during different steps of embryogenesis and adult tissue homeostasis. Therefore, it is an important pathway to development [3]. Furthermore, this pathway is vital to the regulation of the levels of  $\beta$ -catenin.  $\beta$ -catenin is important to EMT as it interacts with the E-cadherin/ $\beta$ -catenin complex that plays an important role in maintaining epithelial cell's integrity. This is due to  $\beta$ -catenin decreasing the levels of E-cadherin by downstream targets of the WNT/ $\beta$ -catenin signaling pathway [4]

Initiation of the Wnt signaling pathway begins with a Wnt-protein ligand binding to a frizzled receptor sending a biological signal to activate the Dishevelled (Dsh) protein family. Activated Dsh directly binds to glycogen synthase kinase 3 beta (GSK3 $\beta$ ) to an inactive form, causing functional  $\beta$ -catenin to accumulate in the cytosol and later translocate to the nucleus. Normally, GSK3 $\beta$  phosphorylates and ubiquitinates  $\beta$ -catenin [6]. With the assistance of T-cell factor / lymphoid enhancer factor (TCF/LEF),  $\beta$ -catenin activates the CD44 gene, Snail, and Cyclo-Oxygenase 2 (COX-2) [7, 8, 9, 10]. CD44 activates the expression of an EMT-associated transcription factor called ZEB1. ZEB1 also activates CD44 causing a positive feedback loop, one that regulates the expression of ZEB1 through the repression of Epithelial Splicing Regulatory Protein 1 (ESRP1) [11]. ZEB1 activates EMT through the downregulation of E-cadherin and upregulation of N-cadherin [12, 13]. ESRP1 regulates CD44 alternate splicing during EMT, causing inhibition of another EMT-associated transcription factor, Snail. Therefore, inhibition of ESRP1 is vital to EMT progression [14]. Activation of COX-2 leads to the increased repression of E-cadherin by activation of Prostaglandin E2 receptor 2 and 4 (EP2, EP4)

[8, 15]. Activated EP2 and EP4, leads to the activation of adenylate cyclase, which increases the levels of intracellular cyclic adenosine monophosphate (cAMP) [15, 16]. Intracellular cAMP leads to the phosphorylation of GSK3 $\beta$  and phosphoinositide 3-kinases (PI3K) making a positive feedback loop for the Wnt signaling pathway and the PI3K/AKT signaling pathway that will be discussed later [17, 18].

### **Integrin Signaling Pathway**

Integrins are members of the cell-extracellular matrix (ECM) and cell-adhesion molecules that play important roles in regulating cell-cell adhesions; they are also involved in making transmembrane connections to the cytoskeleton via activation of intracellular signaling pathways. Furthermore, integrins play key roles in regulating development by controlling cell migration, survival, proliferation, and differentiation [19, 20, 21]. Therefore, when integrins are overexpressed in cancer it promotes signals that allow for cancer cells to survive, grow, and differentiate [21]. Cancer cells also utilize the integrin pathway to disassemble the ECM to allow for angiogenesis to occur [21]. The integrin signaling pathway begins with Integrin-Linked Kinase (ILK) interacts with cytoplasmic domains of  $\beta_1$  and  $\beta_3$  integrins [22]. ILK's downstream targets negatively regulate GSK3 $\beta$  and positively regulate PI3K/AKT via phosphorylation [23]. ILK also directly activates the EMT-associated Transcription Factor Snail [24].

### **PI3K/AKT Signaling Pathway**

The PI3K/AKT signaling pathway is important in regulating cell proliferation [25] and survival [26, 27, 28, 29]. Breast cancer tumors utilize the PI3K/AKT pathway to aid in its own survival, growth, and spread to other parts of the body, which are all downstream effects of this pathway. Specifically, PI3K/AKT leads to the activation of nuclear factor-kappa B (NF- $\kappa$ B), a

crucial protein complex that leads to several EMT-associated transcription factors, angiogenesis and lymphangiogenesis, and activation of other pathways that breast cancer is involved in.

There are a variety of receptors that initiate this signaling transduction pathway such as: human epidermal growth factor receptor 2 (HER 2) [30], receptor tyrosine kinase (RTK) [31], insulin-like growth factor 1 (IGF-1) [32], transforming growth factor beta (TGF- $\beta$ ) [33], KISS1R [34], and receptor activator of nuclear factor kappa-B (RANK) [35]. They all activate the PI3K/AKT signaling pathway by phosphorylating PI3K [36, 37]. PI3K phosphorylates and activates the serine/threonine protein kinase AKT (also known as Protein kinase B), causing a series of downstream effects to occur such as the inhibition of GSK3 $\beta$  [38], activation of endothelial NO synthase (eNOS) [39], phosphorylation of the complex of IKK $\alpha$  via Tyrosine residue at 23 (T23) [40], and activation of mammalian Target of Rapamycin (mTOR) [41]. AKT has also been shown to be activated by TWIST, an EMT-associated factor, in breast cancer cells [42].

The regulation of the WNT/ $\beta$ -catenin pathway via activation of AKT, aids in the induction of EMT in two different ways. First, AKT phosphorylates the serine at residue 552 in  $\beta$ -catenin, increasing its transcriptional activity and aids in the movement of  $\beta$ -catenin from the cytoplasm to the nucleus [43]. Second, AKT also phosphorylates serine at residue 9 in GSK3 $\beta$ , which facilitates the ubiquitination and degradation of GSK3 $\beta$ , thus allowing for production of  $\beta$ -catenin to increase [44, 45].

eNOS is responsible for the synthesis of nitric oxide (NO), which upregulates the WNT/ $\beta$ -catenin by negatively regulating Dickkopf-related protein 1 (DKK1) gene expression, a gene that regulates the WNT/ $\beta$ -catenin pathway [46]. Increased levels of NO in cancerous cells have been shown to promote tumor angiogenesis, via activation of vascular endothelial growth factor

(VEGF). Therefore, promoting VEGF-induced neovascularization, increasing the tumor's ability to metastasize [47]. High levels of NO, *in vivo*, have also been shown to promote tumor lymphangiogenesis in breast cancer cells, via activation of vascular endothelial growth factor c (VEGF-C) [48]. The production of nitric oxide to promote VEGF, via hypoxia, is essential to tumor metastasis, as it allows for channels to the blood and lymphatic stream to be created. NO has also been shown to directly interact with COX-2, causing an increase in its enzyme activity [49].

Phosphorylated AKT phosphorylates T23 in the inhibitor of nuclear factor  $\kappa$ -B kinase subunit  $\alpha$  (IKK $\alpha$ ) sub unit, which in turn, causes the inhibitor of nuclear factor  $\kappa$ -B kinase subunit beta (IkB) protein to be ubiquitinated. This leads to the activation of NF- $\kappa$ B via phosphorylation [40]. Without this cascade of proteins activating each other through phosphorylation, NF- $\kappa$ B is incapable of being activated for this pathway. The NF- $\kappa$ B signaling is vital to the progression of EMT in breast cancer, as the inhibition of NF- $\kappa$ B, *in vivo*, has been shown to block EMT from occurring [50]. This is due to the three EMT-transcription factors that the NF- $\kappa$ B signaling pathway activates: Slug, TWIST1, and Zeb2 [51]. NF- $\kappa$ B may activate Slug through activation of Myb proto-oncogene protein (C-myb), which has been shown to regulate Slug in neuroblastoma cells. Since, C-myb is in high expressions in breast cancer, it may also regulate Slug in breast cancer cells [52, 53, 54]. C-myb also positively regulates COX-2, a protein used in the WNT/ $\beta$ -catenin pathway [55]. NF- $\kappa$ B regulates CD44, another protein used in the WNT/ $\beta$ -catenin pathway, via activation of complement receptor type 1 (CR1) [56]. NF- $\kappa$ B activation forms positive feedback loops with epidermal growth factor receptor (EGFR), promoting each other, through a series of mechanisms discussed by Shostak & Chariot in [57]. An example of one feedback loop would be with the Heregulin- $\beta$ 1 (HRG- $\beta$ 1), a member of the

EGFR family, which upregulates VEGF-C, a lymphangiogenesis transcription factor, through the activation of the NF- $\kappa$ B signaling pathway [58]. NF- $\kappa$ B may also act as a fibroblast growth factor 2 (FGF-2) gene transcription factor, FGF-2 like VEGF, leads to angiogenesis, via increased expression of matrix metalloproteinase-2/9 (MMP-2 and MMP-9) [59, 60, 61]. MMP2 and MMP-9 both are capable of breaking down the ECM, to allow for a new blood vessel to be formed directly to the tumor [In addition, FGF-2 aids in the repression of E-cadherin while inducing expression of vimentin, an EMT biomarker of a mesenchymal-derived cell [61]. NF- $\kappa$ B regulates myc proto-oncogene protein (C-myc), which in turn activates microRNA-9 (miR-9), a repressor of E-cadherin expression [62, 63]. C-myc regulates angiogenesis in tumors by regulating an angiogenic factor, VEGF [64, 65]. C-myc also crosstalks with the WNT/ $\beta$ -catenin pathway by being activated by  $\beta$ -catenin with TCF/LEF transcription factor and inhibits adenomatosis polyposis coli (APC) on GSK3 $\beta$  [66]. NF- $\kappa$ B also regulates hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) by binding directly to HIF-1 $\alpha$ , initiating the hypoxic-related signaling pathway that activates Twist and promotes angiogenesis [67, 68]. Furthermore, NF- $\kappa$ B transcribes the SRY (Sex-Determining Region Y)-Box 9 Protein (SOX9) gene that cooperates with Slug to cause EMT [69, 70]. Finally, NF- $\kappa$ B cross-talks with the Hedgehog signaling pathway by upregulating the expression of the Sonic Hedgehog protein (SHH) [71].

There are two mTOR kinase complexes that AKT activates, mTORC1 and mTORC2 [72, 73]. AKT activates mTORC2 by phosphorylating an mTORC2 subunit known as SIN1. Once SIN1 is phosphorylated, the mTORC2 signaling pathway is activated, leading to the phosphorylation of AKT, forming a positive feedback loop. This interaction between mTORC2 and AKT leads to the full activation of AKT, allowing for EMT-associated transcription factors to be upregulated [72]. Furthermore, mTORC2 aids in EMT progression by regulating MMP-9

expression through Snail, an EMT-transcription factor [74]. TGF- $\beta$  induced mTORC2 has been shown to be required for the progression of EMT in mammary epithelial cells, allowing the cancerous cells to progress from an intermediate state of EMT to an advanced mesenchymal, invasive phenotype [74]. This is due to the vital proteins and kinases that mTORC2 regulates that aid in EMT-associated cell migration and invasion, like MMP-9 and the actin cytoskeleton [74]. AKT activates mTORC1 by phosphorylation of IKK $\alpha$ , which then directly phosphorylates mTORC1 [73]. On the other hand, mTORC1 has been shown to have a negative effect on EMT. As the inhibition of mTORC1 in MCF10A human breast cell line with an active PLK3CA gene, increased the cells' invasiveness and activation of EMT [75]. The role of mTORC1 as an anti-EMT kinase is in agreement with Mikaelian et. al., which showed that mTORC1 suppresses EMT through the negative regulation of ZEB1/ZEB2 and microRNA-200b/c (miR-200b) and miR-200c [76].

### **Ras/MEK Signaling Pathway**

The mitogen-activated protein kinases (MAPK) cascade plays an important role in proliferation, differentiation, development, and apoptosis [77]. Ras/MAPK signaling pathway is important to the progression of breast cancer tumor as ligands like the epidermal growth factor (EGF) is commonly overexpressed in cancer. EGF binds to EGFR, causing an activation of this pathway, which increases cellular proliferation and inhibition of apoptosis [78]. Furthermore, this pathway is important to EMT progression as it directly inhibits E-cadherin expression via activation of several EMT-associated transcription factors.

This signal pathway begins by many ligands, like TGF- $\beta$ , RTK, or EGF binding to its receptor that sends a biological signal through the receptor to recruit the son of sevenless protein (SOS) to Ras. With the assistance of SOS, Ras can exchange its guanosine diphosphate (GDP) for a guanosine triphosphate (GTP), activating Ras [79]. Activated Ras initiates the Raf/MAPK/ERK Kinase 1 (MEK1)/ extracellular signal-regulated kinases (ERK) signaling cascade, known as the MAPK pathway, which is a series of kinases that control one another through phosphorylation [80, 81, 82]. ERK has also been shown to be activated by TWIST in breast cancer cells [42]. Phosphorylated ERK is necessary for EMT as it activates or regulates several EMT-transcription factors like Slug, Snail, and ZEB1/2. Both ERK1/2 phosphorylate Slug [83]. Though only ERK2 has been found to upregulate Snail, which in turn activates ERK2, forming a positive feedback loop, in breast cancer [84]. ERK2 also activates ZEB1/2, with the help of a DEF motif [85].

### **RhoA/Rock**

The Ras homolog gene family, member A (RhoA) signaling pathway is important in EMT due to the pathway's role in controlling the cell's dynamic cytoskeletal organization and cell motility [86]. TGF- $\beta$  rapidly activates RhoA in epithelial cells, inducing the formation of stress fibers that directly affect the actin cytoskeleton and mesenchymal characteristics in them [87, 88]. This occurs because RhoA activates the Rho associated coiled-coil containing protein kinase 1 (ROCK), which then phosphorylates LIM kinase-1 (LIMK), which then phosphorylates cofilin [89, 90, 91]. Cofilin is responsible for tumor cell motility and invasion, as it directly regulates actin polymerization and depolymerization during cell migration; Cofilin also is capable of severing actin filaments [92, 93]. Therefore, high expressions of activated cofilin promotes EMT by causing mammary epithelial cells to gain mesenchymal characteristics [93].

## **Smad Signaling Pathway**

TGF- $\beta$  regulates several cellular functions such as cell proliferation [94], adhesion [95], migration [96, 97], cell-fate determination and differentiation [98], and apoptosis [99, 100]. The Smad signaling pathway has been shown to be crucial to TGF- $\beta$ -induced EMT as it promotes two EMT-associated transcription factors, as well as crosstalk with other non-Smad pathways that are important in EMT but are not described here. A review of these crosstalks, written by Kunxin Luo is described in [101]. Though, the Smad and non-Smad pathways of TGF- $\beta$  must work together to contribute to the establishment of EMT [102].

SMADs are intracellular proteins essential for TGF- $\beta$ , as their role is to transduce a signal from the membrane into the nucleus and regulate gene expression by directly activating it [100]. The pathway begins by a TGF- $\beta$  ligand binding to the TGF- $\beta$  receptor, the TGF- $\beta$  receptor then phosphorylates SMAD family member 2 and 3 (SMAD 2, SMAD 3) [102]. Phosphorylated SMAD2 and SMAD3 come together and recruit SMAD4 to form a trimer, which allows for this complex to transport to the nucleus [10, 103, 104]. In the nucleus, this complex binds to DNA-sequence-specific transcription factors to activate the transcription of Snail [105, 106, 107] and Slug [10, 108, 109].

## **TAK1/JNK Signaling Pathway**

TGF- $\beta$ -activated kinase 1 (TAK1) signaling pathway has been shown to be crucial to the regulation of genes that are involved in inflammation as it regulates expression levels of NF- $\kappa$ B and activator protein 1 (AP-1). TAK1 has been shown to be required for TGF- $\beta$ -induced c-Jun N-terminal kinases (JNK) and NF- $\kappa$ B activation, in vivo [110]; the pathway is also essential for TGF- $\beta$  induce apoptosis, as overexpression of TAK1 caused cells to undergo apoptosis [111, 112,

113]. Furthermore, TAK1 signaling is required for TGF- $\beta$  induced EMT as knockdown of TAK1 has been shown to inhibit TGF- $\beta$ -mediated EMT [80, 114]. The activation of TGF- $\beta$  causes the polyubiquitination of tumor necrosis factor-receptor associated factor-6 (TRAF6). Polyubiquitinated TRAF6 recruits and activates TAK1 [114, 115, 116]. Activated TAK1 ubiquitinates and activates the I $\kappa$ B kinase (IKK), which increases the production of NF- $\kappa$ B by activation IKK $\alpha$  as discussed before in this paper [40, 116]. In addition, activated TAK1 activates Mitogen-Activated Protein Kinase Kinase 3 (MKK3), 4 (MKK4), and 6 (MKK6). MKK3 and MKK6 are responsible for the activation of p38. p38's function in tumor metastasis is still currently debated, with papers showing that p38 is a tumor suppressor while others showing it positively contributes to tumorigenesis [119, 120, 121]. MKK4 is responsible for the activation of JNK. JNK phosphorylates c-Jun which is part of AP-1 [117, 118]. AP-1 is a family of proteins that are involved in inflammation as well as a central transcription factor associated with cancer invasiveness and tumorigenesis [122]. For example, AP-1 regulates the transcription of MMP3/9 expression, which promotes angiogenesis [123]. A molecule known as the epithelial cell adhesion molecule (EpCAM) also works with AP-1 in cancer. Studies have shown that EpCAM works with AP-1 to promote EMT through JNK phosphorylation [122, 124]. The expression of EpCAM has also been associated with increased breast cancer invasion in vitro and in vivo [124]. Furthermore, EpCAM has been shown to be in a double negative feedback loop with ERK2. EpCAM and ERK2 negatively regulate each other, though how this interaction works to promote EMT is still not understood [125].

### **TNF- $\alpha$ Signaling Pathway**

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a cytokine that is mainly utilized by macrophages in response to inflammation [126]. In cancer, TNF- $\alpha$  is a key regulator of a tumor's

microenvironment, while also contributing to tumor proliferation, invasion, survival, and angiogenesis via downstream activation of NF- $\kappa$ B [126]. In breast cancer, increase TNF- $\alpha$  expression level is associated with malignant breast cancer advancement as it aids in cell survival and proliferation [127].

The TNF- $\alpha$  signaling pathway begins with a ligand binding to tumor necrosis factor receptor type 1 (TNF-R1). This changes the receptor, leading to the dissociation of an inhibition protein called silencer of death domain (SODD) allowing for the recruitment of tumor necrosis factor receptor type 1 associated death domain protein (TRADD), tumor necrosis factor receptor associated factor 2 (TRAF2), receptor interacting protein-1 (Rip1), and Fas-associated death domain (FADD). to the receptor [128, 129]. The TRADD/TRAF2/Rip1/FADD complex allows for the initiation of the NF- $\kappa$ B and JNK/AP-1 pathway. TRADD recruits TRAF2, which recruits the IKK complex. The IKK complex phosphorylates and ubiquitinates I $\kappa$ B, which as discussed before increases the production of NF- $\kappa$ B. TRAF2 also recruits MAPK/ERK kinase kinase 1 (MEKK1), which activates MKK7, initiating the JNK/AP-1 cascade [128, 130].

### **Notch Signaling Pathway**

The Notch signaling pathway is involved in cellular proliferation [131, 132], survival [132, 133], apoptosis [132, 133], differentiation [131, 132], and angiogenesis [134]. Notch signaling is important to cells as it maintains the balance of cell proliferation, differentiation, and apoptosis. Therefore in cancer, when this balance is thrown off through upregulations in cell proliferation, Notch is expressed [139]. In addition, Notch has recently been shown to be a key factor in therapy resistance in estrogen receptor alpha (ER- $\alpha$ ) positive breast cancer, as Notch expression has been shown to increase in response to treatment [135, 136].

The pathway begins when ligands from the Delta- like members 1, 3, 4 (DLL1, DLL3, DLL4) or Jagged 1 2 families (JAG1, JAG2) bind and cleave a Notch receptor [137]. This cleaving causes the release of the intracellular domain of the Notch protein (NICD) from Notch through a signaling cascade. NICD translocates to the nucleus and binds to the complex of C protein binding factor 1/Suppressor of Hairless/Lag-1 (CSL) [137, 138, 139]. NICD then recruits transcriptional activators to the CSL changing it from a transcriptional repressor to a transcriptional activator complex. This active CSL complex increases the expression of a variety of Notch target genes like NF- $\kappa$ B, AKT, mTOR, VEGF [137, 138, 139, 140]. Furthermore, NICD directly upregulates the expression of Snail by binding to the Snail promoter [141,142]. Notch also indirectly regulates the expression of Snail by the activation of HIF-1 $\alpha$  which can get recruited to the lysyl oxidase (LOX) promoter resulting in the increased expression of LOX, which stabilizes the Snail protein [142]. The NICD-CSL complex also upregulates the expression of SLUG by directly regulating the SLUG promoter [143,144]. Acar and colleagues discuss more about Notch in breast cancer in [145].

### **Hypoxia Signaling Pathway**

Hypoxia, or oxygen deprivation in the body, is an important condition that plays a part in proper embryonic development [146, 147], controlling tumor angiogenesis [148, 149], and tissue growth [148, 149]. This signaling pathway helps restore oxygen homeostasis by inducing a variety of factors [148]. As a tumor grows, it creates conditions of hypoxia that lead to the activation of HIF-1 $\alpha$  [150]. HIF-1 $\alpha$  directly upregulates the expression of three EMT-transcription factors TWIST [151], Snail [152], ZEB1 [153], by binding to their promoters. HIF-1 $\alpha$  also promotes angiogenesis by increasing the expression of VEGF [154, 155].

### **Hedgehog/Sonic Signaling Pathway**

The Hedgehog (Hh) signaling pathway plays important roles in embryonic development, tissue self-renewal, mammary development, and carcinogenesis [150, 156, 157]. Sonic Hh protein (SHH) has been observed to be expressed in high levels of triple negative breast cancer with poor prognostic pathological features [158]. Furthermore, due to SHH role in the maintenance of cancer stem cells, the Hh signaling pathway aids in the cancer stem cells survival and expansion, by commencing and sustaining proliferation, invasion and metastasis [159,160].

Initiation of this signaling pathway begins with three Hh proteins, Desert (DHH), Indian (IHH), and Sonic (SHH), that all act as a ligand to trigger a specific signal transduction pathway [150, 157]. Specifically, SHH triggers the signaling transduction pathway that induces EMT in breast cancer [161]. SHH binds to protein patched homolog 1(PTCH) stopping the inhibitory effect of PTCH on the smoothed protein (SMO) [161]. Activated SMO translocates to the cilia at the cell membrane and transactivates glioma-associated oncoproteins (GLI proteins: GLI1, GLI2, GLI3) by sending a signal to the cilia to dissociate and release the GLI transcription factor [150, 161, 162]. GLI1 and GLI2 proteins promotes EMT by increasing the expression of Snail [163, 164], VEGFC [165], and VEGF [166, 167]. The activation of GLI1 is necessary for Hedgehog-induced EMT, as knockdown of these oncoproteins leads to significantly reduced proliferation and invasive capabilities in both estrogen receptor negative and triple negative breast cancer [168]. Gli-1 also upregulates the expression of osteopontin, an oncogene that promotes tumorigenesis and metastasis [169].

### **KISS1R Signaling Pathway**

KISS1R signaling pathway has vital key functions in the regulation of placentation and fetal development [233], insulin secretion, cardiovascular function [234], and kidney development [235]. The KISS1 gene is known for its anti-metastatic effects in different types of

cancers such as pancreatic and lung cancer but in breast cancer, it is currently misunderstood [170, 171]. Since, KISS1 has been linked with poor prognosis and breast tumor progression in estrogen receptor positive breast cancer [172]. Though, estrogen receptor alpha (ER $\alpha$ ) negatively regulates KISS1. So, it may be that high expressions of KISS1 occur in breast tumor progression as a result of either ER $\alpha$  losing its ability to negatively regulate KISS1 or KISS1 expression overcoming the downregulation by ER $\alpha$ . [172].

Initiation of the pathway begins when a KISS1 gene encodes for peptide products known as kisspeptin (KP) that serve as ligands that binds to the KISS1 receptor (KISS1R) [173]. This interaction causes a biological signal to be sent through the receptor to transactivate EGFR [174], activate G $\alpha$ q/11 protein, and the Wiskott-Aldrich syndrome family 3 (WASF3). Activated G $\alpha$ q/11, leads to the direct upregulation of RhoA via the activation of a RhoA-specific guanine nucleotide exchange factor known as p63RhoGEF [175, 176]. RhoA is important in tumor metastasis as it regulates the actin cytoskeleton. Increased expression of WASF3 has an inhibitory effect on the KISS1 gene that leads to the release of I $\kappa$ B, increasing NF- $\kappa$ B expression. This leads to the upregulation of ZEB1, ZEB2, MMP-9 via NF- $\kappa$ B [177, 178]. The loss of KISS1 expression, which is common in breast cancer metastases, then may be due to WASF3 [179, 180]. In vitro, WASF3 has been shown to interact with HER2/3 to promote invasion and metastasis in non-metastatic breast cancer cell lines [181]. Therefore, WASF3 may be an oncogene of interest for potential treatment as it is directly involved in EMT and angiogenesis through the upregulation of NF- $\kappa$ B.

To learn more about KISS/KISS1R in breast cancer, refer to Cvetković, Babwah, and Bhattacharya's review paper [182].

## **JAK/STAT3 Signaling Pathway**

The Janus Kinase Family (JAK) / signal transducer and activator of transcription 3 (STAT3) pathway is involved in proliferation, differentiation, immunity, and apoptosis [183]. The signaling pathway is important to the proliferation of breast cancer cells as it regulates the progression of the cell cycle and protects the cancer cells from apoptosis, [184]. Furthermore, STAT3 aids in the tumors metabolism by activating genes that are involved in glycolysis, inducing a hypoxic state [185]. This works by STAT3 directly activating HIF-1 $\alpha$ ; this interaction is necessary for full activation of HIF-1 $\alpha$ -regulated genes [186]. Also, STAT3 promotes angiogenesis by directly binding to the VEGF promoter, increasing production of VEGF [187].

Initiation of the JAK/STAT3 begins with an Interleukin-6 (IL-6) binding to either Interleukin-6 receptor (IL-6R) or an EGF binding to an EGFR [183]. IL-6R with IL-6 then forms a heterotetrameric complex with glycoprotein 130 (gp130) which causes the activation of the JAK1, JAK2, or TYK2 [188, 189]. The JAK family then phosphorylates gp130 that causes the activation of STAT3, MAPK and PI3K/AKT [175, 176, 177]. STAT3 activation leads to the regulation of cell transformation proliferation, survival, and motility [188, 189]. STAT3 aids in EMT by activating both TWIST and Snail in ER- $\alpha$ -positive human breast cancer cells [191]. Though in node negative breast cancer, STAT3 expression is linked with better overall patient prognosis and clinical outcome [192]. Furthermore, TWIST upregulates the production of IL-6, forming a positive feedback loop that drives EMT [193]. Therefore, IL-6 is a receptor of interest for treatment as it is capable of forming this positive feedback loop to continually increase the production of TWIST, causing EMT [190]. STAT3 also directly binds to the WASF3 promoter, an oncogene that is important in the KISS1/KISS1R signaling pathway [194].

## **RANK Signaling Pathway**

The receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) / receptor activator of nuclear factor- $\kappa$ B (RANK) signaling pathway is involved in the regulation of osteoclast differentiation, activation, and proliferation and differentiation of mammary cells during pregnancy [195]. RANKL expression is controlled by female sex hormones like progesterone or estrogen [195], which are found commonly expressed in 70% of breast cancers [196]. Therefore, this signaling pathway is hormone-driven to promote proliferation [196] and is commonly overexpressed in breast cancer [197].

Initiation of the RANK/RANKL pathway begins when a Rank-L binds to RANK. Activated RANK then activates an adapter protein known as TRAF6 [98]. Activated TRAF6 leads to the activation of PI3K/AKT/MTOR, MAPK (ERK, JNK) cascade, and induces the transcription of vascular cell adhesion molecule 1 (VCAM1) or intercellular adhesion molecule 1 (ICAM1) by downstream targets like NF- $\kappa$ B [199, 200, 201]. VCAM1 and ICAM1 are both involved in cell adhesion and motility, which is crucial to the progression of EMT, as VCAM1 upregulation has been shown to be directly correlated with advanced clinical breast cancer [202, 203]. Knockdown of VCAM1 by small interfering RNA (siRNA), inhibited cell growth *in vitro* and *in vivo* and suppressed TGF- $\beta$  or IL-6-stimulated cell migration, *in vivo*. Furthermore, VCAM1 expression has been associated with several EMT related genes like E-cadherin, which suggests its critical role in regulating EMT during breast tumor progression [202]. Though, the mechanism of which VCAM1 regulates EMT is still poorly misunderstood and requires further investigation.

In mammary tumor cell lines, RANK-induced EMT solely by the upregulation of Snail and Twist via the activation of NF- $\kappa$ B. Tsubaki et. al. also found that they had no substantial changes in levels of ERK1/2, AKT, Mtor, STAT3, and JNK after the addition of RANKL to the

mammary tumor cell line. They also found that inhibited NF- $\kappa$ B by Dimethyl Fumarate (DMF), a NF- $\kappa$ B inhibitor, inhibited RANK-induced-EMT. DMF also decreased expression of Snail and Twist, cell migration, and invasion, which proves that RANKL/RANK is dependent on the activation of NF- $\kappa$ B to promote EMT [204].

RANKL may also have a role in the tumor microenvironment in breast cancer, as a chemotactic factor that signals motile breast cancer cells to metastasize to bone [201, 205]. One study which inhibited RANKL expression with Osteoprotegerin, significantly inhibited selective metastasis to the bone, in an *in vivo* melanoma mice model [183]. This may be due to RANKL role in osteoclastogenesis, which is the development of bone cells that break down bone tissue [194, 205]. VCAM1 also is involved in osteoclastogenesis and may act as a chemoattract that causes motile breast cancer cells to metastasize to the bone [206].

### **TIMP-1 Signaling Pathway**

Tissue inhibitor of metalloproteinase-1 (TIMP-1) primarily is involved in the ECM, which is important in development, cell-adhesion, Primarily, TIMP-1 directly regulates the ECM by the inhibition of MMP-9, preventing angiogenesis [207]. In cancer, independent of MMP-9 inhibitory function, TIMP-1 regulates angiogenesis [208], cell survival [209], proliferation [210] and apoptosis [209]. TIMP-1 expression is often linked with poor prognosis and increased in advanced stage breast cancer [211]. There are other TIMPs like TIMP-3, that have been shown to be in high expression in advanced stage breast cancers as well [211]. Though, TIMP-3 is often silenced in cancer, leading to the development of advanced breast cancer [212].

First, TIMP-1 binds with a cell surface protein known as CD63 [213]. The CD63/TIMP-1 complex directly promotes EMT, independent of its MMP-9-inhibitory function, by increasing

the expression of TWIST through the activation of cell survival cellular pathways in human breast epithelial cells [214]. The specific pathways involved are still unclear but PI3K/AKT seems like a likely pathway that CD63/TIMP-1 would activate if it is increasing cell survival and TWIST.

TIMP-1 also crosstalks with the WNT/ $\beta$ -catenin pathway, as when TIMP-1 binds to CD63 activated TIMP-1, it causes the decrease expression of let-7f, which in turn stabilizes the levels of axin 2 [215] Axin 2 is a negative regulator of the WNT/ $\beta$ -catenin pathway as it leads to the phosphorylation and degradation of  $\beta$ -catenin by interacting with GSK3 $\beta$  [215, 216].

### **OPN Signaling Pathway**

Osteopontin (OPN) is a cytokine that is involved in the regulation of cell-matrix adhesion [217], cell survival [217], bone remodeling [217], inflammation [218], and wound healing [218]. OPN is important in metastasis as it upregulates several EMT-associated transcription factors, such as Twist, Zeb1, ZEB2, and Snail [219, 222]. OPN is essential to the initiation of EMT, as inhibition of OPN in a xenograft breast cancer model, has been shown to decrease EMT [220]. In ER positive and triple negative breast cancer, OPN is overexpressed; OPN overexpression has been linked to aggressive breast cancer and poor prognosis [221].

OPN is a hypoxia-responsive gene that activates via HIF-1 $\alpha$ . Activated OPN in hypoxic and normal conditions also directly activates HIF-1 $\alpha$  expression, suggesting a positive feedback loop between OPN and HIF-1 $\alpha$  that drives angiogenesis by the increase of VEGF expression [223]. OPN also directly activates runt-related transcription factor 2 (Runx2) and in turn leads to the activation of Snail in breast cancer [224, 225]. This interaction between Runx2 and Snail is linked with poor prognosis and progression of breast cancer.

OPN phosphorylates nuclear factor inducing kinase (NIK) [226]. Phosphorylated NIK activates the IKK signaling cascades to induce expression of NF- $\kappa$ B. This interaction leads to the upregulation of MMP-9. NIK also is responsible for the activation of the MAPK pathway, which leads to the phosphorylation of TWIST [227]. Then, Twist binds with the B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1) promoter and cooperate with one another, to promote EMT by the repression of E-cadherin [228].

Furthermore, OPN phosphorylates and activates the PI3K/AKT/NF- $\kappa$ B signaling cascade via binding to a cell surface integrin known as alpha-v beta-3 ( $\alpha$ v $\beta$ 3) in breast cancer cells [229, 230]. OPN-NF- $\kappa$ B induced expression leads to the upregulation of ZEB1, ZEB2, and MMP-9 [228, 231]. Activated  $\alpha$ v $\beta$ 3 also activates MEKK1, which phosphorylates JNK, to promote the expression of AP-1. AP-1 leads to the increase expression of MMP-9, an important matrix that aids in angiogenesis [232].

## **Conclusion**

EMT is very important to the progression of breast cancer, as without the process, cancer is incapable of spreading to other parts of the body. EMT is regulated by several of these interconnected signaling pathways that have been discussed here. There are various players involved in the regulation of the process and with the various amounts of cross talk that occurs throughout each of the pathways, mutations in one or two key genes can possibly give rise to metastasis in cancer cells. Since, mutations in these genes can lead to overexpression of any of these pathways that are all capable of decreasing E-cadherin expression directly or indirectly, leading to EMT. Future studies should investigate further into the inhibition of WASF3 and IL-6 as possible treatments to prevent metastasis through their respected pathways. As well as developing methods to prevent E-cadherin loss by directly targeting EMT-associated factors.

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