Elucidating the molecular signaling pathways of WAVE3

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Abstract: Cancer metastasis is a complex, multistep process that requires tumor cells to evade from the original site and form new tumors at a distant site or a different organ, often via bloodstream or the lymphatic system. Metastasis is responsible for more than 90% of cancer-related deaths. WAVE3 belongs to the Wiskott-Aldrich syndrome protein (WASP) family, which regulate actin cytoskeleton remodeling as well as several aspects of cell migration, invasion, and metastasis. In fact, WAVE3 has been established as a driver of tumor progression and metastasis in cancers from several origins, including triple negative breast cancers (TNBCs), which are classified as the most lethal subtype of breast cancer, due to their resistance to standard of care therapy and highly metastatic behavior. In this review, we will attempt to summarize the recent advances that have been made to understand how WAVE3 contributes to the molecular mechanisms that control cancer progression and metastasis. We will also review the signaling pathways that are involved in the regulation of WAVE3 expression and function to identify potential therapeutic options targeted against WAVE3 for the treatment of patients with metastatic tumors.

Keywords: WAVE3; triple negative breast cancer (TNBC); metastasis

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Introduction

The Wiskott-Aldrich syndrome protein (WASP) family includes two subfamilies, WASP (WASP and N-WASP) and WASP family verprolin-homologous WAVE proteins (WAVE1/SCAR1, WAVE2 and WAVE3) (1,2). The WASP and WAVE proteins play a major role in cell motility through the activation of filopodia and lamellipodia formation at the leading edge of migrating cells (3-7) by regulating actin polymerization through binding to the Arp2/3 protein complex (4,5,8-12). The formation of these membrane filament (filopodia) and ruffle (lamellipodia) structures is regulated by molecular switches that are a part of the Rho family of GTP-exchange factors: (RhoA, Cdc42, and Rac) (13-15). While the WASP and NWASP are regulated by Cdc42, WAVE1, 2 and 3 are regulated downstream of Rac (1,2,7,13,15,16). The WAVE regulatory complex (WRC) is a five-subunit protein complex that regulates the activity of the WAVE proteins and it plays an important role in regulating actin polymerization. The WAVE proteins were shown to be sequestered in an inactive state through the formation of a complex with the four other protein members of the WRC, PIR121, Nap125, HSPC300, and Abi1 (17-19). WRC is activated by Rho GTPase Rac1 and sends information to the actin nucleator Arp2/3 complex (20-24). Among the various WASP and WAVE proteins, WAVE3 has been established a major driver of the invasive and metastatic phenotypes in several types of cancers, including the one generating for the breast (3,25). WAVE3, like WAVE1 and WAVE2, contains several

functional domains (I) an N-terminal basic region (BR); followed by (II) a proline-rich domain (PRD) that binds and activates SH2- and SH3-containing proteins kinases such as c-Abl and PI3K-p85; and (III) a verprolin-cofilin-acidic (VCA) domain that binds the Arp2/3 complex and activates actin polymerization (26). Several studies have reported that WAVE3 is found to be present on the leading edge and in the tips of pre-mature and mature filopodia (11,16). This was also demonstrated by immunostaining the filopodia with anti-WAVEs and actin filaments with rhodamine (16). However, WAVE3 is distributed in a discrete manner on the leading edge of lamellipodia and tips of filopodia (27) as opposed to WAVE1 which demonstrated continuous distribution (16). High WAVE3 expression is related to cancer invasion and metastasis (9,28). Loss of WAVE3 inhibits the formation of invadopodia (27), thereby further inhibiting cell migration, invasion, and extracellular matrix (ECM) degradation (5). Ji *et al.* (29) showed that knockdown of WAVE3 inhibits cell migration and invasion in hepatocellular carcinoma (HCC) cells. WAVE3 is largely concentrated in the nucleus and cytoplasm (6). It is found to be upregulated in breast (27), prostate (15), liver (3,29), pancreas (30), and ovarian cancers. Furthermore, it was shown that WAVE3 is co-localized with actin structures in the lamellipodia (6). Reports have shown that WAVE3 protein levels are correlated to different grades of tumor (5,8,31). However, the expression of WAVE3 varies with cell lines. For example, Taylor *et al.* (32) demonstrated that there was high expression of WAVE3 in breast cancer cell lines such as MDA-MB 231 and BT-549. Similarly, the expression of WAVE3 was upregulated in PANC-1, a pancreatic cancer cell line (30). In another study, Lu *et al.* (31) showed that SKOV3 cells showed highest WAVE3 expression among five different human ovarian cancer cell lines whereas A2780 had the lowest expression. The expression levels of WAVE3 upregulated in HCC tissues than adjacent non-cancerous tissues. It was also demonstrated that silencing WAVE3 causes decreased expression of COX-2, VEGF, nuclear factor-kappa B (NFκB) and p38 mitogen-activated protein kinase (MAPK) in SKOV3 cells and elevated expression causes high expression of the same in A2780 cells (31).

One of the tumors where WAVE3 has been heavily investigated is breast cancer. Breast cancer is the second leading cause of cancer-related deaths in women in the United States and worldwide (32-35). Breast cancer causes more than 40,000 deaths in the United States annually (33,36,37). The most abysmal type of breast cancer is triple negative breast cancer (TNBC) which is also disproportionally higher in the African-American population (38-41). Cancer metastasis requires tumor cells to evade from a primary site and form new tumors at a distant site or a different body part, often via bloodstream or the lymphatic system (5,8,9,42,43). Almost 90% of deaths are caused due to metastases (32,44-46). TNBC cells lack estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) (32,47-52). Due to the highly metastatic behavior of these tumors, they are classified as the most lethal subtype of breast cancer (32,33,36). Cancer stem cells (CSCs) have been involved in tumor initiation, development, and metastatic dissemination, especially in TNBCs. They exhibit resistance to chemotherapy and radiotherapy and are capable of self-renewal, often causing recurrence (53-56). Reports have shown that WAVE3 is enriched in the population of CSCs and silencing WAVE3 might reduce chemoresistance in TNBC cells (36).

In this review, we will discuss about the advances made in WAVE3 in the last few decades. We believe that targeting WAVE3 can be one of the approaches for potential therapeutic strategies for treating patients with cancer, especially breast cancer.

Genetic approaches to target WAVE3 expression and activity in cancer cells

There are different approaches to target a specific protein: direct biochemical methods, genetic interaction and genomic methods, and computational inference methods (57). In this section, we describe the various approaches that have been used to successfully target and inhibit WAVE3. Ji *et al.* (29) reported using short interfering RNAs (siRNA) targeting of WAVE3 in HepG2 cells. The expression of WAVE3 protein significantly decreased in si-WAVE3 transfected HepG2 cells than controls. Similarly, Zhu *et al.* (3) found out that the WAVE3 protein significantly decreased after using siRNA to knockdown the proteins in another liver carcinoma cell line, CC-LP-1 cells. All characteristics of a tumor cells such as migration, invasion and proliferation were inhibited after the WAVE3 knockdown using siRNAs (3). The siRNA-mediated knockdown of gene expression is, however, transient since the knockdown effect cannot be sustained beyond 96–120 hours post transfection. Therefore, long-term biological effect of gene knockdown cannot be assessed using siRNAs, including *in vivo* effects. Accordingly, Lu

et al. (31) used short hairpin RNA (shRNA) technique to knockdown WAVE3 in ovarian cancer cells. In another study, Sossey-Alaoui *et al.* (5) used shRNA to overcome the limitation of siRNA. Stable shRNA-mediated WAVE3 knockdown in human MDA-MB-231 and murine 4T1 breast cancer cells was found to significantly inhibit tumor growth and metastasis in mouse models for breast cancer (36). In another study, knockdown of WAVE3 in PC-3 and DU-145 prostate cancer cell lines was carried out using ribozyme transgene method (15,58). The authors also demonstrated that there was a decrease in invasion of the cells after WAVE3 was knocked down using this method. Both studies reported that this technique did not affect the cell adhesion with matrix whereas invasion through Matrigel decreased significantly (8).

CRISPR/Cas9 technology has been used as an efficient and versatile tool to permanently target and edit specific gene at precise locations. CRISPR/Cas9 has proved to be competent than other prevailing gene editing approaches such as siRNA and shRNA. Bledzka *et al.* (36) used CRISPR/Cas9-mediated knockout of WAVE3 where single-guide RNAs (sgRNAs) were used to target exon 2 and exon 3 of the human WAVE3 gene. The number of invadopodia formed and the total area of ECM degradation decreased significantly in WAVE3 knockout cells compared to control (Scram CRISPR) MDA-MB 231.

Another technique to target WAVE3 is achieved by genetic knockdown of CYFIP1 with stapled peptides known as WASF helix mimics (WAHM). Genetic knockdown of CYFIP1 eventually reduces WAVE3 levels, preventing the invasion. Peptide-mediated inhibition of WAVE3 could be a promising therapeutic approach for suppressing tumor invasion and metastasis. However, the level of suppression of invasion by this method is not as much of as compared to the shRNAs (46). Taken together, these studies suggest that the genetic approaches used to knockdown WAVE3 causes inhibition of invasion and migration of cells.

WAVE3 and microRNAs

Small non-coding RNA molecules, also known as microRNAs, are responsible for RNA silencing and post-transcriptional regulation of gene expression (28,59,60). Out of all the miRNAs, nine of them are tumor suppressive in breast cancer. They are miR-30a, miR-30c, miR-31, miR-126, miR-140, miR146b, miR200c, miR-206, and miR-335 (61). miR200 microRNAs are responsible for the expression of WAVE3 protein and regulation of epithelial-mesenchymal transition (EMT) during tumor progression and metastasis (8). WAVE3 expression is suppressed when the miR200 directly targets the 3'-untranslated regions. Invasive and non-invasive cancer cells showed that miR200 and WAVE3 are inversely correlated (28). Mesenchymal cell types such as MDA-MB 231 and PC-3 do not express miR200 whereas epithelial cell types such as MCF7 and HT29 shows overexpression of miR200 (28). Moreover, there is a decrease in miR-200c because of loss of p53. However, loss of WAVE3 causes increase in p53 levels. On the other hand, the downstream protein of p53, Bcl-2 was decreased because of inhibition by p53. It has been reported that downregulation of microRNA miR-31, a metastasis suppressor gene (59), promotes invasion-metastasis cascade (28,60). Furthermore, Sossey-Alaoui *et al.* (8) also showed that there is an inverse correlation between WAVE3 and miR-31 expression level, especially in human breast cancers.

WAVE3 and matrix metalloproteinases (MMPs)

MMPs have been found to be involved in cell invasion and migration (27,62) and they are an essential part of invadopodia (27). MMPs are enzymes that are involved in the breakdown of ECM. Malignant cells use MMPs to enzymatically degrade the ECM through p38 MAPK pathway to facilitate invasion and metastasis (9,63). MMPs play a vital role in different steps of tumor growth and metastasis, angiogenesis, and wound healing (64,65). Previous studies have demonstrated that averting metastasis is possible by targeting and inhibiting the MMPs activity (31). Nuclear factor NFkB, a protein complex involved in invasion and metastasis of cancer, plays a key role in the production of MMPs (MMP-1, MMP-3, and MMP-9) (66). Loss of WAVE3 does not affect MMP-2 but inhibits the expression levels of MMP-1, MMP-3, and MMP-9 (7). However, treatment with MMP activator phorbol myristate acetate (PMA) can bring back the MMP production without altering siRNA-mediated WAVE3 knockdown. Moreover, downregulation of p38 and MMP production is mediated by WAVE3 (7). Low expression of MMP-2 and MMP-9 was observed after WAVE3 knockdown in SKOV3 cells whereas the expression of MMP-2 and MMP-9 was high in A2780 cells. Similarly, there was low expression of MMP-2 in PC-3 cells (58). Further, knockdown of WAVE3 phosphorylation causes decrease in MMP-2 and MMP-9 activity (27).

Pathways contributing to cancer progression and metastasis

WAVE3 and c-Abl

c-Abl, a non-receptor tyrosine kinase, is found in the nucleus and cytoplasm. c-Abl is responsible for the regulation of cell motility and localization of focal adhesions and lamellipodia (67). Sossey-Alaoui *et al.* (4) showed that WAVE3 is downstream of c-Abl tyrosine kinase. Further, treatment with platelet-derived growth factor (PDGF) causes phosphorylation of WAVE3 (6). Interaction of WAVE3 with Ableson (Abl) non-receptor tyrosine kinase promotes the tyrosine phosphorylation. On the other hand, the Abl-mediated phosphorylation of WAVE3 is blocked by STI-571, a specific inhibitor of Abl kinase activity (4). Both WAVE3-Abl interaction and Abl-kinase activity are necessary for the Abl-mediated phosphorylation of WAVE3 (28).

WAVE3 and phosphatidylinositol 3-kinase (pI3K)

Phosphatidylinositol 3-kinases (PI3Ks), also known as phosphoinositide 3-kinases, are enzymes that regulates cellular functions like cell growth, motility, and survival (68,69). PI3K signaling pathway is found to be dysregulated in almost all cancers, including breast cancer (70,71). Targeting this pathway can be a potential therapeutic option for treating TNBC patients (72,73). Reports have shown that PI3K is upstream of WAVE3 (6). Sossey-Alaoui *et al.* (5) demonstrated that knockdown of WAVE3 using siRNA decreases the ability to form lamellipodia at the migrating edge of breast cancer cells. Moreover, LY294002, an inhibitor of PI3K, prevents the formation of lamellipodia and cell migration. Thus, PI3K is essential for WAVE3 activity (6). Further, lamellipodia formation and WAVE3-mediated cell motility in breast cancer cell line (MDA-MB-231) is induced by PDGF which was confirmed by wound healing and migration assays. It was further reported that knockdown of WAVE3 using the same technique causes inhibition in the formation of PDGF-induced lamellipodia in breast cancer cells (6). Direct physical interaction of p85, the regulatory subunit of PI3K, with WAVE3 may be required for WAVE3-mediated lamellipodia formation, cell migration, and invasion. This interaction is facilitated by the BR domain of WAVE3 and the C-terminal SH2 domain of p85. Thus, it is confirmed that PI3K is imperative for the regulation of WAVE3-mediated lamellipodia formation and cell migration (6).

WAVE3 and AKT pathway

Protein kinase B (AKT) has been implicated in a variety of cellular functions such as cell proliferation and migration (74,75). AKT is a downstream effector of PI3K (76). PI3K plays a role in cancer progression and metastasis through both AKT-dependent and AKT-independent mechanisms (77). Inhibition of AKT prevents the formation of invadopodia in breast cancer cells (9,76). There is an increase in phosphorylation of AKT due to overexpression of WAVE3. Knockdown of WAVE3 does not affect the AKT, ERK 1/2, or JNK. NFKB activates AKT signaling and if the AKT is inhibited using AKT inhibitor (MK-2206), NFKB is negatively affected (13). This was proved by treating MDA-MB-231 cells with MK-2206, whereby, there was a significant reduction in phospho-AKT and phospho-p65 whereas total AKT or total p65 levels were not affected (9).

WAVE3 and transforming growth factor-β (TGF-β)

TGF-β, a multifunctional cytokine (32), plays a dual role in breast cancer, acting both as a tumor-promotor and tumor suppressor (78-80). It has been reported that high TGF-β contributes to tumor suppression during early stages of tumor. On the other hand, they lose the growth inhibitory effect, become malignant and aid in promoting tumor towards the later stages (79,81,82). However, the switch of TGF-β from tumor suppressor to tumor promoter remains unelucidated (83,84). Reports have shown that p53 is involved in the switch of TGF-β from tumor suppressor in pre-malignant cells to tumor promotor in cancer cells (83). The tumor suppressor protein p53 inhibits TGF-β induced EMT through activation of miR-200c (85). Taylor *et al.* (32) found out that TGF-β is upstream of WAVE3 and there was an upregulation of WAVE3 by TGF-β in metastatic breast cancer cells (MDA-MB-231 and BT549). However, there was only a slight upregulation detected in non-metastatic cells (MCF7 and T47D). TGF-β receptors not only activate Smads, but they activate other signaling pathways as well. Activation of several receptors such as epidermal growth factor (EGF) through signal transducer and activator of transcription (STAT) and NFkB by tumor necrosis factor-α

(TNF-α) causes inhibition of TGF-β (86). Taylor *et al.* (32) further reported that Smad2 and β3 integrin are important to induce WAVE3 expression in TNBCs by TGF-β.

WAVE3-p38 pathway

p38 MAPKs belongs to the MAPK family and they are responsible for regulating cellular activities such as proliferation, differentiation, migration, and survival (87-89). They are activated by cellular stress and cytokines. TGF-β activates other signaling pathways such as p38 MAPK pathway (86,90). Knockdown of WAVE3 expression using siRNAs causes decrease in cell motility and metastasis-invasion cascade, especially in breast cancer cells. Further, there was a decrease in p38 MAP kinase phosphorylation because of downregulation of WAVE3. Further, it was interesting to note that WAVE3-mediated downregulation of p38 activity does not alter the expression levels of WAVE1 and WAVE2 genes even after knockdown of WAVE3 (7,28). Studies have reported that p38 MAPK is downstream of WAVE3 in cell migration and invasion (5,7). Sossey-Alaoui *et al.* (7) further demonstrated that knockdown of WAVE3 decreases phosphorylation levels of p38 MAPK levels whereas the activity of AKT, ERK1/2, and JNK remains unchanged. It was further demonstrated that Rac1 activates both WAVE3 and p38 MAPK. Thus, WAVE3 is essential for Rac1-dependent activation of p38 MAPK pathway. Reports have indicated that WAVE3 regulates the MMP activity via p38 pathway (7). Overall, WAVE3 may play an important role in cancer cell invasion and metastasis through MMP production and p38 MAPK pathway. Furthermore, WAVE3 phosphorylation by Abl tyrosine kinase is important for MMPs activity.

WAVE3 and NFκB pathway

NFκB is a vital signaling pathway that is involved in pathogenesis as well as invasion and metastasis of several cancer types (91-93). Davuluri *et al.* (9) showed that there is a correlation between WAVE3 and NFκB signaling. They further highlighted the importance of WAVE3 for NFκB activation and demonstrated that knockdown of WAVE3 in MDA-MB 231 causes inhibition of NFκB activity and vice versa. Furthermore, the authors also showed that TNFα-induced stimulation of NFκB signaling activates Akt, and thereby causes cancer cells to undergo apoptosis and finally leads to cell death. In another study, it was revealed that either basic rich or PRD of WAVE3 is required for NFκB signaling (66). The authors showed that loss of WAVE3 in MDA-MB-231 cells causes inhibition of NFκB. This is because there is a nuclear translocation of NFκB. It appears that NFκB activates the production of MMPs, including MMP-1, MMP-3, and MMP-9. WAVE3-mediated modulation of NFκB, in addition to Akt signaling, is essential for formation of invadopodia as well as MMP9 expression. Additionally, phosphorylation of transcription factor p65 subunit and nuclear translocation are required for NFκB-mediated activation (9). Moreover, WAVE3 phosphorylation is important for the nuclear translocation as well as MMP-9 activation (27). Hence, WAVE3 plays a vital role in the regulation of NFκB signaling.

Cytoplasmic vs. nuclear function of WAVE3 and the role of WAVE3: YB1 interaction in the regulation of CSCs

Y-box-binding protein-1 (YB1), also known as Y-box transcription factor, is present in the cytoplasm as well as the nucleus of a cell (94,95). The role of YB1 is to regulate mRNA translation in the cytoplasm and regulate expression of CSC genes in the nucleus. YB1 expression is found to be elevated in various human cancers, especially aggressive breast cancer cell lines (96). A recent study has shown that WAVE3 binds to YB1 through its PRD and translocate YB1 to the nucleus, thereby activating CSC genes. It was also established that WAVE3 and YB1 work together to regulate the breast CSC population and gene expression. Therefore, a novel and never previously described function of WAVE3 in the nucleus, has been found to implicate WAVE3 in the regulation and maintenance of CSCs in breast cancer. Thus, WAVE3 may act as a potential biomarker for CSCs, and preventing WAVE3/YB1 binding may be a novel therapeutic target to treat TNBC (36).

Conclusions

Several signaling pathways have been shown to be associated with WAVE3 in the development, progression, and metastasis of several cancers, as well as the maintenance of CSCs in breast cancer (*Figure 1*). Therefore, targeting WAVE3 may serve as potential therapeutic strategy for treating cancer patients, including those with TNBC tumors. However, the domain of WAVE3 responsible for activating the signaling pathway has not been elucidated.

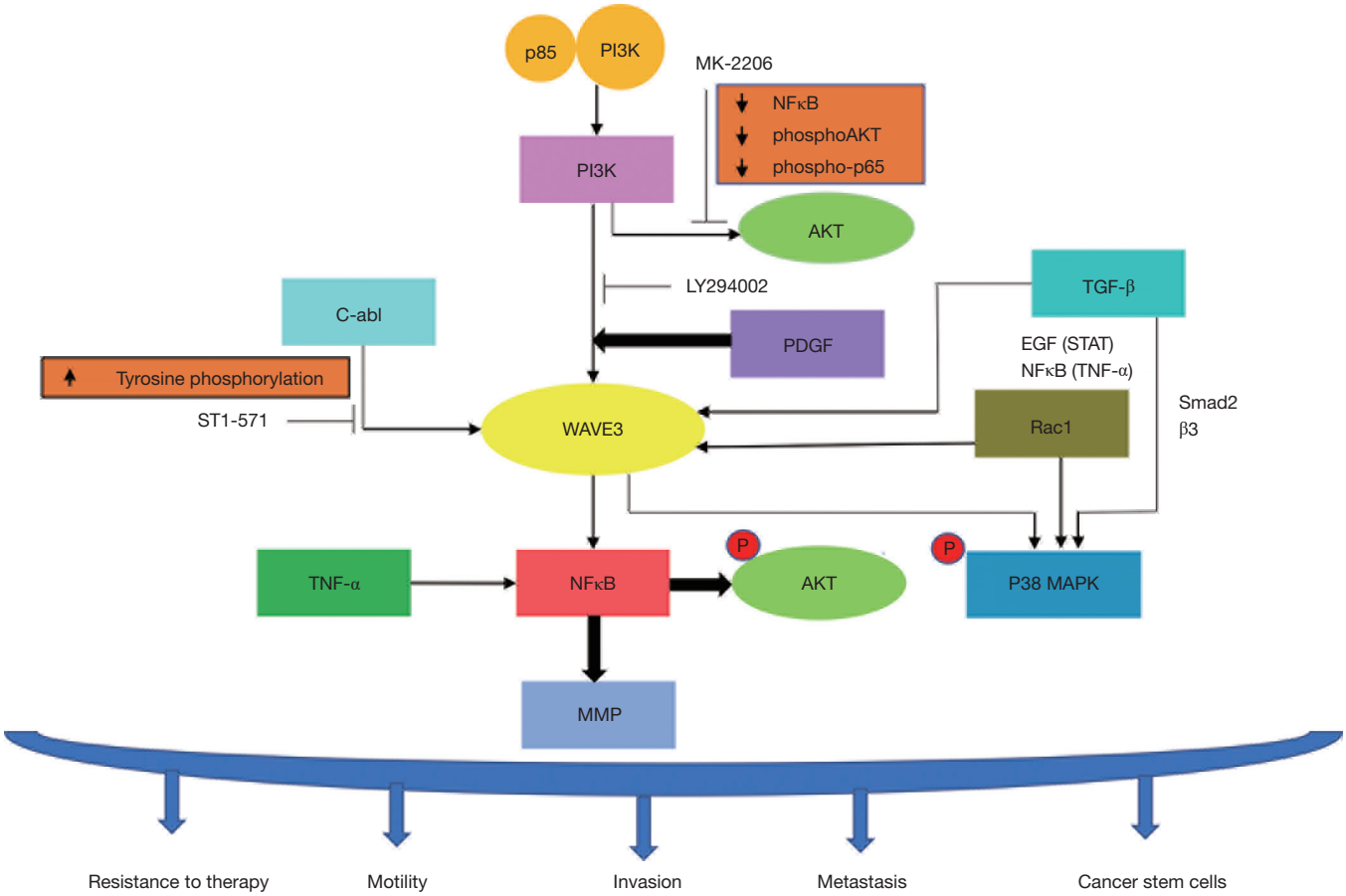

Figure 1 Model representing the molecular signaling pathway of WAVE3 contributing to cancer progression and metastasis.

Identifying the specificity of the different WAVE3 domains is crucial in the regulation of the different molecular signaling pathways and the modulation of the invasion-metastasis cascade.

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