Molecular Mechanisms of Lymph Node Metastasis

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Abstract

Despite improvements in diagnostic and therapeutic modalities, the prognosis of advanced cancer with extensive invasion and metastasis remains poor. The severity of a clinical prognosis depends on whether lymph node metastasis has occurred. For metastasis to occur, tumor cells must undergo a multistep process through a series of sequential and selective events. The metastatic process consists of detachment, local invasion, motility, lymphangiogenesis, lymphatic vessel invasion, survival in the circulation, adhesion to endothelial cells, extravasation, and regrowth in lymph nodes. Among them, the most important process is lymphangiogenesis, which is regulated by members of the vascular endothelial growth factor (VEGF) family and their receptors. In addition to lymphangiogenesis, it is well accepted that cancer stem cells play a significant role in metastasis. Although several types of metastasis-associated molecules have been identified, the expression of these molecules differs among esophageal, gastric, and colorectal cancer. This chapter will review the cellular and molecular mechanisms of lymph node metastasis including lymphangiogenesis and cancer stem cells in these human cancer types.

Keywords

VEGF · PDGF · Migration · Cancer stem cell

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S. Natsugoe (ed.), *Lymph Node Metastasis in Gastrointestinal Cancer*,
https://doi.org/10.1007/978-981-10-4699-5_3

3.1 Lymphangiogenesis

In the multistep process of lymph node metastasis, the most important step is lymphangiogenesis, which plays an important role in both tumor growth and metastasis [1, 2]. Beasley et al. [3] analyzed human head and neck cancers by immunohistochemical staining for the lymphatic endothelial markers LYVE-1 and CD34, and the proliferation marker Ki-67. They observed Ki-67 nuclear staining in a proportion of small intratumoral lymphatic endothelial cells, suggesting that the intratumoral lymphatics do indeed generate new vessels. Furthermore, they found that a high intratumoral lymphatic vessel density is associated with lymph node metastases and an infiltrating margin of tumor invasion. Their study provided evidence that lymphatic growth can occur in human cancers and may in some cases contribute to lymph node metastasis. It has been also reported that tumor lymphangiogenesis correlates with lymphatic metastasis in esophageal squamous cell carcinoma [4], gastric cancer [5, 6], and colorectal cancer [7].

Lymphangiogenesis is regulated by members of the VEGF family and their receptors. The VEGF family includes VEGF-A, -B, -C, -D, -E, and -F and placental growth factor (PlGF) [8]. Among them, VEGF-C and VEGF-D are essential for lymphangiogenesis. Both VEGF-C and VEGF-D are ligands for VEGF receptor (VEGFR)2 and VEGFR3 [9]. Activation of VEGFR3 induces lymphangiogenesis, whereas activation of VEGFR2 is thought to drive angiogenesis. In addition to these molecules, platelet-derived growth factors (PDGFs) and PDGF receptors (PDGFRs) not only promote angiogenesis but are also important players in lymphangiogenesis. These molecules are summarized in Fig. 3.1.

He et al. [10] investigated how tumor cells gain access to lymphatic vessels and at what stage tumor cells initiate metastasis. They showed that VEGF-C produced by tumor cells induces extensive lymphatic sprouting towards the tumor cells as well as dilation of the draining lymphatic vessels in a mouse model. In this model, a significant increase in lymphatic vessel growth occurs between 2 and 3 weeks after tumor xenotransplantation, and lymph node metastasis occurs at the same stage. Lymphatic vessel growth can be blocked by inhibition of VEGFR3 signaling by systemic delivery of a soluble VEGFR3 immunoglobulin. However, VEGFR3 immunoglobulin cannot suppress lymph node metastasis when the treatment is started at a later stage after the tumor cells have already spread. Therefore, tumor cell entry into lymphatic vessels is a critical step during tumor dissemination.

3.1.1 VEGF-C

VEGF-C is an essential chemotactic and survival factor during lymphangiogenesis and is required for the sprouting of the first lymphatic vessels from embryonic veins [11]. Skobe et al. [12] reported that VEGF-C can selectively induce hyperplasia of the lymphatic vasculature in breast cancer in a mouse model. In gastrointestinal cancers, a correlation between VEGF-C expression and lymph node metastasis has been reported. Moreover, expression of VEGF-C protein is observed in cancer cells

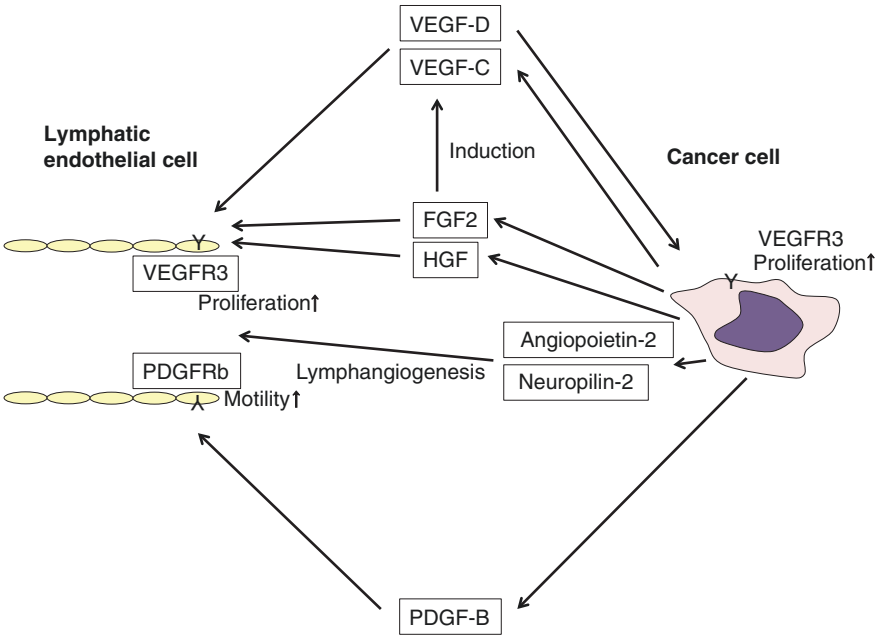

Fig. 3.1 Lymphangiogenesis-associated molecules. Lymphangiogenesis is regulated by members of the VEGF and PDGF families and their receptors

by immunohistochemical analysis. Kitadai et al. [13] examined the expression of VEGF-C in 48 specimens of human esophageal carcinoma tissues by immunohistochemistry. They reported that VEGF-C expression was correlated with depth of tumor invasion, tumor stage, venous invasion, lymphatic invasion, and lymph node metastasis in esophageal squamous cell carcinoma. Matsumoto et al. [14] examined VEGF-C expression in esophageal squamous cell carcinoma and analyzed the relationships between VEGF-C expression and clinicopathological findings such as lymph node metastasis. They demonstrated that VEGF-C overexpression was significantly correlated with depth of tumor invasion, lymphatic invasion, and lymph node metastasis in esophageal squamous cell carcinoma. Moreover, Yonemura et al. [5] studied the expression of VEGF-C in 32 gastric cancer tissue samples by immunohistochemistry. They showed that VEGF-C expression was correlated with lymph node status, lymphatic invasion, venous invasion, and tumor infiltrating patterns in gastric cancer. Furthermore, Amioka et al. [6] analyzed VEGF-C expression in 139 gastric cancer cases. They found that VEGF-C immunoreactivity was associated with a greater depth of tumor invasion, lymphatic invasion, and lymph node metastases in gastric cancers invading the submucosa. Onogawa et al. [15] analyzed the expression of VEGF-C and VEGF-D protein by immunohistochemistry in 139 surgical specimens of human colorectal cancer. They found VEGF-C expression in 46.8% of colorectal cancer cases, which was correlated with the depth of tumor invasion, lymphatic involvement, venous involvement, lymph node metastasis, and

liver metastasis. These reports demonstrate that VEGF-C plays an important role in lymph node metastasis in gastrointestinal cancers.

Tumor-associated lymphatic vessel density is correlated with metastasis to draining lymph nodes and poor prognosis. In gastrointestinal cancers, a correlation between VEGF-C expression and lymphatic vessel density has been reported. Hachisuka et al. [16] investigated VEGF-C expression and lymphatic vessel density in gastric cancer. They found that the expression of VEGF-C was correlated with high lymphatic vessel density. Li et al. [17] evaluated the expression of VEGF-C and VEGFR3 in 147 colon cancer cases. They reported that VEGF-C expression was positively correlated with lymphatic vessel density. These results demonstrate that VEGF-C promotes lymph node metastasis through lymphangiogenesis.

Although overexpression of VEGF-C has been reported, the mechanism that underlies this overexpression in cancers remains unclear. Matsumura et al. [18] investigated DNA methylation and expression of the VEGF-C gene (*VEGFC*) in gastric cancer. Bisulfite DNA sequencing analysis revealed that *VEGFC* was not methylated in nine of 31 gastric cancer samples, while demethylation was not observed in the corresponding non-neoplastic mucosa samples. Overexpression of VEGF-C was frequently found in gastric cancer cases with *VEGFC* demethylation. Thus, these results suggest that demethylation and activation of *VEGFC* is likely involved in lymphangiogenesis in gastric cancer.

3.1.2 VEGF-D

In addition to VEGF-C, VEGF-D also promotes tumor metastasis through lymphangiogenesis [19]. The mature form of VEGF-D shares 61% amino acid sequence identity with VEGF-C, and binds to both VEGFR2 and VEGFR3 [9]. In mice, Vegfd binds only to Vegfr3, indicating that Vegfd might have a somewhat different function in mice and humans [20]. It has been reported that VEGF-D also modulates prostaglandin levels to regulate lymphatic vessel dilation [21]. Expression of VEGF-D protein can be detected in cancer cells by immunohistochemical analysis, and a correlation between VEGF-D expression and lymph node metastasis has been reported in gastrointestinal cancers. Kozlowski et al. [22] reported that VEGF-D expression was significantly correlated with tumor location, tumor size, histological grade, depth of invasion, and lymph node metastasis in esophageal squamous cell carcinoma. Onogawa et al. [23] examined VEGF-C and VEGF-D expression by immunohistochemistry in 140 surgical specimens of submucosally invasive gastric cancer. VEGF-C expression was associated with lymphatic invasion and lymph node metastasis; however, there was no association between VEGF-D expression and clinicopathological features. Arigami et al. [24] analyzed VEGF-C and VEGF-D expression in 80 early-stage gastric cancers. VEGF-C and VEGF-D was detected in 27.5 and 21.3% cases, respectively, and their expression was closely related to lymph node micrometastasis. Furthermore, Onogawa et al. [15] analyzed VEGF-C and VEGF-D protein expression by immunohistochemistry in 139 surgical

specimens of human colorectal cancer. They observed VEGF-D expression in 29.5% of colorectal cancer cases, which was correlated with the depth of tumor invasion, lymph node metastasis, and liver metastasis. These results indicate that in addition to VEGF-C, VEGF-D also plays an important role in lymph node metastasis in gastrointestinal cancers.

Besides VEGF-C, a correlation between VEGF-D expression and lymphatic vessel density has been reported. Wang et al. [25] examined the expression of VEGF-D in 123 patients with gastric cancer. They reported that peritumoral-lymphatic vessel density was significantly associated with lymph node metastasis, lymphatic vessel invasion, VEGF-C expression, and VEGF-D expression. Su et al. [26] analyzed the expression of VEGF-D, SMAD4, and SMAD7 in 251 colon cancer samples. They found that positive expression of VEGF-D was significantly correlated with lymph node metastasis and high lymphatic vessel density. Therefore, VEGF-D also promotes lymph node metastasis through lymphangiogenesis.

3.1.3 VEGFR3

VEGFR3 is a tyrosine kinase receptor that is expressed predominantly in the endothelium of lymphatic vessels [27]. VEGFR3 stimulation alone protects the lymphatic endothelial cells from serum deprivation-induced apoptosis and induces their proliferation and migration. At least some of these signals are transduced via protein kinase C-dependent activation of the ERK1/ERK2 MAPK signaling cascade and via a wortmannin-sensitive induction of AKT phosphorylation. These results demonstrate a critical role of VEGF-C/VEGFR3 signaling in the proliferation and survival of lymphatic endothelial cells [28]. VEGFR3 was originally thought to be expressed specifically in the lymphatic endothelium; however, VEGFR3 is also expressed in a small subset of blood vessels in normal tissues and can be reexpressed in angiogenic blood vessels in certain pathological conditions [29]. Furthermore, VEGFR3 has also been detected in cancer cells, including lung adenocarcinoma [30] and gastric cancer cells. Su et al. [30] reported that the VEGF-C/VEGFR3 axis enhances cancer cell mobility and invasiveness, and contributes to the promotion of cancer cell metastasis through upregulation of the neural cell adhesion molecule contactin-1. Immunohistochemical analyses in lung cancer and colorectal cancer revealed that high levels of VEGFR3 and VEGF-C expression correlated closely with clinical metastasis and patient survival. Kodama et al. [31] found that VEGFR3-specific immunoreactivity was detected on gastric cancer cells. Furthermore, in vitro treatment of a gastric cancer cell line with VEGF-C stimulated cell proliferation and increased expression of cyclin D1, PlGF, and autocrine motility factor. In a mice xenograft model, the tumor growth of VEGF-C-transfected cells was greatly accelerated in comparison with that of control cells. Greater angiogenesis and lymphangiogenesis were also detected in VEGF-C-transfected tumors than in control tumors. Therefore, the VEGF-C/VEGFR3 axis plays a role in the progressive growth of human gastric cancer through both autocrine and paracrine

mechanisms. Tanaka et al. [32] examined the expression and function of the VEGF-D/VEGFR3 axis in human gastric cancer. They found that 34% of gastric cancer cases expressed both VEGF-D and VEGFR3. In vitro treatment of a gastric cancer cell line with VEGF-D increased the expression of cyclin D1 and BCL-2 and stimulated cell proliferation. Therefore, in addition to VEGF-C, VEGF-D is likely to participate in the progression of gastric cancer by acting via autocrine and paracrine mechanisms.

3.1.4 PDGF-B

Members of the PDGF family are often expressed at high levels in many cancers [33]. The PDGF family consists of five isoforms, -AA, -AB, -BB, -CC, and -DD, usually referred to as PDGF-A (AA), PDGF-B (AB and BB), PDGF-C (CC), and PDGF-D (DD) [34]. Their biological activities are mediated by the tyrosine kinase receptors PDGF receptor (PDGFR)a and PDGFRb. PDGFRa binds all possible forms of PDGF except PDGF-DD, whereas PDGFRb preferentially binds PDGF-BB. PDGFs induce tumor growth and stimulate angiogenesis [35]. In addition, Cao et al. [36] showed that PDGF-BB stimulates MAP kinase activity and cell motility of isolated lymphatic endothelial cells. Expression of PDGF-BB in murine fibrosarcoma cells induces tumor lymphangiogenesis, leading to enhanced metastasis in lymph nodes. Matsumoto et al. [37] examined the expression of PDGF-BB and VEGF-C by immunohistochemistry in esophageal squamous cell carcinoma, and found that expression of PDGF-BB and VEGF-C was correlated with lymph node metastasis and lymphatic invasion. Furthermore, they found that lymphangiogenesis in PDGF-BB- or VEGF-C-positive tumors was higher than in negative tumors. Kodama et al. [38] examined the expression of PDGF-BB and PDGFRb in 38 surgical specimens of gastric cancer. They showed that PDGF-B and PDGFRb mRNA expression was significantly higher in patients with lymph node metastasis than in those without, and was also significantly higher in diffuse-type carcinoma than in intestinal-type carcinoma. Expression of PDGF-B was detected in gastric cancer cells, whereas PDGFRb was expressed predominantly in stromal cells. In orthotopic TMK-1 gastric cancer cell line tumors, the cancer cells expressed PDGF-B but not PDGFRb. PDGFRb was expressed by stromal cells, including lymphatic endothelial cells. These data demonstrate that secretion of PDGF-B by cancer cells and expression of PDGFRb by tumor-associated stromal cells are associated with lymphatic metastasis.

3.1.5 Angiopoietin-2

The angiopoietin family growth factors have been identified as ligands for Tie-2. Angiopoietin-1 activates Tie-2, leading to receptor autophosphorylation upon binding, and it simulates endothelial cell migration in vitro, contributing to blood vessel stabilization by recruitment of pericytes [39]. In contrast, angiopoietin-2 is crucial for establishing the lymphatic vasculature. VEGF-C/VEGFR3 signaling is a critical

primary proliferation pathway for lymphatic vessels, whereas angiopoietin-2 is important in later remodeling stages [40].

Jo et al. [41] measured the serum levels of angiopoietin-2 in patients with gastric cancer by immunoassay; elevated serum angiopoietin-2 levels were associated with positive lymph node involvement. Wang et al. [42] analyzed the expression of angiopoietin-2 by immunohistochemistry in 53 gastric cancer and 23 normal gastric mucosa samples. They found that angiopoietin-2 expression was significantly increased in gastric cancer tissues (74%) and was correlated with lymph node metastasis. However, the importance of angiopoietin-2 for lymphatic metastasis of human esophageal cancer or colorectal cancer is still unknown.

3.1.6 Neuropilin-2

Neuropilin-2 was initially identified as a semaphorin receptor and mediator of axon guidance [43]. However, it has been reported that neuropilin-2 binds to VEGF-C [44]. Homozygous neuropilin-2 mutants show a reduction in small lymphatic vessels and capillaries prenatally [45]. Caunt et al. [46] reported that an antibody against neuropilin-2 disrupts VEGF-C-induced lymphatic endothelial cell migration, but not proliferation. It does not affect established lymphatics in normal adult mice but reduces tumoral lymphangiogenesis and functional lymphatics associated with tumors. It also reduces metastasis to sentinel lymph nodes and distant organs.

In normal tissue, neuropilin-2 staining is detected in blood or lymphatic vessels, while staining of neuropilin-2 is identified not only in the vascular or lymphatic endothelial cells, but also in the cytoplasm of cancer cells. Fung et al. [47] examined the expression of neuropilin-2 in esophageal squamous cell carcinoma by immunohistochemistry. They found that levels of neuropilin-2 expression were significantly upregulated in esophageal squamous cell carcinoma, and were correlated with lymph node metastasis. These results suggest that neuropilin-2 plays an important role in lymphatic endothelial cells, as well as in cancer cells. Nonetheless, the importance of neuropilin-2 for lymphatic metastasis of human gastric cancer or colorectal cancer is still unknown.

3.1.7 MicroRNAs

MicroRNAs are 18- to 25-nucleotide-long noncoding RNA molecules that regulate the translation of many genes [48]. Recent studies have indicated that microRNA expression levels are altered in most types of human cancers, and microRNAs are important gene regulators that play critical roles in biological processes and function as either tumor suppressors or oncogenes.

Yang et al. [49] observed altered expression of miRNAs in human lymphatic endothelial cells cocultured with lymphangiogenesis-inducing VEGF-C-transformed gastric cancer cells, with 47 upregulated and 42 downregulated

miRNAs. Upregulated miRNAs included miR-648, miR-5002-3p, miR-4754, miR-4760-5p, miR-4491, miR-4252, miR-5007-3p, and miR-647; and downregulated miRNAs included miR-3178, miR-593-5p, miR-4485, miR-135a-3p, miR-17, miR-1469, and miR-124-5p.

Hu et al. [50] determined that ectopic miR-128 overexpression inhibited VEGF-C expression and reduced the activity of a luciferase reporter containing the VEGF-C 3′-untranslated region. Furthermore, in vivo restoration of miR-128 significantly suppressed the tumorigenicity of A549 cells in nude mice and inhibited lymphangiogenesis of tumor xenografts.

Liu et al. [51] reported that miR-486-5p was significantly downregulated in colorectal cancer tissues compared with adjacent normal tissue by quantitative real-time polymerase chain reaction. They found that neuropilin-2 is a direct functional target of miR-486-5p in colorectal cancer cells, and upregulation of miR-486-5p in colorectal cancer cells was negatively correlated with neuropilin-2 expression. Furthermore, overexpression of miR-486-5p inhibited tumor growth and lymphangiogenesis in nude mice.

3.1.8 Other Factors

Fibroblast growth factor 2 (FGF2, also known as basic FGF) is another factor that is reported to promote lymphangiogenesis. It stimulates lymphangiogenesis in the mouse cornea and upregulates expression of VEGF-C and VEGF-D in this model; this effect was blocked by VEGFR3 antibodies, indicating that FGF2 promotes lymphangiogenesis via induction of VEGF-C expression and activation of VEGFR3 signaling [52]. Mikami et al. [53] analyzed FGF2 expression in human esophageal carcinoma. They found that FGF2 was associated with tumor invasion, lymph node metastasis, and pathological stages. Furthermore, Ueki et al. [54] examined FGF2 expression in gastric cancer. They observed FGF2 expression in 70% of gastric cancer cases, which was confined to the tumor cells. FGF2 expression was correlated with a higher rate of lymph node metastases. It was also shown that hepatocyte growth factor (HGF) can bind to VEGFR3 and induce lymphangiogenesis [55]. Kammula et al. [56] reported that HGF expression is associated with primary colorectal cancer progression and can be used to predict outcome.

3.2 Cell Migration and Lymph Node Metastasis

In addition to lymphangiogenesis, cell migration is also an important process in lymph node metastasis. Cell migration is a process that involves reorganization of the cytoskeleton, formation of protrusions, establishment of adhesive contacts at the leading edge, and cell contraction and detachment at the trailing edge. In gastrointestinal cancers, several genes were found to promote cell migration, thereby enhancing cancer cell invasion and metastasis (Fig. 3.2).

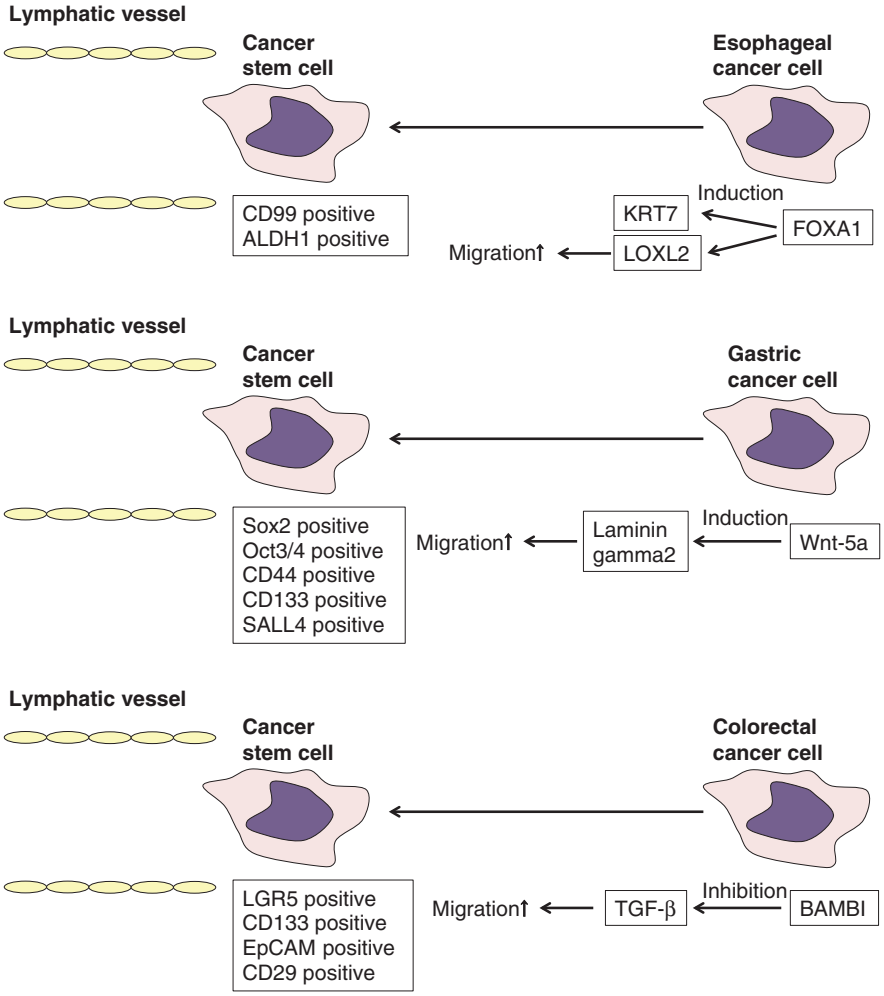

Fig. 3.2 Cell migration-associated molecules in esophageal, gastric, and colorectal cancer. Several markers for cancer stem cells have been reported in esophageal (**a**), gastric (**b**), and colorectal cancer (**c**)

3.2.1 FOXA1 Promotes Lymph Node Metastasis in Esophageal Squamous Cell Carcinoma

Sano et al. [57] compared the gene expression profiles of 24 esophageal squamous cell carcinomas with extensive lymph node metastasis and 11 esophageal squamous cell carcinomas with no metastatic lymph nodes by microarray. They found 209 genes whose expression was associated with lymph node metastasis. Among them, overexpression of *CALB1*, *KRT7*, *MUC1*, and *CEACAM5* in poor prognostic cases with metastatic lymph nodes was confirmed by RT-PCR. They also identified

FOXA1 as a transcriptional factor co-expressed with *KRT7*. FOXA1 is a pioneer factor that possesses the ability to engage with closed chromatin, move nucleosomes, and ultimately allow subsequent binding of other transcription factors [58]. In esophageal squamous cell carcinoma, FOXA1 induces *KRT7* and *LOXL2* expression, both of which are associated with lymph node metastasis.

The *KRT7* gene, which encodes cytokeratin 7, is expressed in several simple ductal epithelia, in mesothelium, and in urothelium. However, cytokeratin 7 is sparsely expressed or absent in gastric foveolar epithelium, intestinal epithelium, hepatocytes, and squamous epithelia [59]. It has been reported that cytokeratin 7 expression is a statistically significant prognostic factor in patients with stage I and II esophageal squamous cell carcinoma [60]. In stage 0–III esophageal squamous cell carcinoma, lymph node metastasis is frequently found in cytokeratin 7-positive cases but not in cytokeratin 7-negative cases [61]. In contrast, a direct association between cytokeratin 7 expression and lymph node metastasis remains unclear. Because cytokeratin 7 expression is regulated by FOXA1, FOXA1 may also regulate several cancer-related genes, such as FOXA1-inducing genes.

The *LOXL2* gene, which encodes lysyl oxidase-like 2 protein, is a member of the LOX family of extracellular matrix-modifying enzymes [62]. Lysyl oxidase-like 2 catalyzes the cross-linking of collagens and elastin [63]. Furthermore, lysyl oxidase-like 2 protein promotes invasion by regulating the expression and activity of the extracellular proteins tissue inhibitor of metalloproteinase-1 (TIMP1) and matrix metalloproteinase-9 (MMP9) [64]. Overexpression of lysyl oxidase-like 2 is observed preferentially in esophageal squamous cell carcinomas with greater than five metastatic lymph nodes [57]. Lysyl oxidase-like 2 overexpression in pancreatic cancer cells enhances the epithelial–mesenchymal transition-like process, and increases migratory and invasive activity [65]. Taken together, these results suggest that FOXA1 plays an important role in lymph node metastasis in esophageal squamous cell carcinoma.

In contrast, Ren et al. [66] reported that expression of FOXA1 was not associated with lymph node metastasis in gastric cancer. Therefore, promotion of lymph node metastasis by FOXA1 may be a specific event in esophageal squamous cell carcinoma.

3.2.2 Wnt-5a Stimulates Cell Migration in Gastric Cancer Cells

Wnt-5a, a member of the Wnt family of proteins, is a representative ligand that activates the β-catenin-independent pathway via mobilization of intracellular Ca^{2+}, and the activation of protein kinase C, resulting in the stimulation of migration of several cultured cell lines, including cancer cells [67]. Kurayoshi et al. [68] analyzed the expression of Wnt-5a in 237 gastric cancer cases. They found that Wnt-5a expression was correlated with the depth of tumor invasion, tumor stage, and lymph node metastasis. They also found that Wnt-5a stimulates cell migration and invasion in gastric cancer cells. Overexpression of Wnt-5a activates focal adhesion kinase, and knockdown of Wnt-5a reduces the turnover of paxillin in focal adhesion.

Yamamoto et al. [69] performed microarray analyses to compare expression patterns between mouse fibroblast L cells that stably express wild-type *Wnt5a* and a mutant form of *Wnt5a*. They found that *Wnt5a* induces the expression of laminin gamma2 through the activation of protein kinase C and c-Jun-N-terminal kinase. The invasive activity of gastric cancer cells depends on laminin gamma2. These results demonstrate that Wnt-5a contributes to gastric cancer metastasis by increasing cell migration activity.

In contrast, Li et al. [70] showed that *WNT5A* is silenced in the highly invasive colon cancer cell line by histone modifications. Dejmek et al. [71] showed that the addition of recombinant Wnt-5a significantly reduces the migratory capacity of SW480 colon cancer cells. Therefore, promotion of lymph node metastasis by Wnt-5a may be a specific event in gastric cancer.

3.2.3 BAMBI Promotes Migration of HCT116 Colon Cancer Cells

Fritzmann et al. [72] compared gene expression patterns between metastatic and nonmetastatic stage-matched human colorectal cancers by microarray analysis. They found that *BAMBI* is highly expressed in approximately half of metastatic primary tumors and metastases but not in nonmetastatic tumors. BAMBI antagonizes the effects of TGF-β superfamily ligands by stably associating with the surface receptors [73], and is directly regulated by β-catenin in colon cancer cells [74]. Fritzmann et al. reported that BAMBI was expressed at a low level in normal colon mucosa and nonmetastatic primary tumors, and was highly expressed in the epithelial compartment of a subset of metastatic primary tumors. Forced expression of BAMBI inhibited TGF-β signaling and increased migration in colon cancer cells. In a mouse model, forced expression of BAMBI caused colon cancer cells to form tumors that metastasized more frequently to the liver and lymph nodes than control cancer cells.

Zhang et al. [75] investigated the expression of BAMBI in 276 gastric cancer tissues by immunohistochemistry. They found that BAMBI expression was significantly correlated with increased depth of invasion, lymphatic invasion, and lymph node metastasis. However, the significance of BAMBI for lymphatic metastasis of human esophageal cancer remains unknown.

3.3 Cancer Stem Cells and Metastasis

In the cancer stem cell model, tumors consist of subsets of cells with functional heterogeneity, and one small subset of cancer cells has the characteristics of stem cells. These cancer stem cells have the capability of both self-renewal and multilineage differentiation into diverse cancer cells, which play a decisive role in maintaining the capacity for malignant proliferation, invasion, metastasis, and tumor recurrence [76, 77]. Cancer stem cells play an important role not only in tumorigenicity but also in cancer metastasis [78]. CD133 is a marker of cancer stem cells or

tumor-initiating cells that can promote human colorectal cancer after being xenografted into immunodeficient mice [79]. A higher percentage of CD133-positive cancer cells is an unfavorable prognostic factor for colorectal cancer patients with locally advanced disease, and CD133-positive cancer cells contribute to tumor progression [80]. Li et al. [81] cultured CD133-positive colorectal cancer cells and analyzed the invasive and metastatic capabilities of CD133-positive single cell-derived progenies in a nude mouse model; all were tumorigenic, and the subcutaneous tumors expanded rapidly, while only one of three CD133-negative single cell progenies developed a minimal tumor in nude mice. They also found that CD133-positive single cell progenies possessed heterogeneity in intestinal wall invasion and lymph node and liver metastases, while CD133-negative single cell progenies did not produce secondary transplanted tumors or intestinal invasion and metastasis. Therefore, the ability of cancer stem cells to initiate new tumors is important for metastatic colonization. Among esophageal, gastric, and colorectal cancers, several markers for cancer stem cells have been reported (Fig. 3.2). It remains unclear which marker is the most important and specific.

3.3.1 Esophageal Cancer Stem Cells

Tang et al. [82] reported that CD90-positive cell populations in esophageal squamous cell carcinoma are endowed with stem cell-like properties and high tumorigenic and metastatic potential. In freshly resected clinical specimens, CD90-positive cells represent a rare cell population, the levels of which correlate with lymph node metastasis.

Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme responsible for oxidizing a variety of intracellular aldehydes to carboxylic acids. ALDH1 is proposed as a common marker for both normal and malignant stem and progenitor cells. Its activity has been employed successfully as a stem cell marker in breast cancer [83]. Wang et al. [84] investigated the expression of ALDH1 protein in human esophageal squamous cell carcinoma tissues by immunohistochemistry. They found that ALDH1 expression was correlated with lymph node metastasis and late pathologic TNM classification staging.

3.3.2 Gastric Cancer Stem Cells

The stemness factors Sox2, Oct3/4, and Nanog are associated with induced pluripotent stem cells, suggesting a correlation between these stemness factors and cancer stem cells [85]. Matsuoka et al. analyzed the expression of Sox2, Oct3/4, and Nanog in gastric cancer by immunohistochemistry. They found a significant correlation between Sox2-positive or Oct3/4-negative expression and invasion depth, lymph node metastasis, and lymphatic invasion.

In addition to ALDH1 and CD133, CD44 can be used to isolate cancer stem cell populations in colorectal cancer [86]. CD44-positive fractions of gastric cancer could

generate spheroid colonies under non-adherent conditions, and small numbers of these cells could generate tumors in mice [87]. Wakamatsu et al. [88] immunohistochemically examined the expression and distribution of representative cancer stem cell markers ALDH1, CD44, and CD133 in primary tumors and lymph node metastases of gastric cancer. They found that CD44 and CD133 expression was associated with lymph node metastasis, and the expression pattern of cancer stem cell markers in lymph node metastases tended to be the same as that in primary tumors.

SALL4 is a member of the *SALL* gene family and acts as a zinc-finger transcription factor. Previous studies have demonstrated that SALL4 has an essential role in maintaining the self-renewal and pluripotency of embryonic stem cells [89]. Zhang et al. [90] analyzed SALL4 expression in gastric cancer. They found that SALL4 levels were highly correlated with lymph node metastasis, and forced expression of SALL4 enhanced the proliferation and migration of human gastric cancer cells.

3.3.3 Colorectal Cancer Stem Cells

Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) is a target of Wnt signaling [91]. LGR5 is a marker for stem cells in the small intestine and colon, and plays a crucial role in the biological function of stem cells [92]. Uchida et al. [93] performed quantitative RT-PCR for *LGR5* expression in 37 colon cancer cell lines. They found that *LGR5* expression was higher in colon cancer cell lines derived from metastatic tumors compared with those from primary tumors. In clinical colorectal cancer specimens, *LGR5* expression was correlated with lymphatic invasion, vascular invasion, tumor depth, lymph node metastasis, and tumor stage.

Silinsky et al. [94] analyzed CD133-positive colorectal cancer cells by fluorescence-activated cell sorting analysis. They found that CD133-positive cancer cells correlated with the presence of lymph node metastasis in colorectal cancer.

Langan et al. [95] examined the expression of non-CD133 colorectal cancer stem cell markers including CD29, CD44, ALDH1A1, ALDH1B1, EpCAM, and CD166 in colorectal cancer tissue samples; of these, EpCAM and CD29 expression was associated with lymph node metastases.

3.4 Biomarkers for Lymph Node Metastasis

A better understanding of the changes in gene expression during invasion and metastasis may lead to new paradigms and possible improvements in the diagnosis and treatment of gastrointestinal cancer. In the past 20 years, numerous genes whose expression is upregulated or downregulated in lymph node metastasis have been reported by microarray analysis. Lymph node metastasis-associated genes identified by microarray analysis are summarized in Table 3.1. Although these genes are useful for the prediction of lymph node metastasis, their functions are largely unknown. Functional analysis of these molecules will further improve our understanding of the basic mechanisms of lymph node metastasis.

Table 3.1 Summary of genetic/epigenetic/gene expression alterations in gastric and intestinal phenotypes of gastric cancer

Cancer	Genes whose expression is upregulated in lymph node metastasis-positive cases	Genes whose expression is upregulated in lymph node metastasis-positive cases	Ref.
Esophagus	CD33, GJB1, ITGA2B, ITGAX, ITGB6, CD2, ERBB4, CSF2RB, MAD1L1, BNIP3, CD38, ARHGDIG, CYP17A1, RVNP2, CRYZ, MMP13	AR, EPHB6, INSR, TLR3, TLR6, CYP24A1, CYP2D6, PDE4A, PDE4C, DFFB, PDCD1, ATR, MDM2, BMP5, CCL1, HSPA4, RXRB, IFNA1	[96]
Esophagus	IL6, DMN, JUND, SDS, PRDX2, PELP1, D8S2298E, PCP4, NDUFA7, ACCN1, GCK, ONECUT1, GPX4, TM7SF2, MAPK8IP1, IDH2, DKFZP434D146	NUP153, DUSP1, PPIC, POT1, LIG4, KIAA1128, MYOG, IFIT4, IL1RAP, PLSCR1, TUBA1, CCNA2, HMGB2, CLCN4, TLR4, MRS2L, GNB5, IFNGR1, KNSL1, FADD, IL1B, GNS, LAMA3, PPFIA1, DAZ, KIAA0562, DDX17, ALOX5, APP, NHLH1, MRS2L, NCYM, CRLF3, ICSBP1, KIAA1128, LOC58525	[97]
Esophagus	NFIL3, SUV39H2, FLJ34941, WNT10B, RIF1, CCNT2, KCNJ14, SCAMP5, SUV39H1, MCM6, NAV2, PCGF6, B2M, EVI1, C7orf26	DUSP22, EHD3, PGS1, SLC26A2, LOC128977, DCTN2, DKFZp564C1964, SYNPO2, RABAC1, GGTL3, AIM2, CXorf40, LRP2, THUMPD1, ATOH1, HPRP8BP	[98]
Esophagus	SPP1, KRT14, TACSTD1, I_1152283, STMN1, COL7A1, COL1A1, BIRC5, H2AFZ, CKS2, CCT5, I_957781, MLF1, CKS1B, STK31, TK1, KIFC1, CTSZ, CENPF, BG1, DEFB105, MCM7, ATP1B3, NM_144668.1, MYNN, ANLN, I_960942, KPNA2, H2AV, HMG4L, SPAG5, LOC152217, LIMS1, ENAH, CDC20, MSH6, I_941570, HSPE1, NDUFB9, SNRPG, RPA3, ATR, MGC10911	MAL, C1orf10, NICE-1, SPINK5, HBB, EMP1, TGM1, SCEL, KRT13, CSTB, IL1RN, I_1110078, RUNX2, CSTA, PPL, SNTA1, CLIC3, LAGY, S100A9, ANXA1, SPRR3, I_955703, HAT, I_958335, RHCG, XRCC5, K5B, SERPINB1, SPRR1A, I_959634, MYO1A, I_966519, BCE-1, I_1002369, FLJ21511, CLCA4, PPP1R3C, LOC84518, TRAPPC1, I_1152130, I_958949, KRT4, PSCA, KIAA0790, CD24, I_928865, IBA2, FLJ20626, MECP2	[99]
Esophagus	CALB1, TFF3, KRT7, SCGB1A1, ITSN2, ADH1A, CLDN10, SETD1B, FOXA1, MAL, NEBL, GDF15, MME, CEACAM5, MUC1, CTBP1, PCP4, FCGR2A, MMP10, CD19, GZMK, BARX2, SELENBP1, RBBP8, CTSD, AQP3, CIB1, FUCA1, CAT, ITPKB, TST, NR1D1, HPCAL1, INDO, MUC1, FBXO7, ASS1	FABP4, LTBR, ENO2, NCAPD2, ZNF384, FTO, KIAA0146, TUSC3	[57]

Stomach	RBP4, OCT2, IGF2, PFN2, KIAA1093, FN1, PCOLCE, FN1	MUC4, LYZ	[100]
Stomach	MMP7, THBS2, FN1, MMP10, TGFB3, IGFBP3, SPARC, COL1A2, MMP1, PDGFRB	–	[101]
Stomach	SCAND1, RGS5, S100A11, RNPC2, APOE, FLJ10815, RNASE1, H3F3B, P24B, CLDN3, MRPL14	BCL2L2, RPLP1, ZYX, RPS17, FLJ11151, CTNND1, CYP20A1, SPC18, LOC339290, ELOVL5	[102]
Stomach	DAG, HMMR, ARL1, CRTAP, EST	–	[103]
Colon	RPS28, SFp32	KRT5, CST4, SAA1	[107]
Colon	RBP1, RPS4Y1, B2M, SERPINA1, PCK1, LRRFIP1, ANXA3, COPS5, TFF3, HIST1H2AE, COL17A1, HRASLS3, BAMBI, RPS12, LSR, SSBP1, APLP2, HSP90B1, NME1, NFIL3, HNRNPA2B1, CFB, RABGGTB, UQCRB, FBL, BTF3, DNAJA1, HNRPM, SUB1, CKS2, HSP90AB1, CD47, ERP29, MDK, EMG1, HERPUD1, GSTP1, DNAJC9, SULT1A3, CCT3, ABP1, TXNDC13, NME1, UBE2E3, TXN, INSIG1, FBL, KANK1, HNRPA3, NACA, TARS, FTH1, PDZK1IP1, PRSS23, COX4I1, STK3, YIPF3, MGST3, RPL31, RPS15A, BSG, SLC25A13, NDUFA1, PPL, BOP1, RPS5, ALDH9A1, COX6C, POLR2H, COX6B1, SLC7A7, LAMB3, HNRNPC, AHNAK2, NDUFS2, MRPL12, RPS12, CALCOCO2, NACA, MSH2, TAF7, SMPD4, PSMD13, C6orf108	SLC25A17, POLD2, RUNX1, BRD3, CRKL, CYB5B, JUND, CASP6, MNT, XRCC5, ATXN2, C5orf21, KLF7, CYP2A13, DKFZP564O0823, RUNX1, FRYL, LAGE3, TMCC1, OGT, ENTPD6, TMEM97, AMOTL2, FOXO3, MYCBP2, CAMTA2, SELENBP1, SAT1, ELISC-1, DDX3X, PTPRD, MGAT5, MAOA	[72]

3.4.1 Esophageal Cancer

Tamoto et al. [96] measured gene expression in 36 esophageal squamous cell carcinoma cases by microarray. They identified 44 genes (including *CD33*, *GJB1*, *ITGA2B*, and *ITGB6*) whose expression was associated with lymph node metastasis. Likewise, Kan et al. [97] analyzed the gene expression profile of 28 esophageal squamous cell carcinoma cases by microarray and found genes whose expression was upregulated in lymph node metastasis-positive cases (including *IL6*, *DMN*, and *JUND*) and genes whose expression is downregulated in lymph node metastasis-positive cases (including *NUP153*, *DUSP1*, and *POT1*). Yamabuki et al. [98] also analyzed the gene expression profile of 19 esophageal squamous cell carcinoma cases by microarray. They found genes whose expression was upregulated in lymph node metastasis-positive cases (including *NFIL3*, *SUV39H2*, and *WNT10B*) and genes whose expression was downregulated in lymph node metastasis-positive cases (including *DUSP22*, *EHD3*, and *PGS1*). Similarly, Uchikado et al. [99] analyzed gene expression in 16 patients with esophageal squamous cell carcinoma using oligomicroarray. They found overexpressed genes correlated with lymph node metastasis (including *SPP1*, *KRT14*, and *TACSTD1*) and suppressed genes correlated with lymph node metastasis (including *MAL*, *SPINK5*, and *HBB*). Sano et al. [57] compared the gene expression profiles of 24 esophageal squamous cell carcinomas with extensive lymph node metastasis and 11 esophageal squamous cell carcinomas with no metastatic lymph nodes. They found overexpression of *CALB1*, *KRT7*, *MUC1,* and *CEACAM5* in cases with metastatic lymph nodes.

3.4.2 Gastric Cancer

Hippo et al. [100] reported that overexpression of *RBP4*, *OCT2*, *IGF2*, *PFN2*, *KIAA1093*, *PCOLCE*, and *FN1* is associated with lymph node metastasis by microarray. Inoue et al. [101] found several genes that were differentially expressed with a significant difference between the two groups with respect to the depth of tumor invasion and lymph node metastasis by cDNA microarray. Upregulation of *MMP7*, *THBS2*, *FN1*, *MMP10*, *TGFB3*, *IGFBP3*, *SPARC*, *COL1A2*, *MMP1*, and *PDGFRB* were associated with lymph node metastasis. Oue et al. [102] performed serial analysis of gene expression (SAGE) on five gastric cancer samples, and reported that upregulation of *FUS* and *APOE* was associated with lymph node metastasis. Marchet A et al. [103] evaluated the gene expression profile in 32 gastric cancer cases. They reported that only three genes (*BIK*, *AURKB*, and *EIF5A2*) could correctly predict lymph node status. Mimori K et al. [104] performed microarray analysis of total RNA from whole bone marrow blood from six cases with metastasis and three cases without metastasis in human gastric cancer. They found that *MT1-MMP*-positive expression in peripheral blood was associated with the incidence of lymph node metastasis. Ueda T et al. [105] analyzed microRNA expression in human gastric cancer. They found 17 microRNAs whose expression was associated with lymph node metastasis. Yamashita et al. [106] examined 242 gastric cancer

patients without or with lymph node metastasis by lectin microarray and found that vicia villosa agglutinin was linked to lymph node metastasis.

3.4.3 Colorectal Cancer

Parle-McDermott et al. [107] performed SAGE on a primary colon cancer cell line (SW480) and an isogenic lymph node metastasis cell line (SW620) and found genes whose expression was upregulated in lymph node metastasis-positive cases (including *KRT5*, *CST4*, and *SAA1*) and genes whose expression was downregulated in lymph node metastasis-positive cases (including *RPS28* and *SFP32*). Bertucci et al. [108] profiled 50 colon cancer tissues using DNA microarray and identified 46 genes as significantly differentially expressed between cancers with and without lymph node metastases. Kwon et al. [109] performed microarray analysis in 12 colorectal cancer cases and found 60 genes possibly associated with lymph node metastasis. Fritzmann et al. [72] compared gene expression patterns between metastatic and nonmetastatic stage-matched human colorectal cancers by microarray analysis. They established a signature of 115 genes that differentiated metastatic from nonmetastatic primary tumors. Among them, *BAMBI* was highly expressed in approximately half of metastatic primary tumors and metastases but not in nonmetastatic tumors. Watanabe et al. [110] analyzed the gene expression profile of 89 colorectal cancer cases. They identified 73 genes whose expression significantly differed between patients with and without lymph node metastasis (including *WSX1*, *GUCY2C*, and *DISPA*).

3.5 Anti-lymphangiogenic Therapies for Gastrointestinal Cancer

Although the efficacy of anti-angiogenic therapy has been extensively studied, the concept of targeting lymphangiogenesis to obtain a therapeutic advantage in cancer is only a recent development. The lymphatic network plays an important role in cancer metastasis, allowing spread to draining lymph nodes. Thus, targeting the induction of tumor lymphangiogenesis and functional alteration of existing lymphatic vessels may help to prevent a route for lymphatic metastasis.

In mice experiments, a neutralizing antibody to VEGFR3 was shown to completely block tumor lymphangiogenesis with no effect on pre-existing vessels [111]. Soluble VEGFR3 fusion proteins as well as monoclonal antibodies targeted to VEGF-C and VEGF-D have been developed, and several preclinical and clinical trials using these agents are currently in progress. Multikinase inhibitors that target VEGFR3 have already been developed and used for the treatment of some solid tumors.

Ang/Tie-2 signaling is another promising target for anti-lymphangiogenesis therapy, and a selective neutralizing antibody against Ang1/2 has been developed. The multikinase inhibitor regorafenib inhibits multiple membrane-bound and intracellular kinases involved in lymphangiogenesis (VEGFR1, 2, and 3, Tie-2), and is used

for the treatment of patients with metastatic colorectal cancer. Takigawa et al. [112] reported that single treatment with regorafenib inhibited tumor growth and metastasis by inhibiting both tumor cells and stromal response in an orthotopic implanted mouse model of colon cancer. Because VEGF-C and PDGF-B expressed by tumor cells are associated with lymphangiogenesis and lymphatic metastasis in gastric cancer, blockage of these factors by regorafenib may be a reasonable approach to the prevention and treatment of lymphatic metastasis. Interestingly, Onoyama et al. [113] examined the effects of PDGFR tyrosine kinase inhibitor (nilotinib) and mTOR inhibitor (everolimus) on tumor stroma in an orthotopic nude mouse model of human gastric cancer. They found that treatment with nilotinib did not suppress tumor growth but significantly decreased stromal reactivity, lymphatic invasion, and lymphatic vessel area. In contrast, treatment with everolimus decreased tumor growth and microvessel density but not stromal reactivity. Nilotinib and everolimus in combination reduced both the growth rate and stromal reaction. These results suggest that targeted molecule-based inhibition of cancer–stromal cell interactions appears promising as an effective antitumor therapy. Further understanding of the cellular and molecular mechanisms that regulate lymphangiogenesis of tumors may facilitate the development of effective anti-lymphangiogenic therapies.

3.6 Conclusion

In this chapter, the cellular and molecular mechanisms of lymph node metastasis in human esophageal, gastric, and colorectal cancers were described. In the multistep development of lymph node metastasis, the most important process is lymphangiogenesis. VEGF-C and PDGF-B expressed by tumor cells plays crucial roles in lymphangiogenesis and lymphatic metastasis. Thus, blockage of the factors inducing lymphangiogenesis could be a reasonable approach to the prevention and treatment of lymphatic metastasis. Interference with the growth of lymphatic endothelial cells via several different signaling pathways should enhance the efficacy of anti-metastatic treatments. In addition to lymphangiogenesis, it is well accepted that cancer stem cells play a significant role in metastasis. A possible therapeutic strategy for eliminating cancer stem cells is to specifically target the signaling pathways and transcription factors that are involved in cancer stem cell maintenance and proliferation. The Wnt, Notch, Hedgehog, and Bmi-1 signaling pathways regulate cancer stem cells. Further understanding of the cellular and molecular mechanisms that regulate lymphangiogenesis and cancer stem cell maintenance and proliferation will facilitate the development of effective anti-metastatic therapies.

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