STUDIES OF THE TUMOR-VASCULATURE INTERFACE: ROLE OF TGF-BETA 1-INDUCED EPITHELIAL TO MESENCHYMAL TRANSITION

Mei-Fong Pang
ABSTRACT

Tumor metastasis is a complex multistep process. Among key steps that occur during metastatic spread are acquisition of tumor cell motility, intravasation of tumor cells into blood or lymphatic vessels and extravasation of tumor cells at distal sites. However, the precise mechanisms that govern these metastatic steps remain elusive. This thesis aimed to bridge the fields of tumor and vascular biology to provide new insights into the metastatic process. Results are presented indicating a role of the cytokine transforming growth factor beta (TGF-β) in activating breast cancer cells for dissemination through the lymphatic system through re-activation of a latent development process termed epithelial to mesenchymal transition (EMT). Furthermore, essential roles of the coxsackie-and adenovirus receptor (CAR) for lymph vessel development, and the sphingosine-1-phosphate receptor (S1PR1) for blood vessel stabilization are presented. We expect the findings to have impact on our understanding of the interface between tumor and vascular biology and to influence future strategies to target cancer metastasis.

In paper I, we present data identifying an essential role of CAR for normal development of lymphatic vessels in the mouse. We show that genetic deletion of the CAR gene (Cxadr) from E12.5 during mouse development leads to subcutaneous edema, hemorrhage and embryonic death. The lymphatic vessels in CAR-deficient mice were dilated and structurally abnormal with the presence of gaps and holes at lymphatic endothelial cell-cell junctions. In addition, blood-filled lymphatics were observed in CAR-deficient mice suggesting an incomplete separation between the blood and lymphatic vascular systems. The data demonstrate that CAR plays a crucial role in the development of lymphatic vasculature in mice through formation of lymphatic endothelial cell-cell junctions.

In paper II, we demonstrate that S1PR1 plays critical role in suppressing angiogenesis and promoting vascular stability during mouse development. S1PR1 signaling promotes cell-cell adhesion and prevents sprouting angiogenesis whereas S1PR1-deficiency leads to hypersprouting angiogenesis. These data suggest that S1PR1 signaling might protect developing blood vessels from abnormal angiogenic signals through promotion of vascular stability.

In paper III, we show that TGF-β-induced EMT promotes chemotactic migration of tumor cells through the lymphatic system by mediating crosstalk between tumor cells and lymphatic endothelial cells through the chemokine receptor 7 (CCR7) and its chemokine ligand, CCL21. Reversal of EMT process through p38 MAPK inhibition inhibited tumor cell invasion in vitro and migration towards the lymphatics in vivo suggesting that p38 MAPK inhibition may be a useful therapeutic approach to inhibit tumor cell dissemination through the lymphatic system.

In paper IV, we describe development of a novel co-culture system to study tumor cell migration and interaction with lymphatic endothelial cells within a 3-dimensional matrix component. This assay allows manipulation of tumor properties or matrix components and can be used as a platform to screen for pharmacological agents which inhibit tumor-endothelial interactions.
LIST OF PUBLICATIONS


III. Mei-Fong Pang, Anna-Maria Georgoudaki, Kazuhiro Hagikura, Malin Jansson, Malin Sund, Christer Betsholtz, Mikael C. I. Karlsson and Jonas Fuxe. TGF-β-induced Epithelial-Mesenchymal Transition Activates Breast Cancer Cells for Lymphatic Dissemination. (In Revision)

IV. Mei-Fong Pang, Yi Jin, Lars Jakobsson and Jonas Fuxe. A Three-Dimensional Beads Invasion Assay to Study Tumor Cell Migration and Interaction with Lymphatic Endothelial Cells. (In Preparation)

Other publications not included in this thesis

I. Joel Johansson, Tove Berg, Ewa Kurzejamska, Mei-Fong Pang, Vedrana Tabor, Malin Jansson, Pernilla Roswall, Kristian Pietras, Malin Sund, Piotr Religa and Jonas Fuxe. Loss of C/EBPβ promotes in breast cancer promotes TGF-β-induced epithelial-mesenchymal transition, invasion and metastasis. (Submitted)

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<table>
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<tr>
<td>Akt</td>
<td>Protein kinase B</td>
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<tr>
<td>CAR</td>
<td>Coxsackie- and adenovirus receptor</td>
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<td>CTC</td>
<td>Circulating tumor cells</td>
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<td>CC</td>
<td>Chemokine</td>
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<tr>
<td>CCR</td>
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<tr>
<td>Co-Smad</td>
<td>Common-mediator of Smad</td>
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<td>EC</td>
<td>Endothelial cell</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<td>EMT-Tf</td>
<td>EMT-transcription factor</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>JAMA</td>
<td>Junctional adhesion molecule–A</td>
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<td>LEC</td>
<td>Lymphatic endothelial cell</td>
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<td>LN</td>
<td>Lymph node</td>
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<td>LYVE-1</td>
<td>Lymphatic vessel hyaluronan receptor-1</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>NMuMG</td>
<td>Namru murine mammary gland</td>
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<tr>
<td>NRP</td>
<td>Neuropilin</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
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<td>Platelet/endothelial cell adhesion molecule–1</td>
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<tr>
<td>PI3K</td>
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<td>S1PR1</td>
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<tr>
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<td>SH2 domain-containing leukocyte protein of 76 kDa</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle actin</td>
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<tr>
<td>SOX18</td>
<td>SRY (sex determining region Y)-box 18</td>
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<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>VEC</td>
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<tr>
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<tr>
<td>ZO-1</td>
<td>Zonula ocludens-1</td>
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1 INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most common type of female malignancy and the most frequent cause of cancer-related death among women [1]. The primary cause of death in breast cancer, and in most other types of cancer, is metastatic spread of cancer cells to vital organs, rather than primary tumors. Therefore, unraveling the underlying mechanisms, which regulate breast cancer metastasis is important for future development of therapeutics drugs that can inhibit metastatic spread, and death in cancer.

1.2 TUMOR METASTASIS

![Model of tumor metastasis](image)

**Figure 1: Model of tumor metastasis.** Metastasis involves multiple steps including detachment of tumor cells from primary site, intravasation, survival during the transit, extravasation and subsequently colonization at distal organs. *Reprinted from Gupta and Massague, Cell 2006, with permission from Elsevier.*
During tumor progression, tumor cells evolve through a combination of genetic alterations and diverse interactions with the tumor microenvironment and gain the ability to migrate, invade and metastasize. Tumor metastasis is a process which involves a series of complex cascades that lead to the detachment of tumor cells from the primary tumor site; intravasation of tumor cells into blood or lymphatic vessels; and extravasation and growth at secondary sites [2,3] (Figure 1). In order for tumor cells to metastasize, tumor cells need to enter the blood or lymphatic vasculature. Still, tumor metastasis may be an inefficient process, with merely 0.01% or fewer circulating tumor cells could form metastases [4,5,6]. Metastatic tumor cells might need to express specific genes to be able to colonize different distant sites during metastasis [7,8,9,10,11]. Identification of mechanisms that regulate tumor cell trafficking in and out of lymphatic and blood vessels is crucial to control the metastatic process.

1.2.1 Blood and lymphatic metastasis

**Figure 2: Pathways for metastasis.** (1) Tumor cells might traffic towards the lymphatics without entering the blood vessels and subsequently end up in venous circulation. (2) Tumor cells might intravasate into the blood and lymphatic vessels to metastasize to distal organs. Reprinted from Wong and Hynes, Cell Cycle, 2006.

Lymphatic fluid from peripheral tissues is transported to lymph nodes where it is filtrated and collected for further transport via the thoracic duct or the right lymphatic duct, and directly drained into the subclavian vein (Figure 2). Thus, no matter whether tumor cells disseminate directly into blood vessels, or via the lymphatic system, they will eventually end up in venous circulation [12] (Figure 2). It has remained a challenge to determine whether tumor cells actively chose to migrate via lymphatic or blood vessels, or whether this is a random process. Numerous studies have shown that most carcinomas spread via lymphatic dissemination [13]. For example, carcinomas and melanomas are often found to develop LN metastases. LN metastasis is an important parameter used for tumor staging and prognosis. Patients diagnosed with LN metastases possess high risk of having, or developing distal metastases. Tumor cells often invade the draining LN and subsequently to the distal LNs [14]. Tumor cells are likely absent at distal LNs if the draining LN remained uninvaded [14]. The presence of CTC in the blood and tumor cells in bone
marrow in patients is another important prognosis factor and indicator of distal metastases and has been associated with poor clinical outcome for cancer patients [14,15,16,17].

1.2.2 Lymphatic metastasis: role of tumor lymphangiogenesis and chemotactic migration

Histopathological analyses of human biopsies often show invading tumor cells or formation of new lymphatic capillaries towards surrounding tumor stroma and peritumoral lymphatics [14]. Malignant tumor cells have been shown to secrete various lymphangiogenic growth factors to stimulate lymphangiogenesis and lymphatic metastasis [18]. Studies have shown that lymphangiogenic growth factors such as VEGF-A, VEGF-C, VEGF-D and PDGFBB play a crucial role in promoting tumor lymphangiogenesis [19,20]. In addition to this, experimental evidence also suggested that active interactions between tumor cells and LEC might be required for lymphatic metastasis [21]. Skobe et al. showed that interaction between tumor cells and LEC can enhance tumor cell invasion into the lymphatics. VEGF-C secreted by the tumor cells can activate LEC and facilitate tumor cells transendothelial migration in human breast cancer [22].

It is suggested that migration of metastatic tumor cells is not random and that various types of cancer have specific metastasis pattern. More recent results indicate that tumor cells, similar to immune cells, may take advantage of certain CCs and CCRs during metastasis [23]. CCs are a superfamily of small peptides produced by various cells in the body promoting directional migration of cells expressing appropriate CCRs. Different tumor cells have been shown to express distinct CCRs subsets. These tumor cells may respond to chemotactic and invasive forces generated by CCs secreted by specific target destinations [24]. Among the CC/CCR pair that has been suggested to be an important mediator of breast cancer metastasis towards the lymphatic is CCL21/CCR7 [24]. CCR7 has been implicated in LN metastasis in various cancers [25,26]. Notably, CCR7 is also involved in the trafficking of dendritic cells (DCs) to LNs during inflammation [27]. This is supported by the findings that activated DCs of CCR7-deficient mice have impaired migration capacity towards the draining LNs [27]. Therefore, it is tempting to speculate that tumor cells may exploit a DC route of LN homing for metastasizing to the lymph. In addition, tumor cells might have more accessibility to the lymphatic vessels due to its ‘leaky’ structure. Unlike blood vessels which have continuous ‘zipper-like’ junctions between ECs, lymphatic vessels have semi-open, discontinued ‘button-like’ junctions between ECs [28] suggesting that tumor cells may take advantage of this and use a similar mechanism of entry into the lymphatics as DCs.

1.3 DEVELOPMENTAL ANGIOGENESIS

The development of blood vasculature involves multistep process such as cell proliferation, differentiation, migration and changes in ECM. These process is being tightly controlled and regulated [29]. Firstly, the mesoderm derived endothelial precursors (angioblasts) will form a primitive network of tubular endothelial structure through a process called vasculogenesis [30]. The primitive vasculature is then being
remodeled into hierarchical network of small and large vessels through a process known as angiogenesis which involves sprouting, branching and pruning [30,31] (Figure 3). Under physiological conditions, sprouting angiogenesis is restricted to wound healing and reproductive cycle. Angiogenesis also occurs during pathological conditions such as during tumor progression, chronic inflammation and vascular diseases [32]. Angiogenesis is predominantly being mediated by VEGF-A through its cognate receptor VEGFR-1 and VEGFR-2, (also known as FLT1 and FLK1) [33]. VEGF co-receptor, NRP, NRP1 and NRP2 enhance VEGFR-2 activity [34]. Mice lacking VEGF-A, FLT1 and FLK1 expression died between E8.5 and E9.5 due to vascular defects [35]. Sprouting angiogenesis ceases to allow neovessel stabilization after the vasculature network reaches sufficient size and configuration [36]. Neovessel stabilization is achieved through maturation process which is mediated through the recruitment of mural cells and pericytes to cover the developing endothelial tubes [37,38]. Among the molecules which has been shown to be important for the recruitment of these cells to vessels wall is S1PR1 which binds to S1P [39,40]. S1P is a sphingolipid produced by erythrocytes, platelets and EC. High concentration of S1P can be found in blood serum and S1P is known to induce cell proliferation, migration and adhesion [41,42,43,44]. Several in vitro analysis also suggest that S1P has a pro-angiogenic role [43,45]. S1P receptor signaling is essential in the regulation of vascular permeability, recruitment of mural cells, trafficking of lymphocytes, inflammation and cardiac function [44]. S1P interacts with its cognate receptor, S1PR1 which is expressed abundantly in differentiating EC [46]. S1PR1 knockout mice had severe hemorrhage and died between E12.5-E14.5 [47]. The process of vasculogenesis and angiogenesis was normal in S1PR1 knockout mice. However, these mice exhibited defects in vascular maturation due to lack of mural cells coverage on the endothelial tubes [40,43,47].

Figure 3: Development of blood vasculature. Angioblasts are derived from mesoderm cells. These cells form lining of the blood vessels through a process called vasculogenesis. The primitive vasculature is being remodeled into hierarchical network of small and large vessels through angiogenesis which involves sprouting, branching and pruning. Reprinted from Oliver, Nature Reviews Immunology, 2004, with permission from Nature Publishing Group.
1.4 DEVELOPMENTAL LYMPHANGIOGENESIS AND TUMOR LYMPHANGIOGENESIS

The lymphatic system plays crucial roles in normal and disease conditions. Lymphatic system regulates tissue homeostasis by re-circulating fluid and cells [48] and involves in fat metabolism by absorbing lipid from the gastrointestinal tract [49]. During inflammation, lymphatic system mediates the trafficking of immune cells from tissues to lymph nodes [50]. Lymphatic system has been shown to be the primary route for lymphogenic spread of metastatic tumor cells [48]. Mutation in one of allele of lymphatic master regulator, Prox-1 leads to late-onset obesity in mice [51]. Lymphatic dysfunction causes lymphedema [52] as well as defects in blood pressure regulation during excessive salt intake [53].

The mammalian lymphatic vasculature is derived from the veins [54]. This is support by the findings that Tie2-Cre;COUP-TFIfl/fl conditional knockout mouse embryo has very few or virtually no LEC due to the absence of the vein endothelial cells [55]. Therefore, lymphatic vasculature only starts to develop from embryonic veins after the formation of the blood vasculature [56]. Venous endothelial cells differentiate into LEC and start to express LEC-specific markers such as: Prox-1, LYVE-1, vascular endothelial growth factor receptor-3 (VEGFR-3) and podoplanin at midgestation [57,58,59,60]. At around E9.5, transcription factors SOX18 induces lymphatic-lineage master regulator, Prox-1 expression in distinct subpopulations of the anterior cardinal vein [55]. These Prox-1-expressing vein endothelial cells acquire LEC fate and start to bud from the vein under the influence of vascular endothelial growth factor (VEGF) –C to form lymph sacs. As LECs migrate from the lymph sacs, the developing lymphatic vasculature should be separated by from the blood vasculature to form two independent circulation systems (Figure 4). It has been shown that expression of Slp 76, Syk, Plcg2 and podoplanin is important for the separation of blood and lymphatic vasculature [54,61,62,63]. After differentiation and maturation steps, LECs sprout and migrate from the lymph sacs to develop into a hierarchical network of lymphatic vessels [54,57,59,64].

Figure 4: Development of lymphatic vasculature. (A) Distinct populations of EC from the vein acquired LEC fate and aggregate to form primary lymph sacs under the influence of VEGF-C produced by mesodermal cells. Further sprouting from the lymph sacs produces a hierarchical network of lymphatic vessels. Collecting lymphatic vessels have SMCs coverage. Reprinted from Schulte-Merker, Sabine and Petrova, JBC 2011.
1.4.1 Lymphatic junction proteins

The mature lymphatic vasculature consists of blind-ended initial lymphatic capillaries and larger collecting lymphatic vessels. Lymphatic capillaries are thin wall vessels which lack pericytes and basement membrane coverage [54,65]. Lymphatic capillaries are composed of single layer of LEC and have unique junction organization where oak leaf-shaped endothelial cells are interconnected by discontinuous button-like junctions at the site of fluid entry [28] (Figure 5). Lymphatic vessels are connected to the ECM through anchoring filaments. Lymphatic anchoring filaments extend under high interstitial pressure. As a consequence, LEC junctions open and fluid enter into the lymphatic vessel [66,67]. The larger collecting lymphatic vessels have continuous zipper-like junctions similar to those of blood vessels [28] (Figure 5). Both button-like and zipper junctions express VE-cadherin and tight junctions including: occludin, JAM-A, claudin-5, ZO-1 and endothelial cell-selective adhesion molecule. Transcriptional profiling of isolated lymphatic endothelial cells also showed that LEC expressed junctional proteins such as PECAM-1 (also known as CD31), VE-cadherin, JAM-A, occludin and CAR [28,68,69,70,71,72].

Figure 5: Buttons and zippers in lymphatic vessels. (A) Initial lymphatics have discontinuous button-like junctions while collecting lymphatics have continuous zipper-like junctions. Reprinted from Baluk and Fuxe et al., 2007 JEM.
1.4.2 CAR

CAR was first identified as a cellular receptor for coxsackie B viruses and type C adenoviruses. CAR is a family of immunoglobulin-like surface molecule which appears to mediate cell-cell contact and adhesion [73, 74]. In polarized epithelial cells, CAR co-precipitates with ZO-1 [73] and other tight junction components [75]. Epithelial tight junctions control the flow of ions and macromolecules across the epithelium. Overexpression of CAR leads to homotypic cell adhesion and inhibit the transepithelial movement of ions and macromolecules [73]. It has been shown that CAR expression is down-regulated soon after birth and predominantly expressed at tight junctions of epithelial cells [76]. During development, CAR expression is indispensable for heart development. CAR is present at the intercalated discs of cardiomyocyte to mediate intercellular contract. CAR deletion in mice leads to embryonic lethality at around E11.5-E13.5 due to functionally impaired cardiomyocyte [77, 78]. Cardiomyocyte-specific deletion of CAR in mice after E11 does not lead to embryonic lethality, suggesting CAR expression is crucial within a specific time frame during heart development [79]. Recently, it has been shown that CAR is expressed by LEC [72] and that CAR plays important roles for LEC cell adhesion, migration, tube formation and the control of vascular permeability [72].

1.5 EMT

Figure 6: EMT model. During EMT process, tumor cells lost their polarity and cell-cell contact. Activation of EMT-Tfs enhances tumor cell migration and invasion and subsequently leads to tumor cell dissemination to distal organs. Reprinted from Peinado, Olmeda and Cano, Nature Reviews Cancer, 2007, with permission from Nature Publishing Group.
EMT is a process in which polarized epithelial cells undergoing profound morphological transformation into highly migratory and invasive mesenchymal-like cells [80] (Figure 6). EMT is a reversible process and involves a cascade of genetic programs in epithelial cells which eventually leads to loss of apical-basal polarity and cell-cell interactions due to the activation of EMT-Tfs such as Snail, Twist, Zeb, Slug and others [81,82,83,84,85]. Cells undergoing EMT has increased deposition of ECM proteins. Activation of integrin signaling by ECM in these cells leads to formation of focal adhesion complexes which enhance cell migration [86]. Initially, EMT was identified as a key process during embryogenesis for generation of tissues and organs. Later, researchers found that EMT also occurs during physiological and pathological conditions such as wound healing, fibrosis and cancer [80,87]. EMT has been suggested to be involved during tumor progression to facilitate the dissemination of tumor cells from primary site to distal organs [88]. EMT induction in tumor cells leads to the acquisition of invasive and metastatic properties [89]. Similar to the EMT process occurred during development, EMT during tumor progression is a dynamic and transient event [90].

1.5.1 TGF-β, TGFβ signaling and TGF-β-induced EMT

TGFβs are polypeptides that governs wide range of cellular processes such as cell motility, cell growth and differentiation, production of ECM and immune functions [91,92]. TGFβ acts as tumor suppressor during early stage of tumorigenesis. At this stage, tumor cells response to TGFβ with growth inhibition, apoptosis and cell cycle arrest. However, at later stage during tumorigenesis, Tumor cells acquire aberrant TGFβ signaling or become unresponsive to TGFβ stimulation. Thus, TGFβ acts as tumor promoter through promotion of cell survival, proliferation, EMT and others [93]. TGFβ expression has been correlated with invasion and metastasis in human breast cancers [94,95].

There are three different TGFβ isoforms with different functions: TGF-β1, TGF-β2 and TGF-β3 [92]. All TGFβ isoforms utilize similar receptor system to transduce signaling. TGFβ has three receptors: TGFβ type I (TGFβRI), TGFβ type II (TGFβRII) and TGFβ type III (TGFβRIII) receptors [92]. TGFβ signaling includes Smad-dependent and Smad-independent pathway (Figure 7). Smad-dependent pathway involves binding of TGFβ ligand to the heterotetrameric complex of TGFβRI and TGFβRII. Activation of TGFβRI phosphorylates R-Smads such as Smad2 and Smad3. Binding of R-Smads to Co-Smad transllocate the resulting complex into nucleus and leads to transcription activation or repression [96,97] (Figure 7). In addition to Smad-dependent pathway, TGFβ can induce Smad-independent pathways which do not involve activation of Smad proteins. Among the Smad-independent pathways which can be activated by TGFβ include: p38, MAPK, Rho-like GTPase and PI3K/Akt [97]. Smad-independent pathways can be activated by receptor interacting proteins as well as non-Smad substrates of the receptor kinases [97] (Figure 7).

TGF-β1 is a potent inducer of EMT [98] and is produced by various cells within the tumor environment [89]. TGF-β1 can also be expressed in tumor cells and act in an autocrine manner [99]. In TGFβ-sensitive or moderately sensitive cells, TGF-β stimulation alone is sufficient to induce an EMT phenotype [100]. Smad
signaling is a crucial mediator of TGFβ-induced EMT [101]. EMT induction is enhanced by Smad3/4 whereas dominant-negative Smad3 inhibits TGFβ-induced EMT in NMuMG cells [102,103]. TGFβ can also work in concert with other signaling pathways such as Ras, Wnt, Hedgehog and Notch to induce EMT [84]. Epithelial cell polarization and barrier integrity is normally regulated by adherens and tight junction components. During TGFβ-induced EMT, these junction proteins are downregulated resulting in loss of cell polarization, cell-cell contacts and context-dependent cell growth [104]. In addition, mesenchymal proteins like vimentin, N-cadherin and α-smooth muscle actin are upregulated during EMT resulting in cytoskeleton remodeling and a highly motile, fibroblast-like mesenchymal phenotype, conferring cells with migratory and invasive properties [105]. Several EMT-promoting transcription factors have been identified to promote repression of junction proteins [10,106,107]. Among these transcription factors, SNAIL1 has been shown to form transcriptional complex with SMAD3/4 to repress junction proteins during TGF-β-induced EMT [108]. Loss of adheren and tight junction proteins is a hallmark of EMT and is a characteristic feature of invasive human carcinomas [97,108,109]. TGF-β signaling has been implicated in tumor progression and metastatic processes [110]. However, it is unclear whether TGF-β-induced EMT could contribute to lymphatic metastasis.

**Figure 7:** TGF-β signaling. Binding of ligand to the heterotetrametric complex of type I and type II TGF-β receptor initiate TGF-β signaling. TGFβ can activates both Smad-dependent and Smad-independent pathways. Smad-dependent pathway involves translocation of Smad complexes into nucleus to activate or repress gene transcriptions. Smad-independent pathways which can be activated by TGFβ include p38, JNK, Akt, MAPK and PI3K.
1.6 METHODS TO STUDY TUMOR CELL MIGRATION AND INVASION

Tumor cell migration and invasion are complex processes involving tumor interaction with stromal components and surrounding tissue architecture. Tumor cell migration and invasion is an essential step in tumor metastasis. In order to metastasize, tumor cells need to change their adhesion properties, migration, and the ability to degrade ECM components [111,112]. Owing to the fact that it is very difficult to study tumor cell invasion and migration in vivo, several in vitro invasion assays have been developed over the years to recapitulate tumor cell invasion and migration in vivo [111]. Scratch or wound healing assay is a simple assay which is being developed to study the capacity of tumor cells to migrate towards an artificially created wound [112] (Figure 8). The most widely used Boyden chamber chemoinvasion assay is being used to evaluate the invasion potential of tumor cells based on its capacity to penetrate through a reconstituted ECM barrier coated on a porous membrane towards a chemoattractant gradient at the lower chamber [113] (Figure 8). Later, tumor cells transendothelial assay has been developed based on modification of Boyden chamber assay to study interaction of tumor cells with endothelial cells [114,115,116,117,118] (Figure 8). In this assay, a monolayer of endothelial cells are cultured on the porous membrane and coated with ECM. Invasion capacity of tumor cells is determined by the ability of tumor cells to transmigrate across the ECM and endothelial monolayer. However, these assays are oversimplified, do not mimic the physiological conditions during tumor invasion and are not able to elucidate detailed mechanisms of tumor cell invasion such as individual or collective invasion [119]. Since assessment of these tumor cells behaviors is crucial in order to provide us with a better understanding on how metastatic tumor cells disseminate, an improved in vitro migration and invasion assay needs to be developed.

**Figure 8: Migration and invasion assays.** (A) Scratch or wound healing assay is used to study the migration capacity of cells. A ‘wound’ is created by scratching the surface of a monolayer cells. Capacity of cells to migrate towards the wound is being evaluated. (B) Chemoinvasion assay is used to study the invasion capacity of cells within a matrix towards a chemoattractant gradient. (C) Transendothelial assay is used to study the ability of cells to penetrate through an EC monolayer.
2 AIMS OF STUDY

The specific aims are:

- To study whether CAR expression is required for development of lymphatic vessel *in vivo* and the consequences of CAR-deletion in the development of lymphatic vasculature *in vivo*

- To investigate the role of S1P1R in sprouting angiogenesis

- To study whether TGF-β-induced EMT plays a role in breast cancer cells dissemination towards the lymphatic vessels and how TGF-β-induced EMT affects the migratory behavior of tumor cells towards the lymphatic *in vitro* and *in vivo*

- To establish a novel 3D coculture assay which allows study of tumor cell migration/invasion and interaction with LEC
3 RESULTS AND DISCUSSION

3.1 PAPER I

Essential role of Coxsackie-and Adenovirus Receptor (CAR) in the development of the lymphatic system in mice

CAR is a tight junction-associated cell adhesion molecule and being expressed predominantly at in epithelial cells in adult tissues. However, during development high levels of CAR expression is detected in the heart and brain. CAR is essential for heart development and localizes to intercalated discs in cardiomyocytes [79]. Genetic deletion of the CAR gene specifically in the heart caused embryonic death at E11 [79]. Recently, it was reported that CAR is expressed in lymphatic endothelial cells (LEC) and that it plays a role in regulating LEC migration and tube formation, in vitro [72]. However, it has not been clear whether CAR plays a role in formation of the lymphatic vasculature in vivo.

In paper I, we aimed to uncover the role of CAR in the development of the lymphatic system in mice. To study this, we generated a tamoxifen-inducible whole-body deletion of Cxadr mice (referred as cKO mice). By administering tamoxifen to pregnant mice we were able to delete the CAR gene (Cxadr) in the mouse embryos at E12.5, which was chosen as a time point since Cxadr deletion at E11 caused embryonic death due to heart abnormalities. We found that deletion of Cxadr from E12.5 caused subcutaneous edema, hemorrhage and embryonic lethality at around E16.5. However, the hearts appeared normal in these mouse embryos. This suggested that CAR might be important for the development of other parts of the cardiovascular system. Analysis of the subcutaneous blood and lymphatic vessels by whole-mount skin preparation of the wild type littermates revealed that CAR was being expressed in the lymphatic vessels but not blood vessels at E14.5-E16.5. CAR expression was localized cell-cell junctions between lymphatic endothelial cells, where it co-localized with CD31 and LYVE-1. However, we failed to detect any CAR expression in the lymphatic vessels in the adult mouse skin. This suggested that CAR could play a critical role in lymphatic vessels during a specific time window during development, when lymphatic endothelial cell-cell junctions are established and lymphatic vessels are formed. However, as the lymphatic system is formed and eventually matures into a network of vessels CAR is no longer needed. It was recently reported that loss of tight junction-associated molecules may be overcome and partially compensated in vivo [120]. Based on this, it is tempting to speculate that CAR might possess redundant but crucial role in lymphatic endothelial cell-cell junction formation during specific time window and might be compensated or taken over by other junction proteins after the specific time window.

We found no difference in distribution of the blood or lymphatic vessels in cKO and control embryos. However, the structure of the lymphatic vessels in cKO embryos was abnormal and dilated compared to the lymphatic vessels of the control embryos. We observed presence of gaps or holes localized at the lymphatic endothelial cell-cell junctions in the lymphatic vessels of the cKO but not in the lymphatic vessels of the control embryos. Further investigation of the subcutaneous lymphatic vessels by using electron microscopy showed that the integrity of the lymphatic vessels was being disrupted in the cKO embryos. There were regions of the lymphatic vessel luminal
surface that lacked LEC coverage in the cKO embryos while lymphatic vessel luminal surface was intact in the control embryos. Together, these data implied a role of CAR in the formation of lymphatic endothelial cell-cell junctions. CAR-deficient caused irregular lymphatic endothelial cell-cell junction formation and eventually loss of LEC. These data were in line with previous findings by Vigl. et al showing that knockdown of CAR in LEC impaired the formation of lymphatic endothelial cell-cell junction in vitro [72].

Immunostaining with a marker for erythrocytes, TER-119, showed the presence of erythrocytes inside blood vessels but not in the lymphatic vessels in the control embryos. However, we detected the presence of erythrocytes in the lymphatic vessel in the cKO embryos. As blood-filled lymphatic vessels might indicate non-separation phenotype [62,63,64], this suggested that CAR-deletion might lead to defect in the separation of lymphatic vessels from the blood vasculature during development. We found that cKO embryos phenotype was reminiscent of podoplanin knockout mice, which also displayed a blood-filled lymphatic phenotype [63]. Similar to CAR expression, podoplanin was exclusively expressed in lymphatic, but not in blood vessels. In addition to podoplanin, depletion of Syk, SLP-76, kindlin-3 had been reported to contribute to non-separation phenotype. Recent studies revealed a new role for platelets in the formation of lymphatic vessels during development. Interaction between LEC and platelets was required for proper closure of the shunt between cardinal vein and the lymph sacs to form two separated circulation system [63]. Podoplanin, Syk, SLP-76 and kindlin-3 were important for platelets to function properly [121]. Since CAR expression recently was identified in human platelets [122], we speculate that loss of CAR might disrupt the interaction between LEC and circulating platelets, resulting a non-separation. It would be interesting for the future to this, which could be done by deletion of Cxadr in LEC and platelets. Further examination of blood-filled lymphatic in cKO embryos showed the erythrocytes were leaking out to the perivascular space from gaps of the defective lymphatic vessels, causing regions of hemorrhage.

In conclusion, we found that CAR plays a critical role in the development of lymphatic system in mouse. CAR-deletion between E12.5-E15.5 lead to edema and hemorrhage due to structurally abnormal and defective lymphatic vessels. Our data suggest that CAR is important both for the formation of lymphatic endothelial cell-cell junctions, and for the separation of blood and lymphatic vasculature. For future studies, it would be interesting to investigate whether CAR expression is re-activated and possibly plays a role in the formation of new lymphatic vessels during pathological lymphangiogenesis, which is a characteristic feature of both chronic inflammatory diseases and cancer.
3.2 PAPER II

The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-Cadherin and VEGFR2

S1P is a sphingolipid metabolite produced by erythrocytes, platelets and EC. S1P acts via its cognate receptors, S1PR1-5. It is reported that EC expressed S1PR1-3 [123]. S1P receptor signaling is critical in the regulation of vascular permeability, recruitment of mural cells, trafficking of lymphocytes, inflammation and cardiac function [44]. S1PR1, S1PR2 and S1PR3 triple knockout mice showed severe vascular phenotype [123]. However, S1PR1 appeared to be the most important receptor [123]. Similarly, depletion of S1P signaling through deletion of sphingosine kinase, Sphk1 and Sphk2 caused neural and vascular defects [124], implying S1P/S1PR1 signaling might be critical for the development of vasculature.

In paper II, we demonstrate that S1PR1 plays critical role in suppressing angiogenesis and promoting vascular stability in the endothelium by using in vivo, ex vivo and in vitro experimental model system.

My part of this project was to performed functional studies with mouse microvascular endothelial cells, MS-1 in vitro to uncover the role of S1PR1 in sprouting angiogenesis. We used well established fibrin bead assay [125] to study the role of S1PR1 in sprouting angiogenesis in vitro. We found that VEGF-A promoted sprouting of MS-1 cells. However, VEGF-induced angiogenic effect was inhibited by S1PR1 agonist, SEW2871 and S1P. Stimulation of SEW2871 and S1P on MS-1 produced less but solid sprouts. In contrast, S1PR1-specific antagonist, (R)-W146 further enhanced the effect of VEGF-dependent sprouting, causing hypersprouting where EC detached from the bead and scattered within the fibrin matrix, suggesting S1PR1 might affect endothelial cell-cell adhesion. The effect of (R)-W146 on sprouting can be inhibited by using VEGFR2 inhibitor SU5416, suggesting VEGF-dependent angiogenic sprouting effect of S1PR1 inhibition. Because of inhibition of S1PR1 caused EC scattering, we suspected that S1PR1 might promote sprouting angiogenesis through regulating endothelial cell-cell adhesion. VE-cadherin has been shown to be a target for S1P signaling in vitro. Stimulation of S1P on EC caused increased VE-cadherin level [126]. We then induced deletion of VE-cadherin in endothelial cells in the mice. We observed hypersprouting phenotype and increase vascular density in the retina of endothelial cell-specific VE-cadherin-deficient mice which mimicked the S1PR1-deficient mice phenotype. We further found that S1PR1-deficient retinas had abnormal VE-cadherin staining pattern. Depleting S1PR1 by using siRNA approach caused loss of VE-cadherin in the EC junctions whereas S1P treatment increased VE-cadherin expression level. We also found downregulation of VE-cadherin induced by VEGF can be stored by S1P or SEW2871, suggesting an opposing role of S1P and VEGF signaling in VE-cadherin regulation. Inhibition of VEGF signaling by sFlt1 and SU5416 had no effect on S1P-induced VE-cadherin expression, indicating regulation of VE-cadherin by S1P was independent of VEGF-induced responses. In contrast, (R)-W146 exerted opposing effect on VE-cadherin regulation compared to S1P and
SEW2871. Data from zebrafish experiments showed that depletion of individual \textit{cdh5} and \textit{s1pr1} using morpholinos caused massive angiogenic hypersprouting in the hindbrain in the embryos. Combination of \textit{cdh5} and \textit{s1pr1} morpholinos injection at low dose caused similar results with high dose of single morpholinos, indicating S1PR1 and VE-cadherin could work together. To study whether S1PR1 and VE-cadherin could cooperate, we performed \textit{in vitro} sprouting angiogenesis assay by using MS-1 cells in the presence or absence of VE-cadherin blocking antibody, BV13. Indeed, we found BV13 potentiated sprouting in MS-1, suggesting inhibitory role of VE-cadherin during sprouting angiogenesis [127]. The angiogenic sprouting and cell scattering effect caused by BV13 was further enhanced in the presence of (R)-W146. On the other hand, SEW2871 reversed the angiogenic sprouting and cell scattering effect caused by BV13. These data provided evidence that S1PR1 and VE-cadherin work in concert to restrict sprouting angiogenesis. Data from in vitro signaling experiments showed that S1PR1 inhibits VEGFR2 signaling events. This suggested that suppression of S1PR1-dependent angiogenic sprouting is linked to suppression of VEGFR2 signaling.

In conclusion, our data suggested that S1PR1 signaling inhibit sprouting angiogenesis through regulation of junctional VE-cadherin stabilization and suppression of VEGFR2 signaling events.
3.3 PAPER III

TGF-β-induced epithelial to mesenchymal transition activates breast cancer cells for lymphatic dissemination

Metastasis is the major cause of death in human cancer. For tumor cells to spread to distal organs, they need to enter either blood or lymphatic vasculature. As lymph will eventually drains into blood [12], tumor cells that disseminate through the lymphatic system will end up in the systemic circulation. Owing to this, it is technically challenging to study how tumor cells disseminate through the lymphatic system. Understanding on how tumor cells gain access to the lymphatic system might lead to novel therapeutic interventions.

Elevated level of TGF-β secretion has been associated with poor prognosis and lymph node metastases in human cancers [91,128,129]. The tumor microenvironment is a rich source of TGF-β [91]. As TGF-β is a potent inducer of EMT process, we speculated that tumor cells undergoing TGF-β1-induced EMT might gain capacity to migrate specifically towards the lymphatic vessels. To test our hypothesis, we used a mouse model of footpad injection to study migration capacity of EMT and non EMT cells towards the draining PLN in vivo. Footpad injection is an established method to study DC and tumor cells trafficking towards the LN [130,131]. Three isogenic cell lines exhibit different EMT phenotype ( Figure 9) and TGFβ responsiveness were used: (i) EpH4 cells, mouse mammary gland epithelial cells which response to TGFβ by growth inhibition but not EMT; (ii) EpRas cells, which is derived from EpH4 but has acquired Ras signalling and can undergo EMT with TGFβ stimulation and (iii) EpXT, which is stable EMT model system driven by both Ras and autocrine TGF-β signalling. An additional TGFβ-induced EMT model system, NMuMG cells were also used. In order to track migration of EMT and non EMT cells in vivo, we labelled EpH4, EpRas and EpXT cells with GFP-expressing lentivirus. Footpad injection showed that GFP-labelled Two days after footpad injection, we detected the presence of GFP-positive EpXT cells in the draining PLN whereas no GFP-positive EpH4 cells can be detected. This suggested that EMT cells possessed significant enhanced migration capacity towards the draining PLN in vivo in comparison to non EMT cells. Reversal of EMT phenotype in EpXT cells by downregulating TGFβRII expression level using shRNA approach impaired the migration capacity of EpXT cells towards the draining PLN, indicating the role of EMT in mediating migration capacity of tumor cells towards the LN in vivo. In contrast, TGF-β1-induced EMT in EpRas cells enhanced migration capacity of EpRas cells towards the LN significantly compared to the untreated control. These data provide evidence that TGF-β1-induced EMT might contribute to lymphatic dissemination of tumor cells.
Figure 9: EMT model systems. EpH4 cells cannot undergo EMT in response to TGF-β1. EpRas cells can undergo TGF-β1-induced EMT and EpXT is a stable EMT model driven by Ras and autocrine TGFβ signalling.

Because TGF-β1-induced EMT can increase migration capacity of tumor cells in general, we wonder whether tumor cells underwent TGF-β1-induced EMT could gain capacity to migrate towards the lymphatic in a directional manner. Immunostaining of EpH4 tumors showed that EpH4 cells appeared to be non-invasive and did not migrate towards blood or lymphatic vessels. Immunostaining of EpXT tumors revealed that EpXT cells displayed different morphology in different regions of tumor. In some regions, EpXT cells appeared to be invasive and elongated whereas in some regions, EpXT cells seems to be ‘round’ shaped and noninvasive. Interestingly, we found that blood vessels were evenly distributed and abundant in all regions of the EpXT tumors whereas lymphatic vessels were concentrated in the invasive areas of the EpXT tumors. High resolution confocal microscopy also showed that EpXT cells appeared to intravasate into lymphatic vessels in the invasive regions of the tumor. Based on this, we speculated that EMT cells might show preferential migration towards the lymphatic vessels. We therefore set out to investigate whether TGF-β1-induced EMT promoted targeted migration towards lymphatic rather than blood vessels. Because it is technical challenging to study tumor cell migration in vivo, we developed a 3D tumor-EC coculture system (Figure 10), which is based on a previously established fibrin beads assay [125]. We demonstrated that GFP-labelled EpXT cells possessed significantly enhanced migration capacity towards the LEC than VEC while GFP-labelled did not migrate towards either VEC or LEC. TGF-β1-induced EMT in EpRas cells enhanced migration capacity of EpRas towards LEC rather than VEC, suggesting tumor cells that undergo TGF-β1-induced EMT gain the capacity to migrate in a directional manner towards lymphatic vessels.

Figure 10: Tumor-EC coculture system. Fluorescently-labelled EMT / non EMT and EC cells are being coated on a microcarrier beads. Migration of EMT / non EMT cells towards VEC/LEC will be observed under fluorescent microscope.
Since CCR7 plays indispensable role in mediating lymphatic homing of immune cells [27] and has been associated with lymphatic metastasis in human cancers [24,25,26,132], we speculated that tumor cells underwent TGF-β1-induced EMT might activates CCR7 expression through EMT process and this allow EMT cells migrate towards the lymphatic vessel in a directional manner. Indeed, CCR7 expression was upregulated in both stable and TGF-β1-induced EMT model systems. In contrast, CCR7 expression level remained unchanged in TGF-β1 treated on non EMT cells, EpH4, suggesting CCR7 induction in response to TGF-β1 is coupled to EMT process. Chemoinvasion assays showed that both stable and TGF-β1-induced EMT model systems invade more efficiently towards CCL21 gradient, a chemokine which is being secreted by LEC. Using a CCR7 neutralizing antibody, we further demonstrated that the invasion and migration of EMT cells towards CCL21 gradient and LEC was CCR7-dependent in the chemoinvasion and bead invasion assay. We also found that the migration capacity of EpXT cells towards the draining PLN were significantly impaired when CCR7 expression was being silenced by using siRNA approach, suggesting a role of CCR7 in mediating migration of EMT cells towards the lymphatic in vitro and in vivo. We also observed upregulation of CCL21 expression in LEC upon TGF-β1 stimulation and upregulation of CCL21 expression in the lymphatic vessels of EpXT tumors compared to the EpH4 tumors. These results demonstrated that TGF-β1 might mediate the directional migration of EMT cells towards the lymphatic vessels through CCR7/CCL21 signalling.

We then investigated the underlying molecular mechanism which regulates CCR7 expression during TGF-β1-induced EMT. We found that CCR7 expression was driven by AP-1 activators during TGF-β1-induced EMT in NMuMG cells and can also be regulated by both Smad3 and p38 signalling. Based on this, we postulated that inhibition of JNK, Smad3 and p38 signalling in EpXT cells might reduce lymphatic dissemination through the reversal of EMT process and downregulation of CCR7 expression. We found that inhibition of JNK signalling in EpXT cells has no effect on CCR7 nor E-cadherin expression whereas inhibition of Smad3 signalling in EpXT cells restored E-cadherin expression but did not affect CCR7 expression. Treatment of EpXT cells with the SB203580 reduced CCR7 expression and increased E-cadherin expression. This suggested that inhibition of p38 signalling was most potent in reverting EMT phenotype in EpXT cells. This might be due to the stable EMT phenotype in EpXT cells were driven by both TGF-β1 and Ras signalling, which converge on p38 MAPK [133]. In addition, we also found that treatment of SB203580 impaired the invasion capacity of EpXT cells towards CCL21 gradient in vitro and migration capacity towards the draining PLN in vivo. Since high level of p38 expression has been associated with poor prognostic of breast cancers [134,135], our data suggested targeting p38 signalling might be a useful strategy to inhibit lymphatic dissemination of tumor cells.

Our data from clinical breast cancer samples suggested a link between TGF-β signalling, lymphatic vessel density and lymphatic node metastasis. This is in accordance with previous findings which showed correlation of lymphatic vessels density and p-Smad3 expression with lymphatic metastasis in breast cancer [136,137]. However, the average staining of Snail/Twist in E-cadherin positive cells was statistically no difference with a tendency to be higher in the LN+ group than the LN-group. In some tumor samples, we were able to detect the presence of some CCR7 positive cells with low E-cadherin expression at the invasive front, suggesting tumor cells underwent EMT might express CCR7. However, we will need to further validate
this finding in more clinical samples. Certainly, it is of interest to be able to identify tumor cells undergoing EMT express CCR7 and correlate EMT markers expression in tumor cells with lymphatic vessels invasion in clinical settings, transgenic mouse model of breast cancer or xenograft mouse model. Since EMT might be a transient process [90,138], capturing of EMT in vivo might be a challenge.

Our finding indicated that TGF-β1 promotes chemotactic migration of tumor cells towards the lymphatic vessels through CCR7/CCL21 signalling by: (i) inducing CCR7 expression in tumor cells through EMT process and (ii) upregulating CCL21 expression in LEC. We also provide evidence that inhibition of p38 MAPK can partly reverse the EMT phenotype of tumor cells and suppress CCR7 expression, suggesting p38 MAPK inhibition might be a useful strategy to inhibit lymphatic dissemination of tumour cells.

As discussed earlier, it is a major challenge to study lymphatic dissemination of tumor cells in vivo. However, with the recent advances in intravital imaging techniques and the availability of lymphatic vessel-specific fluorescent transgenic mouse, it is possible to utilize these tools to study how fluorescently-labelled EMT tumor cells disseminate to the lymphatic vessels or intravasate into the lymphatic vessels in vivo in greater detail. Additional tumor model such as mammary fatpad xenograft model can be used to investigate migration of EMT / non EMT cells towards the draining LN in the mammary fatpad.
3.4 PAPER IV

A three-dimensional beads invasion assay to study tumor cell migration and interaction with lymphatic endothelial cells

Tumor metastasis is a complex process, which involves tumor cell migration/invasion and tumor-endothelial interactions [139]. It is technically challenging and expensive to study these processes in vivo. Therefore, numerous assays have been developed to study tumor cell migration and invasion in vitro [111]. However, most existing in vitro assays have their own drawbacks. For example, scratch or wound healing assay is used to study tumor cell migration towards an artificial 'wound'. This assay only allows study of tumor cell migration in 2D, without the ECM. To date, the classical Boyden chamber assay is the most widely used to study tumor cell migration/invasion. In this assay, the migration/invasion capacity of tumor cells is being evaluated based on the ability of tumor cells to penetrate through the ECM barrier towards a chemoattractant gradient [111,113]. The classical Boyden chamber assay is then modified into transendothelial assay as a method to study the ability of tumor cells to migrate across the EC barrier [114]. However, these assays do not mimic the physiologic conditions and it is difficult to study tumor cell migration/invasion or tumor-endothelial interaction in a greater detail with the existing assays.

In order to overcome these drawbacks in the existing migration/invasion assays, we developed a 3D beads invasion assay based on modifications of existing fibrin bead assay [125]. This 3D beads invasion assay allows one to study tumor cell migration and interaction with LEC. This assay can also be modified to use to study cell-cell migration/invasion or cell-cell interaction with different cell types. In this study, we used invasive and non-invasive tumor cells and LEC expressing different fluorescent proteins to study tumor cell migration/invasion towards LEC and tumor-LEC interaction. Tumor cells/LECs were coated onto a microcarrier beads and then embedded into a 3D fibrin ECM. The use of reconstituted ECM to study tumor cell migration/invasion was critical as ECM is the major constituent of the tumor microenvironment [140]. As fibrin is the main component of tumor stromal [141], we reasoned that the usage of fibrin matrix in this assay might partly mimic the physiological conditions during tumor cell dissemination. In addition, the ability of tumor cells to invade and metastasize was greatly influence by molecular interactions attributed by tumor cells, surrounding ECM and stromal cells [142,143].

Our 3D bead invasion assay holds advantages over the conventional Boyden chamber and transendothelial assay. This assay allows high resolution, real-time imaging of tumor cell migration/invasion and tumor-EC interactions. Migration/invasion of tumor cells within the ECM matrix can be visualized in great detail under time-lapsed high resolution confocal imaging. This enables one to study how tumor cells migrate (e.g. single cell or clustered migration) and how tumor cells interact with other cell types in 3D. This assay is easy to manipulate, as we can grow different type of cells on the beads, and study how cells migrate/invade and interact with various cell types. This assay might also be used as a screening platform to identify novel modulators of tumor cell migration/invasion or tumor-EC interactions through: (i) genetic manipulations through overexpression or knockdown of specific
genes in the target cells or (ii) treatment with different growth factors, small molecules inhibitors or neutralizing antibodies. We provided evidence that anti-CXCR4 neutralizing antibody can block the migration of invasive tumor cells, EpXT cells towards the LEC which secreted SDF-1, a ligand for chemokine receptor, CXCR4. Different ECM such as type I collagen, matrigel or a mixture of different ECM components can be used to study the effect of different ECM components on tumor cell migration/invasion or tumor-endothelial interactions.

In conclusion, we developed a 3D coculture beads invasion assay, which can be used to study cell-cell migration/invasion and cell-cell interaction. The 3D bead invasion assay mimics several aspects of in vivo tumor invasion. Unlike the conventional 2D invasion assay, our assay allows the study of: (i) tumor cells migration/invasion within a 3D ECM and (ii) how other cell type e.g. EC influence tumor cells migration/invasion or vice versa in this coculture system. This assay is inexpensive, easy to manipulate and might serve as a screening platform for potential drug candidates.

Similar to the existing invasion/migration assay, this assay has some drawbacks. This assay is technically challenging and laborious. Some cells appear to be more difficult to attach or grow on the microcarrier beads compared to the ones used in this protocol. Because the embedded beads are randomly distributed within the matrix, the distance between beads could affect the outcome. To overcome this, one has to define a range of distance between beads coated with tumor cells and endothelial cells for quantification.
4 GENERAL CONCLUSION

These studies contribute to better understanding of the underlying mechanisms which regulate:

(i) Development of lymphatic vessels in mice. CAR played critical role for lymphatic vessel development. CAR-deficient lead to structurally abnormal and functionally impaired lymphatic vessels. CAR expression was crucial for formation of lymphatic endothelial cell-cell junction. Loss of CAR-mediated adhesion at lymphatic cell-cell junction lead to presence of gaps or holes in the lymphatic vessels due to improper formation of lymphatic endothelial cell-cell junction. The presence of blood-filled lymphatic in cKO mice suggested that CAR might be involved in the separation of blood/lymphatic vasculature separation (Paper I).

(ii) Vascular stability. S1PR1 played critical role in suppressing angiogenesis and promoting the vascular stability. S1PR1 signaling promoted cell-cell adhesion and prevented sprouting angiogenesis, suggesting S1PR1 signaling might protect developing blood vessels from abnormal angiogenic signals through promotion of vascular stability (Paper II).

(iii) Lymphatic dissemination of tumor cells. TGF-β-induced EMT induces CCR7 expression in tumor cells. Concomitantly, TGF-β also induces CCL21 expression in LEC, suggesting that TGFβ promotes chemotactic migration of tumor cells towards the lymphatic by mediating tumor-LEC crosstalk through the activation of CCR7/CCL21 signaling. Inhibition of p38 MAPK signaling caused reversion of EMT phenotype and loss of CCR7 expression in tumor cells and reduced dissemination of tumor cells towards the lymphatic vessels in vivo, suggesting p38 inhibition as a potential therapeutic strategy (Paper III).

(iv) In addition, the co-culture system that we developed will be an alternative for tumor biologist to study cell migration and interactions within 3D. This assay can also be used as a platform to screen for pharmacological agents (Paper IV).
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