Tumor-induced myeloid cell plasticity toward lymphatic endothelium

Inauguraldissertation

Zur Erlangung der Würde eines Doktors der Philosophie vorgelegt der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel

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Basel, September 2009

Genehmigt von der Philosophisch-Naturwissenschaflichen Fakultät auf Antrag von

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Summary

Tumor-associated lymphangiogenesis contributes to tumor progression by regulating interstitial fluid pressure, transporting immune cells to the tumor site and providing a route for metastatic dissemination. Metastasis to regional lymph nodes via lymphatic vessels represents the first step of tumor dissemination in many human cancers. Therefore, understanding how tumor-associated lymphatic vessels develop may set the stage for the design of novel anti-metastatic therapy.

Thus far, two mechanisms have been described to drive the formation of new lymphatic vessels. While it is widely accepted that newly formed lymphatic vessels arise by sprouting from pre-existing vessels, the recently described context-dependent integration and thus contribution of bone marrow-derived cells to growing lymphatic vessels is rather controversial.

Here, using bone marrow transplantation and lineage-tracing experiments in two tumor mouse models presenting ongoing lymphangiogenesis, we demonstrate that myeloid cells can trans-differentiate into lymphatic endothelial cells and thus contribute to tumor lymphangiogenesis. These data significantly strengthen previous studies on the controversial role of haematopoietic cells in lymphatic vessel formation and provide new insights into the development of tumor-associated lymphangiogenesis.

The molecular mechanism underlying the trans-differentiation process remain unknown. In order to further understand the process, we developed two *in vitro* assays. One assay recapitulates the *in vivo* phenotypical conversion of myeloid cells toward lymphatic endothelial cells, while the other assay mimicks the *in vivo* integration of myeloid cells into lymphatic vessels. The comparison of the transcriptional profiles of cells at different time points of the *in vitro* trans-differentiation process revealed time-specific upregulation of genes representing potential candidates implicated in the different steps of the process. Both *in vitro* assays were then used as a tool to assess the role of selected genes first in the *in vitro* and then in the *in vivo* trans-differentiation process.

Résumé

La lymphangiogenèse tumorale contribue à la progression tumorale en régulant la pression interstitielle, en transportant des cellules immunitaires jusqu'au microenvironnement tumoral ainsi qu'en ouvrant une voie à la dissémination métastatique. Le système lymphatique est en effet considéré dans de nombreux cancers comme la voie primaire de la diffusion des métastases. Pour cette raison, mieux comprendre le développement des vaisseaux lymphatiques associés aux tumeurs constitue un prérequit à la mise en place de nouvelles stratégies thérapeutiques.

Jusqu'à présent, deux mécanismes ont été décrit comme étant impliqués dans la formation *de novo* de vaisseaux lymphatiques. Alors qu'il est largement accepté que les nouveaux vaisseaux lymphatiques proviennent de la division de cellules lymphatiques et du bourgeonnement de celles-ci à partir de vaisseaux lymphatiques préexistants, un nouveau concept, impliquant l'intégration et ainsi la contribution de cellules hématopoïétiques à la formation de nouveaux vaisseaux lymphatiques, est fortement débattu.

Combinant des expériences de transplantations de moelle osseuse et de marquages de lignées cellulaires dans deux modèles de souris présentant une lymphangiogenèse tumorale active, cette étude démontre que les cellules issues de la lignée myéloïde peuvent se différencier en cellules endothéliales lymphatiques et ainsi contribuer à la lymphangiogenèse tumorale. Ces résultats renforcent et élargissent considérablement les données d'études antérieures décrivant le rôle des cellules hématopoïétiques dans la formation de nouveaux vaisseaux lymphatiques.

A ce jour, le mécanisme moléculaire de trans-différenciation cellulaire n'est pas connu. Afin de mieux comprendre ce processus, nous avons développé deux systèmes *in vitro*. Le premier reproduit la transformation phénotypique de cellules myéloïdes en cellules endothéliales lymphatiques observée *in vivo* et le second simule l'intégration de cellules myéloïdes dans les vaisseaux lymphatiques. La comparaison du transcriptome des cellules à différentes étapes de trans-différenciation *in vitro* a permis l'identification et la classification de gènes potentiellement impliqués dans les différentes phases du processus. Les deux systèmes *in vitro* ont alors été utilisés en guise d'outil pour évaluer

l’implication des gènes sélectionnés dans le processus de trans-différenciation, d’abord *in vitro* puis *in vivo*.

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1. Introduction

1.1 Tumor development

1.1.1. The hallmarks of cancer

Cancer is a leading cause of death worldwide. According to the World Health Organization, it accounted for 7.4 million deaths in 2004 (which represents 13% of all deaths), a number which is predicted to rise and reach 12 million deaths in 2030. The main cancer types leading to overall cancer mortality each year are: lung, stomach, colorectal, liver and breast.

The development of a tumor is a multistep process in which a succession of genetic and epigenetic changes leads to the progressive conversion of normal cells into cancer cells, each change conferring one or another type of growth advantage. In 2000, Douglas Hanahan and Robert A. Weinberg suggested that most cancers acquire the same set of functional capabilities during their development and have thus defined six essential alterations in cell physiology that collectively dictate malignant growth [1]. These six capabilities acquired during tumorigenesis are depicted in Figure.1 and summarized in the following paragraphs.

Self-sufficiency in growth signals

Normal cells require mitogenic growth signals to proliferate. In contrast, tumor cells show a greatly reduced dependence on exogenous growth stimulation, as they develop their own proliferative strategies. First, cancer cells can synthesize growth factors acting in an autocrine loop or instruct their normal neighboring cells to produce such factors. Second, they can overexpress growth factor receptors, increasing their responsiveness to growth factors or eliciting ligand-independent signaling. This last feature can also be acquired by structural receptor alterations. Moreover, tumor cells can switch the type of integrins expressed, favoring links to the extracellular matrix that transmit proliferative signals. Both receptor and pro-growth integrin stimulation can activate the proliferative SOS-Ras-Raf-MAP kinase pathway. And third, downstream cytoplasmic circuits that

transmit the proliferative signals can be directly activated in tumor cells. For example, about 50% of colon carcinomas bear a mutant *ras* oncogene.

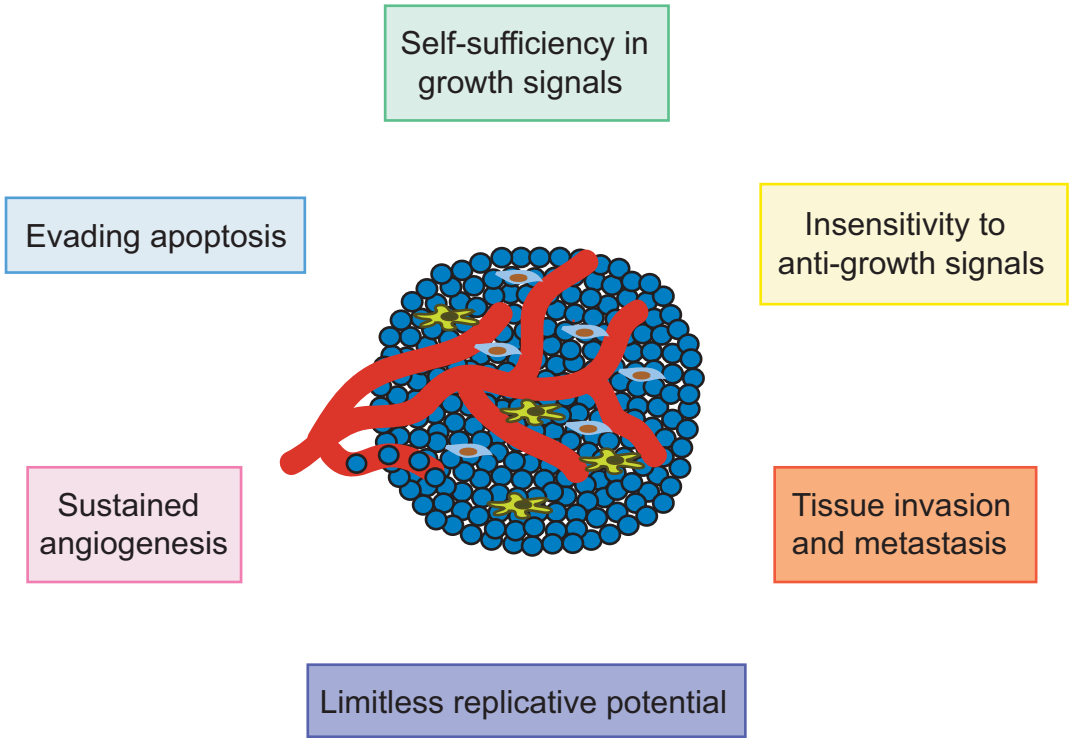

Figure.1: The hallmarks of cancer. Schematic representation of the 6 main molecular, biochemical and cellular traits shared by most types of human cancer.

Insensitivity to anti-growth signals

In normal tissues, tissue homeostasis is maintained by anti-proliferative signals, leading to cell cycle arrest in the quiescent G_0 phase or to entry into post-mitotic differentiated states. Most anti-proliferative signals pass trough the retinoblastoma protein (pRb), which controls the entry in the S phase of the cell cycle. pRb, in its hypophosphorylated state, sequesters the transcription factors E2Fs that control the expression of genes necessary to progress from the G_1 to the S phase. Phosphorylation of pRb results in the release of E2Fs and on the concomitant entry in the S phase. In cancer cells, loss of function mutations in the *pRb* gene can lead to inactivation of this tumor suppressor gene and so to deregulated cell cycle control. Alternatively, in

papillomavirus-induced cervical carcinogenesis, pRb function is abrogated through its sequestration by the oncogenic protein E7. In addition, cancer cells can turn off the expression of cell adhesion molecules sending anti-growth signals and turn on the expression of those delivering pro-growth signals.

In order to avoid terminal differentiation, *i.e.* the entry into an irreversible post-mitotic state, cancer cells have developed various strategies. For example, the overexpression of the *c-myc* oncogene impairs cellular differentiation and promotes cancer cell proliferation by shifting the balance toward the formation of proliferation-specific Myc-Max transcription factor complexes over differentiation-specific Mad-Max complexes. Similarly, inactivation of the APC/β-catenin pathway in colon carcinogenesis, blocks the differentiation of colonic crypt enterocytes.

Evading apoptosis

Programmed cell death massively takes place in rapidly growing tumor cells, constituting a major barrier to tumor progression that has to be circumvented. Tumor cells have thus developed various resistance strategies to apoptosis. The most common one consists in mutation-ensued loss of function of the tumor suppressor gene *p53*, which is observed in more than 50% of human cancers. In response to DNA damage, p53 elicits either cell cycle arrest to allow DNA repair to take place or apoptosis if the damage is excessive. Abrogating death receptor signaling represents another anti-apoptotic strategy generated by tumor cells. For example, lung and colon cancer cells have been reported to produce a non-signaling decoy receptor for the FAS ligand, impairing thus FAS receptor activation and subsequent death signaling. Additionally, the PI3 kinase-AKT/PKB pathway, transmitting anti-apoptotic survival signals, is involved in overcoming apoptosis. This pathway can be activated by extracellular factors like IGF-1/2, by intracellular signals originating from Ras activation or by loss of the phospholipid phosphatase PTEN.

Limitless replicative potential

Normal cells carry an intrinsic program that limits their multiplication, allowing 60-70 doublings before entering senescence. The inactivation of tumor suppressor genes like

p53 or *pRb* enables additional multiplication till the entry into a crisis state characterized by end-to-end fusion of chromosomes and concomitant massive cell death. This karyotypic disarray is associated with the appearance of unprotected chromosomal ends arising through telomere shortening upon cellular division, a process attributed to the inability of DNA polymerases to completely replicate the 3' ends of chromosomal DNA during DNA replication. Tumors cells, mainly by upregulating telomerase expression, maintain telomere length above a critical threshold, which permits unlimited multiplication. Thus, when carcinogens are applied to tumor prone mice deficient for the cell cycle inhibitor $p16^{INK4A}$ and also lacking telomerase, tumor incidence is reduced in comparison to mice harboring telomerase activity. This reduction in tumor burden is concomitant with telomere shortening and genomic instability.

Sustained angiogenesis

The early stages of tumor formation arise from cumulated genetic and epigenetic alterations that activate oncogenes and/or inhibit tumor suppressor genes. Consequently, cell proliferation is increased and apoptosis is decreased, concomitant processes resulting in early hyperplastic growth. However, once the tumor mass has reached the critical size at which tumor cells located in the center become necrotic due to a lack of oxygen and nutrients, further growth is impaired. In order to overcome this growth inhibition, tumor cells recruit blood vessels, an event known as tumor angiogenesis. This well-characterized multistep process contributes to tumor progression from primary tumor growth to metastasis formation. The newly formed blood vessels provide oxygen and nutrients to hypoxic tumor cells and offer a route for tumor cell dissemination to distant organs. The transition from a quiescent pre-vascular to a growing vascularized tumor is called the angiogenic switch and this time-restricted step in the tumor angiogenesis process as well as anti-angiogenic therapies and concomitant development of resistance mechanisms will be discuss in details in chapter "1.1.2. Tumor angiogenesis".

Tissue invasion and metastasis

Metastasis constitutes the cause of 90% of human cancer death. Tumor cell invasion through surrounding tissue constitutes the first step to metastasis formation. It can occur either by the dissemination of single tumor cells having acquired migratory

capacities or by the movement of large tumor cell sheets, a process referred to as collective cell migration. The mechanism by which collective cell migration occurs is still elusive. In contrast, for single cell migration, activation of extracellular proteases and changes in the physical coupling of cells to their microenvironment have been reported to be central in the acquisition of invasiveness and metastatic features. Tumor cells upregulate proteases, downregulate protease inhibitors and convert inactive zymogen forms into active enzymes. Besides their direct matrix-degrading role, these active proteases also contribute to tumor angiogenesis and growth signaling, contributing in turn to the invasive/metastatic capability. The most widely observed alteration of cell-environment interactions involves the homotypic cell-cell adhesion molecule E-cadherin. In a majority of epithelial tumors, E-cadherin function is lost by mechanisms including mutational inactivation of the E-cadherin gene, transcriptional repression or proteolysis of the extracellular cadherin domain. Changes in expression of cell-cell adhesion molecules as well as integrin switch are also involve in the process of single cell invasion/migration. For example, in various cancers, it has been reported that N-CAM expression changes from a highly adhesive isoform to poorly adhesive ones and that shifts in the spectrum of integrin α or β subunits occur in order to enable the migrating cells to interact with their novel environmental matrix components. Migrating tumor cells can disseminate to distant organs through the blood vessels or, at least in a first step, to regional lymph nodes through lymphatic vessels. Tumor angiogenesis and lymphangiogenesis are discussed in more details in the following chapters (1.1.2. and 1.1.3.).

1.1.2. Tumor angiogenesis

The following review focuses on the molecular and cellular players implicated in the onset of angiogenesis as well as on the anti-angiogenic therapeutic strategies and the concomitant development of resistance mechanisms studied in mouse models of cancer.

Baeriswyl and Christofori: "*The angiogenic switch in carcinogenesis*" Seminars in Cancer Biology, 2009, in press.

Abstract

Coined in the late eighties, the term “angiogenic switch” refers to a time-restricted event during tumor progression where the balance between pro- and anti-angiogenic factors tilts towards a pro-angiogenic outcome, resulting in the transition from dormant avascularized hyperplasia to outgrowing vascularized tumor and eventually to malignant tumor progression. The molecular players and mechanisms underlying the angiogenic switch have been intensely investigated. In particular, a large number of pro-angiogenic factors and angiogenic inhibitors activated and repressed, respectively, in their activities during the angiogenic switch have been identified and characterized. Part of this research has lead to the development of various pro- and anti-angiogenic therapies that are currently tested in clinical trials or are already in clinical use. More recently, transgenic mouse models of cancer have been instrumental in revealing that inflammatory responses within the tumor microenvironment are critically contributing to the onset of tumor angiogenesis. These mouse models closely recapitulate multistage carcinogenesis in cancer patients and represent reliable tools to study the molecular and cellular players implicated in the onset and maintenance of tumor angiogenesis. Furthermore, they also offer the opportunity to assess the efficacy of novel anti-angiogenic cancer therapies and the nature of developing resistance mechanisms. These experiments have provided first important concepts to improve anti-angiogenic therapy and thus directly contribute to their translation to the clinical setting.

Keywords: angiogenesis, cancer, drug resistance, therapy, transgenic mice

Abbreviations: BMDC, bone marrow-derived cells; CAF, cancer-associated fibroblasts; EPC, endothelial progenitor cells; HPV, human papilloma virus; HSPC, haematopoietic stem/progenitor cells; LV, lentiviral vector; MMP, matrix metalloproteinase; RCC, renal cell cancer; SCC, squamous cell carcinoma; TAM, tumor-associated macrophages; TEM, Tie-2-expressing macrophages

Introduction

The early stages of tumor formation are based on a combination of genetic and epigenetic alterations that activate oncogenes and/or inhibit tumor suppressor genes. Consequently, the rate of cell proliferation is increased while apoptosis is diminished,

concomitant processes resulting in early hyperplastic growth. However, once the tumor mass has reached a critical size, tumor cells located in a distance to blood vessels lack an appropriate supply of oxygen and nutrients and thus undergo apoptosis or necrosis, and further tumor growth is impaired. Unfortunately, tumor cells can overcome this growth inhibition by inducing the formation of new blood vessels from pre-existing blood vessels, a process known as tumor angiogenesis. This well-characterized multistep process contributes to tumor progression not only by providing oxygen and nutrients for tumor outgrowth but also by offering a route for tumor cells to disseminate via the blood stream to distant organs and to form metastases.

The transition from pre-vascular hyperplasia to highly vascularized and progressively outgrowing tumors is referred to as the “angiogenic switch”. Discovered approximately 30 years ago, many molecular aspects of the angiogenic switch have been summarized by excellent recent review articles (see for example references [2,3]). In this review, we specifically focus on recent exciting discoveries on the molecular mechanisms underlying the angiogenic switch, as revealed by elegant experimentation in transgenic mouse models of cancer, and the implications of these findings on patient treatment. As the angiogenic switch is controlled by changes in the fine-tuned balance between pro- and anti-angiogenic factors secreted either by tumor cells or by cells of the tumor microenvironment, we discuss both the functional contribution to the angiogenic switch of the various angiogenic factors on one side and the different cells of the tumor microenvironment on the other side. Moreover, we summarize recent findings on the nature of developing resistance against anti-angiogenic therapy in experimental systems and in cancer patients and discuss their clinical relevance.

Pro- and anti-angiogenic factors

Observations on the growth of explanted experimental tumors lead the late Judah Folkman to formulate the hypothesis that tumor progression depends on the active recruitment of blood vessels to the tumor bed in response to the secretion of specific angiogenic factors. It was Judah Folkman himself who spearheaded the discovery of the first angiogenic factors and who first experimentally demonstrated the occurrence of the induction of angiogenesis by secreted factors, the angiogenic switch (see below; reviewed in reference [4]). The angiogenic switch is a discrete component of multistage

tumor development, usually initiated by an alteration in the balance between pro- and anti-angiogenic factors. While an equilibrium between pro- and anti-angiogenic activities or a prevalence of anti-angiogenic factors prevents tumor outgrowth, a status also referred to as tumor dormancy, tilting the balance towards pro-angiogenic activities results in increased tumor angiogenesis and thus tumor growth.

In the past decades, a plethora of angiogenic factors have been identified that directly or indirectly induce proliferation and differentiation of endothelial cells. Of the polypeptide factors, Vascular Endothelial Growth Factor-A (VEGF-A) is a prototypic pro-angiogenic factor and a major regulator of physiological and pathological angiogenesis. VEGF-A is a member of a gene family that includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E, all of which bind with varying specificities and affinities to their *bona fide* tyrosine-kinase receptors, VEGFR-1, 2 and 3 [5]. While VEGF-A, by binding to VEGFR-2 on blood vessel endothelial cells, promotes blood vessel angiogenesis, VEGF-C and D preferentially bind to VEGFR-3 expressed predominantly on lymphatic endothelial cells and thus induce lymphangigenesis [6]. However, VEGF-C and D have also been shown to contribute to tumor angiogenesis by binding to VEGFR-2 and 3, with VEGFR-3 being expressed in the tip cells of growing tumor blood vessels [7,8]. Based on alternative splicing of the mRNA, there are five main isoforms of VEGF-A which bind with high affinity to VEGFR-1 and 2. VEGF-A145, 165, 189 and 206 are heparin-binding and require an enzymatic release from the cell surface and the extracellular matrix to achieve their angiogenic activities, whereas VEGF-A121 is freely diffusible and thus does not necessitate any further activation [9-11]. Fibroblast growth factor (FGF)-1 and 2 [12] and platelet-derived growth factor (PDGF)-B and C [13,14] are also important positive regulators of angiogenesis. They induce endothelial cell proliferation and migration by their direct interaction with their specific receptors expressed on endothelial cells. Angiopoietins contribute to angiogenesis by cooperating with other angiogenic factors in modulating the activation status of endothelial cells by binding to the Tie-2 tyrosine kinase receptor expressed by endothelial cells [15]. Angiopoietin-1 (Ang-1) induces the final maturation of blood vessels and thus counteracts angiogenesis, whereas Angiopoietin-2 (Ang-2) antagonizes Ang-1 activity and keeps the growing vasculature responsive to additional angiogenic factor stimulation. In addition, there is a plethora of polypeptide factors, hormones and metabolites that have been reported to directly or indirectly stimulate physiological and pathological angiogenesis.

Opposing the activities of angiogenic factors, a large number of endogenous anti-angiogenic factors have been functionally characterized. One class comprises components and proteolytic fragments of the extra-cellular matrix (ECM) or the basement membrane. For example, thrombospondin-1 (TSP1), a large ECM glycoprotein, and proteolytic fragments thereof have been identified as potent inhibitors of angiogenesis [16]. Other matrix-derived anti-angiogenic factors are endostatin, a proteolytic cleavage product of collagen XVIII [17], and canstatin and tumstatin, two proteolytic fragments of collagen IV [18,19]. A second class of endogenous angiogenesis inhibitors includes soluble factors like interferon-α and β (IFN-α and β) and angiostatin, a cleavage product of plasmin [20,21].

Like many pro-angiogenic factors, the activities of the various anti-angiogenic factors are regulated at the level of gene expression, secretion, and proteolytic activation. The expression of pro-angiogenic factors can be induced by environmental stress, such as hypoxia, glucose deprivation, formation of reactive oxygen species (ROS), cellular acidosis or iron deficiency, or by the activation of oncogenes or the loss of the function of tumor suppressor genes [22-26]. As will be discussed below, pro- and anti-angiogenic factors can be derived from both tumor cells themselves and from tumor-infiltrating inflammatory cells. Thus, the angiogenic switch, as a change in the balance between pro- and anti-angiogenic factors, is an intrinsic event of multistage tumorigenesis. Thereby, genetic and epigenetic events within tumor cells cooperate with inflammatory responses and cells of the tumor stroma to define the ultimate cocktail of pro- and anti-angiogenic factors and thus directly or indirectly shift the balance in favor of an onset of tumor angiogenesis.

Angiogenic factors in mouse models of cancer

Many transgenic mouse models of cancer represent reliable experimental systems to investigate the process of angiogenesis during multistage tumorigenesis. First insights into the angiogenic switch came from a study in the Rip1Tag2 mouse model of pancreatic β-cell carcinogenesis, where Judah Folkman, Doug Hanahan and co-workers elegantly demonstrated the occurrence of the angiogenic switch during the progression from hyperplasia to hypervascularized neoplasia [27]. Rip1Tag2 mice express the Simian Virus 40 large T antigen oncoprotein under the control of the rat insulin promoter,

resulting in the specific expression of the oncogene in the β-cells of the islets of Langerhans and the subsequent development of β-cell tumors [28]. Distinct stages of tumorigenesis are identifiable: normal islets, hyperplastic islets, differentiated adenoma and invasive carcinoma. Notably, co-culturing hyperplastic islets of Langerhans together with endothelial cells in a three-dimensional collagen matrix revealed two subsets of hyperplastic islets. While some did not elicit any response by the endothelial cells, so-called angiogenic hyperplastic islets provoked endothelial cell proliferation, migration and tube formation, all hallmarks of angiogenesis [27]. These experiments also indicated that diffusible angiogenic factors are secreted by the angiogenic tumor stages. VEGF-A is the primarily pro-angiogenic factor implicated in the angiogenic switch in the Rip1Tag2 model. Crossing these mice with transgenic mice expressing human VEGF-A165 in pancreatic β-cells resulted in earlier onset of tumor angiogenesis [29]. Conversely, inhibiting the function of VEGF-A either by the use of a chemical inhibitor of VEGFR signaling [30-32], by adenoviral delivery of a soluble form of VEGFR-1 or 2 [33,34], or by genetically depleting VEGF-A in β-cells [35] all resulted in an impaired angiogenic switch and thus in impaired tumor growth. However, VEGF-A is expressed in normal islets, and its expression is only slightly increased in later stages of tumorigenesis [36]. Treating Rip1Tag2 mice with a chemical inhibitor of matrix metalloproteinase-9 (MMP9) or crossing them with mice lacking MMP9 prevented the angiogenic switch, revealing a critical function for MMP9 in the proteolytic release of matrix-bound VEGF-A [37]. Comparably, blocking heparanase function with a small molecule inhibitor mimicking its physiological ligand also resulted in an impaired onset of angiogenesis, indicating an additional mechanism of VEGF-A release from the ECM for its activation [38]. Two members of the cystein protease family, cathepsin-B and S, also appear to play an important role in the angiogenic switch, as crossing cathepsin-B or S-deficient mice with Rip1Tag2 mice resulted in a decreased number of angiogenic islets [39]. Together, these reports clearly demonstrate that it is the bioavailability of pro-angiogenic factors which constitutes the determining element in the onset of angiogenesis (Figure.2).

Members of the FGF family, together with VEGF-A, have also been shown to contribute to tumor angiogenesis in Rip1Tag2 mice. Adenoviral delivery of a soluble FGF receptor construct (FGF-trap) reduced tumor angiogenesis to the same extent as a VEGF trap [34]. Notably, inhibition of VEGF function resulted in hypoxia-mediated up-regulation of FGFs and to unbowed angiogenesis and re-growth of tumors. Only concomitant

blockade of VEGF and FGF signaling impeded such anti-VEGF therapy-resistant angiogenesis [33,34].

In a second transgenic mouse model of β-cell carcinogenesis, caused by the inducible expression of the oncogene c-Myc, the onset of tumor angiogenesis is triggered by the secretion of the pro-inflammatory cytokine interleukin-1β (IL-1β). This cytokine activity is necessary and sufficient to induce the release of pre-existing sequestered VEGF-A from the ECM, which then initiates the rapid and synchronous onset of endothelial cell proliferation after c-Myc activation [40].

Another spontaneous tumor model where the angiogenic switch has been intensively investigated is the K14-HPV16 transgenic mouse model of squamous cell carcinoma (SCC). In these transgenic mice, the human papilloma virus type 16 (HPV) early region, in particular the E6 and E7 oncogenes, are expressed under the control of the keratin 14 promoter in the basal cells of the epidermis to induce multistage development of SCC. First, keratinocyte hyperplasia occurs accompanied by a mild increase in microvascular density, progressing to dysplasia with abundant neovascularization, and finally leading to highly angiogenic carcinomas [41]. Chronic exposure of these mice to estrogen (termed then K14-HPV/E_2 mice) specifically induces neoplastic progression in the squamous epithelium of the cervix and vagina [42]. Similar to the Rip1Tag2 model, VEGF and FGF signaling have been implicated in the onset of angiogenesis, as VEGF-A and FGF-1 were up-regulated during the early stages of carcinogenesis [43,44]. Crossing MMP9-deficient mice with K14-HPV16 or K14-HPV/E_2 mice also demonstrated the requirement of MMP9 activity for tumor progression [45,46]. Moreover, PDGF exerts an indirect pro-angiogenic effect in this model [47]. Experiments with pharmacological or antibody-mediated blockade of PDGF receptors revealed that PDGF produced by transformed epithelial cells stimulated cancer-associated fibroblasts (CAF) to express FGF-2, which then exerts its pro-angiogenic action on endothelial cells. Similarly, forced expression of PDGF-C in transplanted B16 melanoma cells stimulated CAF to secrete FGF-2 and also osteopontin [48], a soluble extracellular matrix protein known to act in concert with FGF-2 to promote tumor angiogenesis and metastasis [49].

In a transgenic mouse model expressing the bovine papillomavirus genome (BPV.16), dermal fibrosarcomas develop in three stages: mild fibromatosis, aggressive fibromatosis, and fibrosarcoma. Evidence of new blood vessel growth and thus an

angiogenic switch becomes apparent at the stage of aggressive fibromatosis and correlates with altered FGF-2 localization, i.e. a switch from cell-associated to secreted FGF-2 [50]. This observation is intriguing, since FGF-2, as well as the other potent angiogenic FGF, FGF-1, lack a signal sequence for secretion. In fact, β-tumor cells of Rip1Tag2 mice have also been shown to secrete FGF-1 by a non-conventional secretion pathway, the details of which remains to be elucidated [51]. These results suggested that the angiogenic switch may, at least in part, rely on a switch in FGF secretion.

In the MMTV-PyMT mouse model of breast cancer, in which expression of the polyoma virus middle T antigen (PyMT) is expressed under the control of the mouse mammary tumor virus LTR enhancer (MMTV), mammary lesions progress through four tumor stages, including benign hyperplasia, adenoma/mammary intraepithelial neoplasia (MIN), and early (EC) and late (LC) carcinoma stages [52]. The angiogenic switch takes place during the transition from the premalignant to the malignant EC stage and has been shown to depend on VEGF-A. Temporal expression of VEGF-A in the mammary epithelium restored tumor angiogenesis in MMTV-PyMT mice in which stromal cell delivery of VEGF-A was abolished [53]. In a similar manner, expression of pleiotrophin in tumors of MMTV-PyMT mice resulted in increased intra-tumoral microvascular density, demonstrating a functional role of this secretory cytokine, expressed in many human breast cancers, in tumor angiogenesis [54].

Taken together, spontaneous tumor mouse models have been instrumental in revealing the functional role of various angiogenic factors in the angiogenic switch, among which VEGF-A is certainly the most prominent one (Figure.2).

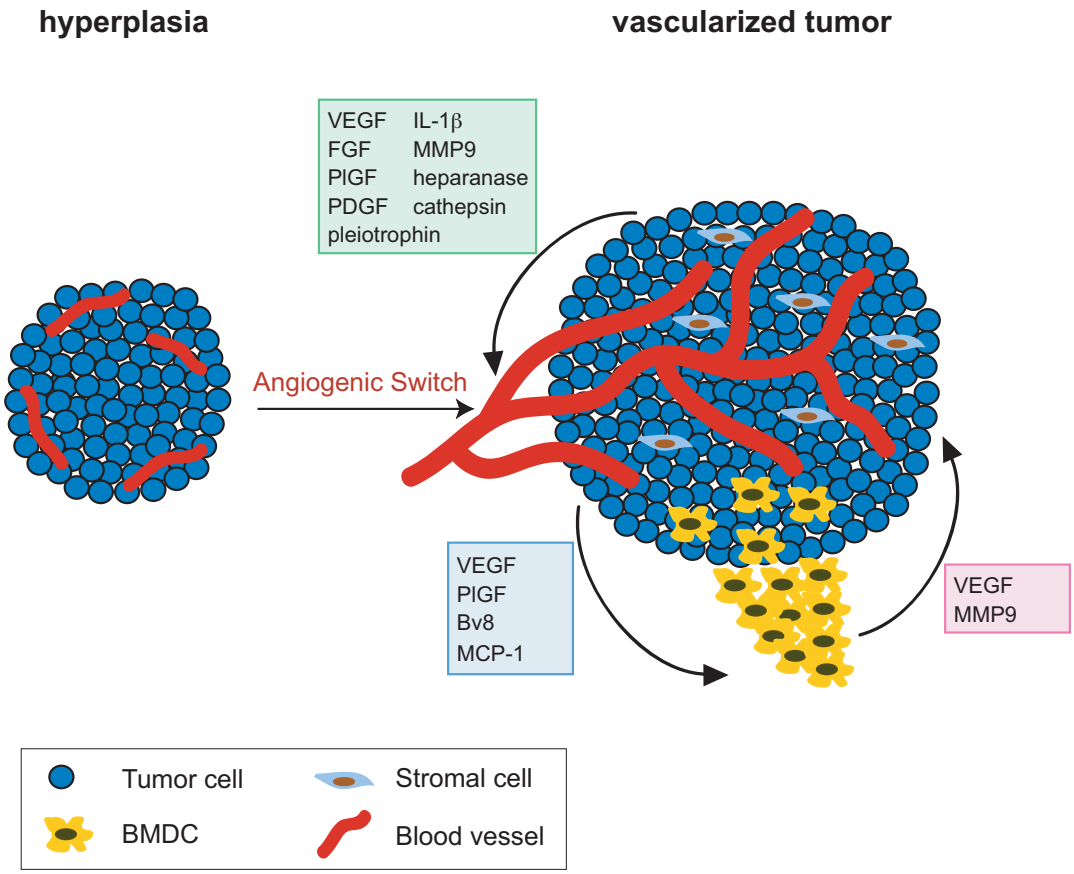

Figure.2: Simplified scheme of the molecular players of the angiogenic switch in tumor mouse models. The angiogenic switch takes place when the balance between pro- and anti-angiogenic factors swaps in favor of the pro-angiogenic factors, resulting in the transition from a dormant avascularized to a growing vascularized tumor. Here are emphasized the pro-angiogenic factors and proteases secreted by the tumor cells themselves (green box) or by the recruited-immune cells (pink box), as well as the factors secreted by the tumor cells to recruit immune cells (blue box) .

Clinical implications: therapy targeting angiogenic factors

Clinical evidence for the existence of an angiogenic switch in cancer patients came first from immunohistological detection of endothelial cell markers in preneoplastic lesions associated with cancers of the mammary duct and the uterine cervix [55,56]. Side-by-side comparison of endothelial specific-immunostainings of human and transgenic mouse tumor biopsies before and after the occurrence of the angiogenic switch revealed remarkably similar patterns in the changes in microvessel density between pre-angiogenic and angiogenic lesions [57].

Thanks to their close recapitulation of the multistep process of carcinogenesis in patients, transgenic mouse models offer the testing and optimization of therapeutic

approaches targeting the various stages of tumor progression, including the angiogenic switch. Indeed, a study testing inhibitors of angiogenic factor signaling and matrix metalloproteases at different stages of pancreatic islet carcinogenesis in Rip1Tag2 transgenic mice demonstrated that their inhibitory effects can be limited to a certain stage of tumorigenesis. BB-94, a broad-spectrum inhibitor of matrix metalloproteinases and endostatin specifically reduced the incidence of angiogenic switching in a prevention trial. BB94, endostatin, AGM-1470, a small molecule inhibitor of endothelial cell proliferation and angiostatin repressed tumor growth in intervention trials, while only AGM1470 showed efficacy in regression trials [32].

The identification of the VEGF-A/VEGFR-2 axis as a key regulator in the onset of tumor angiogenesis has led to the development of several agents targeting VEGF-A-mediated signal transduction of which a few are in routine clinical use and over 20 are in clinical trials [58]. These therapeutic candidates include neutralizing antibodies to VEGF-A or VEGFR-2 and a variety of small chemical tyrosine kinase inhibitors (TKI) with high but not exclusive selectivity to all three VEGFR. Bevacizumab (Avastin), a humanized monoclonal antibody against VEGF-A, is currently used in combination with chemotherapy for treating patients with late-stage colon cancer, non-small-cell lung cancer, breast cancer and others [59]. Sunitinib and sorafenib, two TKI mainly targeting VEGFR-2 have been approved for the treatment of renal carcinoma, gastrointestinal stromal tumors and hepatocellular carcinomas [60]. Depending on cancer type, these anti-angiogenic treatments can lead to a 3-6 months increase in progression-free survival, but fail to provide enduring clinical responses, with transitory improvements being followed by a relapse phase in tumor angiogenesis and subsequent tumor growth.

This adaptive resistance to anti-angiogenic therapy has also been observed and studied in spontaneous tumor models. Treatment of Rip1Tag2 mice with DC101, a monoclonal antibody blocking VEGFR-2 signaling, in a first phase resulted into reduced microvessel density, followed by a second phase of re-initiation of tumor angiogenesis and tumor growth [33]. The relapsing tumors exhibited increased levels of other pro-angiogenic factors, including FGFs, in response to the treatment-induced hypoxia, and blocking FGF signaling with an FGF-trap overcame the resistance against the inhibition of VEGFR signaling. A phase II clinical trial with a pan-VEGFR tyrosine kinase inhibitor pointed to an analogous FGF-dependent resistance in glioblastoma [61]. The patients who experienced tumor progression while on VEGFR blockade presented an increase in

plasma levels of FGF-2 and SDF-1, seemingly correlating with the re-initiation of tumor angiogenesis. Recently, another caveat to the inhibition of VEGF-driven angiogenesis has been raised [62,63]. Treatment of Rip1Tag2 mice or syngeneic and xenograft tumor transplantation models with the VEGF signaling inhibitors DC101 or sunitinib led to an increase in local invasiveness and metastasis formation. Notably, treatment of the mice before tumor implantation also promoted metastasis, raising important concerns about anti-angiogenic therapies currently used in the clinics.

In cancer patients treated with VEGFR blockade or radio-immunotherapy plasma levels of PlGF, a VEGF-family member also involved in the regulation of the angiogenic switch in pathological conditions, has been reported to be elevated, suggesting a role for PlGF in the angiogenic compensation process [64,65]. This hypothesis was tested by employing VEGF-trap therapy in combination with neutralizing antibodies against PlGF in a number of syngeneic tumor models [66]. Besides its direct anti-angiogenic effect on endothelial cells, anti-PlGF therapy also acted through the inhibition of macrophage recruitment to the tumor site, a facet of tumor-driven pro-angiogenic strategies that will be discussed in detail below. Notably, in contrast to VEGF blockade, PlGF blockade did not lead to the development of resistance and efficiently repressed tumor angiogenesis over extended time periods. While these results highlight anti-PlGF therapy as a promising approach, its applicability in patients needs to be assessed.

Recently, elegant experiments showed that fibroblasts isolated from tumors with developed resistance against VEGF signaling blockade had up-regulated PDGF-C expression in comparison to fibroblasts isolated from tumors still sensitive to anti-VEGF-A therapy [67]. Here, a neutralizing antibody against PDGF-C reduced tumor angiogenesis and growth of resistant tumors and exerted additive effects to anti-VEGF-A therapy.

An obvious possibility to prevent the development of resistance against anti-angiogenic therapy is interfering with alternative angiogenic circuits that may be induced as compensatory resistance mechanisms. Therefore, it will be interesting to see the final results of clinical trials with drugs simultaneously targeting a number of angiogenic signals [68,69].

As already mentioned above, some angiogenic factors require proteolytic cleavage for their full activity, raising the attractive idea of indirectly interfering with angiogenic factors by targeting their activating proteases. Unfortunately, clinical trials of MMP

inhibitors have been thus far rather unsuccessful, mainly due to poor efficacy and to toxicity [70]. In contrast, treatment of Rip1Tag2 mice in a prevention trial with the broad-spectrum cathepsin inhibitor JPM-OEt, resulted in 50% reduction in the number of angiogenic islets in comparison to mock-treated animals. Furthermore, cathepsin inhibition lead to decreased tumor growth in a late stage regression trial. This effect was even more pronounced upon combination with chemotherapy [71,72]. These results, together with the lack of apparent toxicity, reveal cathepsins as new exciting target candidates. But how can these promising results been conciliated with the disappointing results obtained with MMP inhibitors in clinical trials? First, most MMP inhibitors tested in the clinics were broad-spectrum inhibitors acting on a very large family of proteases, which not only contained pro-tumorigenic MMP but also so-called anti-targets, such as proteases displaying essential roles in normal cells or in anti-tumor defense mechanisms. Second, despite preclinical results showing the crucial role of MMP in the onset of angiogenesis, a rather early event in tumor progression, the clinical trials tested MMP inhibitors in patients with advanced disease, possibly explaining their inefficiency in these trials [70]. Along these lines, crossing isoform-specific cathepsin-null mice with Rip1Tag2 mice revealed that cathepsin B and S were active at early stages of carcinogenesis, while cathepsin L was important to sustain later stages of tumor progression. These isoform-specific functions of cathepsins may explain why in this case a broad-spectrum inhibitor was effective also in late stage intervention/regression trials [39].

Cellular players in the angiogenic switch

It is now well appreciated that stromal cells of the tumor microenvironment exert an important role during the angiogenic switch. In addition to tumor cells, pro-angiogenic factors are also secreted by pericytes [73], cancer-associated fibroblasts (CAF) [74], and cells of the immune system [75,76]. Notably, the tumor-driven recruitment of cells of the innate immune system to secrete pro-angiogenic factors and to trans-differentiate into endothelial cells and to integrate into tumor blood vessels appear to critically contribute to tumor angiogenesis (Figure.3).

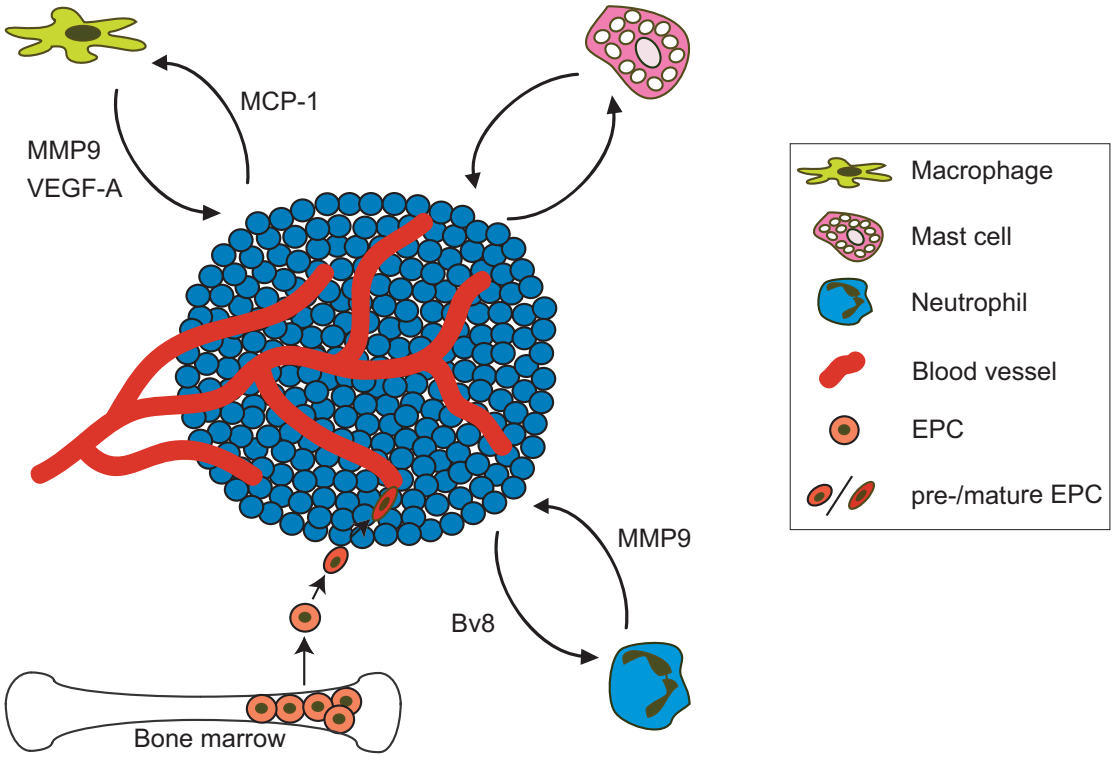

Figure.3: Cellular players and related pro-angiogenic factors implicated in the angiogenic switch in the different tumor mouse models.

Tumor associated-macrophages (TAM) play a pivotal role in modulating tumor angiogenesis, and also tumor progression, in a paracrine manner [77]. They are recruited from the bone marrow through chemoattractants secreted by tumor cells, including pro-angiogenic factors (VEGF-A and PlGF), chemokines (CC-chemokine ligand 2, CCL2), cytokines (granulocyte macrophage/macrophage colony-stimulating factor, GM/M-CSF), and interleukins (IL3, IL6). Depending on the cytokine/chemokine composition of the tumor microenvironment, TAM can either fulfill their primary immuno-stimulatory functions in an anti-tumor defense (referred to as M1 state of macrophages) or, conversely, they can be polarized into a so-called M2 state, thereby exerting immuno-suppressive functions and supporting tumor progression by the secretion of pro-angiogenic cytokines and proteases like VEGF-A, FGF-2 and MMP9. Consistent with this notion, clinical studies have shown that a high extent of leukocyte infiltration in solid tumors correlates with poor prognosis [78]. Within the TAM population, a subpopulation of pro-angiogenic macrophages expressing the angiopoietin receptor Tie-2 has been identified [79]. Interestingly, these Tie2-expressing macrophages (TEM) were exclusively present inside

the tumor bed and not detectable in non-neoplastic tissues in mouse models of cancer and in cancer patients [80]. Similar to TAM and TEM, neutrophils and mast cells have also been reported to induce and sustain tumor angiogenesis by the paracrine secretion of pro-angiogenic factors [81,82].

During tumor angiogenesis, in addition to the formation of new blood vessels from preexisting vessels, it has been proposed that endothelial progenitor cells (EPC), residing in the adult bone marrow, are recruited by tumor-secreted factors to sites of neovascularization where they incorporate into growing blood vessels. However, the actual contribution of EPC to tumor angiogenesis remains controversial, since the extent of their incorporation into the growing tumor vasculature varies remarkably between different experimental settings. Moreover, a consensus on the actual identity of EPC is still to be found [83]. Besides the contribution of progenitor cells distinct from the haematopoietic lineage, myeloid cells have been reported to be able to trans-differentiate into endothelial cells. For example, myeloid progenitors have been found integrated into the portal veins of bone marrow-transplanted or parabiotic mice [84]. Moreover, dependent on pleiotrophin function in a tumorigenic environment, monocytes are able to trans-differentiate into blood endothelial cells [85]. Thus far, the MMTV-PyMT mouse model of breast cancer appears to be the only experimental system where incorporation of bone marrow-derived cells into growing blood vessels are required for tumor neovascularization [86].

Such myeloid-endothelial cell plasticity, *i.e.* the trans-differentiation of cells of myeloid origin into endothelial cells, has also been reported in the context of inflammation-driven lymphatic vessel growth [88,89]. However, the functional contribution of myeloid-endothelial trans-differentiation to tumor blood vessel angiogenesis and lymphangiogenesis remains to be elucidated.

Cellular players in mouse models of tumor angiogenesis

A number of studies have addressed the functional contribution of tumor-infiltrating cells of the immune system to tumor angiogenesis, for example by investigating the cellular composition of the tumor microenvironment and by genetic or pharmacological ablation of cells of the immune system (Figure.4). For example, following the discovery

that MMP9 critically contributes to the onset of angiogenesis in the Rip1Tag2 transgenic mouse model of multistage pancreatic β-cell tumorigenesis by releasing matrix-sequestered VEGF-A [37], $CD11b^{+}Gr\text{-}1^{+}$ neutrophils were identified to express MMP9 inside angiogenic tumor stages, and transient depletion of these cells repressed the onset of tumor angiogenesis, supporting the hypothesis that myeloid cells are required for the angiogenic switch by providing pro-angiogenic MMP9 [81]. $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells also confer refractoriness to anti-angiogenic treatment. Inhibition of VEGF-A function results in the recruitment of these cells to the tumor site, where they produce several angiogenic factors, including Bv8, also known as prokineticin-2, a regulator of myeloid cell mobilization and mitogen for endothelial cells [90,91]. Early treatment of tumor-transplanted mice and Rip1Tag2 transgenic mice with anti-Bv8 antibodies resulted in an impaired angiogenic switch and concomitant inhibition of neutrophil mobilization [92]. The expression of Bv8 appears to be regulated by increased levels of G-CSF, and G-CSF and Bv8 correlate with refractoriness to anti-VEGF treatment, revealing the role of G-CSF and Bv8 in the recruitment of pro-angiogenic neutrophils to the tumor site and in overcoming anti-VEGF treatment [93].

MMP9-expressing neutrophils have also been reported to sustain tumor angiogenesis in the K14-HPV/E_2 mouse model of cervical carcinogenesis [94]. However, significant neutrophil contribution to the tumor microenvironment was only observed, when the recruitment of macrophages was impaired by genetic ablation of the expression of CC-chemokine receptor-2 (CCR2), the receptor for the monocyte chemoattractant protein-1 (MCP-1 = CCL2). Actually, in this model, TAM constituted the primary source of MMP9 required for the onset of angiogenesis [46]. When present at the tumor site, they repressed neutrophil recruitment by the secretion of still undefined soluble factors. However, when TAM were suppressed, recruited neutrophils replaced TAM by providing an alternative paracrine support for tumor angiogenesis [94]. In the K14-HPV16 mouse model of SCC of the skin, bone marrow transplantation of $MMP9^{+/+}$ bone marrow cells into lethally irradiated MMP9-deficient K14-HPV16 mice restored tumor angiogenesis and tumor growth, demonstrating that also in this model, bone marrow-derived cells constitute an important source of proteolytic activity [45]. Furthermore, in this mouse model, recruitment of mast cells and neutrophils was dependent on oncogene-specific, B cell-derived immunoglobulin deposits in the neoplastic tissue, revealing a crosstalk between the adaptive and the innate immune system in promoting tumor angiogenesis [95].

Critical functions of TAM in tumor angiogenesis and tumor progression have also been revealed by genetic experiments in the MMTV-PyMT mouse model of breast carcinogenesis [96]. Crossing MMTV-PyMT mice with macrophage-deficient $CSF1^{op/op}$ mice resulted in a delayed angiogenic switch and impaired tumor progression. This delay could be recovered, concomitant with the recruitment of macrophages, by tumor-specific expression of CSF-1, revealing the crucial pro-angiogenic role of TAM in this mouse model of breast cancer. Moreover, the temporary expression of VEGF-A in the mammary epithelium of $CSF1^{op/op}$/MMTV-PyMT composite mice restored the angiogenic switch, revealing TAM as a source of this pro-angiogenic factor [53]. Interestingly, when VEGF-A expression was exclusively ablated in myeloid cells, tumor blood vessel density was reduced, yet the otherwise highly leaky and barely functional blood vessels of the tumors were normalized to optimal perfusion, resulting in an acceleration and not an impairment of tumor progression [97]. The different outcomes can be explained by the fact that the complete elimination of macrophages affects all and not only one of the many functions of macrophages in tumor progression.

Mast cells may also contribute to tumor angiogenesis. Pharmacological inhibition of their function in a mouse model of c-Myc-induced pancreatic β-cell tumorigenesis resulted in a block of tumor angiogenesis [82]. These experiments revealed that mast cells acted in addition and distinct from the initial oncogene-induced, IL1β-dependent angiogenic switch described in this model [40], however, their paracrine role remains to be elucidated.

Even if the experimental studies described above have lead to the identification of cellular players critical for the onset of tumor angiogenesis, the complexity of the tumor microenvironment and its cancer type-dependent variability pose a major problem for the development of efficient anti-angiogenic therapies. Notably, the fact that ablation of one angiogenic factor or cells secreting them leads to a compensation of the angiogenic activities by other infiltrating cells of the immune system indicates an urgent need for further investigations.

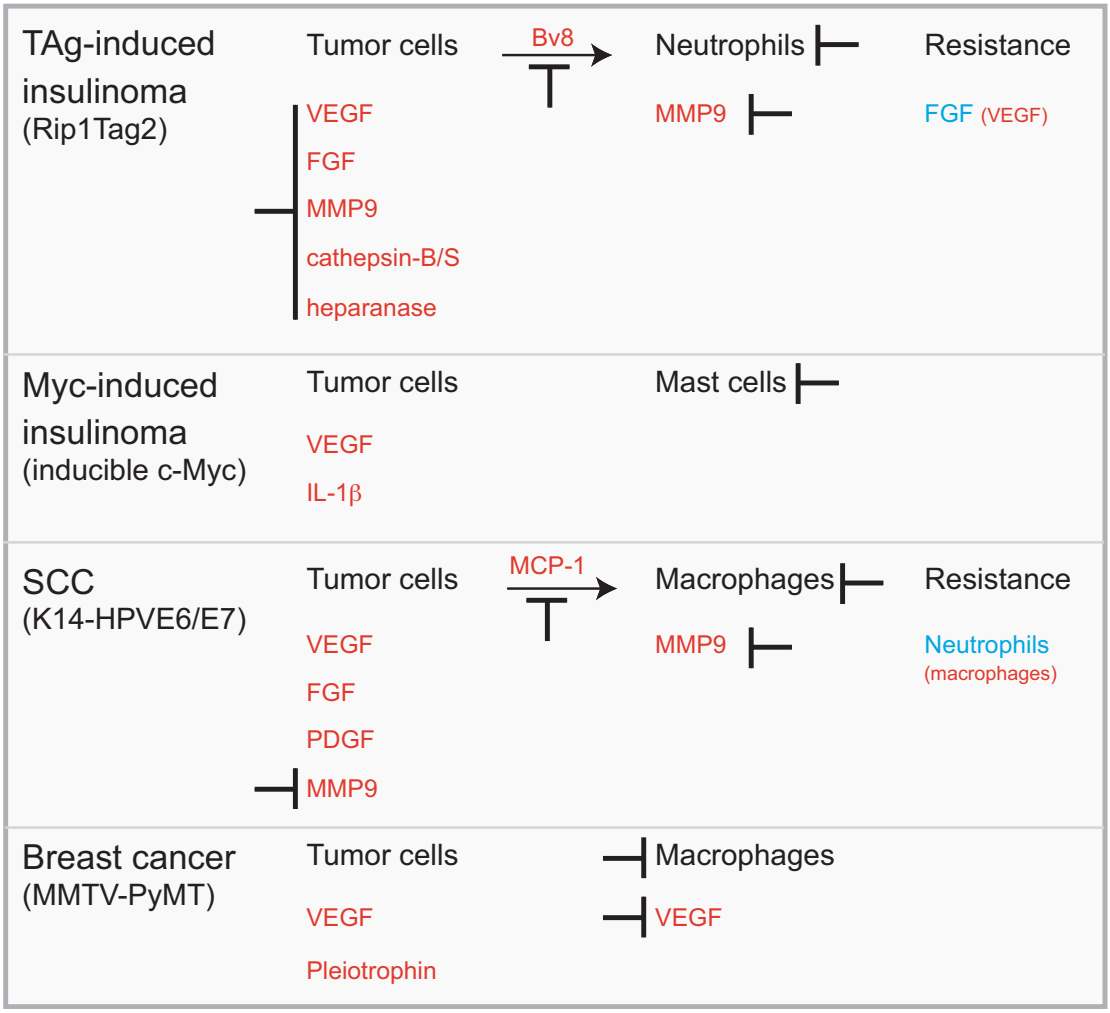

Figure.4: Targeting of the molecular and cellular players to inhibit the angiogenic switch in the different tumor mouse models and consecutive resistance mechanisms.

Cellular players as therapeutic targets

The discovery of the importance of the cells of the inflammatory tumor stroma in promoting tumor angiogenesis raised the attractive possibility of therapeutically targeting the cellular players instead of their secreted pro-angiogenic factors (Figure.4). The effect of pharmacologically targeting TAM, either by the inhibition of their pro-angiogenic function or by their complete removal, has been assessed in a number of tumor models and clinical studies. Zoledronic acid (ZA) is a nitrogen-containing (amino-) bisphosphonate that is approved by the FDA to reduce skeletal complications of bone metastasis [98]. Treating K14-HPV/E2 mice with ZA inhibited MMP9 activity and expression by TAM, resulting in reduced VEGF-A mobilization and subsequent impairment of angiogenesis and cervical carcinogenesis [46]. Consistent with these data,

clinical studies demonstrated that ZA administrated either as a single dose or weekly at low dosage effectively reduced circulating levels of VEGF-A in patients with advanced solid tumors [99-101]. Liposome-encapsulated clodronate (Clodrolip), a non-amino-bisphosphonate, has been used to systematically deplete macrophages in syngeneic tumor transplantation models resulting into significantly impaired tumor angiogenesis and tumor growth [102]. However, this approach has not been tested in cancer patients yet, and potential toxic side effects are not clear. Also, targeting other cell types that have been demonstrated to be functional in the angiogenic switch in pre-clinical models, such as neutrophils or mast cells, have not yet been adapted for clinical use [81,82].

Another way to interfere with the activities of pro-angiogenic tumor-infiltrating cells of the immune system involves blockade of their recruitment to the tumor site. PlGF, by its interaction with VEGFR-1, is a critical factor in inducing monocyte chemotaxis [103]. PlGF is frequently found at high levels in cancer patients and in tumor-bearing mice, and inhibiting PlGF activity with a neutralizing antibody against PlGF significantly reduced intra-tumoral macrophage recruitment and repressed tumor angiogenesis and tumor growth in various transgenic and tumor transplantation mouse models of cancer [66].

Other approaches to design innovative therapies aimed to interfere with the angiogenic switch involve thwarting the plasticity of the tumor-promoting inflammatory environment, which allows adaptive resistance against anti-angiogenic therapy to occur. As mentioned above, impairment of macrophage recruitment in a mouse model of cervical carcinogenesis lead to a compensatory neutrophil response, indicating that interfering with the recruitment of a particular inflammatory cell type may be compensated for by the pro-angiogenic action of another cell type [94]. However, the nature and variability of such compensatory responses in cancer patients are unknown. Hence, targeting the inflammatory context in its entirety instead of focusing on the particular cellular players may offer an alternative route to the problem. For example, Cyclooxygenase-2 (COX-2) expression is found to be upregulated in most cancer types, and COX-2 inhibitors have been reported to present tumor type-dependent effects on tumor progression. COX-2 is an enzyme involved in the production of inflammatory mediators, such as prostaglandins and leukotrienes. Whereas in colorectal cancers, COX-2 inhibition significantly reduced tumor growth, it did not show any benefit in combination with conventional chemotherapy in pancreatic and cervical cancer [104,105]. Similarly, Lenalidomide, an FDA-approved immunomodulatory drug for 5q-

myelodysplasia and multiple myeloma [106], has also been documented to bear anti-angiogenic properties by directly inhibiting endothelial cell function [107]. Its efficacy on renal cell cancer (RCC) has been assessed in phase II clinical trials showing response criteria to treatment comparable to other targeted therapies for RCC, including anti-VEGF-A therapy with bevacizumab [108-110].

Recently, the brilliant idea has been proposed to employ cells of the immune system that are being recruited to the tumor site as vehicles for the delivery of therapeutic agents [111]. Exploiting the tumor-homing specificity of TEM, IFN-α delivery to the tumor microenvironment was accomplished by forcing the expression of IFN-α in TEM through the transduction of haematopoietic stem/progenitor cells (HSPC) with a lentiviral vector (LV) encoding IFN-α under the control of the Tie-2 promoter, a promoter specifically active in TEM and also in activated endothelial cells. IFN-α, at the appropriate dosis, is known to exert anti-proliferative, angiostatic and immune cell-activating functions and, in fact, bone marrow reconstitution of irradiated MMTV-PyMT mice with Tie2-IFN LV-transduced HSPC resulted in a significant inhibition of tumor growth. These results are encouraging, yet for clinical application, refined LV with time-specific transgene expression or tightly regulated expression of a suicide gene to eliminate the modified cells will be required.

The recent breakthroughs described above underscore the need for a more detailed characterization of the inflammatory components of the tumor stroma in order to identify, based on cellular markers, the various cell populations recruited to the tumor microenvironment. Understanding their nature and their function will then allow the development of more specific approaches to either deplete these pro-tumorigenic cell subsets or to employ them as vehicles for anti-cancer drug delivery at a time, when the angiogenic switch is occuring.

Conclusions

Since twenty-five years, transgenic mouse models of spontaneous carcinogenesis are employed as reliable tools to study stage-specific events, such as the angiogenic switch as the initiator of tumor angiogenesis. Importantly, these mouse models are not only amenable to molecular and biochemical analysis of the processes underlying the

angiogenic switch, they also allow the assessment of potential anti-angiogenic therapy under defined and reproducible conditions. However, despite encouraging results in preclinical studies, only few anti-angiogenic drugs are currently in use in the clinics. The low success rate of the translation from bench to bedside may be explained by a combination of different factors. First, in preclinical models, tumor growth is rather fast and accompanied by the rapid formation of new blood vessels. Thus, the tumor vasculature in mouse models tends to be more responsive to anti-angiogenic therapy than the human tumor vasculature [112]. Second, the combination of pro-angiogenic factors and cellular players involved in the onset of tumor angiogenesis might be cancer type-dependent. Hence, to target a broader range of molecular pathways, the use of tyrosine kinase inhibitors may be advantageous over specific monoclonal antibody therapies, as they usually target multiple tyrosine kinases which, in addition to their angiogenic functions, are also directly involved in cancer cell proliferation and survival [113]. Third, experimental drugs that in preclinical early stage prevention or intervention trials have been proven to efficiently impair the onset of tumor angiogenesis may not exert any anti-angiogenic activity when tested in late stage intervention or regression trials, as usually performed in the clinics. Avascular tumors are rather small and usually not detected before undergoing the angiogenic switch, and blocking the angiogenic switch *per se* in a prevention treatment may be rather irrelevant in a clinical setting. Hence, one way to improve anti-angiogenic therapy may be based on a better understanding of the factors and processes underlying the onset vs. the maintenance of tumor angiogenesis. Moreover, research on the tumor microenvironment in its entirety should be intensified to obtain a complete understanding of its complexity, thus paving the way for the design of new anti-angiogenic strategies.

One of the initial arguments in favor of targeting tumor-associated endothelial cells to impair tumor progression was based on the assumption that endothelial cells are genetically stable and, in contrast to transformed cancer cells, would not easily develop resistance to therapy. However, the clinical and pre-clinical experience of the past few years has taught us that interfering with VEGF-A as the main angiogenic factor or with particular stromal cells producing angiogenic factors leads to adaptive resistance and compensatory mechanisms. These observations have raised a number of new questions important for overcoming these unexpected hurdles. Again, we need to know whether there are different factors involved in the onset versus the maintenance of tumor

angiogenesis, their nature and functions, and their regulation to develop multi-targeting of parallel pro-angiogenic signaling circuits, however, with keeping in mind the potential accumulation of side effects.

Finally, reliable surrogate markers allowing, in a non-invasive manner, the accurate determination of the different stages of tumor angiogenesis and concomitant progression, are urgently needed [113]. After all, being able to effectively interfere with the angiogenic switch may keep primary tumors and metastasis in a dormancy state.

1.1.3. Tumor lymphangiogenesis

Lymphatic development

The lymphatic system is composed of a vascular network of thin-walled collecting vessels that drain lymph from extracellular spaces within most organs into larger thicker-walled collecting trunks which return the lymph back to the blood circulation through the thoracic and the right lymphatic ducts. Initial lymphatic capillaries are composed of a single cell layer of overlapping endothelial cells attached on the sides by specialized button-like junctions. Through these junction opening fluid enters, driven by hydrostatic and colloidal osmotic pressure gradients [114]. Anchoring filaments connect the vessels to the ECM. Larger collecting vessels contain in addition a continuous muscular layer, an adventitial layer, a basement membrane and valves to prevent retrograde flow. They present continuous zipper-like junctions which, as button-like junctions, are composed of vascular endothelial cadherin and tight junction-associated proteins like occludin, claudin-5, ZO-1, JAM-A and endothelial cell-selective adhesion molecule [114]. Besides returning extravasated tissue fluid back to the blood circulation, the lymphatic system also plays a role in immune cell transport and in the absorption of lipids from the intestine.

The development of the mammalian lymphatic vasculature has been proposed to take place in four stages: lymphatic endothelial cell (LEC) competence, LEC bias, LEC specification and finally lymphatic vessel differentiation and maturation (Figure.5) [115]. Accordingly, one of the first events taking place during embryonic lymphatic development is that venous endothelial cells become competent to respond to a still unknown initial lymphatic-inducing signal. The expression of the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) by some of the endothelial cells lining the anterior cardinal vein at

E9.0-9.5 is one of the first morphological indication of this competence [116]. In a second step, polarized expression of the homeobox protein Prox-1 becomes detectable in a subpopulation of competent LYVE-1-expressing cells. Progressively, the number of Prox-1-expressing cells increases and endothelial cells positive for this lymphatic-specific transcription factor begin to be visible in more caudally located embryonic veins. Around E11.5-12.5, during the third stage of lymphatic development, in response to a VEGF-C signal in the surrounding mesenchyme [117], VEGFR-3/Prox-1 double-positive LEC migrate from the veins to form the primitive lymph sacs which are scattered along the anteroposterior embryonic axis. The fact that Prox-1 expression by venous endothelial cells is considered as the first indication that a LEC phenotype is specified comes from the observation that in Prox-1-null embryos, blood endothelial cells (BEC) fail to acquire a lymphatic-specific profile [118]. Furthermore, forced expression of Prox-1 in cultured BEC is sufficient to upregulate LEC markers and concomitantly downregulate BEC markers [119]. Thus, *prox-1* may be a master control gene of LEC differentiation, acting by turning off the BEC program while turning on the LEC program. Correspondingly, during the process of LEC specification and migration, the expression of other lymphatic markers, such as neuropilin-2, Podoplanin and secondary lymphoid chemokine (SLC), can be detected in the budding Prox-1 expressing LEC [118,120,121]. In a final step, concomitant with lymph sac formation and lymphovenous separation, LEC sprout from the sacs to give rise to the entire lymphatic network. The signaling molecules Slp-76, Syk and PLCγ2 are key players in lymphovascular separation, as functional inactivation of any of these genes in mice results in the appearance of blood-filled lymphatic vessels [122,123]. During later stages of embryonic and postnatal lymphangiogenesis, the expression of additional lymphatic markers can be detected in the forming lymphatics. For example, ephrinB2 and its typrosine kinase receptor EphB4 have been reported to be expressed on LEC. The Ephrin-Eph system can function bidirectionally, the ligand binding inducing a so-called “forward” signaling as well as a “reverse” signaling. The “forward” signaling acts through receptor phosphotyrosine-mediated pathways and the “reverse” signaling through either phosphorylation of the ligand’s cytoplasmic tail, providing docking sites for intracellular signaling molecules, or through the ligand C-terminal motif for the binding of PDZ-domain containing proteins. EphrinB2-mutant mice, lacking the C-terminal PDZ interaction site, fail to remodel their primary lymphatic capillary plexus into a hierarchical vessel network, revealing the ephrin system as an

essential regulator of lymphatic development [124]. The forkhead transcription factor Foxc2 has also been described to be expressed in LEC. Loss of Foxc2 leads to defects in lymphatic remodeling, failure to form lymphatic valves and increased pericyte coverage [125]. Recently, Foxc2 has been reported to exert its effect on lymphatic collecting vessel formation and maturation through its cooperation with the transcription factor NFATc1, a member of the nuclear factor of activated T cell family [126]. Additionally, the joint action of Foxc2 and Foxc1 has been shown to be required for lymphatic sprouting during vascular development [127]. Ang-2 is also involved in postnatal lymphatic remodeling and maturation as revealed by studies on Ang-2-null mice, an effect which can be substituted by Ang-1 expression [128]. Finally, desmoplakin and β-chemokine receptor D6 have been reported to be expressed in final stages of lymphatic differentiation and maturation [129,130].

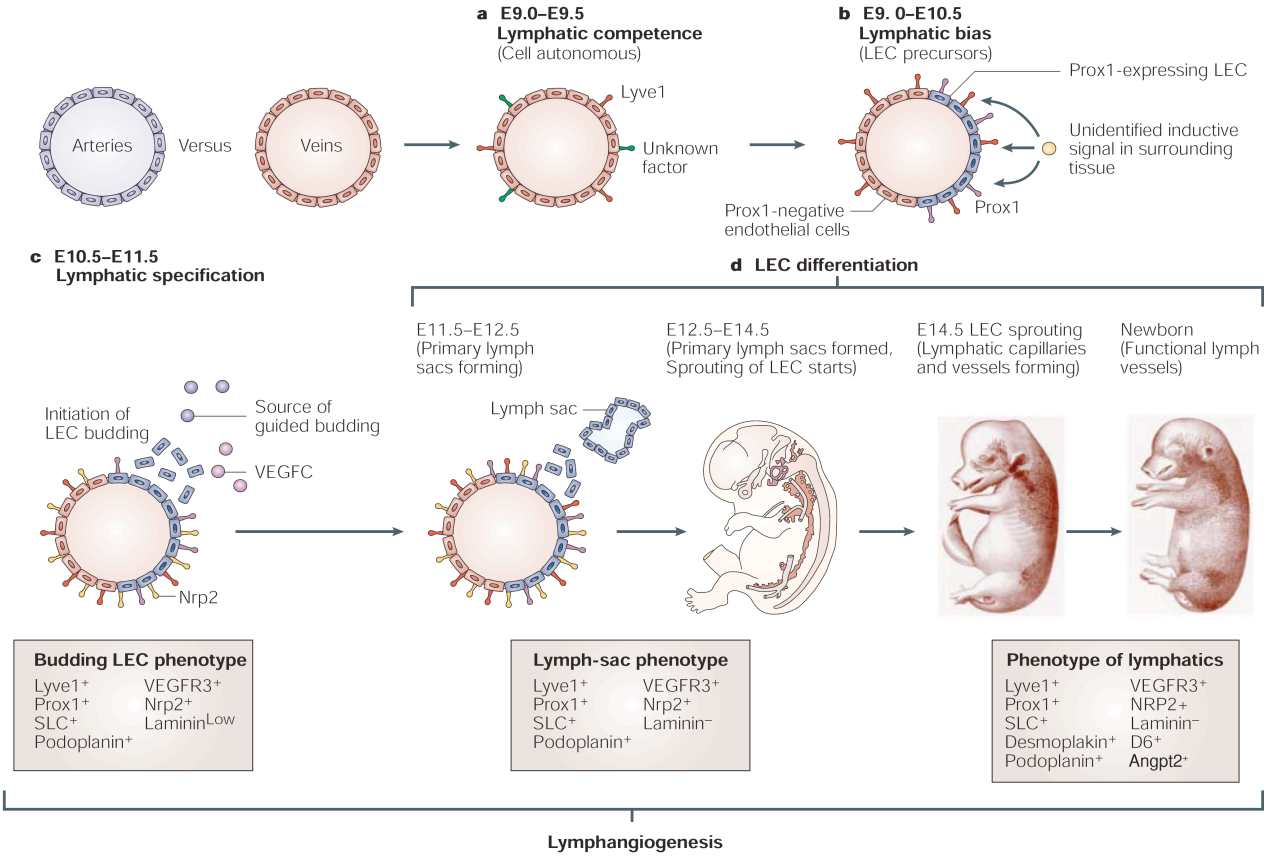

Figure.5: A proposed four-step model of lymphatic vasculature formation. First LEC competence is acquired, followed by LEC bias and LEC specification and finally lymphatic vessel differentiation and maturation. (Modified From [115]).

Tumor-associated lymphatic vessels

In adult organisms, lymphangiogenesis takes only place under pathological conditions, such as tissue repair, inflammation and tumor growth. VEGF-C and its structurally closely related family member VEGF-D are the main lymphangiogenic growth factors which, predominantly through binding to VEGFR-3, promote growth, migration and survival of LEC [131]. Notably, after proteolytic cleavage, these growth factors can also bind to VEGFR-2 [132,133]. A lymphangiogenic potential for its ligand VEGF-A has also been revealed in *in vitro* and *in vivo* studies [134]. Thus, transgenic overexpression of VEGF-A in the mouse skin promoted at wound healing sites the formation of new lymphatic vessels strongly expressing VEGFR-2. Systemic blockade of VEGFR-2 prevented VEGF-A-induced lymphangiogenesis, showing that VEGF-A promotes lymphatic vasculature formation via its direct interaction with VEGFR-2 present on newly formed lymphatic vessels [135]. Additionally, it has been proposed that VEGF-A may also act indirectly by attracting VEGF-C/-D producing inflammatory cells [136]. In order to dissect VEGFR-3 independent mechanisms of lymphangiogenesis, signaling through this receptor was blocked in postnatal and adult mice using a ligand trap (a VEGFR-3-Ig fusion protein). Inhibition of the VEGF-C/-D/VEGFR-3 axis revealed that this signaling pathway is only required for the maintenance of small lymphatic vessels during the first weeks of life, as thereafter, lymphatic vessels were able to regenerate despite constant VEGFR-3 inhibition [137]. Consistent with the idea that the VEGF-C/-D/VEGFR-3 axis plays a specific time/condition-restricted role in adult lymphangiogenesis, antibody-mediated VEGFR-3 blockade completely prevented VEGF-C-enhanced lymphangiogenesis in the adult mouse but had no effect on the survival or function of existing lymphatic vessels [138]. Taken together, these studies indicate that other molecular factors may be implicated in the growth and maintenance of lymphatic vessels during adult life. Indeed, other growth factors able to promote lymphangiogenesis have been identified, including Ang-1 [139], hepatocyte growth factor (HGF) [140], fibroblast growth factor-2 (FGF-2) [141], insulin-like growth factor-1 and 2 (IGF-1/2) [142], platelet derived growth factor (PDGF) [143] as well as recently described, growth hormone (GH) [144] and adrenomedullin (AM) [145] (Figure.6).

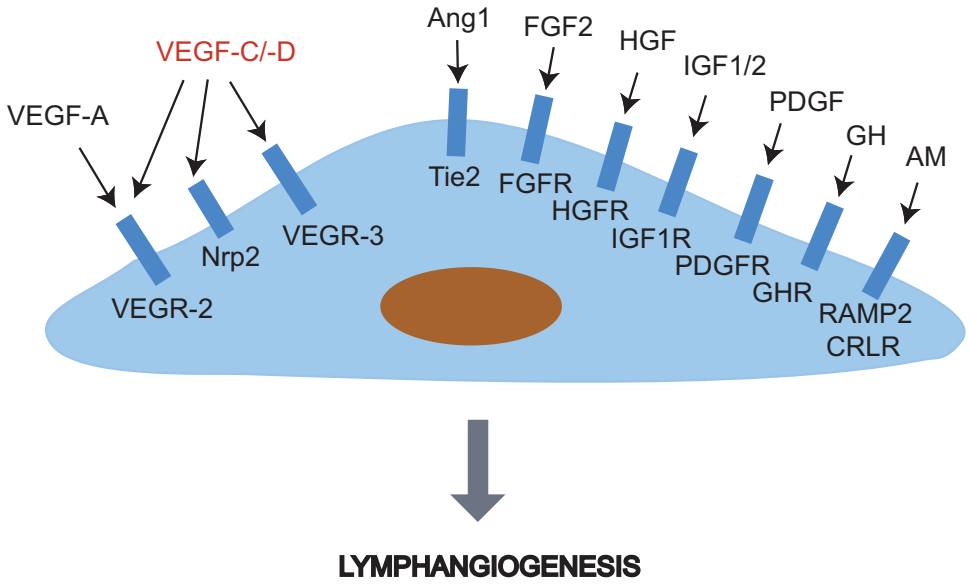

Figure.6: Molecular control of lymphangiogenesis. Schematic representation of the major currently identified lymphangiogenic factors and their receptors on lymphatic endothelium.

Metastasis to regional lymph nodes via lymphatic vessels represents the first step of tumor dissemination in many human cancers and is an important prognostic indicator for disease progression. It has been initially assumed that lymph node metastasis arise from a passive dislocation of tumor cells into pre-existing lymphatic vessels present in their vicinity, along with interstitial fluid. As established by several studies in animal tumor models, it is now clear that tumors can actively induce tumor-associated lymphangiogenesis either by the direct secretion of lymphangiogenic factors, like VEGF-C [146], VEGF-D [147] and VEGF-A [148], or by the recruitment of lymphatic growth factor-secreting immune cells [149]. Increased lymphatic vessel density promotes the spread of tumor cells to regional lymph nodes. In human cancers, a strong correlation between expression levels of VEGF-C and lymph node metastasis has been found in more than thirty retrospective studies [150]. Moreover, VEGF-C and VEGF-A have been reported to induce expansion of the lymphatic network in the sentinel lymph nodes, even before the arrival of the metastatic cancer cells. Once having reached the sentinel lymph nodes, metastatic tumor cells continue to induce local lymphatic vessel growth, resulting in increased drainage of growth factors and lymphatic expansion in distant lymph nodes [148,151]. Another mechanism developed by tumor cells to actively disseminate to lymph nodes implicates the CCL21/CCR7 axis. *In vitro* and *in vivo* studies have reported that

LEC attract CCR7-expressing cancer cells, by the expression of the CCR7 receptor ligand CCL21, a chemokine also shown to be involved in the trafficking of immune cells to lymph nodes [152,153]. Moreover, CCR7 expression has been correlated with lymph node metastasis of several types of carcinomas [154-156]. Recently, autologous secretion of CCR7 ligands has been shown to be used by CCR7-expressing tumor cells as an “interstitial flow sensing” mechanism to home to lymphatic vessels. Under the influence of the flow, the CCR7 ligand pericellular distributions are biased in its direction, causing downstream gradients that then guide tumor cells to the draining lymphatic vessel [157]. Additionally, a synergistic activity between VEGF-C and CCR7 expression in promoting invasion toward lymphatics has been proposed. VEGF-C would then enhance tumor cell chemo-invasion by inducing LEC to increase their CCL21 secretion, so resulting in increased paracrine signaling by lymphatic vessels to CCR7-expressing tumor cells [158].

As tumor-associated lymphangiogenesis correlates with lymph node metastasis, its inhibition might be of therapeutic interest. Indeed, blocking the VEGF-C/-D/VEGFR-3 axis, either by the use of neutralizing antibodies or ligand traps [159,160], or by small interfering RNA-mediated *vegf-c* gene silencing [161], resulted in reduced tumor lymphangiogenesis and in concomitant reduction of lymph node metastasis. As VEGFR-3 is expressed on tumor blood vessels, anti-VEGFR-3 therapy may represent an additional beneficial effect by concomitantly inhibiting tumor angiogenesis [162]. Finally, because VEGF-C/-D can activate VEGFR-2 and VEGF-A promotes tumor lymphangiogenesis, anti-VEGFR-2 and anti-VEGFR-3 antibodies in combination have been shown to decrease lymph node and lung metastasis more efficiently than either antibody alone [159].

1.2. Cell plasticity

1.2.1. Concept, hopes and controversies

It has first been thought that cells arising from the segregation into germ layers (ectoderm, mesoderm, endoderm) during embryogenesis irreversibly maintain their tissue lineage specification into and throughout adulthood. Furthermore, homeostatic cell replacement and tissue regeneration in the adult have been considered to maintain such

tissue specificity, so that rare tissue-resident stem cells would only be able to generate mature cell types corresponding to their tissue of origin, and not cell types of different lineages. However, over the past decade, experiments have challenged this notion of lineage commitment by suggesting that, under certain circumstances, cells may “trans-differentiate” and, in doing so, contribute to a much wider spectrum of differentiated progeny than previously anticipated. This suggestion has given rise to the concept of stem cell plasticity, which holds that the lineage determination of an adult stem cell may not be rigidly defined but instead flexible, allowing these cells to respond to a variety of microenvironmental regenerative cues [163]. The term trans-differentiation is not restrictively used in the context of stem cell plasticity, but similarly describes the conversion of one differentiated cell type into another, supporting a more general concept of cell plasticity (Figure.7).

That adult cells may have the ability to switch fates from one cell type to another, has recently sparked the idea to experimentally reprogram adult mammalian cells and with it has raised new hopes for designing therapeutic approaches for tissue regeneration. The primary goal of regenerative medicine is to produce new cells for the repair or replacement of diseased and damaged tissues. The conversion of abundant adult cells into medically important cells would go toward this goal. Up to now, two different approaches have been used to reprogram cells. In the first one, adult cells were converted to pluripotent stem cells, which were then differentiated in the cell type of interest. In the second approach, the intermediate pluripotent state is skipped and adult cells are directly converted into other progenitor or mature cells.

Reprogramming differentiated cells back to a pluripotent stage has been initiated by pioneering experiments on somatic nuclear transfer (SNT) in amphibians in the 1960s [164]. Transplanting the nucleus of an adult cell into the cytoplasm of an unfertilized egg in order to reverse its differentiation process and reach a pluripotent state has since then been accomplished in many mammalian species [165]. Recently, a burst of enthusiasm is born following the discovery by Yamanaka and colleagues that adult skin fibroblasts can be converted into pluripotent cells (named induced pluripotent cells or iPS) by the expression of a small set of transcription factors [166]. The use of retroviral or lentiviral vectors, which integrate into the genome and thus may create mutations, to deliver the reprogramming factors into the differentiated cells constituted on of the main concern regarding the use of iPS for clinical applications. To circumvent this limitation, vector

integration-free mouse iPS have been derived from liver cells using adenoviral vectors [167], as well as from embryonic fibroblasts using repeated plasmid transfections [168]. Although promising, the low frequencies of iPS obtained raised the concern that these approaches may not be suitable for human cells as they require longer exposure to the reprogramming factors [166,169]. Three alternative approaches have been described to remove transgenes from mouse and human iPS. First, Cre/LoxP recombination was successfully used to excise integrated transgenes but, as with the application of this method residual vector sequences remain, insertional mutations cannot be excluded [170]. Second, seamless excision of piggyBac transposons achieved efficient production of vector and transgene-free mouse iPS [171], however this approach has not yet been tested on human iPS. And third, Yu *et al.* reported that human iPS free of vector and transgene sequenes can be derived from fibroblasts by a single transfection with oriP/EBNA1 (Epstein-Barr nuclear antigen-1)- based episomal vectors [172]. Other limitations for iPS clinical application are the use of oncogenic transcription factors to achieve reprogramming as well as the low efficiency of the process. For these reasons, together with the concern that the use of viral vectors integrating in the host genome should be avoided, the interest in finding small molecules which would compensate for viral transduction of critical factors and facilitate reprogramming is currently raising. Shi *et al.* reported that a combination of BIX-01294, a G9a histone methyltransferase inhibitor, with Bayk8644, a L-channel calcium agonist, could compensate for the lack of the transcription factor Sox2, usually necessary for inducing pluripotency [173]. Along those lines, Huangfu *et al.* showed that valproic acid, a histone deacetylase inhibitor, enables reprogramming of human fibroblasts without the need for oncogenic transcription factors [174]. Furthermore, BIX-01294, valproic acid as well as 5'azacytidine enhanced the reprogramming efficiency [175-177]. Encouraged by these results, the quest to ultimately find a chemical cocktail that would allow reprogramming of somatic cells into pluripotent cells is actively ongoing. Moreover, identifying these small molecules might lead to the identification of general pathways involved in the reprogramming process.

As mentioned above, a secondary approach to convert one cell into another consists in skipping the intermediate pluripotent step and in directly converting an adult cell into another mature or progenitor cell. This approach is referred to as lineage reprogramming in distinction to pluripotent reprogramming (Figure.7). The fact that tissue regeneration is preceded by reactivation of embryonic genes normally functioning during

organ development raised the hypothesis that expression of embryonic genes would be important for lineage reprogramming. With this idea in mind, Zhou *et al.* performed a genome-wide analysis to identify transcription factors with specific expression in precursor cells of the β-cell lineage [178]. Combining this approach with results from knock-down studies, they could identify three transcription factors required for β-cell fate specification. Mature exocrine cells of the adult pancreas were chosen as target cells for reprogramming. The transcription factors were delivered into the murine pancreas in adenoviral vectors, as it has been shown that adenovirus preferentially infects pancreatic exocrine cells but not islet cells [179]. By re-expressing these key developmental regulators *in vivo*, differentiated exocrine cells gave rise to β-cells, which were indistinguishable from endogenous islet β-cells regarding their size, shape and ultrastructure. Furthermore they expressed genes essential for β-cell function and could ameliorate hyperglycemia by remodeling local vasculature and secreting insulin [180]. Based on the same principle, other studies have shown that lineage reprogramming can be achieved using factors being well-studied developmental regulators. For example, MyoD, a transcription factor critical for the specification of the skeletal muscle lineage, can convert cultured embryonic fibroblasts, chondroblasts, and retinal epithelial cells into contracting muscle cells [181]. Furthermore, B cells can be converted to macrophages by the transcription factor CEBP [182] and inner ear support cells are reprogrammed to hair cells by Math1 [183]. This last report together with the report of Zhou *et al.*, where reprogramming takes place directly in the native tissue environment, suggest the optimistic prediction, that it will once be possible to rescue motor neuron deficiencies or acute heart injuries by converting central nervous system glial cells into motor neurons or by using cardiomyocytes reprogrammed from skeletal muscle or skin fibroblasts, respectively. Finally, it is interesting to note that current examples of lineage reprogramming mostly occur between closely related cell types. The fact that cells closely related share much of their developmental history and therefore much of their epigenetic marks should make the interconversion easier, as only a small portion of their epigenomes would have to be rearranged [184]. Similarly, as terminal differentiation involves epigenetic changes “locking in” the differentiated program, it may be more difficult to reprogram mature than immature cells [185].

Taken together these reports underline the remarkable plasticity of cells, even terminally differentiated, and raise the question: besides experimental manipulated

cellular reprogrammation, can trans-differentiation also be observed *in vivo*? The most commonly accepted examples of “spontaneous” trans-differentiation are limb regeneration in the amphibian [186] and the conversion of iris pigmented epithelial cells into lens cells after injury [187]. In these instances, differentiated cells de-differentiate to a progenitor stage prior to their metamorphosis into other differentiated cell phenotypes. Moreover, it is well established that cellular reprogramming may be central to certain types of pathological metaplasia, a term referring to the transformation of one tissue type into another. Metaplasias arise in tissues that have been subjected to chronic injury, infection or abnormal hormonal stimulation, hence undergoing continuous regeneration. For example, bronchi of smokers, which are normally lined only with columnar epithelium, often present patches of squamous metaplasia [188]. The basics of the metaplasia theory have been formulated for the first time in the 1980s by Jonathan M.W. Slack [189], and evidence supporting his theory has gradually accumulated over the past years. The main principle says that tissue type changes are due to changes in the combination of expressed regulatory genes. More precisely, under regenerative conditions, tissue-resident stem or progenitor cells may express a different set of genes encoding transcription factors determining tissue identity, giving rise to a focus of metaplasia [190]. Besides these trans-differentiation cases recognized as such by the majority of the scientific community, an important number of other studies, claiming that adult cells can switch fates from one cell type to another have been looked at with pronounced skepticism. Bone marrow cell contributions to non-blood cells as well as non-haematopoietic cell contributions to haematopoiesis have been reported. But after a first wave of great excitement, concerns about the possibility that cell contamination or cell fusion could account for the results as well as failures to reproduce these results and the low frequency at which trans-differentiation events occurred led to questioning whether the observed processes were really trans-differentiation. For example, Gussoni *et al.* described that skeletal muscle stem cells could produce blood cells when injected intravenously into lethally irradiated recipient mice [191]. When the blood-forming cells derived from muscle cells were better characterized, they turned out to be haematopoietic in origin [192]. As circulating haematopoietic stem cells (HSC) contaminate many non-haematopoietic tissues, the observed haematopoietic activity of muscle cells was in fact due to a contamination of itinerant HSC present in the injected fraction. Furthermore, cell-cell fusion constitutes a major concern regarding HSC plasticity as it has been reported that bone marrow-derived cells can fuse with Purkinje

neurons, cardiomyoctes and hepatocytes [193]. By transplanting lethally irradiated mice bearing a fatal genetic liver disease caused by a mutation in the fumaryl-acetoacetate hydrolase (FAH) gene, with male HSC carrying a reporter gene, Lagasse *et al.* were able to generate donor-derived hepatocytes which rescued the liver deficiency. Some years later, southern blotting and cytogenetic analyses revealed that what had been interpreted as HSC trans-differentiation into hepatocytes [194], was the result of cell fusion [195]. These misinterpretations have emphasized the urgent need of strict criteria to demonstrate trans-differentiation [196], such as ensuring that the starting cell population is not contaminated by any progenitor cell which could give rise to the potential trans-differentiated cells and that no cell fusion events occurred. Although important controversies raise important questions on the existence of trans-differentiation, recent well-conducted studies demonstrate that the conversion of on cell type into another can be observed under certain conditions, although at low frequency (Figure.7). In the following chapter reports considering haematopoietic/endothelial cell plasticity will be discussed in more details.

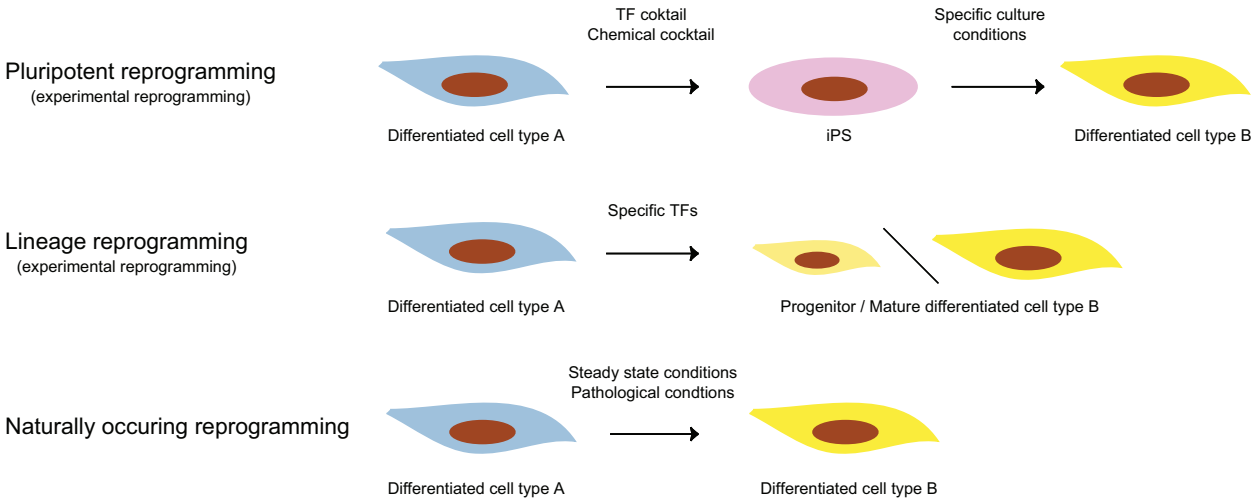

Figure.7: Cell plasticity. Experimental versus naturally occurring reprogramming of one cell type into another.

1.2.2. Haematopoietic/endothelial cell plasticity

The hemangioblast concept

The existence of a common precursor cell, called the hemangioblast, giving rise to both haematopoietic and endothelial cells as been proposed about hundred years ago. Evidence for this concept was first obtained by experiments in the chick yolk sac, where it has been observed that a mesoderm-derived cell could produce red blood cells as well as endothelial cells. However, the real proof of the existence of a common precursor cell for both lineage came at the end of the 1990s from a study by Gordon Keller's group. They developed an *in vitro* assay allowing single-cell analysis of so-called blast colony-forming cells (BL-CFC), which are derived from differentiating mouse embryonic stem cells. Upon specific culture conditions, blast colonies generated both haematopoietic cells and adherent cells displaying typical characteristics of the endothelial lineage. Mixing studies then demonstrated that the haematopoietic and endothelial precursors within the blast colonies developed from the same cell, the BL-CFC. This observation together with insights into the molecular regulation of the development and differentiation of colonies that emerge from a BL-CFC provided strong evidence that the BL-CFC represents the long-hypothesized hemangioblast [197].

Recently, dissecting the cellular events happening during the generation of blood cells from BL-CFC revealed that HSC are formed from a subset of early endothelial cells, constituting the so-called hemogenic endothelium. The fact that in many species, during embryonic development, HSC appear as clusters attached to the endothelium that lines the ventral wall of the abdominal aorta, further supports the implication of the endothelium as the source of developing blood cells. Using time-lapse microscopy, two distinct stages in the formation of mature blast colonies could be observed: cells formed first tightly adherent clusters on the top of which then round, non-adherent cells appeared [198]. Among the adherent cell clusters, a transient cell population expressing various endothelial markers from which blood cell colonies were formed, could be detected. This transient cell population corresponds to the hemogenic endothelial intermediate through which HSC arise from hemangioblasts. Tracking the fates of all cells present in mature blast colonies revealed that one or more endothelial cells per colony directly gave rise to non-adherent haematopoietic cells. These findings did not reflect an in vitro-restricted phenomenon as the existence of hemogenic endothelial cells *in vivo* could be

demonstrated by the direct isolation of primary endothelial cells with hemogenic potential from early mouse embryos [199]. Furthermore, the expression of the transcription factor Runx1 within the endothelium, from embryonic day 8.25 to embryonic day 11.5, has been shown to be required for the formation of HSC [200]. Not only embryonic HSC but also most fetal liver cells and adult bone marrow cells have been reported to originate from the hemogenic endothelium [200,201]. Taken together, these recent findings convincingly show that cells of the endothelial lineage can give rise to cells of the haematopoietic lineage. Would it then be possible, under specific conditions, to observe the reverse situation: haematopoietic cells giving rise to endothelial cells?

Myeloid/endothelial cell plasticity

Indeed, yes. Evidence that HSC can give rise to both blood and vascular endothelial cells *in vivo* has been demonstrated by transplantation of single HSC carrying reporter genes into lethally irradiated recipient mice. Thus donor-derived cells have been shown to contribute to retinal neovascularization [202], to tumor vasculature [203] as well as to the vasculature of the liver, the lung, the heart, the skeletal muscle and the intestine [204]. All these studies were well conducted. Reconstitution at the clonal level ensured that HSC and not bone marrow derived-EPC gave rise to endothelial cells, and fusion events were carefully excluded. Let's have a closer look at the study of Bailey *et al.*. As mentioned above, single GFP+ HSC were able to contribute to the vasculature of several tissues. 3-4% of endothelial cells were HSC-derived. To assess potential fusion events, trans-differentiated cells were FACS sorted from tissues of sex-mismatched transplanted mice based on their GFP+/CD31+/CD45- (haematopoietic marker) pattern, and DNA content as well as X/Y chromosome composition were examined by FACS and FISH respectively. No significant number of cells with 4N DNA content or more than 2 sex chromosomes could be detected. Furthermore, the integrated cells not only expressed endothelial cell markers like CD31 or von Willebrand factor but were also uniformly CD45- and presented the ability to take up low-density lipoprotein, reflecting thus a functional trans-differentiation of HSC into endothelial cells.

These findings raised the question whether the HSC-derived endothelial cell progenitors reside in an established haematopoietic lineage or represent a novel

progenitor population. About two years after the publication of their first study, Bailey *et al.* assessed this question by transplanting irradiated mice with FACS-sorted fractions of tagged-bone marrow [84]. The expression of the progenitor marker c-kit was revealed to be necessary for the contribution of bone marrow-derived cells to the liver endothelium; however, cells expressing mature haematopoietic lineage markers (Lin+) were also able to give rise to donor-derived endothelial cells. This, in combination with the failure to generate endothelial cells from lymphoid progenitors, suggested that endothelial cell progenitors might arise from myeloid lineage progenitors. Subsequently, tagged common myeloid progenitors (CMP) as well as granulocyte/macrophage progenitors (GMP) were transplanted into irradiated recipients. GMP constitute a further differentiated progeny of CMP. About 1% of portal vein cross-sections contained donor-derived endothelial cells, showing that endothelial cells can arise from well-defined populations of myeloid lineage-restricted bone marrow progenitors and that the endothelial potential persists at least until the GMP stage of myeloid differentiation. Similar results were obtained by parabiosis experiments, demonstrating that acute radiation injury was not a prerequisite for the trans-differentiation process to occur. In the first study, despite the lack of detection of increased DNA content or number of sexual chromosomes, rare cell fusion events which would be followed by a reduction division yielding to diploid cells, could not be excluded. In contrast, in the second study, fusion events were completely ruled out by genetic tracing experiments. HSC isolated from tagged Cre recombinase transgenic mice were transplanted into mice harboring a β–galactosidase (β–gal) gene inactivated by a floxed stop codon, so that only host cells that fuse with donor cells would undergo DNA recombination and express β–gal. None of the donor-derived cells expressed the β–gal reporter gene, consistent with direct differentiation of the transplanted cells. Taken together, their results reveal that endothelial cells represent a previously unrecognized differentiation potential of the myeloid lineage. Recently, the role of the tumor-secreted angiogenic factor pleiotrophin (PTN) in the conversion of myeloid to endothelial cells has been reported [85]. When co-injected with human multiple myeloma into severe combined immunodeficient mice, tagged-human monocytes were found incorporated into tumor blood vessels and expressed vascular markers, a process blocked by the use of anti-PTN antibodies. Thus, tumor production of PTN seems to orchestrate the trans-differentiation process, at least in the case of human multiple myeloma.

Such evidence that haematopoietic cells can give rise to vascular endothelial cells brought up the hypothesis that these cells may also be able to give rise to lymphatic endothelial cells. To assess this presumption, similar experimental procedures were used. Transplantation of GFP+ HSC into irradiated recipient mice or parabiosis-induced chimerism revealed the presence of donor-derived lymphatic endothelial cells, defined as GFP+ LYVE-1+ CD45- and F4/80- (macrophage marker) cells, into the lymphatic endothelium of the liver, kidney, stomach and intestine [205]. Under these steady state conditions, HSC-derived LEC contributed to 3-4% of lymphatic vessels. In contrast to the reported differentiation potential of myeloid cells towards blood endothelial cells, no donor-derived LEC could be detected post CMP or GMP transplantations, suggesting that the lymphatic endothelium, at least under physiological conditions, does not arise from haematopoietic cells comprised within the myeloid lineage. A study on human samples confirms as well as partially contradicts the previous results [87]. Analysis of biopsies of human normal skin and intestine derived from individuals with gender-mismatched bone marrow transplants, did not reveal any contribution of donor-derived cells to the lymphatic endothelium. However, following rejection of sex-mismatch human kidney transplants, host-derived LEC could be observed within the lymphatic vessels present in the rejected organs, confirming the existence of lymphatic progenitor cells in the presence of an inflammatory context. About 4.5% of renal explants lymphatic endothelial cells were derived from lymphatic progenitor cells and no fusion events could be detected by FISH analysis of more than 7000 lymphatic endothelial cell nuclei. Moreover, under inflammatory conditions, myeloid cells have been reported to be able to contribute to *de novo* lymphangiogenesis. First, following cornea suture placement, CD11b+ (myeloid marker) cells were found integrated into newly formed inflammation-induced lymphatic vessels. Systemic depletion of macrophages using clodronate liposomes consequently suppressed corneal lymphangiogenesis [88]. And second, it has been reported that lymphatic vessels that form during the acute phases of excisional wounds are comprised largely of cells co-staining for the macrophage marker F4/80 and the lymphatic markers LYVE-1 and Podoplanin [89]. Interestingly, double-positive cells were not detected in the pre-existing lymphatics in the non-injured tissue at the wound edge. Actually, F4/80/LYVE-1 double-positive cells only persisted 10 to 14 days after wounding, though in lower numbers, and lymphatic structures that remained in the granulation tissue stained for LYVE-1 only. These results suggest that the new lymphatic vessels were formed from F4/80+ cells and so underline the specific inflammation-

induced contribution of macrophages to the lymphatic endothelium. In support to these *in vivo* data, several studies have recapitulated *in vitro* the ability of myeloid cells to give rise to lymphatic endothelium. For examples, under specific culture conditions, human VEGFR3+ monocytes could be stimulated to express LYVE-1 and Podoplanin [87] and mouse thioglycollate-stimulated CD11b+ cells showed the ability to form LYVE-1/Podoplanin double positive tube-like structures [88].

In the inflammatory context of tumors, are there also specific subsets of bone marrow-derived cells contributing to lymphatic vessels? Depending on the tumor models and the analytic methods used to assess this question, divergent answers have been given. Investigation of human biopsies of two rare carcinomas with low rates of lymphatic endothelial proliferation derived from gender-mismatched bone marrow transplanted individuals, did not show any incorporation of donor-derived cells into tumor-associated lymphatic vessels [87]. Similarly, subcutaneous injection of B16-F1 or LLC tumor cells into syngeneic GFP-tagged bone marrow-transplanted mice did not reveal any contribution of GFP+ cells to tumor lymphatics [206]. However, the study of such rare events requests intense analysis as well as appropriate tumor models presenting consistent tumor-associated lymphangiogenesis. Thus, 3-dimensional analysis of confocal Z-stacks of tumors sections of GFP-tagged bone marrow-transplanted mice subcutaneously injected with T241 fibrosarcoma cells, demonstrated the presence of bone marrow-derived cells into peritumoral lymphatic vessels [207]. Furthermore, the transplantation of FACS-sorted tagged HSC into $Apc^{Min/+}$ mice, an endogenous mouse model of multiple intestinal neoplasia, resulted in the incorporation of donor-derived LEC into the lymphatic vessels of spontaneously arising intestinal tumors, showing that bone marrow-derived cell contribution to the tumor lymphatic vasculature is not restricted to transplantation tumor models [205]. Even if these two last studies show that bone marrow-derived cells can give rise to tumor lymphatic endothelium, they do not determine which cell population within the different bone marrow cell populations contributes to tumor lymphatic vessels. Moreover, confirmative studies are needed to assess if this process is a general mechanism of tumor lymphatic development or if it is restricted to certain tumor types.

2. Aim of the study

As mentioned above, the physical contribution of bone marrow-derived cells to the tumor lymphatic endothelium is rather controversial. Moreover, the studies reporting the existence of bone marrow-derived tumor lymphatic endothelial cells are purely descriptive and no mechanistic insights into how this trans-differentiation process occurs have been provided. Since tumor lymphatic vessels promote tumor progression by regulating interstitial fluid pressure, transporting immune cells to the tumor microenvironment and finally providing a route for metastatic dissemination, assessing the existence of bone marrow-derived tumor lymphatic endothelial cells in different tumor mouse models as well as understanding their functional role is warranted.

Along these lines, the principal aims of this study have been the following:

a) To assess the potential physical contribution of bone marrow-derived cells to the tumor lymphatic vasculature in transgenic and syngeneic tumor transplantation mouse models. The goal being to determine if the conversion of bone marrow cells toward tumor lymphatic endothelial cells represents a general process contributing to tumor lymphatic growth.

b) To characterize the bone marrow-derived cells integrating into tumor lymphatics by determining if these cells reside within a specific bone marrow cell population.

c) To get insights into the mechanism by which bone marrow cells could give rise to lymphatic endothelium.

3. Experimental design

The potential physical contribution of cells derived from the bone marrow to tumor lymphatic endothelium as well as the characterization of these cells were assessed by transplantation of lethally irradiated tumor-bearing mice presenting ongoing tumor lymphangiogenesis with GFP-labeled bone marrow or bone marrow fractions and subsequent tumor lymphatic confocal microscopy and FACS analysis. Two tumor mouse models were used, a transgenic tumor model, the Rip1Tag2; Rip1VEGF-C (RT2;VC) mice as well as a syngeneic tumor transplantation model, the *s.c.* injection of prostate adenocarcinoma cells (TRAMP-C1 cells) into C57Bl/6 mice. The Rip1Tag2 mice (RT2) express the Simian Virus 40 large T antigen oncoprotein under the control of the rat insulin promoter, resulting in the specific expression of the oncogene in the β-cells of the islets of Langerhans and the subsequent development of β-cell tumors [28]. When crossed to Rip1VEGF-C (VC) mice, double-transgenic RT2;VC mice develop tumors with high peritumoral lymphangiogenesis and lymph node metastasis [146] (Figure.8). TRAMP-C1 is a murine prostate adenocarcinoma cell line which has been shown to induce intratumoral lymphangiogenesis upon transplantation into syngeneic C57Bl/6 mice [208,209] (Figure.8).

The phenotypical conversion of bone marrow cells into lymphatic endothelial cells was recapitulated in an *in vitro* assay, offering a useful tool to study the trans-differentiation mechanism. More precisely, trancriptome comparison of cells at different time points in the trans-differentiation process revealed time-specific upregulated genes representing potential important players at different steps of this process. Some of these potential candidates were then assessed for their role first in the *in vitro* and then in the *in vivo* trans-differentiation process by knock down and blocking experiments.

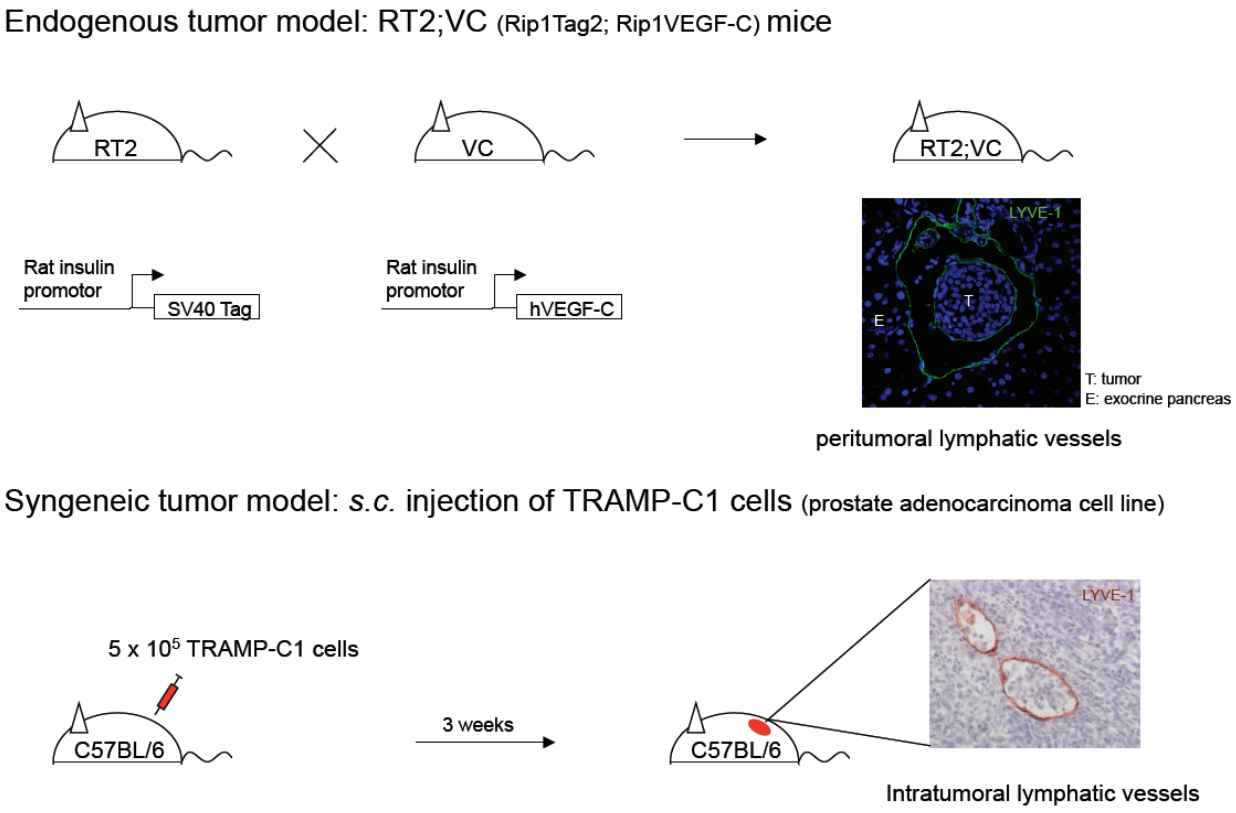

Figure.8: Schematic representation of the two tumor mouse models presenting ongoing lymphangiogenesis used to assess the existence of bone marrow-derived tumor lymphatic endothelial cells. RT2;VC mice develop insulinomas surrounded by lymphatic vessels as C57BL/6 mice transplanted with TRAMP-C1 cells develop subcutaneous tumors presenting intra-tumoral lymphatic vessels.

4. Results

4.1. Myeloid cells contribute to tumor lymphangiogenesis

Running title: Myeloid cells and lymphangiogenesis

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Keywords: angiogenesis, bone marrow cells, lymphangiogenesis, macrophages, myeloid cells, tumorigenesis

4.1.1. Abstract

The formation of new blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) promotes tumor outgrowth and metastasis. Previously, it has been demonstrated that bone marrow-derived cells (BMDC) can contribute to tumor angiogenesis. However, the role of BMDC in lymphangiogenesis has largely remained elusive. Here, we demonstrate by bone marrow transplantation/reconstitution and genetic lineage-tracing experiments that BMDC integrate into tumor-associated lymphatic vessels in the Rip1Tag2 mouse model of insulinoma and in the TRAMP-C1 prostate cancer transplantation model, and that the integrated BMDC originate from the myelomonocytic lineage. Conversely, pharmacological depletion of tumor-associated macrophages reduces lymphangiogenesis. No cell fusion events are detected by genetic tracing experiments. Rather, the phenotypical conversion of myeloid cells into lymphatic endothelial cells and their integration into lymphatic structures is recapitulated in two *in vitro* tube formation assays and is dependent on fibroblast growth factor-mediated signaling. Together, the results reveal that myeloid cells can contribute to tumor-associated lymphatic vessels, thus extending the findings on the previously reported role of haematopoietic cells in lymphatic vessel formation.

4.1.2. Introduction

In the adult, the vascular network is usually expanded and remodeled by sprouting and proliferation of endothelial cells from pre-existing blood and lymphatic vessels, processes called angiogenesis and lymphangiogenesis, respectively. In addition to tissue resident cell types, several studies have demonstrated that BMDC are recruited to angiogenic sites to support the establishment of new vessels [79,210,211]. BMDC are typically sub-classified into haematopoietic progenitor cells (HPC) and endothelial progenitors cells (EPC). In various tumor models, HPC have been shown to contribute to blood vessel angiogenesis by secreting angiogenic factors and proteases required for the activation of latent forms of angiogenic factors [45,136]. HPC have also been implicated in the preparation of a pre-metastatic niche in organs that are colonized by disseminating cancer cells [212]. EPC on the other hand have been shown to directly integrate into growing blood vessel walls, however, to varying extents, ranging from 0 to 50% and thus raising questions about their functional contribution to blood vessel angiogenesis in

various physiological and pathological conditions [79,213]. Recently, it has been reported that also cells of the myeloid lineage are able to differentiate into *bona fide* blood endothelial cells [84].

Only few studies have addressed the role of BMDC in lymphangiogenesis. Haematopoietic stem cells (HSC) and BMDC have recently been shown to contribute to lymphatic endothelium in various organs and during embryonic development [123,205,214]. BMDC contribution to lymphatic vessels has also been reported under inflammatory conditions. For example, experiments employing a cornea angiogenesis model have revealed incorporation of BMDC in newly formed lymphatic vessels [207]. Furthermore, following rejection of human kidney transplants, lymphatic vessels within the rejected organs have been described to contain host-derived lymphatic endothelial cells, supporting the existence of bone marrow-derived lymphatic endothelial progenitor cells [87]. More specifically, myeloid cells present in the murine inflamed conjunctiva were found to express the lymphatic endothelial specific marker VEGFR-3 and to integrate into lymphatic structures that develop in mouse cornea transplants [88,215]. In addition, macrophage depletion appeared to cause reduced lymphangiogenesis and impaired wound healing in diabetic mice [89].

The contribution of BMDC to tumor lymphangiogenesis is rather controversial. While two independent studies report a BMDC contribution to tumor lymphatics [205,207], transplantation of Lewis Lung Carcinoma or B16-F1 melanoma cells in syngeneic mice has not revealed any integration of BMDC into newly formed lymphatic vessels [206]. Here, we have employed the Rip1Tag2 transgenic mouse model of pancreatic β-cell carcinogenesis as well as subcutaneous transplantation of TRAMP-C1 murine prostate cancer cells in syngeneic C57Bl/6 mice to demonstrate that cells derived from the myeloid lineage can contribute to tumor lymphangiogenesis by integrating into tumor-associated lymphatic vessels. Moreover, *in vitro* culture assays reveal that macrophages can convert into lymphatic endothelial cells and integrate into cord-like structures formed by lymphatic endothelial cells. These data support and extend previous findings on the controversial role of haematopoietic cells in newly formed lymphatic vessels.

4.1.3. Results

BMDC integrate into tumor lymphatics

We have used the Rip1Tag2 (RT2) mouse model of multistage pancreatic β-cell carcinogenesis to investigate the contribution of BMDC to tumor angiogenesis and lymphangiogenesis [28]. RT2 transgenic mice recapitulate hallmarks of tumor progression, including the regulated onset of tumor angiogenesis, the functional contribution of tumor-infiltrating immune cells to a pro-angiogenic tumor microenvironment, and the transition from adenoma to carcinoma [37,81,216]. When crossed to Rip1VEGF-C (VC) mice, double-transgenic RT2;VC mice develop tumors with high peritumoral lymphangiogenesis and lymph node metastasis [146].

To investigate whether BMDC integrate into tumor blood and lymphatic vasculature in the RT2 model, lethally irradiated single transgenic RT2 and double-transgenic RT2;VC mice were transplanted with bone marrow isolated from actin-GFP transgenic mice (Figure.9A). FACS analysis of peripheral blood (PB) showed efficient haematopoietic reconstitution with more than 90% chimerism (data not shown). Immunofluorescence analysis of tumor sections revealed that the proportion of GFP^+ tumor-infiltrating BMDC was invariant in the range of 3.5% of total cellularity, independent of the transplantation of single transgenic RT2 mice or double-transgenic mice expressing VEGF-C (Figure.10). From the GFP^+ BMDC within the tumors, approximately 80% were $F4/80^+$ macrophages (Figure.10). Immunofluorescence co-staining for F4/80 and the hyualuronan receptor LYVE-1 identified $LYVE\text{-}1^+$ macrophages in the tumor periphery with relatively large size compared to intra-tumoral macrophages (data not shown) [217,218]. In contrast, Podoplanin or Prox-1 were not expressed by these tumor-associated macrophages (TAM). These observations instructed us to carefully differentiate between tumor lymphatic endothelium, defined as a continuous $LYVE\text{-}1^+$ vessel lining, and isolated, peritumoral $LYVE\text{-}1^+$ TAM.

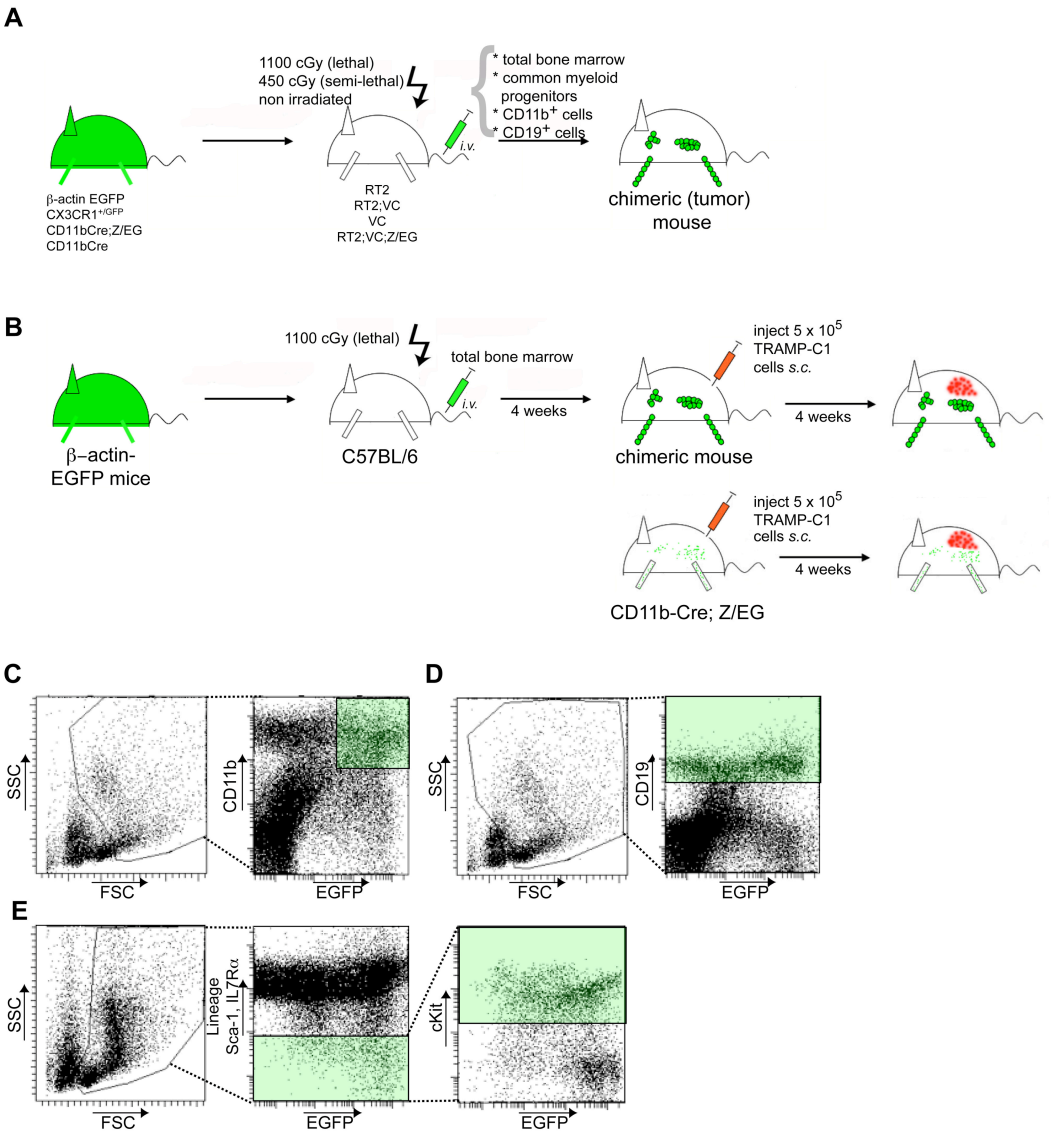

Figure.9: Bone marrow transplantation strategies. (A) For total bone marrow transplantations, 5 x 10^6 T cell-depleted total bone marrow cells from donor mice were injected *i.v.* into lethally irradiated (2 x 550 cGy) mice, as indicated. Semi-lethally irradiated (450 cGy) mice were injected with FACS-sorted 4 x 10^5 $CD11b^+$ myeloid cells, 4 x 10^5 $CD19b^+$ B-cells or 4 x 10^4 common myeloid progenitors (CMP) cells. 4 x 10^5 $CD11b^+$ myeloid cells were also transferred into non-irradiated mice. After 3-8 weeks mice were sacrificed, engraftment of transplanted bone marrow was evaluated by FACS and pancreata were analyzed by histology for the presence of bone marrow-derived cells at the tumor site. (B) Schematic illustration of syngeneic TRAMP-C1 tumor experiments. 5x10^5 TRAMP-C1 cells were injected into the flank of either C57BL/6 previously reconstituted with bone marrow of beta-actin-GFP transgenic mice or bone marrow of double-transgenic CD11b-Cre;Z/EG mice, and tumors were allowed to grow for 3 to 4 weeks. FACS analysis was used to assess bone marrow reconstitution or Cre recombinase-mediated GFP expression, respectively. Histological sections from TRAMP-C1 tumors were analyzed by immunofluorescence for the presence of GFP^+ cells. (C-E) Flow cytometry-based strategy for cell sorting. (C) Within a scatter gate excluding lymphocytes, $CD11b^{high}$/ GFP^{high} cells were isolated by FACS. (D) $CD19^+$ was used as marker for the isolation of B lymphocytes. (E) CMP cells were sorted as lin^-/ $Sca\text{-}1^-$/ $IL7Ra^-$/ $cKit^+$ as described in Methods.

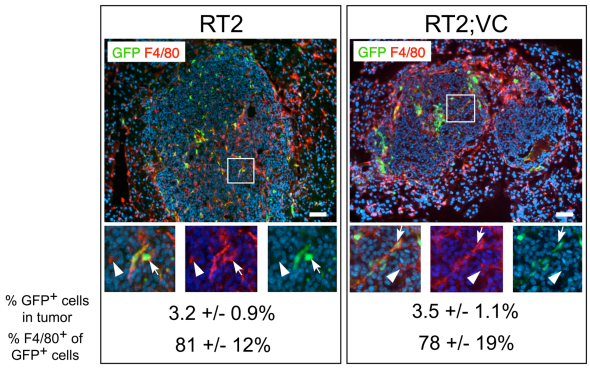

Figure.10: Infiltration of transplanted BMDC in RT2 tumours. Lethally irradiated RT2 and RT2;VC mice were transplanted with GFP-labeled bone marrow, as indicated. Approximately 3-3.5% of tumour-constituting cells were GFP+ (green) and thus bone marrow-derived, and approximately 80% of GFP+ cells co-expressed the monocyte/macrophage marker F4/80 (red). White rectangles indicate area of higher magnification shown below, with merge picture on the left, F4/80 in the middle, and GFP on the right. F4/80+ macrophages are either donor-derived (co-expressing GFP, indicated by arrows) or host-derived (no GFP expression, arrowheads). 3 mice per genotype with 16-29 tumours each were analyzed. DAPI was used for nuclear counterstaining (blue). Scale bar : 50 µm.

The potential contribution of BMDC to intra-tumoral blood vessels was analyzed by confocal microscopy and subsequent 3D reconstitution on pancreatic sections of transplanted RT2 and RT2;VC mice stained for the endothelial marker CD31 and for GFP. Bone marrow-derived, GFP^+ cells were mainly found in close proximity of tumor blood vessels, yet a significant direct incorporation of BMDC into the blood vasculature was not detectable (data not shown).

In contrast, BMDC had incorporated into lymphatic vessels surrounding VEGF-C expressing β-cell tumors of transplanted RT2;VC mice. Pancreatic sections from these mice were stained for the three lymphatic markers Podoplanin, Prox-1 and LYVE-1 and for GFP. Confocal imaging revealed that 3% of $Podoplanin^+$ tumor lymphatic endothelial cells (TLEC) as well as 3.5% of $Prox\text{-}1^+$ or $LYVE\text{-}1^+$ TLEC co-expressed GFP, indicating that approximately 3% of tumor-surrounding lymphatic endothelial cells are derived from the bone marrow (Figure.11A). Routine 3D reconstitution analysis by compiling the Z-stacks of the confocal images enabled us to distinguish integrated GFP^+ BMDC cells from cells located in the close vicinity of lymphatic vessels or transmigrating through the lymphatic endothelial barrier, as shown in Supplemental Videos 1 and 2. Furthermore, VE-cadherin, an endothelial-specific adherens junction molecule reported to connect lymphatic endothelial cells in lymphatic vessels [114], was expressed on host as well as on bone marrow-derived TLEC, further demonstrating a functional integration of BMDC into tumor lymphatic vasculature (Figure.12). Note that in contrast to blood endothelial cells, where VE-cadherin principally clusters at cell-cell junctions (Figure.12, arrows), VE-cadherin staining on lymphatic endothelium was more homogenously distributed throughout the membrane.

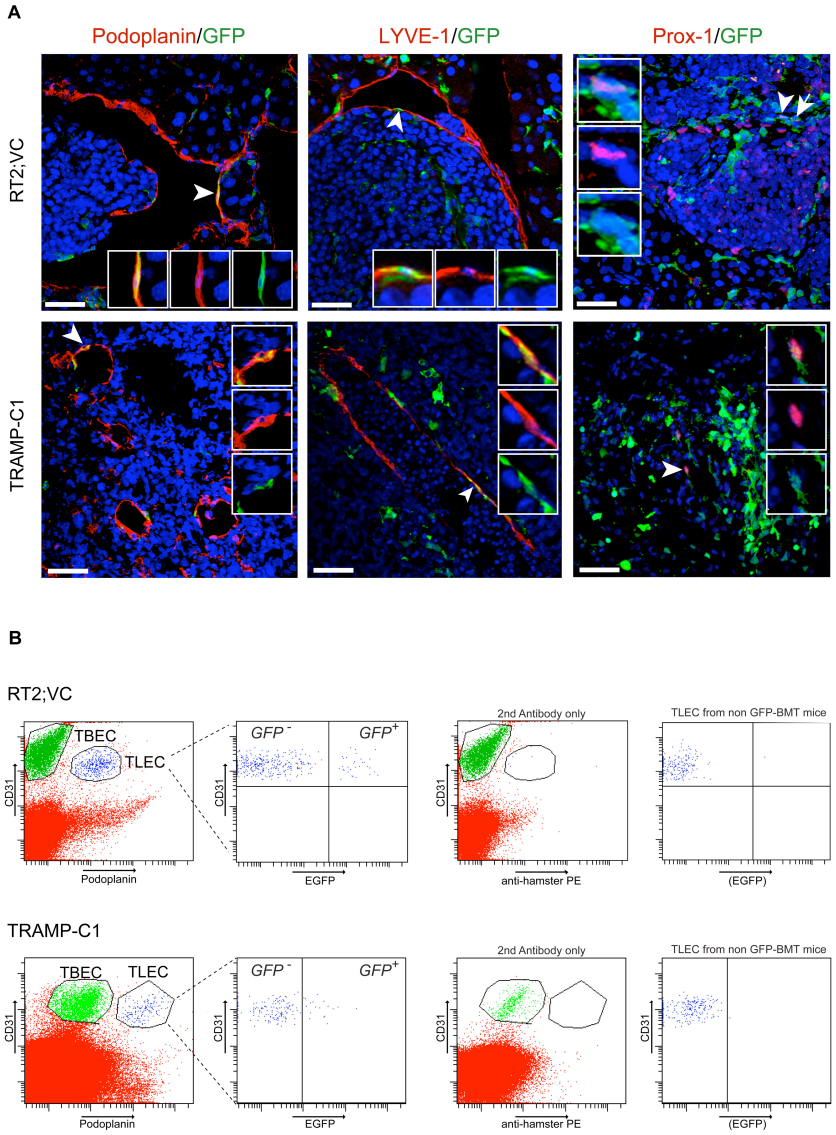

Figure.11: BMDC integrate into tumor-associated lymphatic vessels. (A) Lethally irradiated RT2;VC mice (5 mice) were reconstituted with GFP-labeled bone marrow. 20 μm histological pancreatic sections were stained for the lymphatic markers Podoplanin, Prox-1, LYVE-1 and for GFP as indicated and analyzed by confocal microscopy and subsequent 3D reconstitution. Representative tumor sections per lymphatic marker are shown. 3% of Podoplanin$^+$ TLEC (7 Podoplanin$^+$/GFP$^+$ cells out of 227 Podoplanin$^+$ cells) as well as 3.5% of Prox-1$^+$ or LYVE-1$^+$ TLEC (14 Prox-1$^+$/GFP$^+$ cells out of 400 Prox-1$^+$ cells and 17 LYVE-1$^+$/GFP$^+$ cells out of 485 LYVE-1$^+$ cells) are bone marrow-derived. TRAMP-C1 tumors were subcutaneously implanted in C57BL/6 mice (4 mice) previously reconstituted with GFP-labeled bone marrow. 7 - 20 μm histological tumor sections were stained as described above. 4.1% of Podoplanin$^+$ TLEC (14 Podoplanin$^+$/GFP$^+$ cells out of 334 Podoplanin$^+$ cells) as well as about 2.8% of LYVE-1$^+$ TLEC (11 LYVE-1$^+$/GFP$^+$ cells out of 395 LYVE-1$^+$ cells) are bone marrow-derived. Arrows indicate double-positive cells and arrowheads indicate double-positive cells shown in inset magnifications. Insets show merged and individual channels. DAPI stains nuclei (blue). Scale bars: 40 μm. (B) Tumors of GFP-labeled bone marrow-transplanted RT2;VC mice or TRAMP-C1 tumors grown in GFP-labeled bone marrow-transplanted C57BL/6 mice were enzymatically digested (3 mice each). Single cell suspensions were stained for the pan-endothelial marker CD31 and the lymphatic endothelial marker Podoplanin and analyzed by FACS (left panels). 9.4 +/- 4.1% (RT2;VC) and 10 +/- 4.6% (TRAMP-C1) of CD31$^+$/ Podoplanin$^+$ TLEC were GFP$^+$, indicating their bone marrow origin (middle left panels). As control, the anti-Podoplanin antibody was omitted resulting in no separation between TLEC and TBEC (middle right panels). Furthermore, similar analysis of tumors grown in non-transplanted mice showed no GFP$^+$ cells within TLECs (right panels).

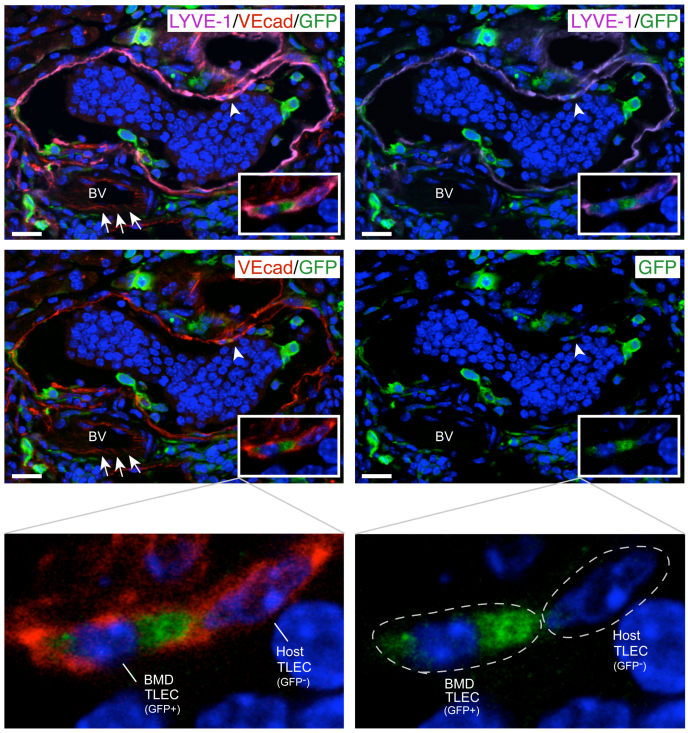

Figure.12: BMDC integrated into tumor lymphatics express vascular endothelial cadherin. A representative tumor section from RT2;VC mice previously reconstituted with GFP-labeled bone marrow was stained for the lymphatic marker LYVE-1 (purple), for the cell junction molecule VE-cadherin (red), and for GFP (green) and analyzed by confocal microscopy and subsequent 3D reconstitution. As indicated by arrowheads and shown magnified in insets, VE-cadherin expression, indicative for homophilic cell-cell contact, is observed between bone marrow-derived (BMDTLEC) and host-derived TLEC (HTLEC). Note the continuous VE-cadherin staining between lymphatic endothelial cells in contrast to the cell-cell contact restricted staining of blood endothelial cells depicted by arrows. DAPI was used for nuclear counterstaining(blue). BV : blood vessel. Scale bars : 20 µm.

To assess the general significance of the findings in the RT2 insulinoma model as well as to test whether the observed integration of BMDC occurred also in the absence of transgenic expression of VEGF-C, we employed the TRAMP-C1 murine prostate adenocarcinoma cell line previously shown to induce robust tumor lymphangiogenesis upon transplantation into syngeneic C57Bl/6 mice [208,209]. TRAMP-C1 cells were injected *s.c.* into one flank of C57Bl/6 mice that had been previously transplanted with GFP-labeled bone marrow (Figure.9B). In the resulting tumors, the number and morphology of BMDC that had integrated into tumor lymphatic vessels were comparable to the results obtained with RT2;VC mice. GFP^+ cells were detected in lymphatic vessels staining for LYVE-1 and Podoplanin (Figure.11A) and constituted 2.8% of LYVE-1^+ and 4.1% of Podoplanin$^+$ cells within lymphatic vessel structures. GFP expression was also detected in Prox-1^+ TLEC (Figure.11A), however to a lower extent as compared to LYVE-1 or Podoplanin. This might be explained by the fact that overall only a subset of LYVE-1^+ TLEC express Prox-1 (data not shown).

To corroborate the simultaneous expression of lymphatic markers and GFP in individual cells, single cell suspensions from tumors of GFP^+ bone marrow-transplanted or control non-transplanted mice were analyzed by FACS (Figure.11B). TLEC were identified by co-expression of CD31 and Podoplanin (Figure.11B, left panels; note that

similar to blood vessel endothelial cells TLEC express CD31, albeit at slightly reduced levels). In tumors derived from RT2;VC and TRAMP-C1 mice, 9.4 +/- 4.1% and 10 +/- 4.6% of TLEC, respectively, were GFP^+, confirming the immunofluorescence data. As expected, GFP^+ TLEC could not be observed in non-transplanted mice (Figure.11B, right panels). In order to avoid detecting false positives by cell duplets containing GFP^+ BMDC and TLEC that would appear as $CD31^+$/$Podoplanin^+$/GFP^+ triple-positive, such events were rigidly excluded by forward scatter pulse width (data not shown).

We next investigated whether BMDC integration into newly formed lymphatic structures occurred only in a tumor microenvironment by transplanting non tumor-bearing, single-transgenic VC mice with GFP-labeled bone marrow. Notably, no GFP^+ cells were found incorporated into the lymphatic vessels surrounding normal islets of Langerhans in these mice [146] (Figure.13). These results demonstrate that BMDC only incorporate into β-cell associated-growing lymphatic vessel in a tumor context.

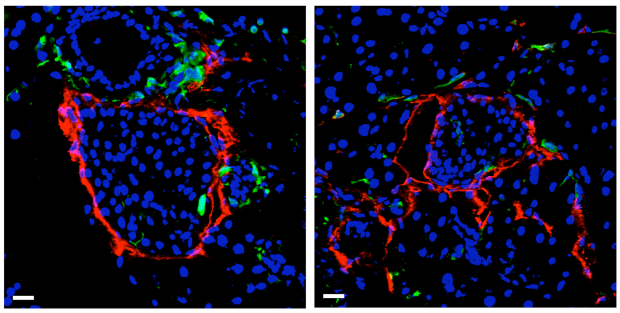

Figure.13: BMDC do not integrate into lymphatic vessels surrounding normal islets. VC mice were transplanted with GFP-labeled bone marrow. Pancreatic sections of transplanted mice were stained for the lymphatic markers LYVE-1 (red) and GFP (green) and analyzed by confocal microscopy. Two representative tumour sections are shown. No GFP+ cells were found integrated into islet surrounding lymphatic structures. DAPI stains nuclei (blue). Scale bar : 20 µm.

Integrated BMDC are of myeloid origin

Myeloid cells have been reported to give rise to blood endothelium and, under inflammatory conditions, to lymphatic endothelium [84,88,89]. To investigate whether BMDC contributing to tumor lymphangiogenesis express macrophage markers, pancreatic sections of transplanted RT2;VC mice were stained by immunofluorescence for the lymphatic marker LYVE-1, the macrophage marker F4/80 and GFP (Figure.14A). Triple-positive GFP^+/$LYVE\text{-}1^+$/$F4/80^+$ cells were readily observed within the lymphatic vessel lining surrounding the tumors. Interestingly, not all BMDC that had integrated into the lymphatic vasculature expressed F4/80, suggesting that macrophages physically

contributed to tumor lymphatics but eventually lost their macrophage features upon integration, as previously reported [89].

Next, we performed various independent lineage-tracing experiments to assess whether cells of the myeloid lineage were indeed able to incorporate into tumor lymphatic vessels. First, lethally irradiated RT2;VC mice were transplanted with bone marrow isolated from either CX3CR1$^{+/GFP}$ mice or CD11b-Cre;Z/EG mice (Figure.9A). In CX3CR1$^{+/GFP}$ mice, the coding region for EGFP had been inserted in the *CX3CR1* gene, a receptor expressed mainly by monocytes and to a minor extent by a subset of lymphocytes, resulting in monocyte-specific GFP expression [219] (Figure.15A). The Z/EG transgene contains, under the control of an ubiquitous promoter, a *lacZ* gene/stop cassette flanked by loxP recombination sites and followed by EGFP [220]. When crossed to CD11b-Cre mice, expressing Cre recombinase under the control of the myeloid specific CD11b promoter, Cre-mediated excision of the *lacZ* gene/stop cassette induced permanent GFP expression exclusively in cells having passed through a CD11b-positive, myeloid stage [221]. Pancreatic sections of transplanted RT2;VC were stained for the lymphatic markers Podoplanin or LYVE-1 and for GFP, and double-positive cells were scored. In both transplantation settings, GFP$^+$ cells were found integrated into the tumor lymphatic vasculature, demonstrating that cells of the myeloid lineage physically contributed to tumor lymphangiogenesis (Figure.14B, upper panels).

We also tested whether CD11b$^+$ cells integrated into tumor lymphatics without prior bone marrow transplantation by transplanting TRAMP-C1 cells into CD11b-Cre;Z/EG mice (Figure.9B). Specific Cre-mediated recombination within the myeloid lineage of these mice was confirmed by FACS analysis of PB cells (Figure.15B). In the resulting tumors, GFP$^+$ cells were found incorporated into LYVE-1$^+$ and Podoplanin$^+$ lymphatic vessel lining (data not shown). Triple-staining for LYVE-1, Prox-1 and GFP further showed that formerly myeloid cells express two lymphatic markers simultaneously (Figure.14C, arrowhead), indicating a significant differentiation towards a lymphatic endothelial phenotype. Thus, integration occurred independently of prior irradiation, which had been previously reported to increase macrophage infiltration in human cancer [222].

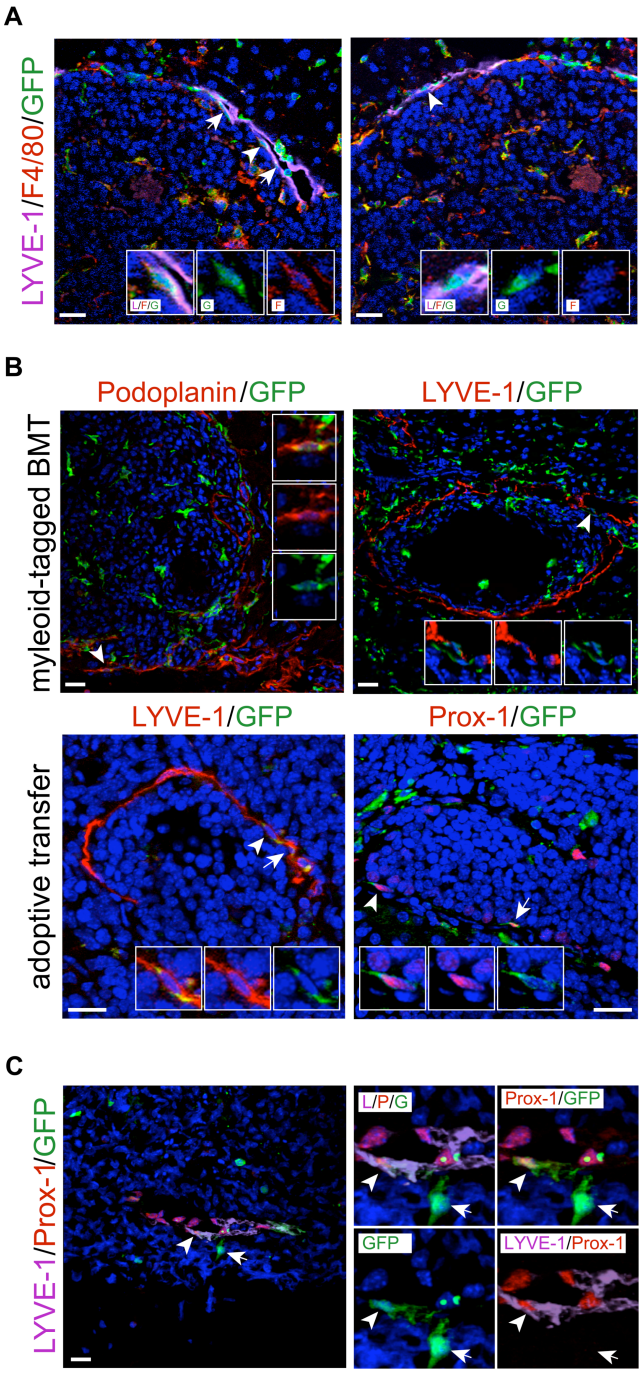

Figure.14: Myeloid origin of bone marrow-derived TLEC. (A) 20 µm histological pancreatic sections of GFP-labeled bone marrow-transplanted RT2;VC mice were stained for the lymphatic marker LYVE-1 (purple), for the macrophage marker F4/80 (red), and for GFP (green) as indicated and analyzed by confocal microscopy and subsequent 3D reconstitution. Two representative tumor sections are shown. Left panel: BMDC that have integrated into tumor lymphatics express the macrophage marker F4/80. Arrows indicate triple positive cells and the arrowhead indicate a triple-positive cell shown in inset magnifications. Right panel: not all integrated cells express F4/80. The arrowhead indicates an integrated cell (LYVE-1+/GFP+) that does not express F4/80. Inset magnifications of this double-positive cell are shown. Insets show merged and individual channels. (B) 20 µm histological pancreatic sections of RT2;VC mice transplanted with bone marrow isolated from either CX3CR1+/GFP (2 mice) or CD11bCre;Z/EG mice (3 mice), or adoptively transferred with FACS-sorted CD11b+ cells were stained for the lymphatic markers LYVE-1, Podoplanin or Prox-1 (red) as well as for GFP (green) and analyzed by confocal microscopy. Representative tumor sections are shown. In both transplantation settings, myeloid cells were found integrated into the lymphatic vasculature surrounding the tumors. Arrowheads indicate double-positive cells shown in inset magnifications (first inset : merged channels, second and third insets: red and green channel, respectively). DAPI stains nuclei (blue). Scale bars: 20 µm. (C) CD11b+ lineage tracing experiments demonstrate the myeloid origin of TLEC in a transplantation-independent setting. TRAMP-C1 tumors were grown subcutaneously in CD11b-Cre;Z/EG mice (3 mice). In these mice, cells that have passed through a CD11b+ myeloid lineage express GFP. Fluorescent triple staining of histological tumor sections reveal co-expression of LYVE-1 and Prox-1 by myeloid-derived GFP+ cells integrated into lymphatic vessels (arrowhead). The right panel represents a magnification of the relevant region with individual channels combined. Note that GFP+ cells not connected to vascular structures do express neither LYVE-1 nor Prox-1 (arrow). DAPI stains nuclei (blue). Scale bars : 20 µm.

In a second series of lineage-tracing experiments, FACS-sorted $CD11b^+/GFP^+$ cells were *i.v.* injected into semi-lethally or non-irradiated RT2;VC mice (Figure.9A and C). 3 weeks after injection, adoptively transferred GFP^+ cells were observed integrated into tumor lymphatics, identified by LYVE-1 or Prox-1 expression (Figure.14B, lower panels). The fact that the adoptive transfer of $CD11b^+/GFP^+$ cells into non-irradiated RT2;VC mice resulted into an integration of the injected cells into tumor lymphatics indicates that a full

reconstitution of the haematopoietic system by stem cells is not a prerequisite for BMDC contribution to tumor lymphangiogenesis.

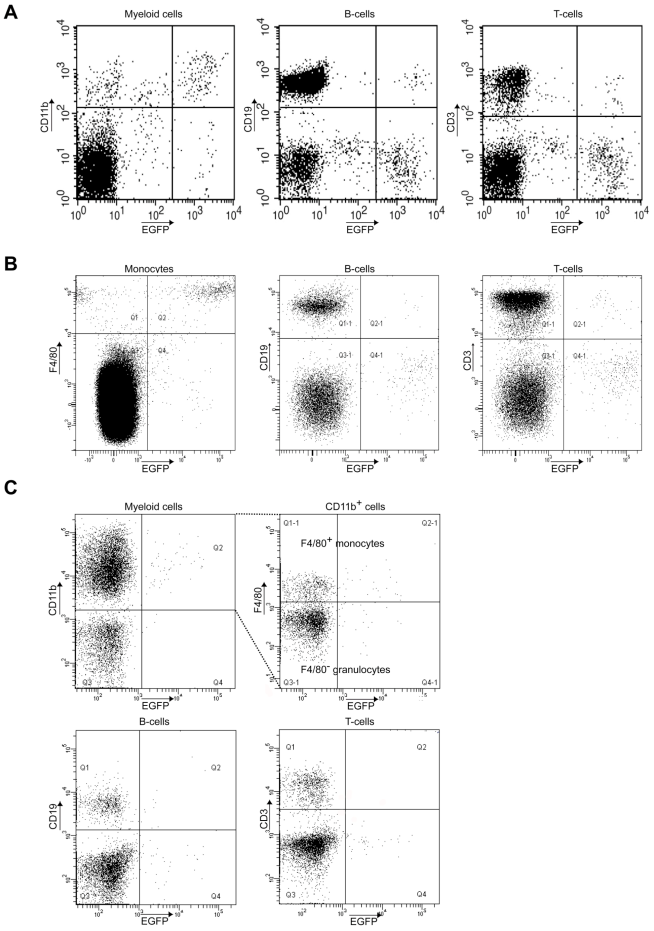

Figure.15: FACS analysis of the lineage tracing experiments. (A) FACS analysis of peripheral bood cells from a representative RT2;VC mouse reconstituted with bone marrow isolated from CX3CR1+/GFP mice indicates GFP expression mainly in CD11b+ cells. A minor fraction of CD19+ B-cells and CD3+ T-cells also expressed GFP. (B) FACS analysis of peripheral blood cells from CD11b-Cre;Z/EG mice transplanted with TRAMP-C1 tumours indicates effective Cre-mediated recombination and subsequent expression of GFP predominantly in F4/80+ monocytes and to lesser extent in B or T lymphocytes. (C) FACS analysis of peripheral blood cells from RT2;VC mice reconstituted with common myeloid progenitor (CMP) cells indicates that GFP+ cells are present within the CD11b+/F4/80+ monocyte fraction and the CD11b+/F4/80- granulocyte fraction but not in B or T lymphocytes.

To assess whether common myeloid progenitor cells (CMP) [223] provide the cells that incorporate into tumor lymphatics, FACS-sorted CMP cells ($lin^-/Sca\text{-}1^-/IL7R\alpha^-/cKit^+/GFP^+$; Figure.9E) were adoptively transferred into semi-lethally irradiated RT2;VC mice. FACS analysis of PB cells 3 weeks post-injection revealed that transplanted CMP contributed to the generation of $CD11b^+/F4/80^+$ monocytes and $CD11b^+/F4/80^-$ granulocytes but not to $CD19^+$ B lymphocytes or $CD3^+$ T lymphocytes (Figure.15C). Also here, GFP^+ cells were found integrated into tumor-associated lymphatic endothelium, detected by LYVE-1 or Podoplanin expression (Figure.16). In contrast, adoptive transfer of FACS-sorted $CD19^+/GFP^+$ B cells (Figure.9A and D) did not result in any incorporation of these cells into tumor lymphatic vessels (Figure.17), underscoring the exclusive ability of myeloid cells to contribute to tumor lymphangiogenesis and excluding the possibility

that minor contaminations of haematopoietic stem cells in the FACS-sorted fractions may have contributed to the GFP^+ cells that incorporated into tumor lymphatics. Finally, FACS analysis of tumors from non-transplanted RT2;VC mice revealed that some *bona fide* $CD31^+/LYVE\text{-}1^+$ TLEC express the myeloid marker CD11b (Figure.18), indicating that the integration of cells of the myeloid lineage into tumor lymphatics and their simultaneous expression of lymphatic endothelial cell markers occurs also in the absence of any bone marrow transplantation.

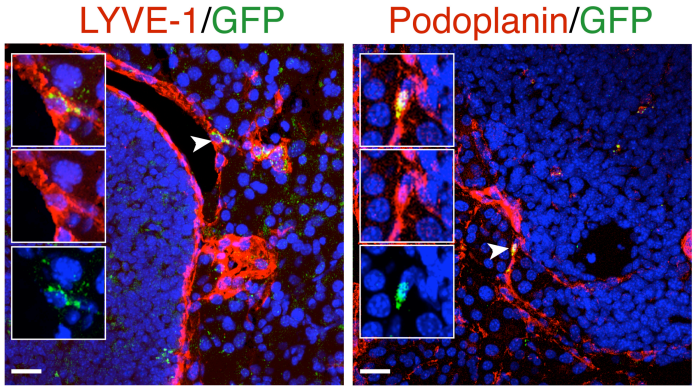

Figure.16: Pancreatic sections of RT2;VC mice adoptively transferred with GFP+ CMP (3 mice) were stained for LYVE-1 or Podoplanin as well as for GFP and analyzed by confocal microscopy. Representative tumor sections are shown. Double positive cells for GFP and LYVE-1 or Podoplanin could be observed, showing that CMP can provide the cells that incorporate into tumor lymphatics. DAPI stains nuclei(blue).Scale bars: 20 µm.

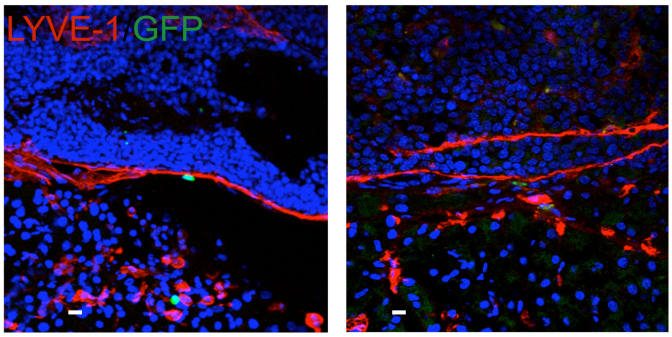

Figure.17: CD19+ B lymphocytes do not integrate into tumour-associated lymphatics. FACS sorted CD19+/GFP+ cells were adoptively transferred into semi-lethally irradiated RT2;VC mice (2 mice). 3 weeks after transfer, mice were sacrificed and tumour sections were stained for the lymphatic marker LYVE-1 (red) and for GFP (green) and analyzed by confocal microscopy. No GFP+ cells co-expressing LYVE-1 could be observed. DAPI was used for nuclear counterstaining (blue). Scale bars: 20 µm.

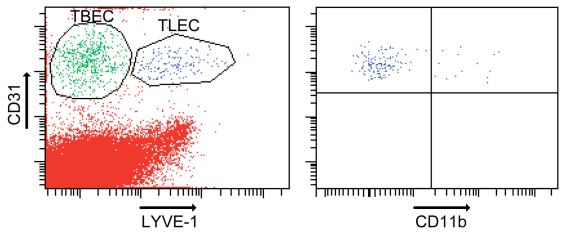

Figure.18: Tumors of non bone marrow-transplanted RT2;VC mice were enzymatically digested. Single cell suspension were stained for the pan-endothelial marker CD31, the lymphatic endothelial marker LYVE-1 and the myeloid marker CD11b and analyzed by FACS. 6.2 +/- 4.5% of CD31+/ LYVE-1+ TLEC co-expressed CD11b.

In order to assess potential fusion events between bone marrow-derived cells and pre-existing lymphatic endothelial cells, lethally irradiated triple-transgenic RT2;VC;Z/EG mice were transplanted with bone marrow isolated from CD11b-Cre mice (Figure.9A). Fusion of $CD11b^+$-BMDC, expressing the Cre recombinase, with host (tumor lymphatic endothelial) cells would result in GFP expression from the recombined Z/EG locus. Seven weeks after transplantation, no GFP^+ cells were detected in or around lymphangiogenic insulinomas, indicating that Cre-expressing, bone marrow-derived myeloid cells had not fused with RT2;VC;Z/EG lymphatic endothelial cells or any other host cell (data not shown).

These results demonstrate that cells found integrated into growing tumor lymphatic vessels can have a myeloid origin and that bone marrow-derived lymphatic progenitor cells are at least in part derived from the already myeloid committed haematopoietic lineage.

Depletion of macrophages

To investigate the functional contribution of macrophages to tumor lymphangiogenesis, RT2;VC mice were treated with liposome-encapsulated Clodronate (ClodroLip) or PBS as vehicle-control for 4 weeks to ablate TAM [224,225]. Successful macrophage depletion was achieved as shown by reduced F4/80 immuno-reactivity in ClodroLip treated mice (Figure.19A). Peri-tumoral lymphatic vessel density (LVD) was significantly decreased in ClodroLip vs. PBS treated mice (Figure.19B; treated: median 70%, mean: 61% vs. control: median 90%, mean 74.9%; $P < 0.01$). Notably, the formation of lymph node metastasis was not affected by the significant but rather moderate reduction of tumor lymphangiogenesis (data not shown). In contrast to a recent study where ClodroLip reduced tumor growth of xenotransplants in immuno-compromised mice [102], average tumor volume, tumor incidence and blood vessel density were not significantly reduced in our experiments (Figure.20). To evaluate the amount of VEGF-C, VEGF-D, FGF-1 and FGF-2 provided by TAM, $CD11b^+$ cells were FACS-isolated from RT2;VC tumors and mRNA levels were assessed by quantitative RT-PCR and compared to levels in total tumors and FACS-isolated tumor cells. The expression of endogenous murine VEGF-C, VEGF-D and FGF-1 in total tumors and tumor cells (not considering the high levels of transgenic human VEGF-C expression in

RT2;VC mice) was higher than in TAM (Figure.19C). FGF-2 was not found expressed at significant levels in any of the samples. From these results we conclude that macrophages contribute to tumor lymphangiogenesis in RT2;VC mice by processes other than the secretion of the main lymphangiogenic factors.

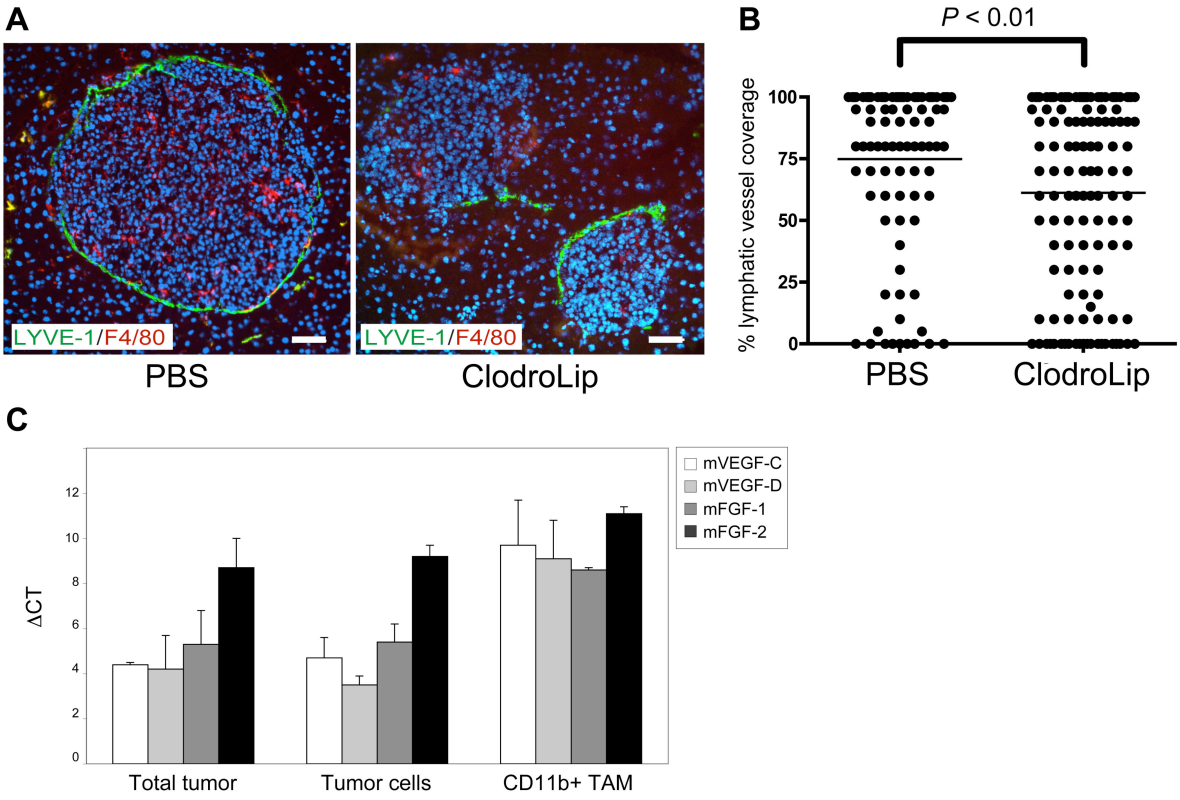

Figure.19: Depletion of macrophages reduces peritumoral lymphatic vessel density. (A) RT2;VC mice were treated with liposome-encapsulated Clodronate (ClodroLip). Pancreatic sections representing a total of 5 PBS vehicle control-treated mice (97 tumors) and 6 ClodroLip-treated (132 tumors) mice were analyzed. Successful depletion of intra- and extra-tumoral macrophages in ClodroLip-treated mice is illustrated by the reduction of F4/80 immunoreactivity (red). Co-staining with the lymphatic endothelial marker LYVE-1 (green) reveals a reduced coverage of tumors by lymphatic vessels in ClodroLip-treated mice vs. in PBS-treated mice. DAPI was used for nuclear counterstaining (blue). T: tumor. Scale bar: 50 μm. (B) Tumors of ClodroLip and control-treated mice were analyzed by immunofluorescence staining with antibodies against LYVE-1 for the extent of lymphatic vasculature surrounding the perimeter of the tumors. Tumors of control-treated mice were surrounded by 90% or more with lymphatic vessels (median 90%, mean 74.9%), whereas tumors of ClodroLip-treated mice had significantly lower coverage (median 70%, mean 61.1%; $P < 0.01$, Mann-Whitney test). (C) Tumor-associated $CD11b^+$ macrophages (TAM) and tumor cells were isolated from tumors of RT2;VC mice by flow cytometry, and mRNA levels for murine VEGF-C, VEGF-D, FGF-1 and FGF-2 were determined using quantitative RT-PCR and compared to levels in total tumors. Shown is the result of three independent cell isolations. ΔCT represents the normalized to internal control (RPL19) CT value. Note that low ΔCT values represent high mRNA levels.

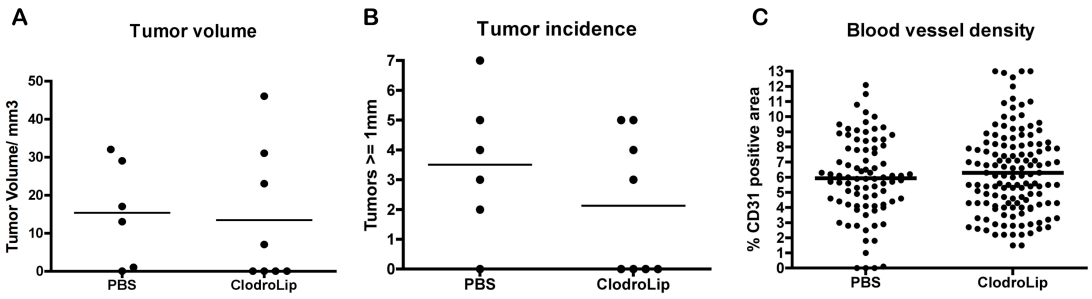

Figure.20: Macrophage depletion does not affect tumor growth. RT2;VC mice were treated for 4 weeks either with PBS (vehicle control) or with ClodroLip as described in Methods in order to deplete intra- and peritumoral macrophages. Tumor volume has been determined as the total volume of tumors per mouse (A), tumor incidence is the number of tumors larger than 1mm per mouse (B), and blood vessel density is the % area fraction of CD31 staining (C), as determined using ImageJ image analysis Software. None of these parameters was significantly altered between ClodroLip and control-treated mice.

Macrophages form and contribute to lymphatic-like structures in vitro

We next investigated whether bone marrow-derived-macrophages had an intrinsic capability to form lymphatic vessel-like structures. Bone marrow cells were cultured for 7 days in 30% M-CSF containing-medium to induce the specific differentiation of progenitor cells into non-activated macrophages [226]. Flow cytometric analysis confirmed the macrophage identity ($CD11b^+$/ $F4/80^+$) of these cells (Figure.21A). The bone marrow-derived-macrophages were then activated with LPS and seeded on Matrigel to monitor differentiation and tube formation. After two days in endothelium-specific medium supplemented with defined growth factors, macrophages associated in clumps, before forming tube-like structures with increasing connections between days 3 and 15 (Figure.21A). Confocal immunofluorescence microscopy analysis at day 12 revealed that only macrophages that had formed tube-like structures and not single isolated cells expressed the lymphatic marker Podoplanin (Figure.21B). Furthermore, quantitative RT-PCR analysis of mRNA from macrophages isolated either before or after the tube formation process revealed a marked up-regulation of the lymphatic markers LYVE-1, Prox-1, VEGFR-3, FoxC2 and FoxC1 as well as a down-regulation of the haematopoietic/monocytic markers CD45 and CX3CR1 during tube formation (Figure.21B). Exclusion of individual growth factors revealed the requirement of FGF-2 for tube formation (Figure.21C), whereas the other supplemental growth factors (VEGF-A, IGF-1, EGF, hydrocortisone) were dispensable. Accordingly, mRNA levels of FGF receptor-1 and 2 were up-regulated during tube formation, as revealed by quantitative RT-PCR analysis (Figure.21C).

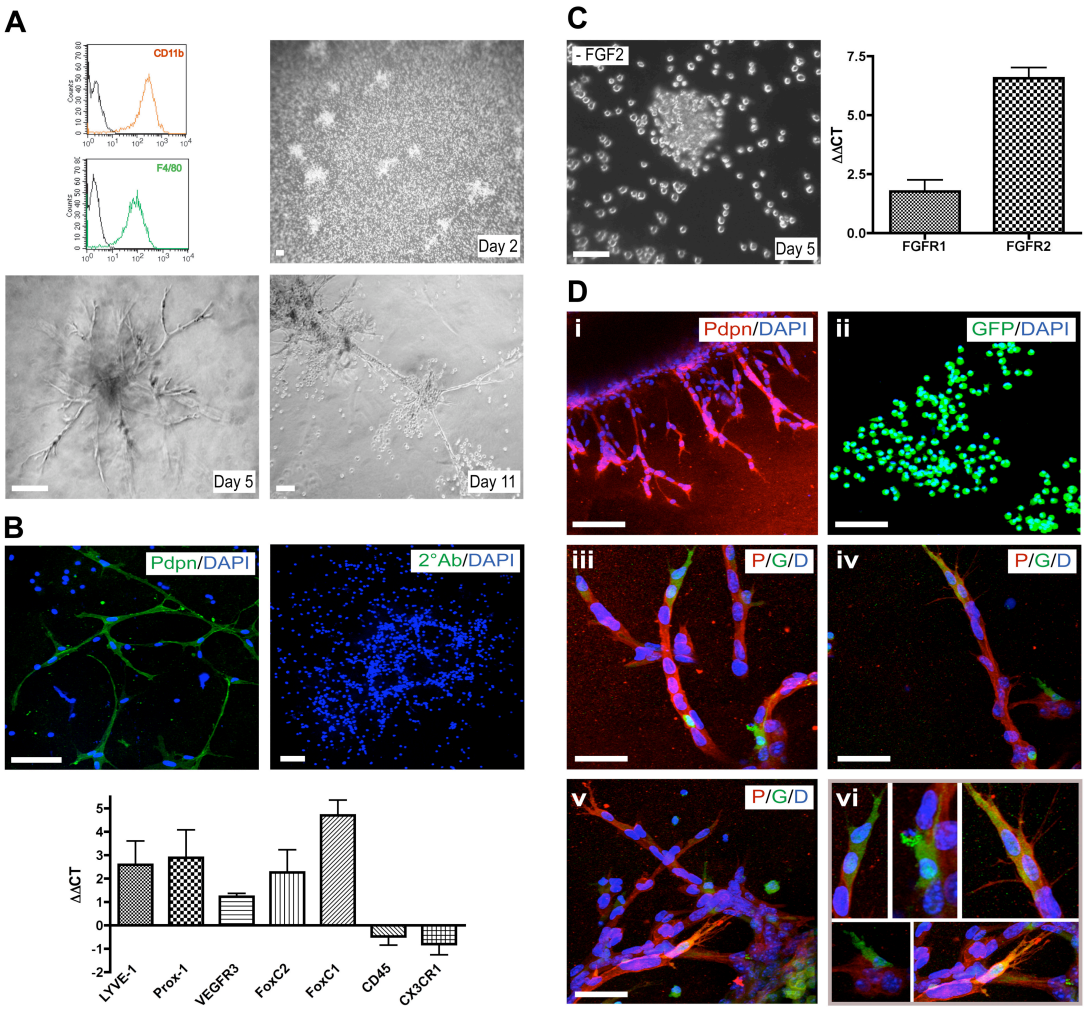

Figure.21: Bone marrow-derived-macrophages form and contribute to lymphatic-like structures *in vitro.* (A) *In vitro* generated macrophages showed a specific marker expression profile ($CD11b^+$/ $F4/80^+$) (upper left panel). Tube formation on Matrigel was monitored by phase-contrast microscopy. At day 2, macrophages formed clusters. Between days 3 and 15, they developed into tube-like structures with numbers of branches increasing over time. Scale bar: 100 μm. (B) Immunofluorescence staining against Podoplanin (Pdpn) revealed that macrophages having formed tube like structures express Podoplanin whereas single cells do not. Staining of the tubular structures in the absence of any primary antibody was used as a control (2ºAb). DAPI stains nuclei (blue). Scale bar: 100 μm. Quantitative RT-PCR analysis revealed that upon tube formation, macrophages up-regulate lymphatic markers (LYVE-1, Prox-1, VEGFR-3, Foxc2, Foxc1) and down-regulate haematopoietic/myeloid marker (CD45, CX3CR1). ΔΔCT corresponds to the difference between the normalized CT values of macrophages forming tubes (day 8) and macrophages not having yet formed tubes (day 1). (C) FGF-2 is required for the formation of cord-like structures by macrophage, as its specific exclusion from culture medium abrogated this process (left panel). Furthermore, analysis of mRNA levels revealed up-regulation of FGF receptors -1 and 2 during tube formation. (D) Immortalized $Podoplanin^+$ murine lymphatic endothelial cells (SV-LEC) (i), GFP-labeled bone marrow-derived-macrophages (ii), and mixed cultures of macrophages and SV-LEC (iii-vi) were seeded in Matrigel. At day 5, cells were stained for Podoplanin (red) and analyzed by confocal microscopy. Mixed cultures demonstrate that bone marrow-derived macrophages contribute to SV-LEC-mediated tube formation: GFP^+ cells (green) are found integrated into $Podoplanin^+$ tube-like structures (iii-vi). Note the preferential integration of bone marrow-derived macrophages at the tips and branch points of sprouting tube-like structures formed by SV-LEC (magnified in panel vi). DAPI stains nuclei (blue). Scale bars: 100 mm (i-ii) and 50 mm (iii-vi).

In order to explore the capacity of myeloid cells to integrate into lymphatic structures *in vitro*, GFP-labeled macrophages were generated as described above from bone marrow of actin-GFP transgenic mice and subsequently cultured on Matrigel alone or in combination with SV40 T antigen-immortalized murine lymphatic endothelial cells (SV-LEC) [227]. Five days later, the cultures were stained for Podoplanin. Cultured SV-LEC formed tube-like structures positive for Podoplanin expression (Figure.21D/i), and cultured *in vitro* activated macrophages were positive for GFP (Figure.21D/ii). In mixed cultures, bone marrow-derived macrophages lined up with SV-LEC, incorporating into tube-like structures and expressed Podoplanin (Figure.21D/iii-vi). Interestingly, GFP^+ macrophages were predominantly located at the tips and at branch points of growing tube-like structures (Figure.21D/iii-vi) and seemed to guide SV-LEC to form a new sprout as observed by time-lapse video microscopy (Figure.22, Supplemental Video 3). The live visualization of GFP+ macrophages guiding LEC together with the observation that macrophages located at the tip of the lymphatic sprout exhibit filopodia-like structures (Figure Figure.21D/iii-vi) strongly suggest that instead of capping the exposed ends, they actively instigate the new sprout.

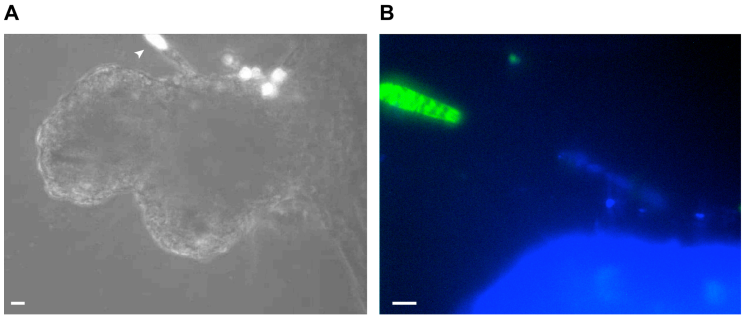

Figure.22: Macrophages initiate lymphatic endothelial cell tube formation in an in vitro co-culture system. (A) Phase contrast microscopy of SV40 large T antigen-immortalized lymphatic endothelial cells (SV-LEC) co-cultured with GFP+ macrophages in Matrigel. GFP+ macrophages are detected by their bright signal in phase-contrast microscopy. As an initial step, SV-LEC associate into compact cell aggregates with concomitant recruitment of macrophages. As indicated by an arrowhead, after 1 day, GFP+ macrophages could be observed leading outgrowing SV-LEC tubes. (B) Immunofluorescence microscopy of a magnification of the same field depicted in (A) showing the tube-leading GFP+ macrophage followed by four Hoechst-stained SV-LEC. Scale bars: 10 µm.

These results demonstrate that bone marrow-derived macrophages have the ability to form lymphatic-like structures *in vitro*, a process requiring FGF signaling. Their preferred incorporation at tips and branchpoints of pre-existing lymphatic cord-like structures suggests a role of macrophages in lymphatic endothelial cell sprouting.

4.1.4. Discussion

Research on BMDC in patho-physiological processes, such as atherosclerosis, limb/heart ischemia and cancer, has in the past mainly focused on the importance of haematopoietic cells in promoting or attenuating inflammation, in clearing cancer cells, or in inducing immunological tolerance to neoplastic lesions. However, recent findings indicate that the bone marrow is also a rich source of progenitor cells with mesenchymal and endothelial potential [83,228]. In the case of endothelial progenitor cells, the lineage relationship to the haematopoietic system is not clear. While some experiments have recently revealed that during development haematopoietic cells arise from a specialized endothelium named the haemogenic endothelium [198-200], other reports provide evidence that the reverse direction of cellular conversion is also possible, *i.e.* that myeloid cells can contribute to the formation of blood endothelial cells [84,229].

Here, we have used bone marrow transplantation experiments in two different mouse models of carcinogenesis to demonstrate that BMDC significantly contribute to tumor lymphangiogenesis, but rarely integrate into tumor blood vessels. About 3% of lymphatic endothelial cells in lymphangiogenic tumors are of bone marrow origin, a contribution comparable to findings with lymphatics in rejected human kidney transplants [87] and in normal liver, stomach and intestine of HSC-transplanted mice [205]. We have performed lineage-tracing experiments to obtain insights into the ontogeny of bone marrow-derived TLEC. First, transplantations of FACS-sorted bone marrow fractions representing different haematopoietic lineages or of total bone marrow expressing GFP under a myeloid specific promoter indicate that integrated BMDC are derived from the myeloid lineage. Second, genetic tagging of myeloid cells with GFP confirms this notion; cells that have passed through the myeloid lineage are found integrated into the lymphatic vasculature surrounding tumors. Third, depletion of tissue macrophages using ClodroLip significantly reduces peritumoral lymphatic vessel density, demonstrating a functional role of macrophages in tumor lymphangiogenesis. Fourth, the intrinsic ability of myeloid cells to give rise and incorporate into lymphatic-like structures is recapitulated in two *in vitro* assays. Taken together, *in vitro* and *in vivo* experimentation strongly suggest that cells of the myeloid lineage physically contribute to tumor lymphangiogenesis.

The statement that BMDC can also contribute to lymphangiogenesis in a paracrine-independent manner is highly debated. As with any controversial scientific discussion,

well-controlled studies conducted in different laboratories and leading to similar conclusions constitute the basis to overcome skepticism. Along these lines, the present study is consistent with previously described observations that haematopoietic cells can contribute to lymphatic endothelium, in normal organs, during embryonic development, in inflammatory conditions, and in a tumor microenvironment [87-89,123,205,207,214,215]. The experimental results presented here extend these findings by identifying that cells of the myeloid lineage can contribute to lymphatic endothelial cells in a tumorigenic context.

The existence of specific lymphatic progenitor cells (LPC), distinct from haematopoietic as well as blood endothelial progenitor cells, has not been established. Based on a number of control experiments, such as the transplantation of FACS-sorted $CD19^+$ B-cells or the adoptive transfer of $CD11b^+$ myeloid cells into non-irradiated recipients, we exclude the possibility that FACS-sorted cell fractions may have contained haematopoietic stem cells that also reconstitute potential LPC. Rather, our data indicate a myeloid origin of cells that integrate into tumor-associated lymphatic endothelial cells, thus supporting the notion that LPC reside at least partially within an already committed haematopoietic lineage. It is interesting to note that the myeloid contribution to lymphatic vessels has thus far only been described to occur under inflammatory conditions, such as corneal transplantation and wound healing [88,89]. In contrast, the existence of LPC within the haematopoietic stem cell population, but distinct from the myeloid lineage, has been reported to play a role in steady state lymphangiogenesis [205]. The contribution of haematopoietic cells to lymphangiogenesis has been also shown during embryonic development. Mice lacking the haematopoietic signaling molecules SLP-76, Syk and PLCγ2 fail to separate emerging lymphatic vessels from blood vessels [122,123]. Notably, this phenotype depends on the expression of these signaling molecules in haematopoietic progenitor cells that give rise to circulating endothelial progenitor cells, thus demonstrating a cell-autonomous contribution of haematopoietic cells to vascular development [123,214].

The low frequency of bone marrow-derived lymphatic endothelial cells is a recurrent observation among the different studies, raising questions towards the functional contribution of these cells to lymphangiogenesis. However, the pharmacological depletion of tumor-associated macrophages results in a decrease in *de novo* lymphangiogenesis [88] (Figure.19). Moreover, our results indicate that macrophages are not the main source of lymphangiogenic factors in the RT2 tumor model, leading us to

conclude that macrophages contribute to tumor lymphangiogenesis, at least in this model, by processes other than the paracrine secretion of lymphangiogenic factors. Rather, when co-cultured *in vitro* with lymphatic endothelial cells, bone marrow-derived macrophages incorporate predominantly at the tips and branch points of growing tube-like structures. *In vitro* time-lapse video microscopy confirms this notion and shows that macrophages, after being recruited to lymphatic endothelial cells, are able to instigate lymphatic sprouts. These observations suggest that myeloid-derived lymphatic endothelial cells may exert a specific functional role, which may explain the need of only a low number of these cells for the complete process of lymphangiogenesis.

In summary, we demonstrate here that in the context of tumor growth, cells of the myeloid lineage contribute to the formation of tumor-associated lymphatic endothelium. Since tumor lymphatic vessels provide a route for metastatic dissemination, understanding the functional role of bone marrow-derived tumor lymphatic endothelial cells seems warranted.

4.2. Molecular players of the trans-differentiation process

4.2.1. Transcriptional changes upon *in vitro* trans-differentiation

In order to get insights into the mechanism by which myeloid cells can give rise to lymphatic endothelium, the transcriptome of macrophages (Mϕ) was determined at different stages of the *in vitro* trans-differentiation process.

For this purpose, LPS-activated bone marrow-derived macrophages were cultured on Matrigel in complete endothelium-specific medium (EGM2-MV) for 1 (E1), 3 (E3), 6 (E6) and 8 (E8) days and for 1 (D1) day in complete DMEM medium. After 1 day in endothelium-specific medium, macrophages associate in clusters, but do not yet form tube-like structures. After 3 days tube-like structure formation is initiated and after 6-8 days, most of the macrophages give rise to tubular structures. In contrast, when macrophages are cultured in complete DMEM medium, no tube formation is observed. Therefore macrophages cultured for 1 day in complete DMEM medium were used as reference. Total RNA was extracted at each time point and mRNA was amplified and hybridized as cDNA to its specific DNA oligonucleotides present on the microarray (Figure.23). The experiment was performed in biological duplicates, *i.e.* two independent experiments of *in vitro* macrophage differentiation from haematopoietic progenitor cells followed by two independent time-course cultures.

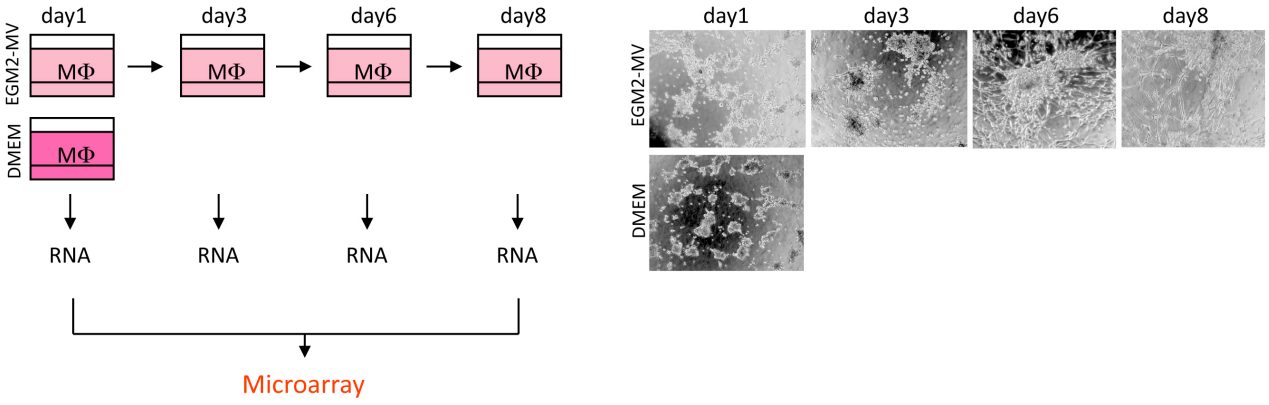

Figure.23: Transcriptome comparison of macrophages at different stages of the *in vitro* trans-differentiation process. The experiment setting is summarized on the left panel and on the right panel light microscopy pictures of the time-course cultures are shown.

Pearson's correlation signals indicate how similar the data between samples are. Squares depicted in red represent high correlation between two samples, while squares depicted in blue represent low correlation between samples. As shown on Figure.24, Pearson's correlation signals revealed a satisfactory similarity between duplicates as well as important changes in gene expression upon tube-like structure formation. Only few changes between the last time points, E6 and E8, could be observed. Similarities/disparities observed here corresponded to similarities/disparities observable in cell morphology.

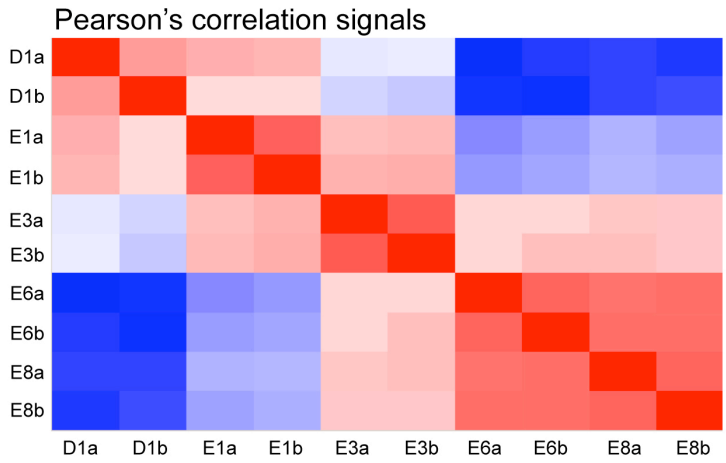

Figure.24: Pearson's correlation signals. a and b represent duplicates. Red corresponds to high similarity, blue corresponds to poor similarity between samples.

The data were then analyzed for statistical significance using GeneSpring GX 9.0. One-way analysis of variance (ANOVA) revealed that more than 2'600 genes were significantly differently expressed (cut-off: 1.5X) upon tube-like structure formation when the p value was set at $p<0.01$. At least 2X and/or 4X and 10X differently expressed genes between two specific time points were listed (all lists of upregulated genes are available on the attached CD):

	Differentially expressed genes	Upregulated genes
2X between D1 and E1	403 genes	171 genes
2X between E6 and E8	23 genes	10 genes
2X between E1 and E6	1439 genes	916 genes
4X between E1 and E6	512 genes	416 genes
10X between E1 and E6	139 genes	117 genes

In concordance with the Pearson's correlation only few genes were differently expressed between E6 and E8. This result was expected as no significant change in macrophage morphology could be observed between these two time points. Potential candidate genes implicated in the *in vitro* trans-differentiation process were selected among the genes significantly upregulated between E1 and E6, the time window between the initiation and the end of the process. Genes differently expressed between D1 and E1 were taken into consideration during the selection of candidate genes in order to subtract the effect of the culture medium on gene expression.

The phenotypical conversion of myeloid cells toward lymphatic endothelial cells was confirmed by the upregulation of genes implicated in lymphatic vessel formation and the concomitant downregulation of genes implicated in typical macrophage functions. More precisely, between E1 and E6, genes described to be expressed or required for lymphatic vessel development [129,141-144,230], genes recently identified in our lab to be lymphatic-specific markers (Paralemmin-1 and insulin growth factor binding protein-5, unpublished observation from Imke Albrecht), as well as guidance molecules described to be implicated in vascular remodeling and vessel navigation [231], and proteases needed for tube formation were upregulated (listed below). In contrast, haematopoietic/macrophage markers as well as genes implicated in fundamental macrophages functions like antigen presentation, pattern recognition, phagocytosis and chemokine/cytokine response and expression [232] were downregulated upon tube-like structure formation (listed below).

Gene expressed or required during LV formation

Lymphatic vessel endothelial hyaluronan receptor1 (Lyve1): 3.5X up

Desmoplakin (Dsp): 2X up

Forkhead box C1 (FoxC1): 15X up

Forkhead box C2 (FoxC2): 4X up

Neuropilin-1 (Nrp1): 3X up

Neuropilin-2 (Nrp2): 1.5X up

Vascular endothelial growth factor-C (Vegfc): 2.6X up

Vascular endothelial growth factor-D (Figf): 23X up

Adrenomedullin (Adm): 21X up

Emilin-1 (emilin1): 1.7X up

Ephrin B2 (efnb2): 5X up

Angiopoietin-1 (Angpt1): 5.7X up

Angiopoietin-2 (Angpt2): 8X up

Paralemmin-1 (Palm): 2.5X up

Insulin-like growth factor-2 (Igf2): 3X up

Insulin-like growth factor binding protein-5 (igfbp5): 3.3X up

Insulin-like growth factor-1 receptor (Igf1r): 3.2X up

Growth hormone receptor (Ghr): 18X up

Platelet-derived receptor alpha (Pdgfra): 30X up

Platelet-derived receptor beta (Pdgfrb): 10.5X up

Fibroblast growth factor receptor-1 (Fgfr1): 4.6X up

Fibroblast growth factor receptor-2 (Fgfr2): 3.7X up

Vascular guidance molecules and proteases

Ephrin B1 (efnb1): 2.6X up

Ephrin receptor B4 (EphB4): 4X up

Unc-5 homolog B (Unc5b): 2.6X up

Neogenin (Neo1): 4X up

Round about axone guidance rec-1 (Robo1): 2.8X up

Slit homolog-2 (Slit2): 8X up

Semaphorin-3A (Sema3A): 18X up

Semaphorin-5A (Sema5A): 11.3X up

Semaphorin-6D (Sema6D): 2.8X up

Semaphorin-7A (Sema7A): 4.3X up

Matrix metalloproteinase-11 (Mmp11): 4X up

Endopeptidase (Mme): 56X up

Matrix metalloproteinase-2 (Mmp2): 32X up

ADAM metallproteinase 1 (Adamts1): 24X up

ADAM metallproteinase 5 (Adamts5): 24X up

Macrophage markers

CD45 (Ptprc): 1.7X down

F4/80 (Emr1): 1.5X down

CD11b (Itgam): 1.5X down

CD18 (Itgb2): 2X down

PU.1 (Sfpi1): 2X down

CD86 (Cd86): 4.6X down

CD80 (Cd80): 3X down

CD14 (Cd14): 4X down

Gr-1 (Ly6g): 1.5X down

Chemo/cytokine signaling

Chemokine C-C rec-5 (Ccr5): 1.6X down

GM-CSF rec-α (Csf2ra): 2.6X down

GM-CSF rec-β (Csf2rb): 3.7X down

GM-CSF rec-β2 (Csf2rb2): 4.3X down

Tumor necrosis factor-α (Tnfa): 1.5X down

Interleukin-1β (Il1b): 2.6X down

Interleukin-6 (Il6): 1.6X down

Pattern recognition molecules

Toll-like receptor-2 (Tlr2): 1.5X down

Toll-like receptor-6 (Tlr6): 2X down

Toll-like receptor-7 (Tlr7): 1.5X down

Toll-like receptor-8 (Tlr8): 1.9X down

Toll-like receptor-9 (Tlr9): 2.6X down

Toll-like receptor-13 (Tlr13): 1.7X down

MyD 88 (Myd88): 2X down

Fcγ receptor-1 (fcgr1): 2.6X down

Fcγ receptor-2 (fcgr2): 1.7X down

Phagosome formation and maturation

Rab-4b (Rab4b): 1.6X down

Rab-8a (Rab8a): 1.5X down

Rab-20 (Rab20): 2.3X down

Rab-27a (Rab27a): 5X down

Rab-32 (Rab32): 1.7X down

Syntaxin-11 (Stx11): 2.8X down

Dynein heavy chain-12 (Dynhc12): 11X down

Ag presenting molecules

H2-Aa (H2-aa): 24X down

H2-Ab1 (H2-ab1): 21X down

H2-D1 (H2-d1): 1.6X down

H2-Eb1 (H2-eb1): 7.5X down

H2-M2 (H2-m2): 2.5X down

H2-M3 (H2-m3): 2.5X down

H2-Q1 (H2-q1): 1.6X down

H2-Q6 (H2-q6): 4.5X down

H2-Q8 (H2-q8): 4.5X down

H2-T9 (H2-t9): 3X down

H2-T10 (H2-t10): 3.7X down

H2-T23 (H2-t23): 2X down

H2-T24 (H2-t24): 3.7X down

As initial analysis, the top 50 upregulated genes, corresponding to at least 15X upregulation between E1 and E6 were listed (see below). An enrichment for extracellular matrix (ECM) proteins as well as proteases degrading the ECM could be observed. Some genes implicated in lymphatic vessel formation and mentioned above were also part of this highly upregulated gene list. Three soluble modulators of the Wnt signaling, secreted frizzled-related protein (SFRP)-1, 2 and 4 were as well present, among which SFRP-1 and 4 have been recently reported to play a role in neovessel formation by inducing endothelial cell spreading through actin cytoskeleton reorganization [233]. Finally, leukocyte chemoattracting cytokines appeared as interesting candidates, as they may potentially regulate the coordinate movement of cells necessary for tube-like structure formation.

TOP 50 upregulated genes

Proteases

Endopeptidase (Mme): 56X up
Matrix metalloproteinase-2 (Mmp2): 32X up
ADAM metallproteinase 1 (Adamts1): 24X up
ADAM metallproteinase 5 (Adamts5): 24X up
Carboxypeptidase E (Cpe): 19X up
Serin peptidase inhibitor-f1 (Serpinf1): 18X up
Serin peptidase inhibitor-a3n (Serpina3n): 17X up

Growth factors/Soluble modulators

Osteoglycin (Ogn): 45X up
Secreted frizzled related protein-2 (Sfrp2): 30X up
Secreted frizzled related protein-1 (Sfrp1): 21X up
VEGF-D (Figf): 23X up
Adrenomedullin (Adm): 21X up
Semaphorin-3A (Sema3A): 18X up
Secreted frizzled related protein-4 (Sfrp4): 15X up

Chemokines

Stromal cell-derived factor-1 (Sdf1 or Cxcl12): 24X up
Chemokine ligand-5 (Cxcl5): 21X up
Chemokine ligand-13 (Cxcl13): 18X up

Adhesion molecules

Gap junction protein alpha-1(Gja1): 21X up
Caherin-11 (Cdh11): 19X up
Procadherin-9 (Pcdh9): 18X up

Extracellular matrix proteins

Fibulin-3 (Efemp1): 111X up
Thrombospondin-2 (Thbs2): 49X up
Procoll C-endopeptidase enh (Pcolce): 26X up
Nidogen-1(Nid1): 24X up
Collagen type-1 alpha-2 (Col1a2): 21X up
Matrix Gla protein (Mgp): 19X up
Lumican (Lum): 17X up
Matrilin-2 (Matn2): 15X up
Fibrillin-1 (Fbn1): 15X up

Receptors

Platelet-derived receptor alpha (Pdgfra): 30X up
Growth hormone receptor (Ghr): 18X up
Oncostatin M receptor (Osmr): 18X up
Leptin receptor (Lepr): 16X up
TGF-β receptor-3 (Tgfbr3): 15X up

Metabolism

Epoxide hydrolase-1 (Ephx1): 21X up
Nedd-4 (Nedd4): 17X up
Pyruvate kinase dehydrogenase-4 (Pdk4): 17X up
Cyclin-dependant kinase inhibitor-2B (Cdkn2b): 17X up
Ectonucleotide pyrophosphatase/phosophdiesterase-2 (Enpp2): 17X up
Lysyle oxidase-like-1(Loxl1): 16X up
Adenylase kinase-1 (Ak1): 15X up

Others

Sned-1 (Sned1): 37X up
Internal membrane prot-2A (Itm2a): 32X up
Matrix remodeling-a8 (Mxra8): 19X up
Cellular retinoic acid BP-1 (Crabp1): 18X up
Insulin-like GF BP-4 (igfbp4): 17X up
Cysteine rich protein-2 (Crip2): 15X up
AE binding protein-1 (Aebp1): 16X up
GFR bound protein-10 (Grb10): 16X up

More detailed analysis performed on the microarray data using the gene ontology tool of GeneSpring GX 9.0 and Ingenuity Pathway Analysis Software revealed that an important number of molecules being part of different signaling pathways are implicated in *in vitro* trans-differentiation. However this analysis did not indicate the specific implication of any particular pathway. Thus, to better sort out the important quantity of data obtained, differentially expressed genes were classified by their specific upregulation at different stages in the tube-like structure formation process. Two lists were then generated: one regrouping the genes specifically upregulated during the initiation phase and the other one regrouping the genes specifically upregulated during the elongation phase. The initiation phase corresponds to the time window between E1 and E3, when clustered macrophages initiate tube-like structure formation. The elongation phase corresponds to the time window between E3 and E6, when initial sprouts elongate to give rise to “mature” tube-like structures. Genes classified in the initiation process list were at least upregulated 2X between E1 and E3, were not changed more than 2X between D1 and E1 and were not upregulated more than 2X between E3 and E6 and between E6 and E8. Genes classified in the elongation process list were at least upregulated 2X between E3 and E6, were not changed more than 2X between D1 and E1 and between D1 and E3 and were not upregulated more than 2X between E6 and E8. Thus, 144 genes demonstrated a specific role in the beginning of the *in vitro* trans-differentiation process as 394 genes showed specific implication in the second part of the process. The typical gene expression profiles presented by genes classified in both lists (available on attached CD) as well as the criteria to fulfill to enter in one of the two lists are depicted on Figure.25.

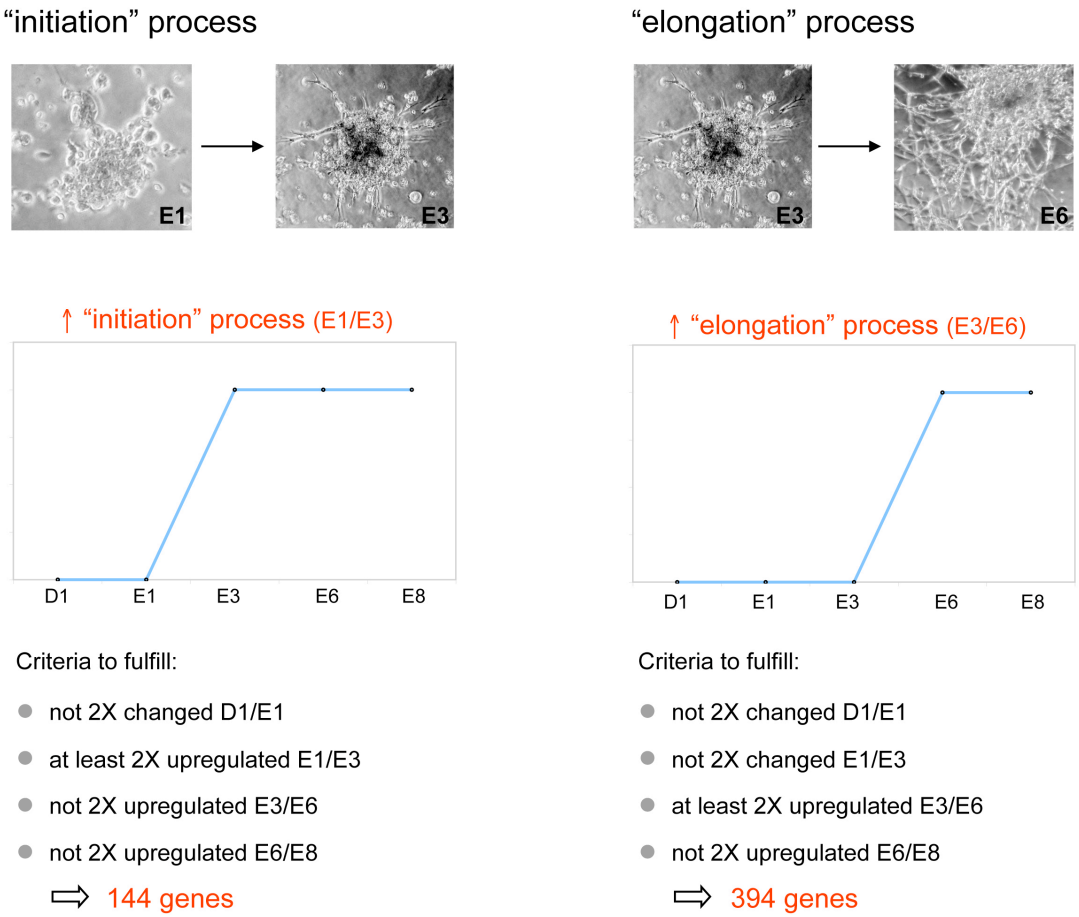

Figure.25: Typical gene expression profile of genes specifically implicated either in the initiation or in the elongation phase of the *in vitro* trans-differentiation process. 144 genes and 394 genes presented a typical "initiation" and "elongation" profile, respectively.

Step-specific upregulated genes were classified in these two lists with the idea to identify among the initiation process list, transcription factors which would represent potential master switches of *in vitro* trans-differentiation. The six transcription factors listed below were specifically upregulated at the initiation of the process and thus represent potential master switches:

- Forkhead box C2 (**Foxc2**): 4X up
- Twist homolog-2 (**Twist2**): 3.5X up
- Sex determining region Y-box 9 (**Sox9**): 3.5X up
- High mobility group AT-hook2 (**Hmga2**): 3.3X up
- Myc (**Myc**): 2.3X up
- GLI-Kruppel family member-3 (**Gli3**): 2.1X up

Foxc2 is highly expressed and required during lymphatic vessel development [125,234]. It has been described to cooperate with its closely related family member Foxc1 in the regulation of early lymphatic vascular development [127]. Indeed, Foxc1 and Foxc2 are required for lymphatic sprouting as compound Foxc1$^{+/-}$; Foxc2$^{-/-}$ embryos show reduced lymphatic vascular density in the areas of initial spouting of LEC from the primary lymph sacs. Interestingly, while Foxc2 is specifically upregulated during the initiation step of the trans-differentiation process, Foxc1 is highly upregulated (15X) during the second part of the process (Figure.26).

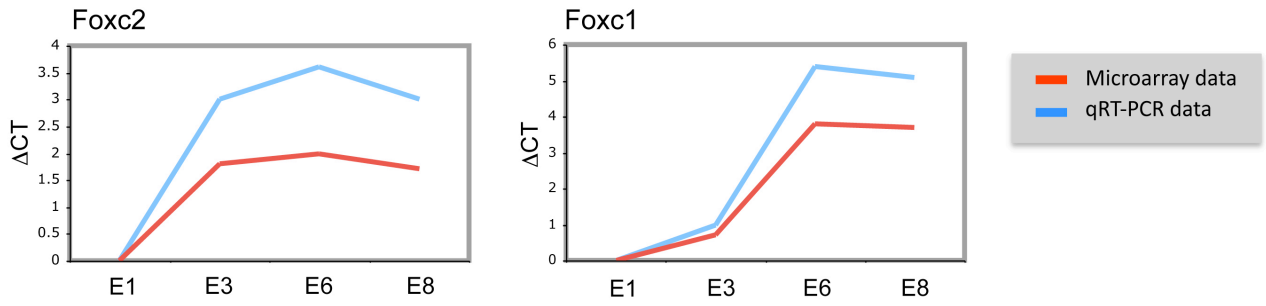

Figure.26: Foxc2 and Foxc1 upregulation upon *in vitro* trans-differentiation. As revealed by microarray analysis and confirmed by qRT-PCR, Foxc2 was specifically upregulated at the initiation of the process as Foxc1 was highly upregulated in the second part of the process. ΔΔCT represents the ΔCT value (CT value normalized against RPL19) of each time point subtracted from the ΔCT value of E1.

For all these reasons Foxc2 is of particular interest as a potential master switch of the trans-differentiation process.

4.2.2. Candidate gene selection and microarray data validation

Two candidate genes upregulated upon *in vitro* trans-differentiation were selected for further analysis:

- Fibroblast growth factor receptor (FGFR)-1 and 2 (4.6 and 3.7X up respectively)
- Stromal cell-derived factor-1 (SDF-1) (24X up).

In addition to their marked upregulation upon tube-like structure formation, FGFR-1 and 2 were selected as potential candidate genes as when FGF-2, which is a ligand for these receptors, was depleted from the complete endothelium-specific culture medium, *in*

vitro trans-differentiation was impaired (Figure.21C); thus strongly suggesting the implication of FGF signaling in this process. SDF-1 was chosen as second to be tested for its potential role in *in vitro* trans-differentiation. First, because SDF-1 was among the top 50 upregulated gene list, presenting an upregulation of 24X between E1 and E6. And second, because it has been reported that SDF-1 promotes endothelial cell clustering and movements of these clustered cells in order to form a network [235]. The microarray data were first validated by qRT-PCR (Figure.27). Then the requirement of FGF and SDF-1 signaling for the trans-differentiation process was assess, first *in vitro* and then *in vivo* (for FGF signaling only).

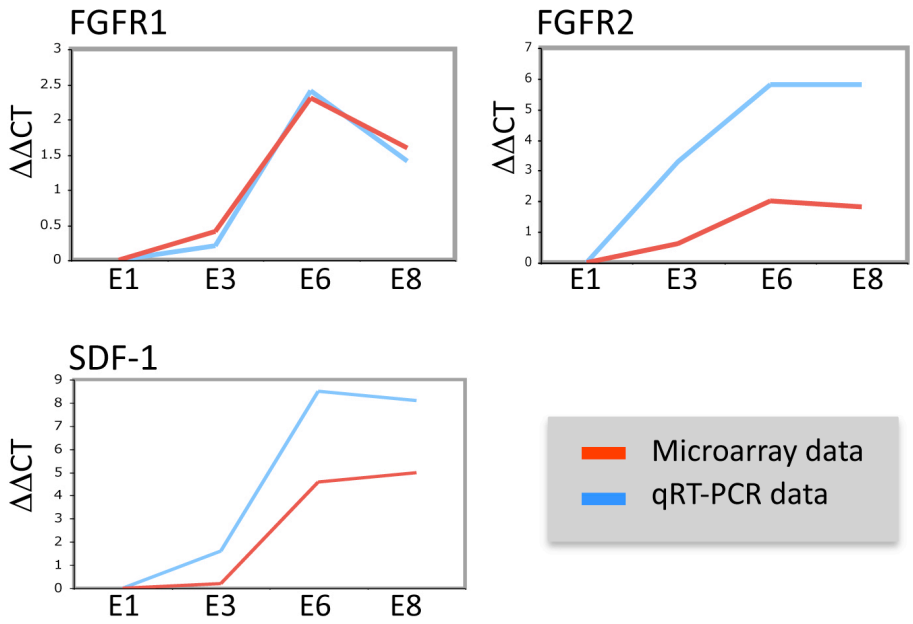

Figure.27: qRT-PCR validation of the microarray data. ΔΔCT represents the ΔCT value (CT value normalized against RPL19) of each time point subtracted from the ΔCT value of E1.

4.2.3. FGF signaling requirement

In vitro trans-differentiation

To assess whether FGF signaling is implicated in the *in vitro* phenotypical transition of myeloid cells toward lymphatic endothelial cells, macrophages were knocked-down for FGFR-1 and/or 2 using shRNAs and tube-like structure formation was monitored. Macrophages are particularly refractory to transfection/transduction. To overcome this obstacle, bone marrow progenitor cells were transduced with lentiviral vectors encoding shRNAs against FGFR-1 and/or 2 and then *in vitro* differentiated into macrophages. The experimental setting used is summarized in Figure.28 and briefly described here. C57BL/6 mice were treated with 5-Fluorouracil (5-FU) in order to increase their bone marrow in progenitor cells. Six days post 5-FU injection, progenitor-enriched bone marrow cells were collected and cultured over night in transplant medium (TM). On two consecutive days the cells were infected with lentiviral vectors encoding either an shRNA control (LVshctr) or shRNAs against FGFR-1 (LVshFGFR1) and/or 2 (LVshFGFR2). All lentiviral vectors used encoded a puromycine selection cassette. One day post the last infection, transduced bone marrow cells were selected with puromycine for two days prior to nine day culture in 30% M-CSF containing-medium to induce the specific differentiation of progenitor cells into macrophages. Knocked-down or control macrophages were then seeded on Matrigel in complete endothelium-specific medium and tube-like structures formation was monitored for six days.

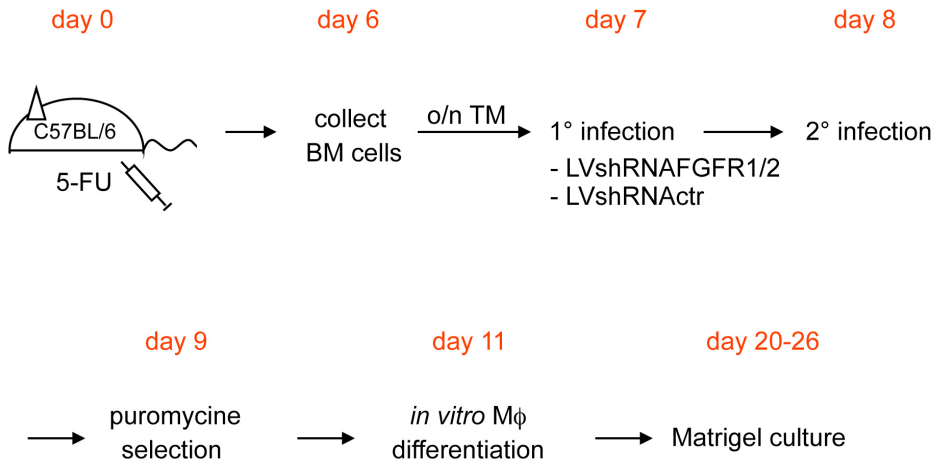

Figure.28: Experimental setting used to assess the effect of downregulating FGFR-1 and/or 2 on the *in vitro* trans-differentiation process.

The knockdown efficiency was tested by qRT-PCR pre- and post-Matrigel culture. No difference could be observed between the two different time points. In the case of an infection with a single lentiviral vector (LVshFGFR1 or LVshFGFR2), the knockdown efficiency for FGFR-1 was 75% on average (ranging from 60-90% between 3 independent experiments), and for FGFR-2 it was 88% on average (ranging from 80-95% between 2 independent experiments). In the case of an infection with two lentiviral vectors (LVshFGFR1 and LVshFGFR2), the knockdown efficiency for FGFR-1 was 60% on average (ranging from 40-80% between 2 independent experiments) and for FGFR-2 77% on average (ranging from 75-80% between 2 independent experiments). The average knockdown efficiency was lower in the case of an infection with two lentiviral vectors. This can be explained by the fact that, as both lentiviral vectors encode the same selection cassette, a double infection does not automatically result in a double transduction.

When cultured for six days on Matrigel and complete endothelium-specific medium, knocked-down macrophages presented a significant impairment in tube-like structure formation in comparison to control macrophages (Figure.29).

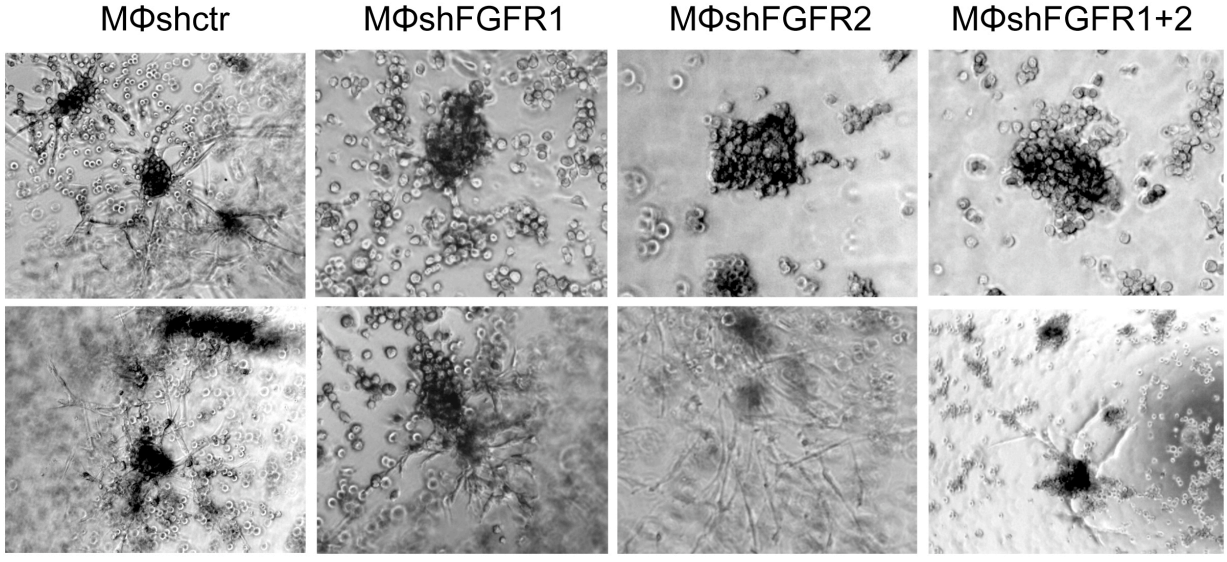

Figure.29: FGF signaling is required for an efficient *in vitro* trans-differentiation. Macrophages knocked-down for either FGFR-1 or FGFR-2 or for both receptors were only rarely able to give rise to tube-like structures.

Out of three independent experiments, 80-100% of the clusters formed by macrophages transduced with the lentiviral vector encoding the shRNA control (Mϕshctr) gave rise to tube-like structures. In contrast, for macrophages knocked-down for FGFR-1 (MϕshFGFR1), out of three independent experiments, two did not give rise to any tube-like structure and in one only 10% of the clusters presented such structures. Similarly, for macrophages knocked-down for FGFR-2 (MϕshFGFR2), out of two independent experiments, one did not give rise to any tube-like structure as in the other only 10% of the clusters presented such structures. And finally, for double-knocked-down macrophages (MϕshFGFR1+2), out of two independent experiments, one did not give rise to any tube-like structure as in the other only 15% of the clusters presented such structures. Taken together these results demonstrate that FGF signaling is required for an efficient *in vitro* trans-differentiation process.

In vivo trans-differentiation

FGF signaling is clearly implicated in *in vitro* trans-differentiation but does this signaling pathway also play a role in *in vivo* trans-differentiation?

To assess this question, RT2;VC mice were transplanted with GFP-labeled bone marrow knocked-down for FGFR-1 and 2 (to avoid a potential redundancy of the receptors) and BMDC contribution to tumor lymphatic vessels was assessed by FACS on the reconstituted mice. The experimental setting used is summarized in Figure.30 and briefly described here. 5-FU treated bone marrow cells collected from β-actin-GFP mice were incubated overnight in TM and infected on two consecutive days with either LVshctr or with both LVshFGFR1 and LVshFGFR2. One day post the last infection, transduced bone marrow cells were selected with puromycine for two days and dead cells were removed using a Ficoll gradient prior to bone marrow transplantation.

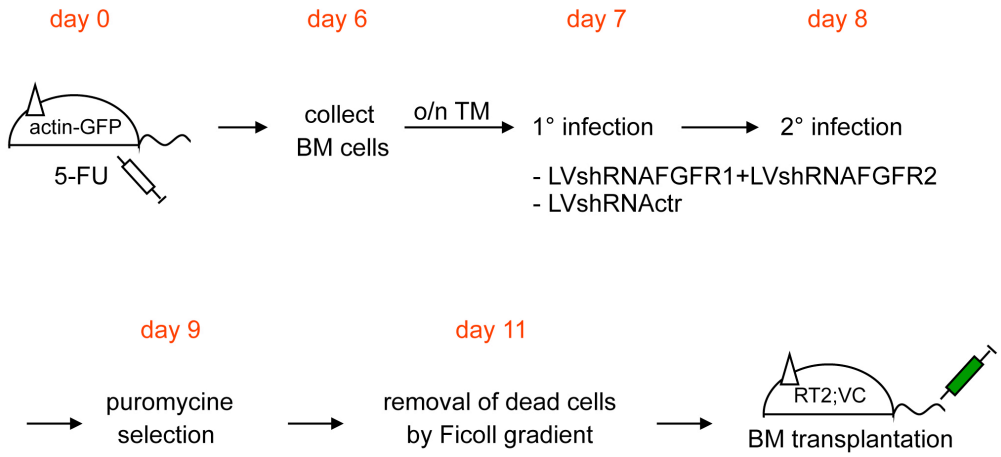

Figure.30: Experimental setting used to assess the effect of knocking-down FGFR-1 and 2 on the *in vivo* trans-differentiation process.

Two mice were transplanted with bone marrow cells infected with LVshctr (shctr mice) and three mice with double knocked-down bone marrow cells (shFGFR1+2 mice). The knockdown efficiency prior bone marrow transplantation was assessed by qRT-PCR. It reached 70% for FGFR-1 but could not be determined for FGFR-2 as this receptor is expressed at too low levels in bone marrow progenitor cells. Mice were sacrificed 6-7 weeks post bone marrow transplantation. To assess if knocking-down FGFR-1 and 2 did not affect haematopoietic reconstitution post bone marrow transplantation, the proportion of myeloid, T and B cells in bone marrow and peripheral blood of shFGFR1+2 mice was compared to that of control mice. Control mice include shctr mice as well as mice transplanted with non-infected cells and non-transplanted mice. As depicted in Figure.31, shFGFR1+2 mice presented a normal haematopoietic composition both in the bone marrow and in the periphery. Moreover, efficient bone marrow reconstitution by the transplanted GFP-labeled cells was confirmed by a chimerism of more than 80%.

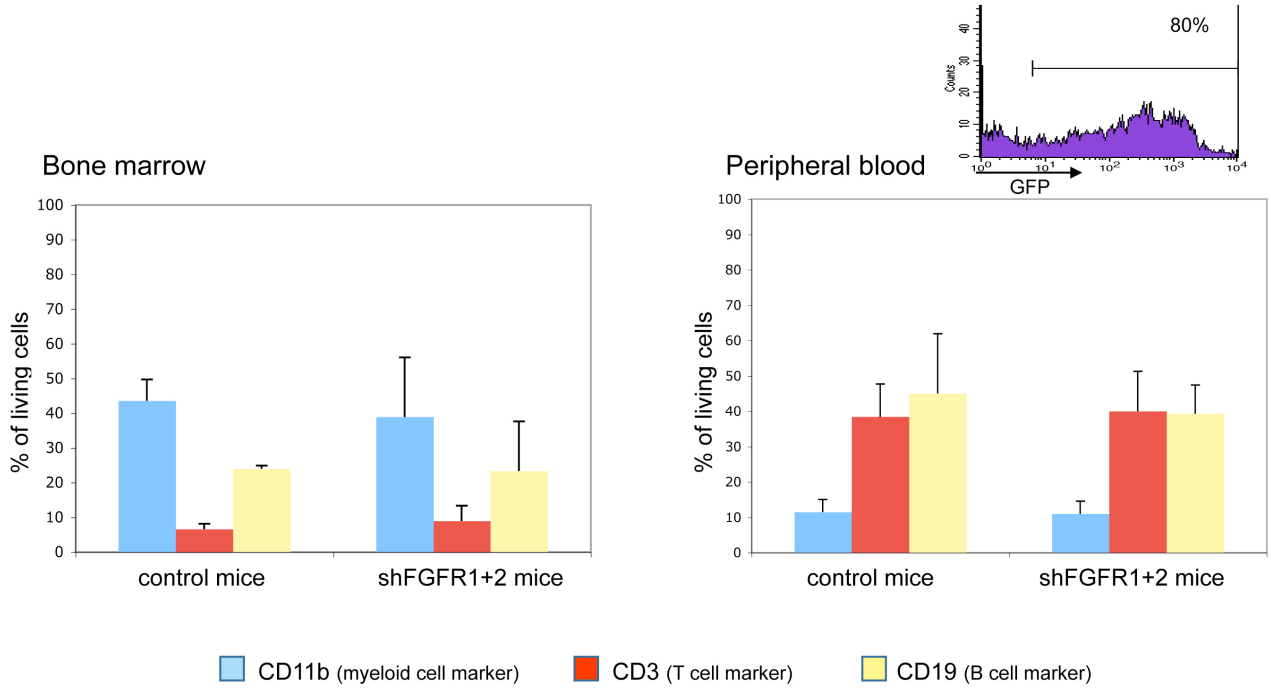

Figure.31: FACS analysis of bone marrow and peripheral blood mononuclear cells revealed efficient haematopoietic reconstitution in shFGFR1+2 mice with more than 80% chimerism and normal haematopoiesis in both haematopoietic comportments in comparison to control mice.

After having checked that the mice were correctly reconstituted, BMDC contribution to tumor lymphatic vessels was assessed by FACS. Tumor single cell suspensions were stained for CD31 and Podoplanin and the percentage of GFP positive TLEC ($CD31^+Podoplanin^+$ cells) was compared between shFGFR1+2 mice and shctr mice. No significant change in the bone marrow-derived TLEC (BMDTLEC) population could be observed (Figure.32A). Consistently with these results, tumor lymphatic vessel coverage (TLVC) was neither affected upon FGFR-1 and 2 knockdown (Figure.32B).

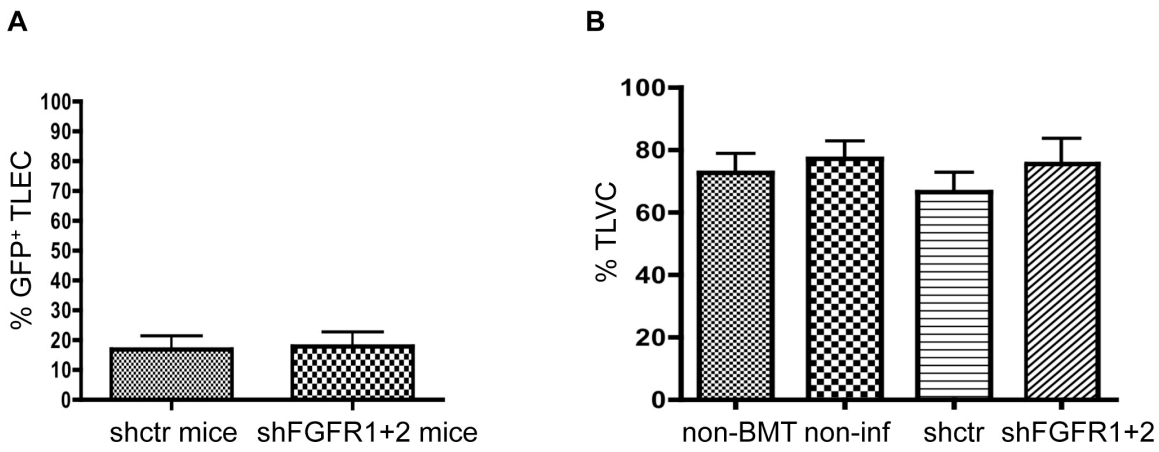

Figure.32: Knocking-down FGFR-1 and 2 does neither affect BMDC contribution to tumor lymphatic vessels nor tumor lymphatic vessel coverage. (A) Single-cell suspensions of tumors isolated from either shctr mice (N=2) or shFGFR1+2 mice (N=3) were stained for CD31 and Podoplanin and the proportion of BMDTLEC was assessed by FACS. (B) Tumors of non-transplanted mice (non-BMT, N=2), of mice transplanted with non-infected bone marrow cells (non-inf, N=3), of shctr mice (N=2) and of shFGFR1+2 mice (N=3) were analyzed by immunofluorescence staining with antibodies against LYVE-1 for the extent of lymphatic vasculature surrounding the perimeter of the tumors (TLVC).

As the shRNAs against FGFR-1 and 2 were delivered by lentiviral vectors, thus insuring a stable integration in the host genome, the knockdowns observed pre-bone marrow transplantation should remain stable. However, to confirm that the lack of visible effect on the proportion of BMDTLEC upon FGFR-1 and 2 knockdown was not due to a rather unlikely silencing of the shRNAs, knockdown efficiency was re-assessed by qRT-PCR post-bone marrow transplantation in macrophages *in vitro* differentiated from isolated bone marrow cells at the day of sacrifice. The knockdown remained stable upon bone marrow transplantation/reconstitution as the knockdown efficiency for FGFR-1 and 2 was on average 60% and 70%, respectively.

Taken together these data demonstrate that, even if FGF signaling is clearly implicated in the *in vitro* trans-differentiation process, it does not seem to play a critical role in the *in vivo* trans-differentiation process.

4.2.4. SDF-1 signaling requirement

A potential SDF-1 signaling requirement for trans-differentiation was assessed *in vitro* by first chemically inhibiting its receptor, CXCR4, on macrophages and monitoring tube-like structure formation. Macrophages were pre-incubated with a chemical inhibitor against CXCR4 (ICXCR4) at concentrations ranging from 0.3 to 5μM and then seeded on Matrigel in complete endothelium-specific medium in the presence or in the absence of the inhibitor at the same concentration used for the pre-incubation. Blocking SDF-1/CXCR4 signaling resulted in a dose-dependent decrease in tube-like structure formation (Figure.33).

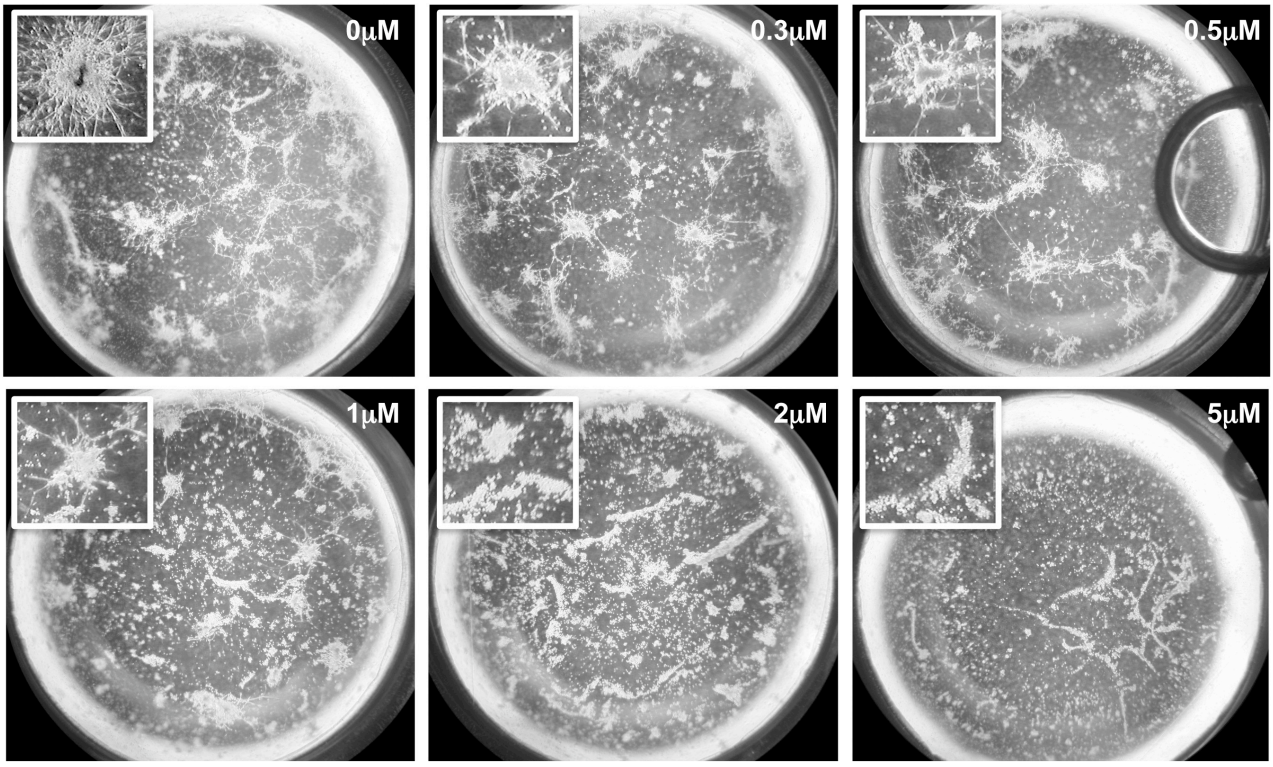

Figure.33: Blocking SDF-1/CXCR4 impairs tube-like structure formation in a dose-dependent manner. Macrophages were pre-incubated and cultured in the presence or absence of CXCR4 inhibitor, at concentrations varying from 0.3 to 5μM.

More precisely, macrophage clustering as well as the percentage of clusters giving rise to tube-like structures were reduced upon inhibitory conditions, in a dose-dependent manner (Figure.34 and Figure.35). A cluster is defined as a tight circular agglomerate of cells.

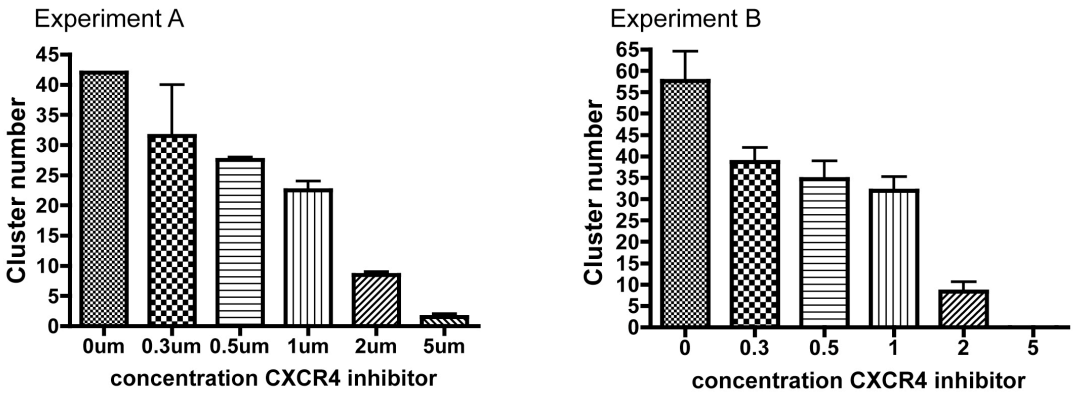

Figure.34: Inhibiting CXCR4 resulted in reduced macrophage clustering, in a dose-dependent manner. Here are shown the results of two independent experiments done in duplicates.

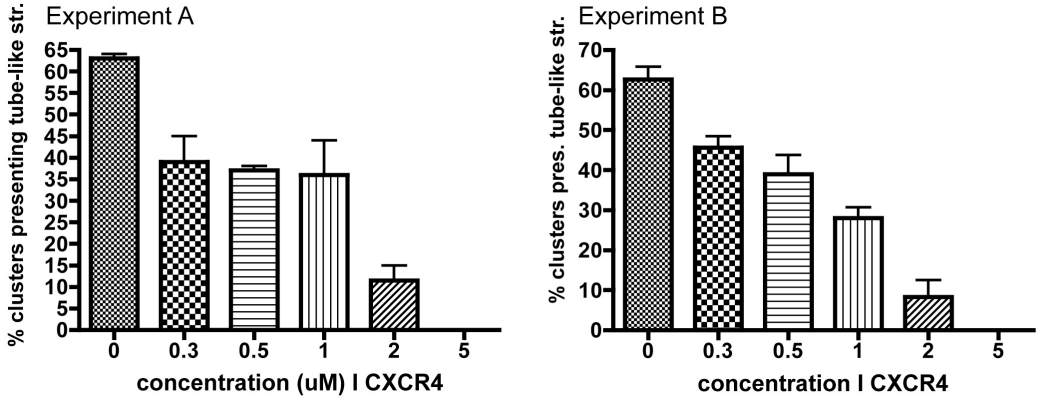

Figure.35: The percentage of clusters giving rise to tube-like structures was reduced in the presence of the inhibitor. Here are shown the results of two independent experiments done in duplicates.

To exclude the eventuality that impaired tube-like structure formation upon SDF-1/CXCR4 signaling inhibition would be due to cell death induced by a potential toxic effect of the inhibitor, macrophages were cultured for eight days in the presence or in the absence of the inhibitor and cell viability was assessed by FACS using propidium iodide (PI staining). Independently of the different culture conditions, 80% of the cells were PI negative, demonstrating no cytotoxic effect of the inhibitor.

SDF-1 has been reported to be implicated in the recruitment of endothelial progenitor cells to site of neovascularization [236,237]. Considering that myeloid cells can be context-dependent lymphatic endothelial progenitor cells, the role of SDF-1/CXCR4 signaling in the recruitment of macrophages to lymphatic endothelial cells was assessed *in vitro*. SV40 T antigen-immortalized murine lymphatic endothelial cells (SV-

LEC) were co-cultured with macrophages, pre-incubated or not with ICXCR4 (0, 5 or 10μM), on Matrigel in complete endothelium-specific medium in the presence or in the absence of the inhibitor at the same concentration used for the pre-incubation. Eight hours post seeding, in the absence of the inhibitor, SV-LEC clustered together and attracted macrophages to them (Figure.36, left panel). In contrast, when SDF-1/CXCR4 signaling was blocked using an inhibitor concentration of at least 5μM, macrophages recruitment to LEC was impaired (Figure.36, middle and right panels).

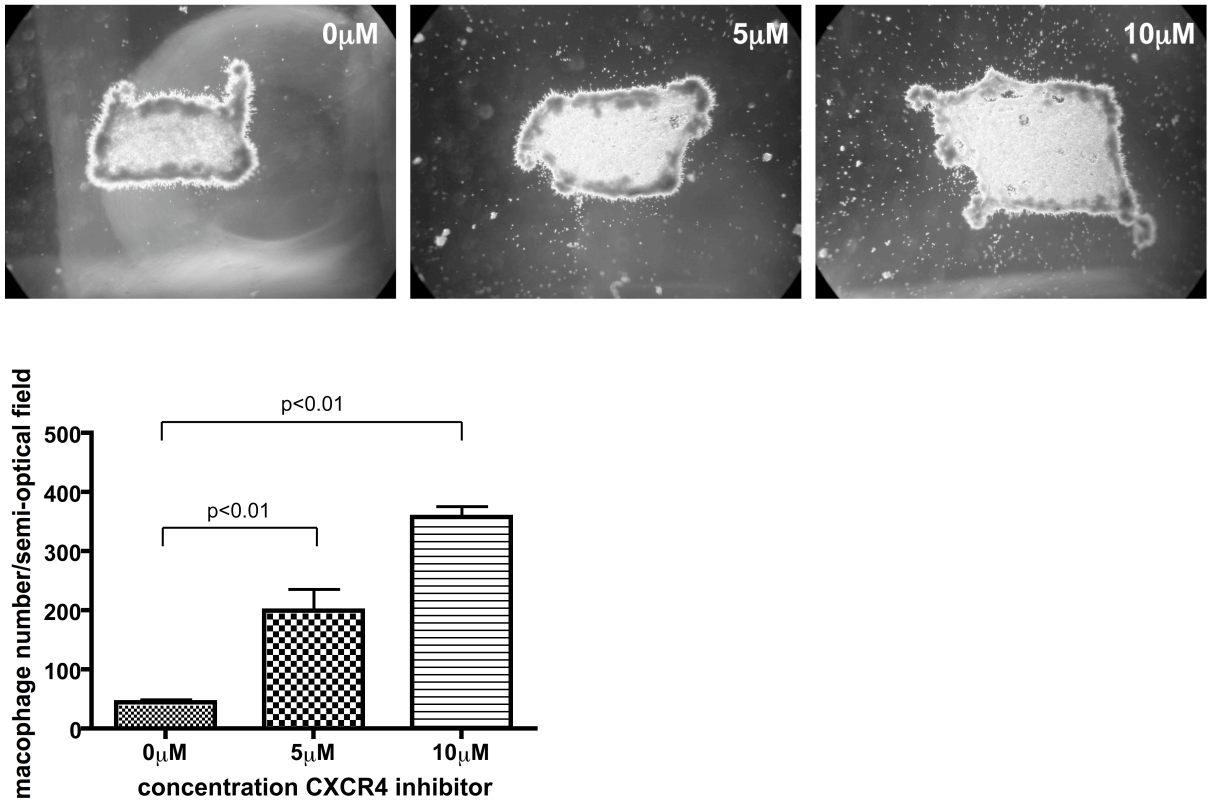

Figure.36: Blocking SDF-1/CXCR4 impairs macrophages recruitment to lymphatic endothelial cells in a dose-dependent manner. Macrophages were pre-incubated and co-cultured with SV-LEC in the presence or absence of CXCR4 inhibitor (5 or 10μM). The number of macrophages, which were not recruited to SV-LEC, was quantified (by semi-optical field, N= 4-8).

Taken together, these results show that SDF-1 signaling is implicated in the *in vitro* trans-differentiation process and in the recruitment of macrophages to lymphatic endothelial cells. Therefore, interfering with SDF-1 signaling in myeloid cells may negatively affect the *in vivo* trans-differentiation process by directly acting on the capacity of the cells to

undergo trans-differentiation but also by preventing their recruitment to actively growing tumor lymphatic vessels. This hypothesis would need to be assessed in reconstituted GFP-labeled bone marrow-transplanted RT2;VC treated with ICXCR4.

5. Material and methods

Mouse strains

Generation and phenotypic characterization of Rip1Tag2, Rip1VEGF-A and Rip1VEGF-C mice have been described previously [28,29,146]. C57BL/6-Tg(ACTB-EGFP)mice [238] and Z/EG mice [220] were provided by K. Hafen (University of Basel). CD11b-Cre mice [221] and CX3CR1$^{+/GFP}$ mice [219] were obtained from J. Vacher (University of Montreal) and C. Ruegg (CePO Lausanne), respectively. All experiments involving mice were performed in accordance with the guidelines of the Swiss Federal Veterinary Office (SFVO) and the regulations of the Cantonal Veterinary Office of Basel-Stadt.

Total bone marrow transplantations

Bone marrow cells were extracted under sterile conditions from femurs and tibiae from donor mice indicated in Figure 1. After T cell depletion [239], 5 x 10^6 cells were injected in the tail vein of lethally irradiated (2 x 550 cGy) 6 week old mice which were sacrificed for further analysis 5 to 7 weeks after transplantation.

TRAMP-C1 subcutaneous tumor model

5x10^5 TRAMP-C1 cells [240] (provided by N. Greenberg, FHCRC, Seattle) were injected into the flank of either GFP-labeled bone marrow transplanted C57BL/6 mice (4 weeks after transplantation) or CD11b-Cre;Z/EG mice and grown for 3 to 4 weeks.

Flow cytometric analysis

Cells were washed in PBS supplemented with 5% FBS, Fc-blocked with a monoclonal antibody against mouse CD16/CD32 (Clone 2.4G2, Pharmingen), and stained with directly-labeled monoclonal antibodies against mouse CD19 (Clone MB19-1, eBioscience), CD3 (Clone 145-2C11, eBioscience), CD11b (Clone M1/70.15, CALTAG), F4/80 (Clone CI:A3-1, Serotec), LYVE-1 (Clone ALY7, CliniSciences), CD31 (Clone 390, eBioscience). Podoplanin expression was revealed by hamster anti-mouse Podoplanin (Clone 8.1.1), followed by biotinylated anti-hamster-IgG antibody and streptavidin-PE (eBioscience). Stained cells were analyzed on a FACSCanto II using DIVA Software (Becton Dickinson). Dead cells were excluded by a combination of light scatter and PI fluorescence. Cell duplets were excluded by forward scatter pulse width. Peripheral blood

mononuclear cells were isolated by Ficoll–Histopaque (SIGMA) density-gradient centrifugation. Bone marrow cells were extracted from mouse femurs and tibiae by flushing. Tumor single cell suspensions were obtained by digestion for 45 minutes at 37° C using the following digestion buffers: TRAMP-C1: HEPES buffered saline, 0.1mg/ml DNaseI (Roche), 1mg/ml collagenase I (SIGMA); RT2: DMEM, 5% NU serum (Becton Dickinson), 0.2mg/ml DNaseI, 1.2U/ml DispaseII (Roche Applied Science).

CD11b$^+$ and CMP cell sorting and adoptive transfer

Bone marrow cells were extracted from femurs and tibiae of female C57BL/6-Tg(ACTB-EGFP) mice, washed in PBS/ 2% BSA, Fc blocked and stained with a phycoerythrin (PE)-conjugated monoclonal antibody against mouse CD11b (CALTAG) or, for CMP isolation, lineage markers, CD3, CD4 (Clone GK1.5), CD8 (Clone 53-6.7), Ter119 (Clone TER-119), B220 (Clone RA3-6B2), CD19, Gr-1 (Clone RB6-8C5), Sca-1 (Clone D7), IL7Ra (Clone A7R34) and Allophycocyanin-labeled anti-cKit (Clone 2B8) (all from eBioscience). CD11b$^+$ GFP$^+$ or CMP (lineage$^-$/ Sca-1$^-$/ IL7Ra$^-$/ cKit$^+$) cells were sorted on a FACSAria (Becton Dickinson) with a purity > 98%. 4 x 10^5 CD11b$^+$ or 4 x 10^4 CMP were injected in the tail vein of semi-lethally (450 cGy) or non irradiated 9 week old RT2;VC mice, which were sacrificed 3 weeks after transplantation.

Histological analysis

7 or 20 μm cryosections from pancreata or TRAMP-C1 tumors were prepared and stained as described [84]. Briefly, harvested tissues were fixed in 4% paraformaldehyde for 2 hours at 4°C, incubated in 30% sucrose overnight and then cryopreserved in OCT medium. Tissue sections were incubated at RT for 30 minutes with blocking buffer (5% goat serum in PBS) prior to overnight incubation at 4°C with the primary antibodies. When required, PBS/0.2% Triton-X-100 was used for permeabilization. The following primary antibodies were used at the dilutions specified in brackets: rat anti-mouse LYVE-1 (1:200) (Clone ALY-7, MBL, Japan), rabbit anti-mouse LYVE-1 (1:200) (Reliatech, Germany), rabbit anti-mouse Prox-1 (1:100) (K. Alitalo, University of Helsinki), goat anti-human Prox-1 (1:100) (R&D Systems), rabbit anti-Podoplanin (1:100) (D. Kerjaschki, Medical University Vienna), hamster anti-mouse Podoplanin hybridoma supernatant (1:20) (Clone 8.1.1), rat anti-mouse VE-Cadherin hybridoma supernatant (1:50) (Clone B14, E. Dejana, University of Milano), rat anti-mouse F4/80 (1:200) (Clone CI:A3-1, Serotec), rat anti-mouse CD11b (1:100) (Clone M1/70.15, Serotec) and rat anti-mouse

CD31 (1:50) (Clone MEC 13.3, Pharmingen). Alexa Fluor 488-, 568- and 633-labeled secondary antibodies (Molecular Probes) were used (1:400). Alexa Fluor 488-conjugated rabbit anti-GFP antibody (1:500) (Molecular Probes) was employed for the detection of GFP. DAPI (SIGMA) was used for nuclear counterstaining. Sections were analyzed on a Nikon Diaphot 300 immunofluorescence microscope (Nikon) using Openlab 3.1.7. Software (Improvision) or with a LSM 510 Meta confocal microscope using LSM Software for 2D and 3D analysis (Zeiss). Videos were created using Imaris 6.1.1 Software (Bitplane Scientific Solutions, Zurich, Switzerland).

ClodroLip-mediated macrophage depletion.

Eight week old RT2;VC mice were injected *i.p.* every 4 days for 4 weeks with 80mg/kg body weight (first injection) or 40mg/kg body weight (following injections) ClodroLip or with an equal volume of PBS as control. 2 days after the last injection, mice were sacrificed, pancreata were embedded in OCT and snap frozen in liquid nitrogen. Tumor macrophage depletion and tumor lymphatic vessel coverage were determined by immunofluorescence stainings with anti-F4/80 antibodies and anti-LYVE-1 antibodies, respectively, and ImageJ Software (http://rsb.info.nih.gov/ij/). Statistical analysis and graphs were performed with GraphPad Prism Software (GraphPad Software Inc.). Non-parametric Mann-Whitney tests were used to compare tumor lymphatic vessel coverage of treated versus control mice.

Isolation of tumor-associated macrophages (TAM) and tumor cells

Single cell suspensions of tumors from 13-14 week old RT2;VC mice were obtained as described above, washed in FACS buffer (PBS/ 2% BSA/ 5mM EDTA) and stained with anti-CD11b-PE and anti-CD31-APC. 20'000 – 50'000 $CD11b^+$ cells (TAM) or $CD11b^-$ $CD31^-$ cells (tumor cells) were sorted on a FACSAria directly into TRIZOL reagent (Invitrogen).

Quantitative RT–PCR

Total RNA was prepared using TRIZOL (in the case of RNA isolation from Matrigel cultures, two consecutive rounds of TRIZOL purification were performed), and reverse transcribed with random hexamer primers using M-MLV reverse transcriptase (SIGMA). cDNA was quantified on a ABI Prism 7000 Taqman (Applied Biosystems) using SYBR green PCR MasterMix (Fermentas) using the following primers: mVEGFC: fwd: 5'-

AGCAGCCACAAACACCTTCTT-3', rev: 5'-TCAAACAACGTCTTGCTGAGG-3'; mVEGFD: fwd: 5'-GCACCTCCTACATCTCCAAACAG-3', rev: 5'-GGCAAGCACTTACAACCCGTAT-3'; mFGF1: fwd: 5'-CCGAAGGGCTTTTATACGG-3', rev: 5'-TCTTGGAGGTGTAAGTGTTATAATGG-3'; mFGF2: fwd: 5'-CGGCTCTACTGCAAGAACG-3', rev: 5'-TGCTTGGAGTTGTAGTTTGACG-3'; mFGFR1: fwd: 5'-TGTTTGACCGGATCTACACACA-3', rev: 5'-CTCCCACAAGAGCACTCCAA-3'; mFGFR2: fwd: 5'-TCGCATTGGAGGCTATAAGG-3', rev: 5'-CGGGACCACACTTTCCATAA-3'; mLYVE-1: fwd: 5'-GGTGTCCTGATTTGGAATGC-3', rev: 5'- AGGAGTTAACCCAGGTGTCG -3'; mProx-1: fwd: 5'-AAGAGAGAGAGAAAGAGAGAGAGTGG-3', rev: 5'-TGGGCACAGCTCAAGAATC-3'; mVEGF-R3: fwd: 5'-CGTGTGTGAAGTGCAGGATAGG-3', rev: 5'-TCACTCACGTTCACCAGGAGGT-3'; mFoxC1: fwd: 5'-GCTTTCCTGCTCATTCGTCTT-3', rev: 5'-AAATATCTTACAGGTGAGAGGCAAG-3'; mFoxC2: fwd: 5'-GACCCTAGCTCGCTGACG-3', rev: 5'-CACCAGCCCTTCCGAGT-3'; mCD45: fwd: 5'-CAAAAGCAGATCGTCCGGA-3', rev: 5'-TGTCGGCCGGGAGGTT-3'; mCX3CR1: fwd: 5'-AAGTTCCCTTCCCATCTGCT-3', rev: 5'-CAAAATTCTCTAGATCCAGTTCAGG-3'; mSDF-1: fwd: 5'-CTGTGCCCTTCAGATTGTTG-3', rev: 5'-TAATTTCGGGTCAATGCACA-3'; mRPL19: fwd: 5'-ATCCGCAAGCCTGTGACTGT-3', rev: 5'-TCGGGCCAGGGTGTTTTT-3'. Ct values were normalized against ribosomal protein L19 (RPL19).

Tube formation assay using bone marrow-derived-macrophages

Bone marrow cells were extracted from femurs and tibiae of C57BL/6 or C57BL/6-Tg(ACTB-EGFP) mice and cultured on Teflon plates for 7 days in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin and 30% L929 cell conditioned media containing M-CSF. Bone marrow-derived-macrophages were collected with PBS/ 1 mM EDTA. Matrigel (Becton Dickinson) was mixed 1:1 with endothelial cell medium (EGM-2 MV, Cambrex) and allowed to solidify for 1 hour at 37°C in 8-chamber slides. 2-3 x 10^5 bone marrow-derived macrophages or immortalized lymphatic endothelial cells (SV-LEC) or a mixture of each 1.5 x 10^5 cells each in EGM-2 MV supplemented with 1 μg/ml LPS were seeded on the polymerized Matrigel and tube formation was monitored up to 20 days. For inhibitory studies, macrophages were pre-incubated for 30 minutes at 37°C with a chemical inhibitor against CXCR4 (Novartis, Basel) at concentrations ranging

from 0.3 to 10μM and then seeded in the presence or in the absence of the same concentration of inhibitor used for the pre-incubation. Immunofluorescence staining of tube-like structures was performed as described [241]. For time-lapse video microscopy, Hoechst labeled SV-LEC and GFP^+ macrophages were co-cultured as described above and pictures were taken every 10 minutes for a period of 12 hours using a Zeiss Axiovert 35M microscope (Zeiss), Princeton Instruments CCD camera and Metamorph Imaging Software (Universal Imaging Corporation).

Microarray processing and data analysis

Total RNA was isolated from Matrigel cultures 1, 3, 6 and 8 days post seeding using two consecutive rounds of TRIZOL purification. RNA quality and quantity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples used for analysis presented a RNA integrity number (RIN) higher than 6.5 and were comparable between samples, showing appropriate quality. The manufacturer's protocols for the GeneChip platform by Affymetrix (Santa Clara, CA) were used. Methods included synthesis of first- and second-strand cDNAs, synthesis of cRNA by *in vitro* transcription, subsequent synthesis of single-stranded cDNA, biotin-labeling and fragmentation of this cDNA and subsequent hybridization to the microarray slide (GeneChip® Mouse Gene 1.0 ST array), posthybridization washings, and detection of the hybridized cDNAs using a streptavidin-coupled fluorescent dye. Hybridized Affymetrix arrays were scanned with an Affymetrix GeneChip 3000 scanner. Image generation and feature extraction were performed using Affymetrix GCOS Software and quality control was performed using Affymetrix Expression Console Software. Raw microarray data were normalized with Robust Multi-Array (RMA) and analyzed using Gene Spring GX 9.0 Software. One-way analysis of variance (ANOVA) and asymptotic analysis were used to identify significantly differentially expressed genes, with a Benjamini-Hochberg false discovery rate corrected-p value set at $p<0.01$. The gene ontology (GO) tool from Gene Spring GX 9.0 Software as well as the Ingenuity Pathway Analysis Software (http://www.ingenuity.com) were used for further data analysis.

Knockdown studies

Lentiviral vectors encoding shRNAs against FGFR-1 and 2 were kindly provided by N.Hynes (FMI, Basel). The lentiviral vector encoding the shRNA control was purchased at SIGMA (Mission® pLKO.1-puro Control Vector). All vectors encode a puromycine

selection cassette. rmSDF-1 was purchased at PeproTech (London) while IL-3 and IL-6 were kindly provided by T.Rolink (University of Basel).

Progenitor-enriched bone marrow cells were extracted from femurs and tibiae of C57BL/6 or C57BL/6-Tg(ACTB-EGFP) mice injected *i.p.* with 5-Fluorouracil (150mg/kg) 6 days before cell collection. After one night in transplant media (RPMI supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, 125ng/ml SCF-1, 75ng/ml IL-3 and 125ng/ml IL-6), 4 x 10^6 cells/condition were infected with lentivirus particules produced as described [242]. More precisely, collected cells were seeded in 6 well plates (4 x 10^6 cells/well). 4μg/ml polybrene, 7.5mM Hepes buffer (SIGMA) and the supernatant of producing 293T cells cultured in 6 well plates were added to the bone marrow-derived cells (the supernatant of 1 well of producing cells was added to 1 well of bone marrow-derived cells) and plates were centrifuged at 30°C, 2'500 rpm for 90 minutes. Cells were incubated for 3 hours post-centrifugation at 37°C before replacement of the medium by fresh transplant medium. The infection procedure was repeated a second time on the next day. 24 hours post the last infection, transduced cells were selected using 3μg/ml puromycine (SIGMA) for 2 days.

For *in vitro* assays, transduced cells were cultured on Teflon plates for 9 days in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin and 30% L929 cell conditioned media containing M-CSF. The ability of *in vitro* produced macrophages to form tube-like structures was then assessed using the tube formation assay described above. For *in vivo* assays, living transduced cells were isolated by Ficoll–Histopaque (SIGMA) density-gradient centrifugation and 5 x 10^5- 1 x 10^6 cells were transplanted in lethally irradiated 7 week old RT2;VC mice sacrificed for further analysis 7 weeks after transplantation.

6. Discussion

Using tagged-bone marrow transplantations into two different tumor mouse models presenting ongoing lymphangiogenesis, we could demonstrate that bone marrow-derived cells (BMDC) can trans-differentiate into lymphatic endothelial cells and thus contribute to tumor lymphangiogenesis. Importantly, these data support previous findings on the role of haematopoietic cells in lymphatic vessel formation, showing that tumor-induced haematopoietic cell plasticity toward lymphatic endothelium is a general component of tumor-associated lymphatic vessel development. Moreover, lineage-tracing experiments revealed that bone marrow-derived tumor lymphatic progenitor cells reside, at least partially, within the myeloid lineage. Finally, the phenotypical conversion of myeloid cells toward lymphatic endothelial cells was recapitulated in two *in vitro* assays, offering the opportunity to assess the trans-differentiation mechanism.

The fact that haematopoietic cells are able to give rise to lymphatic endothelium is not a completely unexpected discovery. Indeed, the generation of haematopoietic cells from a so-called hemogenic endothelium has recently been well characterized [198-200]. It is therefore conceivable that if endothelial cells can give rise to haematopoietic cells, plasticity in the reverse direction is also possible. Moreover, as these two cell types share their developmental history and in consequence may share epigenetic marks, the interconversion between endothelial and haematopoietic cells may be facilitated as only a small part of their epigenomes would have to be rearranged in contrast to unrelated cell types [180]. However, while HSC are able to contribute to lymphatic vessel formation under steady state conditions [205], myeloid lineage committed cells require an inflammatory context to achieve such plasticity (Figure.14) [88,89]. Thus the conversion of myeloid cells toward lymphatic endothelium seems to be dependent on local signals provided by the microenvironment, which in this study could be supplied by tumor cells, tumor lymphatic endothelial cells (TLEC) or educated tumor-associated stromal cells. Pleiotrophin (PTN) is thus far the only factor reported to play a role in myeloid to endothelial cell plasticity. Culturing monocytes in the presence of PTN resulted in tube-like structure formation and expression of vascular endothelial cell markers, while co-injecting tagged-monocytes and multiple myeloma cells into severe combined immunodeficient (SCID) mice led to the incorporation of monocytes into tumor blood

vessels. These trans-differentiation processes were abrogated by the use of anti-PTN neutralizing antibodies [85]. Interestingly, this secreted factor was also significantly upregulated (1.5X) upon *in vitro* phenotypical conversion of myeloid cells toward lymphatic endothelium in my experiments. As PTN expression is very limited in healthy adult tissues, treating bone marrow-transplanted RT2;VC mice with anti-PTN antibodies might reveal the potential implication of this factor in myeloid cell contribution to tumor lymphatic vessels.

Recapitulating the *in vivo* trans-differentiation process *in vitro* and assessing gene expression changes upon phenotypical conversion allowed the identification of an important number of candidate genes potentially implicated in this process. The role of selected candidate genes could be further evaluated by setting up specific *in vitro* and *in vivo* experimental protocols. FGFR-1/2 and SDF-1 were shown to be clearly required for the *in vitro* trans-differentiation process. However, *in vivo*, the requirement of FGFR-1 and 2 could not be confirmed. This may be explained by the fact that, *in vitro*, the FGFR ligand FGF2 is added to the culture medium, possibly artificially rendering the *in vitro* trans-differentiation process dependent on this signaling pathway. The well-defined *in vitro* growth conditions may not reflect the *in vivo* situation, where the ablation of FGF signaling may be compensated for by other factors present in the tumor microenvironment. Furthermore, as the bone marrow-derived tumor lymphatic endothelial cells (BMDTLEC) only represent a small percentage of tumor-associated lymphatic endothelial cells, variations in this population cannot be easily detected by our current analytic methods. The complete abrogation of the trans-differentiation process would be necessary to be clearly visualized *in vivo* and therefore, the contribution of FGF signaling in collaboration with other signaling pathways cannot be excluded. Along these lines, it is also important to consider that the *in vivo* knockdown efficiency for FGFR-1 and 2 of around 65% represents the downregulation efficiency of a pool of cells. Depending on where and in how many copies the shRNA construct integration took place, cells may present important variations in their knockdown efficiency, rendering individual cells more or less able to undergo trans-differentiation. Supporting this hypothesis, clusters of knocked-down macrophages for which some tube-like structures could be observed *in vitro* always correlated with a downregulation efficiency located in the lower range. Taken together, these considerations outline the fact that an efficient interference with the *in vitro* trans-differentiation process will not necessarily clearly affect the *in vivo* trans-

differentiation process. Therefore, for the experimental reasons mentioned above, the use of knockdown studies in BMDC to impair trans-differentiation must be considered carefully. Alternatively, blocking SDF-1 signaling by the use of a chemical inhibitor against its receptor CXCR4 may, based on the *in vitro* studies, allow the detection of a clear impairment of the *in vivo* trans-differentiation process. However, it is important to keep in mind that pharmacological inhibition might be less specific than knockdown studies directly targeting BMDC. Another approach to obtain insights into the myeloid to lymphatic endothelial cell plasticity consisted in comparing the transcriptomes of BMDTLEC, host TLEC, and BMDC present at the tumor site but not integrated into TLEC. Unfortunately, it has not been possible to obtain a sufficient number of cells to perform these experiments. Thus, despite these limitations, the *in vitro* trans-differentiation assay remains a very interesting and unique tool to identify genes required for the different steps of myeloid cell conversion into lymphatic endothelium.

Depletion of tumor-associated macrophages resulted in a 14% reduction of tumor lymphatic vessel coverage, demonstrating a functional implication of myeloid cells in tumor-associated lymphatic vessel formation. It was, however, surprising to see that the depletion of BMDTLEC, which constitute only a small proportion of TLEC, could significantly affect overall tumor lymphangiogenesis. Our results show that myeloid cells do not contribute to tumor lymphangiogenesis through paracrine secretion of the main lymphangiogenic factors but rather suggest that BMDTLEC may fulfill a tip cell-like function by instigating lymphatic sprouts. Depleting these specialized cells would thus substantially affect tumor lymphangiogenesis. Specialization of cells to confer a critical function to a biological process is not without precedence. It has been reported that carcinoma-associated fibroblasts (CAF) promote squamous cell carcinoma (SCC) cell collective invasion by remodeling the matrix and thus making a path that SCC cells can use to invade [243]. Normal dermal fibroblasts are in contrast unable to promote SCC invasion, underlying the necessity of fibroblasts to be converted into specialized cells to fulfil their leader function. In addition to the activation of quiescent fibroblasts and bone marrow recruitment, trans-differentiation has been shown to constitute a source of CAF [244]. Drawing a parallel to these studies, it is tempting to hypothesize that, in order to be able to interact with pre-existing TLEC as lymphatic tip cell-like cells, tumor-associated myeloid cells have to acquire lymphatic characteristics. Macrophages are phagocytic cells able to produce degradative enzymes. Upon *in vitro* tube-like structure formation,

diverse matrix metalloproteinases (MMP-2/11, Adamts-1/5) are highly upregulated, suggesting that BMDTLEC may go through an intermediate trans-differentiation state. In such a state, their newly acquired lymphatic features would allow them to interact with pre-existing TLEC, while their remaining and enhanced macrophage-specific properties would enable them to generate tracks for instigating sprouting. Terminal trans-differentiation would then be achieved once sprouting is completed. The *in vitro* trans-differentiation assays may be used as a tool for verifying these hypotheses. For example, by first identifying chemokines or adhesion molecules upregulated upon tube-like structure formation and which could theoretically play a role in the coordinated movement of BMDTLEC-induced lymphatic sprouting. And then by interfering with these molecules in an *in vitro* co-culture assay. Interestingly, Unc5b, a well-characterized endothelial tip cell marker as well as an important number of guidance molecules implicated in vascular navigation were upregulated upon *in vitro* trans-differentiation.

In conclusion, this study demonstrates that myeloid cells can give rise to tumor lymphatic endothelium. As tumor lymphangiogenesis promotes metastatic dissemination, understanding the signaling pathways which orchestrate this trans-differentiation process seems to be warranted. A quest which is facilitated by the transcriptional characterization of an *in vitro* lymphatic-like tube formation assay as well as by the development of an *in vitro* co-culture assay mimicking the *in vivo* trans-differentiation process.

7. References

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8. Curriculum Vitae

Personal data

- Birthday: 20.08.1980, Geneva
- Nationality: Swiss
- Languages: French, English, German, Portuguese (spoken), Italian (beginner level)

Education

- 1995-1999: “Maturité Fédérale”, scientific section (type C), Ste-Croix high school, Fribourg
- 2000-2004: Studies in Biology at the University of Lausanne
- 2004-2005: Diploma thesis and graduation as biologist from the University of Lausanne

Experience in research

- Summer semester 2003: Semester certificate in the laboratory of Prof. Dr. B. Rossier, Department of Pharmacology and Toxicology, University of Lausanne. Project: “Testing the inducible tet-operator system in mpkCDD cells”
- Winter semester 2003/4: Semester certificate in the laboratory of Prof. Dr. D. Nardelli-Haefliger, Medical Microbiology Institute, University of Lausanne. Project: “Construction of new recombinant Salmonella strains expressing HPV genes and analysis of the immune response with the improvement of an *in vitro* neutralization test”
- 09/2004-06/2005: Diploma thesis in the laboratory of Prof. Dr. A. Wodnar-Filipowicz, Exerimental Haematology, ZLF, Basel. Project: “Genetic modification of human NK cells by lentiviral mediated transfer of siRNA directed against NKG2D receptor”
- Since 08/2005: PhD studies in the laboratory of Prof. Dr. G. Christofori, Department of Biomedicine, Basel.

Publications

Baeriswyl V* / Zumsteg A*, Imaizumi N, Schwendener R, Ruegg Curzio, Christofori G * equally contributing authors

Myeloid cells contribute to tumor lymphangiogenesis.

PLoSONE, September 2009

Baeriswyl V, Christofori G

The angiogenic switch in carcinogenesis.

Seminars in Cancer Biology, in press

Schomber T, Kopfstein L, Djonov V, Albrecht I, Baeriswyl V, Strittmatter K, Christofori G

Placental growth factor-1 attenuates vascular endothelial growth factor-A-dependent tumor angiogenesis during beta cell carcinogenesis.

Cancer Research, November 2007

Kren A, Baeriswyl V, Lehembre F, Wunderlin C, Strittmatter K, Antoniadis H, Fässler R, Cavallaro U, Christofori G

Increased tumor cell dissemination and cellular senescence in the absence of beta1-integrin function.

EMBO Journal, June 2007

Kopfstein L, Veikkola T, Djonov VG, Baeriswyl V, Schomber T, Strittmatter K, Stacker SA, Achen MG, Alitalo K, Christofori G

Distinct roles of vascular endothelial growth factor-D in lymphangiogenesis and metastasis.

American Journal of Pathology, April 2007

Baeriswyl V, Wodnar-Filipowicz A, Kalberer CP

The effect of silencing NKG2D through RNA interference on receptor functions in interleukin-2-activated human natural killer cells.

Haematologica, November 2006

Conferences

- 05/2006: *ESH, 6th EuroConference on Angiogenesis*
  Cannes, France
  Oral and Poster presentations
- 09/2006: *Gordon Research Conference on Molecular Mechanisms in Lymphatic* Function
  Les Diablerets, Switzerland
  Poster presentation
- 10/2006: *BioValley Life Science week 2006*
  Basel, Switzerland
  Poster presentation
- 05/2007: *EuCC*
  Strasbourg, France
  Oral presentation
- 08/2007: *Gordon Research Conference on Angiogenesis*
  New Port, RI, USA
  Poster presentation
- 10/2007: *BioValley Life Science week 2007*
  Basel, Switzerland
  Poster presentation
- 02/2008: *USGEB 2008*
  EPFL, Lausanne
  Poster presentation
- 04/2008: *Oncoday 2008*
  Kaiseraugst, Switzerland
  Oral presentation
- 09/2008: *5th International Kloster Seeon Meeting on Angiogenesis*
  Basel, Switzerland
  Poster presentation
- 09/2008: *Embo Workshop and ESH EuroConference on Lymph/Angiogenesis*
  Helsinki, Finland
  Poster presentation

Awards

Roche Poster Award (10/2007)
BioValley Science Day, Basel.
Poster title: “Myeloid cells contribute to tumor lymphangiogenesis”.
Award received together with A.Zumsteg

Paul Basset Oral Presentation Award (05/2007)
EuCC Meeting, Strasbourg, France.
Oral presentation title: “Myeloid cells contribute to tumor lymphangiogenesis”.

9. Acknowledgements

I first would like to thank my principal investigator Gerhard Christofori for giving me the opportunity to do my PhD studies in such a motivating laboratory, regarding to the scientific themes as well as to the collaborative and overall stimulating atmosphere. I specially appreciated its constant support in letting me explore new approaches and set the required collaborations, which rendered my studies even more diverse and exciting. I was also impressed by his scientific enthusiasm and his capacity in developing new ideas even sometimes from, regarding to me, disappointing results. An attitude that helped me coming out of his office with the motivation I had lost before entering.

I really would like to thank Adrian for being “my postdoc” by sharing with me part of its substantial scientific experience. I learned a lot from him getting thus closer to one of the main goal of PhD studies, which consists in learning how to scientifically think and work. Besides the scientific collaboration, its was also very amusing and positively challenging to share a project with him. And finally, thanks a lot for filling my music library with new fantastic discoveries!

Thanks to Roland Jost for being such a nice and helpful animal care taker and specially for the nice and funny words that would stick a smile on your face for the rest of the day after having had him on the phone.

Thanks to Karin Strittmatter, Helena Antoniadis, Petra Schmidt und Ursula Schmieder for their availability and consistent technical help.

Thanks to Katrin Hafen and Jason Gill for their precious help and teaching regarding to the “art” of bone marrow transplantation.

And finally, thanks a lot to Miguel, Pascal, Maura, Ralph, Dorothea, Chantal, Karin, Akiko, Ernesta, Jörg, François, Mahmut, Fabienne, Angie, Lucie, Tibor, Imke, Lukas, Natalie, Lorenz, Anna, Ulrike, Andreas and Neha for such an excellent lab atmosphere as well as for the nice moment spent together far away from the Mattenstrasse building.