Stem Cells and Their Niches in Angiogenesis:

Vascular Endothelial Stem Cells, Hematopoietic Progenitors and Hematopoietic Effector Cells

Shentong Fang

Department of Pathology
Haartman Institute
Faculty of Medicine
&
Graduate Program in Molecular Biology and Biotechnology
University of Helsinki
Finland

ACADEMIC DISSERTATION

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Thesis Supervisor
Petri Salvén, M.D., Ph.D.,
Docent
Department of Pathology
Haartman Institute
University of Helsinki
Helsinki, Finland

Thesis follow-up committee
Jorma Keski-Oja, M.D., Ph.D.,
Professor
Translational Cancer Biology, Biomedicum Helsinki
Haartman Institute
University of Helsinki
Helsinki, Finland

Pentti Tienari, M.D., Ph.D.,
Docent
Research program for molecular neurology, Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Reviewers appointed by the Faculty
Juha Partanen, Ph.D.,
Professor
Department of Biosciences
University of Helsinki
Helsinki, Finland

Klaus Elenius, M.D., Ph.D.,
Professor
Medical biochemistry and genetics
University of Turku
Turku, Finland

Opponent appointed by the Faculty
Michele De Palma, Ph.D.,
Assistant Professor
School of Life Sciences - Swiss Federal Institute of Technology Lausanne (EPFL)
The Swiss Institute for Experimental Cancer Research (ISREC)
Switzerland

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To my family and friends

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ORIGINAL PUBLICATIONS

This Thesis is based on the following original publications, which are referred to by their Roman numerals in the text.

I. **Fang, S.,** Wei, J., Pentinmikko, N., Leinonen, H., and Salven, P. (2012). Generation of functional blood vessels from a single c-kit+ adult vascular endothelial stem cell. PLoS Biology 10(10): e1001407. doi:10.1371/journal.pbio.1001407

II. **Fang, S.*,** Pentinmikko, N.*, Ilmonen, M., and Salven, P. (2012). Dual action of TGF-beta induces vascular growth in vivo through recruitment of angiogenic VEGF-producing hematopoietic effector cells. Angiogenesis 15, 511-519

III. **Fang S.,** Louhimo R., Pentinmikko N., Leinonen H., Virolainen S., Heikkilä P., Hautaniemi S., and Salven P.. Initiation of an ectopic tumor hematopoietic microenvironment supporting angiogenesis and cancer growth. manuscript

ABBREVIATIONS

APC	antigen presenting cell
ALK	activin receptor-like kinase
Ang	angiopoietin
AGM	aorta-gonad-mesonephros
BEC	blood endothelial cell
bFGF	basic fibroblast growth factor
BM	bone marrow
BMDC	bone marrow derived cell
BMP	bone morphogenetic protein
BMSEC	bone marrow sinusoidal endothelial cell
CFC	colony forming cell
CFU-EPC	EPC colony-forming units
CCL	cysteine-cysteine chemokine ligand
CSF-1	colony stimulating factor-1
CXCL	cysteine-X- cysteine chemokine ligand
CXCR	CXCL-receptor
DC	dendritic cell
Dll4	delta-like ligand 4
EC	endothelial cell
ECFC	endothelial colony forming cell
ECM	extracellular matrix
EF	ejection fraction
EOC	endothelial outgrowth cell
EPC	endothelial progenitor cell
Eph	ephrin
GM-CSF	granulocyte macrophage colony-stimulating factor
HAEC	human aortic endothelial cell
HIF-1α	hypoxia induced factor-1α
HPC	hematopoietic progenitor cell
HPP-ECFC	high proliferative potential endothelial colony forming cell
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
HUVEC	human umbilical vein endothelial cell
IL	interleukin
INF-γ	interferon-γ
JNK	c-Jun N-terminal kinases
LLC	Lewis lung carcinoma
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MDSC	myeloid derived suppressor cell
MMP-9	matrix metalloproteinase -9
MNC	mononuclear cell

MS-1	Mile SVEN 1
MSC	mesenchymal stem cell
NG2	neuron-glial antigen 2
Nrp	neurophilin
OB	osteoblast
PBMNC	peripheral blood mononuclear cell
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PFS	progression free survival
PIGF	placental growth factor
PI3K	phosphoinositide 3-kinase
PPC	pericyte progenitor cell
Sca-1	stem cell antigen-1
SCF	stem cell factor
SCL-1	stem cell leukemia-1
SDF-1	stromal cell derived factor-1
SMC	smooth muscle cell
SMPC	smooth muscle progenitor cell
Sl	steel
TAM	tumor associated macrophage
TAN	tumor associated neutrophil
TEM	Tie-2 expressing monocytes/macrophages
TF	tissue factor
TGF-β	transforming growth factor-β
Th-1	T helper cell type 1
Tie-2	tyrosine kinase with immunoglobulin and EGF homology domains
TKI	tyrosine kinase inhibitor
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VESC	vascular endothelial stem cell
VCAM	vascular cell adhesion molecule
VE-cadherin	vascular endothelial cadherin
VPC	vascular progenitor cell
vSMC	vascular smooth muscle cell
vWF	von Willebrand factor
Wt	wild type
β-gal	β-galactosidase

ABSTRACT

Most tissues possess tissue-specific stem cells that allow them to maintain tissue integrity. Stem cell niches provide an ideal regulatory microenvironment to support the maintenance and proliferation of adult stem cells. However, adult stem cells and stem cell niches have not been identified for all tissues.

Adult stem cells that give rise to the vascular endothelium are still unknown. In this thesis work, we identified a rare population of vascular endothelial stem cells (VESC) on the vascular wall by the phenotype $lin^{-}CD31^{+}CD105^{+}Sca\text{-}1^{+}c\text{-}kit^{+}$. A single c-kit expressing VESC with highly proliferative capacity generated functional blood vessels in vivo. A genetic defect in endothelial c-kit resulted in an abolished colony-forming ability of VESCs and impaired tumor angiogenesis and tumor growth.

Angiogenesis, the growth of new blood vessels from pre-existing vessels, is actively involved in many physiological and pathological processes such as wound repair, female reproductive cycling, ischemic disease and tumor development. There are two major groups of cells involved in the adult vascular growth, cells that contribute directly by composing the blood vessels, such as vascular endothelial stem cells, and cells that contribute indirectly in a paracrine manner such as infiltrating hematopoietic cells.

Infiltrating hematopoietic cells from the bone marrow contribute to angiogenesis in a paracrine manner by secreting angiogenic factors or by remodeling the extracellular matrix. In this thesis work, we found that transforming growth factor-β (TGF-β) recruited a massive amount of hematopoietic cells to local microenvironment. TGF-β stimulated vascular endothelial growth factor (VEGF) expression on these hematopoietic effectors and thus induced vascular growth. This stimulation was regulated by p38 and p44/p42 mitogen-activated protein kinase (MAPK) signaling pathways. These results together provided evidence for a dual action mechanism for TGF-β-induced angiogenesis in vivo. In malignant tumors, we found that the tumor expressed osteoblastic and vascular hematopoietic stem cell (HSC) niche molecules and enclosed multipotent hematopoietic progenitors. The proliferating hematopoietic progenitors generated hematopoietic effector cells and supported angiogenesis and tumor growth by secreting matrix metalloprotease 9 (MMP-9) and VEGF. HSPCs were found to be in proximity to tumor vasculature. Tumor microenvironment shared features of HSC niche in the bone marrow. Therapeutic ablation of hematopoietic cells including proliferating hematopoietic cells from tumor using AMD3100 in vivo resulted in inhibited tumor angiogenesis and growth.

In conclusion, we identified and characterized a rare population of c-kit expressing VESCs that give rise to the vascular endothelium in adult. Further purification and detailed characterization of VESCs will provide a better understanding of VESCs and hierarchy of endothelial lineage. Our observations on the in vivo angiogenesis induced by TGF-β elucidated the mechanisms of action of TGF-β in promoting vascular growth. Together with current findings on the ectopic tumor–hematopoietic microenvironment, the proliferating hematopoietic progenitors and angiogenic hematopoietic effector cells, these results provided potential therapeutic targets to treat cancer and other diseases.

中文摘要

在哺乳动物的成体组织中，成体干细胞利用自我更新和其分化能力修复再生身体各组织器官， 从而使组织和器官维持生长和老化衰退的动态平衡。成体干细胞，如上皮干细胞和造血干细胞，通常位于一个特定的微环境中。这样的干细胞微环境与干细胞相互作用，控制监管并支持着干细胞的更新，分化和增殖。研究人员已经在多种组织和气管内发现成体干细胞及微环境，但是还存在一些未知的成体干细胞。

例如，在血管组织中，负责再生血管细胞的成体干细胞仍旧未被研究人员有所定论。在这篇论文中的工作，我们已经发现在血管壁上表型为$lin^{-}CD31^{+}CD105^{+}Sca\text{-}1^{+}c\text{-}kit^{+}$的血管内皮细胞中有及其少量的血管内皮干细胞(VESC)存在。$c\text{-}kit^{+}$血管内皮干细胞的具有高度增殖能力，并且能在体内生成功能性血管。同时通过研究c-kit在内皮细胞中的功能， 我们发现，c-kit的遗传缺陷可以导致血管内皮干细胞的细胞集落形成能力大大的减弱，并且抑制了肿瘤生长和肿瘤血管的新生。

血管新生时从已有血管发芽生成新血管的过程。 在成体内，血管新生积极参与了许多生理和病理过程，如创面修复，女性生殖循环，缺血性疾病和肿瘤的发展。参与血管新生的细胞可以主要分为两类，一类是直接构成的血管的细胞，如血管内皮干细胞；另一个类是以旁分泌的方式促进血管新生的细胞，如造血细胞。骨髓造血细胞通过分泌血管生成因子或细胞外基质重塑以旁分泌的方式参与成体中的血管新生。在本论文工作中，我们发现，转化生长因子- β (TGF –β)吸引了大量的造血效应细胞来到局部微环境中。这些组织浸润的造血效应细胞通过p38和p44/p42丝裂原活化蛋白激酶(MAPK)信号转导通路分泌血管内皮生长因子(VEGF)从而诱导血管生长。我们的研究结果阐明了TGF-β在体内诱导血管生成的双重作用机制。而在恶性肿瘤中，我们发现肿瘤组织不仅表达用于鉴别造血干细胞所处微环境的蛋白分子同时包含了造血干细胞以及前体细胞。这些肿瘤组织中造血干细胞增殖产生的造血效应细胞通过分泌MMP-9和血管内皮生长因子促进血管生成和肿瘤生长。我们进一步发现，当肿瘤组织中的造血干细胞被AMD3100驱除后，肿瘤血管生成和肿瘤生长都受到抑制。

INTRODUCTION

Vascular development and maintenance are fundamental for life. In adulthood, angiogenesis is transiently switched on in some physiological events, such as female reproductive process, and pathological situations, such as tumorigenesis. Various tissue-specific stem cells and progenitors are actively participating in angiogenesis to generate daughter cells or secrete various angiogenic related factors. There are two major categories of stem cells/progenitors involved in angiogenesis. One category of cells is stem cells and progenitors that generate daughter cells that compose the blood vessels, such as endothelial cells and vascular mural cells. The endothelial lineage hierarchy in adult was previously dominated by studies on circulating endothelial progenitor cells (EPC) (Asahara et al., 1997). Recent studies have provided solid evidence on the existence of resident stem cells on the vascular wall (Goligorsky and Salven, 2013; Purhonen et al., 2008; Rinkevich et al., 2011). However, the identity of the resident vascular endothelial stem cells is still not known. The other category is stem cells and progenitors that generate progeny and participate in angiogenesis indirectly, such as hematopoietic progenitors. Hematopoietic cells contribute to angiogenesis mostly in a paracrine manner (Fang and Salven, 2011; Murdoch et al., 2008).

The emphasis of the current work was to identify the cellular origin of endothelial cells that directly contribute to the newly formed blood vessels in adult and further understand the role of hematopoietic effector cells in angiogenesis. We identified a rare population of VESCs within the c-kit expressing ECs on the vascular wall. The c-kit expressing VESCs were capable of generating millions of daughter cells and undergoing a clonal expansion in vitro. In vivo, VESCs were able to form functional blood vessels and self-renewal. Next, we characterized how the hematopoietic effector cells supported angiogenesis. We elucidated the dual action mechanisms of TGF-β in promoting angiogenesis by recruiting hematopoietic cells and inducing them secreting VEGF. On the other hand, malignant tumors initiated an ectopic hematopoietic microenvironment to support proliferating hematopoietic progenitors by generating hematopoietic cells and induce them to secrete VEGF and MMP-9.

REVIEW OF THE LITERATURE

1. BLOOD VASCULATURE

The blood vascular system in vertebrates is a closed circulatory network of blood vessels to transport oxygen, carbon dioxide, nutrients and metabolic waste. Blood endothelial cells (BEC) line to form a monolayer on the luminal surface of blood vessels. Tight conjunctions and adherens junctions connect adjacent BECs with each other. BECs have heterogeneous phenotypes and molecular signatures (Aird, 2007a, b; Nolan et al., 2013). There are different gene expression patterns in arterial and venous ECs. For instance, the arterial ECs are preferably expressing ephrin (Eph) B4 whereas EphB2 is highly expressed on venous ECs (reviewed in Swift and Weinstein, 2009). Furthermore, ECs also obtain functional, structural and morphological differences, even organ specific phenotypes (Aird, 2007a, b; Nolan et al., 2013). Structurally, ECs can be categorized into three types, continuous non-fenestrated, continuous fenestrated and discontinuous ECs. In endocrine organs, the vascular endothelium is more fenestrated, while in the bone the vascular endothelium is discontinuous and in the brain and lung it is more non-fenestrated (reviewed in Aird, 2012).

1.1 EMBRYONIC VASCULAR DEVELOPMENT

Both vascular development and its maintenance are very important for life. The cardiovascular system is the primary organ to develop during embryonic development in mammals. Any major defects or disruptions in the vascular development during embryogenesis will lead to embryonic lethality (Adamo and García-Cardeña, 2012; Risau and Flamme, 1995). The yolk sac is the first site of hematopoiesis and blood vessel formation in embryonic development. Vasculogenesis is referred to as a process of the vascular growth from de novo endothelial cells differentiated from precursor cells (Fig. 1A). Hemangioblasts are the precursors of hematopoietic stem cells and angioblasts (Adamo and García-Cardeña, 2012; Auerbach et al., 1997). Angioblasts are a population of endothelial progenitor cells capable of differentiating into endothelial cells in the embryo. In the embryo and yolk sac of mammals, angioblasts are recruited to the site and differentiate to form a primitive vascular labyrinth (reviewed in Risau and Flamme, 1995; Swift and Weinstein, 2009).

Hematopoiesis is tightly associated to vascular development in embryogenesis (Dieterlen-Lievre, 1975). There are two waves of hematopoiesis during development, primitive hematopoiesis and definitive hematopoiesis (reviewed in Orkin and Zon, 2008). The primitive hematopoiesis produces primarily red blood cells in yolk sac, followed by definitive hematopoiesis. Hematopoietic progenitors are first found to be located in the developing aorta in aorta-gonad-mesonephros (AGM) region (Godin et al., 1993; Medvinsky et al., 1993). In addition to their spatial association, angioblasts and hematopoietic stem cells (HSC) are considered to originate from hemangioblasts or hemogenic endothelium. VEGFR-2^{+} cells first emerged from mesodermal precursors is thought to represent hemangioblasts (Adamo and García-Cardeña, 2012). VEGFR-2 is indispensable for both vascular development and hematopoiesis in embryo. VEGFR-2 knock-out mouse has defects in both hematopoiesis and vascular development (Shalaby et al., 1995). Besides VEGFR-2, ECs and HSCs also share certain other antigenic determinants, such as Tie-2 and CD34 (Adamo and García-Cardeña, 2012). However,

contradictory evidence on hemangioblasts is later provided by clonal analysis in vivo in mice where the investigators show that the contribution of single hemangioblast to both hematopoietic and endothelial lineages is infrequent (Ueno and Weissman, 2006).

The developing vessels during embryonic development are also heterogeneous. In the umbilical arteries, hematopoietic cells are found to be co-localized with endothelial cells whereas umbilical veins do not have hematopoietic potential (Inman and Downs, 2007). A possible hierarchy may exist during definitive hematopoiesis. Studies have shown that during development there are two distinct endothelial lineage, hemogenic and non-hemogenic (Adamo and García-Cardeña, 2012). During definitive hematopoiesis, hemogenic endothelium generates hematopoietic cells. Zovein et al. (2008) have shown that VE-cadherin^{+} progenitors from the endothelium of the aorta at AGM can differentiate into HSCs. The capacity of hemogenic endothelial cells to produce hematopoietic progeny is Runx1 dependent (Lancrin et al., 2009). Furthermore, recent studies using time-lapse confocal imaging have shown that aortic hemogenic endothelial cells generate de novo HSCs in vivo in zebrafish embryos and mouse aorta explants (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010).

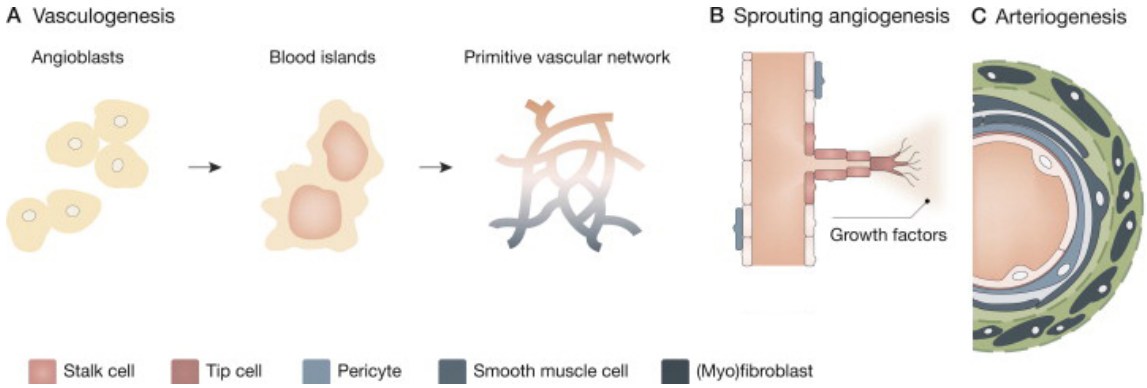

Fig. 1: Vascular blood vessel growth. (A) Vasculogenesis: vascular network is formed by de novo ECs differentiated from angioblasts. (B) Sprouting angiogenesis: blood vessels sprout from pre-existing vasculature. ECs are specialized into tip cells and stalk cells. Tip ECs sprout out towards growth factors to guide the stalk cells. (C) Arteriogenesis: the nascent vasculature develops distinct morphological identities as arteries, veins and capillaries during vascular development (adapted from Leite de Oliveira et al., 2011).

Subsequently, the new blood vessels sprout from the pre-existing vasculature and form the vascular networks. This sprouting process is termed as angiogenesis (Fig. 1B). The formed primary vascular plexus remodels during development and becomes a mature vasculature under the different pressure of blood circulation (Adams and Alitalo, 2007; Jain, 2003; Torres-Vazquez et al., 2003). Mural cells- pericytes and smooth muscle cells (SMC), as well as the basement membrane, wrap around the nascent EC tubes so that the vessels become mature and stabilize. Vascular mural cells provide physical support to the EC tubes and confer signals from microenvironment to the endothelium. The blood vessels adopt three distinct morphologically distinct identities as arteries, veins and capillaries during vascular development (Fig. 1C) (Adams and Alitalo, 2007; Jain, 2003; Torres-Vazquez et al., 2003). Commonly, vascular SMCs (vSMC) cover larger vessels and arteries while pericytes tend to appear on smaller vessels. Additionally, vSMCs and pericytes are sparser on capillaries than larger vessels (reviewed in Armulik et al., 2005; von Tell et al., 2006). Later on, the vasculature further remodels to meet the requirements

of the developing organs by angiogenic sprouting, intussusception, splitting and enlargement (reviewed in Djonov et al., 2000).

The growth of the vascular network, including cell differentiation, proliferation and migration, is tightly coordinated via various signaling pathways (Carmeliet and Jain, 2011; Heinke et al., 2012; Park et al., 2013). VEGF signaling pathway plays a pivotal role in regulating vascular development (Carmeliet and Jain, 2011; Heinke et al., 2012). VEGF-A is one of the most prominent proteins in the VEGF family including VEGF-B, VEGF-C, VEGF-D and PlGF. VEGF-A is the first growth factor that shows an endothelial specific effect (Keck et al., 1989; Leung et al., 1989). Haplodeficiency of VEGF in mice results in embryonic lethality with severe vascular defects (Carmeliet et al., 1996; Ferrara et al., 1996). Furthermore, the balance among different VEGF-A isoforms can regulate vascular growth and remodeling (reviewed in Ferrara et al., 2003; Ladomery et al., 2007). For instance, specific knockouts of $VEGF_{164}$ and $VEGF_{188,}$ isoforms of VEGF, lead to impaired myocardial angiogenesis and decreased vascular density in other organs (Carmeliet and Collen, 1999; Carmeliet et al., 1999). Peripheral nerve-derived VEGF-A in mouse embryonic skin promotes arterial differentiation (Mukouyama et al., 2005). VEGFs bind to three major receptors, VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-2 is the chief receptor for VEGF-A. In the yolk sac, the expression of VEGFR-2 indicates the developing endothelial lineage in vasculogenesis (Rossant and Howard, 2002; Yamaguchi et al., 1993). VEGFR-2 expression is down regulated in mature blood vessels whereas elevated in adult angiogenesis (Goede et al., 1998; Hanahan and Folkman, 1996). VEGFR-1, a VEGF receptor that binds to VEGF-A and VEGF-B, is specifically expressed on developing vessels (Dumont et al., 1995). Neurophilins (Nrp) are another group of important receptors that bind to VEGF. There are two neuropilins, Nrp1 and Nrp2. Nrp2 is specifically expressed on veins and lymphatic vessels while arterial ECs express Nrp1 (Gu et al., 2003; Yuan et al., 2002). Double knock-outs of Np1 and Np2 in mice cause embryonic lethality and the mutant embryos are avascular (Takashima et al., 2002).

Angiopoietin (Ang) /Tie axis is another key pathway in vascular remodeling during development. Tie-2 mutant embryos are lethal due to major defects in vascular remodeling. Ang/Tie signaling pathway mediates EC cell survival, sprouting, EC/ECM interaction and EC chemotaxis (reviewed in Loughna and Sato, 2001).

Many other signaling pathways, such as Notch and TGF-β, also regulate vascular development in the embryo. Mutations in molecules in TGF-β pathway in mice result in embryonic lethality caused by severe vascular defects (reviewed in Dunker and Krieglstein, 2000). Endoglin, a type III receptor expressed by ECs, reduces the activation of TGF-β signal (Barbara et al., 1999; Letamendía et al., 1998). Studies have also found that knockout mice lacking Notch pathway molecules, such as Notch 1, Notch 4 and Delta-like ligand 4 (Dll4), result in embryonic death with major vascular defects (reviewed in Rossant and Howard, 2002).

1.2 PHYSIOLOGICAL ANGIOGENESIS IN ADULTHOOD

The vasculature in adult is mostly quiescent. Endothelial cell proliferation in adulthood holds at a very low rate. The cellular turnover of all ECs takes years (Engerman et al., 1967; Hobson and Denekamp, 1984). In a healthy adult, new blood vessels are seldom formed, except in some physiological processes such as female reproductive process and wound healing when the angiogenic stimuli are switched on. Angiogenesis is initiated by a low oxygen microenvironment (Fraisl et al., 2009). Similar to the vascular growth during

embryonic development, VEGF is also important in adult angiogenesis. Under hypoxic conditions, the hypoxia inducible factor-1α (HIF-1α) is activated and then induces the expression of VEGF which subsequently promotes the vascular growth (Stone et al., 1995). The mechanisms of angiogenesis can be generally divided into two categories, sprouting angiogenesis and non-sprouting angiogenesis (Adams and Alitalo, 2007).

Since 2003, the molecular mechanisms on the formation of new vascular sprouts have been elucidated and extensively studied. Gerhardt et al. (2003) first showed that VEGF gradients induced by hypoxia enroll ECs in a mode called sprouting angiogenesis. ECs in the angiogenic sprouts are specified into two different types, tip cells and stalk cells (Gerhardt et al., 2003). The tip cells lead the growing sprout during angiogenic growth. This selection of ECs is strictly regulated, as an indiscriminate reaction of all ECs in the vessels would cause defects and disintegrated vascular network. Dll4 is expressed in the tip cells. The balance of EC specialization between tip cells and the stalk cells is controlled primarily by Dll4/Notch signaling pathway and is influenced by the gradient level of angiogenic factors, especially VEGF, in the environment (Bentley et al., 2008; Carlier et al., 2012; Carmeliet et al., 2009; De Smet et al., 2009; Gerhardt et al., 2003). VEGF-A could induce Dll4 expression in ECs, whereas VEGFR-2 expression is suppressed by activation of Notch signaling (Williams et al., 2006). Disruptions in Notch signaling lead to increased vascular sprouts and more tip cells (Hellstrom et al., 2007; Stenzel et al., 2011).

During angiogenic sprouting, the basement membrane degrades and pericytes detach from the vascular tube to allow the proliferation and migration of ECs (Adams and Alitalo, 2007). From the observations of angiogenic sprouting in aortic ring culture model, the first cells migrating towards the angiogenic stimuli are fibroblasts and adventitial macrophages ($CD45^+CD11b^+$ and $CD68^+$ macrophages) (Nicosia, 2009). Adventitial macrophages provide paracrine stimulations for ECs, and fibroblasts direct the vascular sprouts via mechanical forces (Nicosia, 2009). The tip cells follow the migrating fibroblasts and adventitial macrophages, and extend more filopodia towards the angiogenic signals. $VEGF_{164}$, an isoform of mouse VEGF-A, functions as a chemoattractive signal and promotes the polarized extension of tip cell filopodia. In addition to VEGF-A, platelet-derived growth factor B (PDGFB) is another important angiogenic gradient during angiogenic sprouting. Pericytes express the receptor for PDGFB. High levels of PDGFB in the growing sprouts induce the recruitment of pericytes and thus stabilize the growing vessels. Further steps of sprouting angiogenesis involve the migration, proliferation and elongation of ECs and establishment of a vascular lumen (Bentley et al., 2008; Bentley et al., 2009; Blanco and Gerhardt, 2013; Gerhardt et al., 2003). The stalk ECs in the base are very proliferative while the tip cells are quiescent (Gelati et al., 2008; Nicosia, 2009; Nicosia et al., 2011).

Intussusception, a non-sprouting angiogenesis process, is another distinct mode of vascular formation and remodeling (Fig. 2A). In intussusceptive angiogenesis, new blood vessels are formed by transcapillary pillars which allow a rapid expansion and increasing in the capillary plexus (Djonov et al., 2000). Different from ECs in sprouting angiogenesis, ECs in intussusceptive angiogenesis undergo an extensive morphological remodeling instead of proliferation (Burri et al., 2004). The molecular mechanisms on intussusceptive angiogenesis are not clear. VEGF is a potential modulator of intussusceptive vascular growth (Hagedorn et al., 2004).

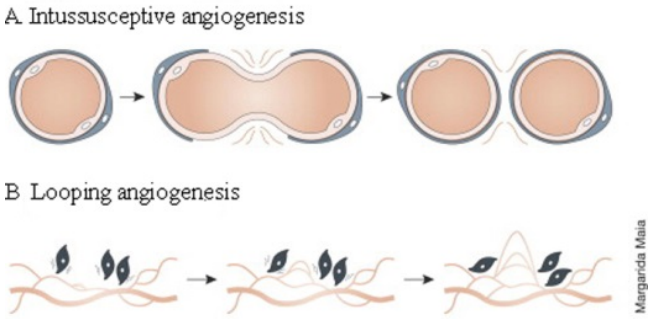

Fig. 2 (A) Intussusceptive angiogenesis: a non-sprouting angiogenesis process in which ECs undergo extensive remodeling and form transcapillary pillars. (B) Looping angiogenesis: a vascular growth process during the wound healing where pre-existing vessels translocate into the wound area and form loops (adapted from Leite de Oliveira et al., 2011).

More recently, another distinct mechanism of vascular growth called looping angiogenesis (Fig. 2B) was proposed (Kilarski et al., 2009). This proposed strategy for vascular growth describes the vascular growth in wound healing. Activated fibroblasts or myofibroblasts mediate the translocation of pre-existing vessels into the wound area as vascular loops (Kilarski et al., 2009).

1.3 TUMOR ANGIOGENESIS

Tumor vasculature has an abnormal structure and function compared to normal healthy blood vessel networks (reviewed in Jain, 2005a; Potente et al., 2011). The tumor blood vessels often lack proper coverage and support from vascular mural cells, resulting in abnormal permeability, disorganized vascular network and defective maturation (reviewed in Armulik et al., 2005). These common abnormal characteristics of the vasculature are often due to defective vascular remodeling. The vascular abnormality leads to insufficient oxygen and nutrients supply, and subsequently the onset of tumor hypoxia (De Bock et al., 2009; Jain, 2005b). Furthermore, tumor-associated ECs have also been found to contain frequent chromosomal abnormalities (Akino et al., 2009).

Tumor angiogenesis is important for tumor growth since the tumor vasculature supports the tumor with oxygen and nutrients (reviewed in Folkman, 1997a, b). The growth of vessels in tumor has emerged as a hallmark of cancer (Hanahan and Weinberg, 2000, 2011). The tumor microenvironment is composed of a heterogeneous population of cells that together modulate angiogenesis. Once emerging from dormancy, tumor cells exploit their microenvironment by secreting various factors to activate stromal cells and expand. When tumor reaches certain size, the tumor microenvironment suffers from hypoxia and nutrient deprivation. As a consequence, tumor angiogenesis is switched on by various pro-angiogenic factors in order to obtain sufficient blood supply, oxygen and nutrients for growth (Weis and Cheresh, 2011).

2. STEM CELLs/PROGENITORs CONTRIBUTING TO ANGIOGENESIS

Self-renewal, differentiation and homeostatic maintenance are three key characteristics of stem cells. In the stem cell hierarchy, stem cells generate progenitor cells with transient proliferative abilities to produce daughter cells, and progenitor cells are gradually more lineage restricted. Progenitors, unlike stem cells, do not have the capacity of self-renewal. Adult stem cells are fundamental to tissue maintenance and regeneration in adult. Most

adult tissues in mammals contain resident stem cells or tissue specific stem cells which allow adult tissues to undergo tissue regenerations or maintenance (Li and Xie, 2005). Hematopoietic stem cells in the bone marrow, mesenchymal stem cells, and epithelial stem cells in skin and intestine are the most studied adult tissue stem cells (reviewed in Li and Xie, 2005). However, adult stem cells and stem cell niches have not been identified for all tissues.

There are two major categories of cells implicated in angiogenesis: (1.) cells that contribute directly by generating vascular daughter cells, such as vascular smooth muscle cell progenitors, pericyte progenitors and potential vascular endothelial stem cells, (2.) cells that contribute indirectly by acting mostly as modulators (Fig. 3). The role of bone marrow derived cells (BMDC) in angiogenesis has been extensively studied (reviewed in Murdoch et al., 2008). Abundant evidence have been shown that bone marrow derived cells contribute to angiogenesis mostly by secreting angiogenic factors (reviewed in Fang and Salven, 2011).

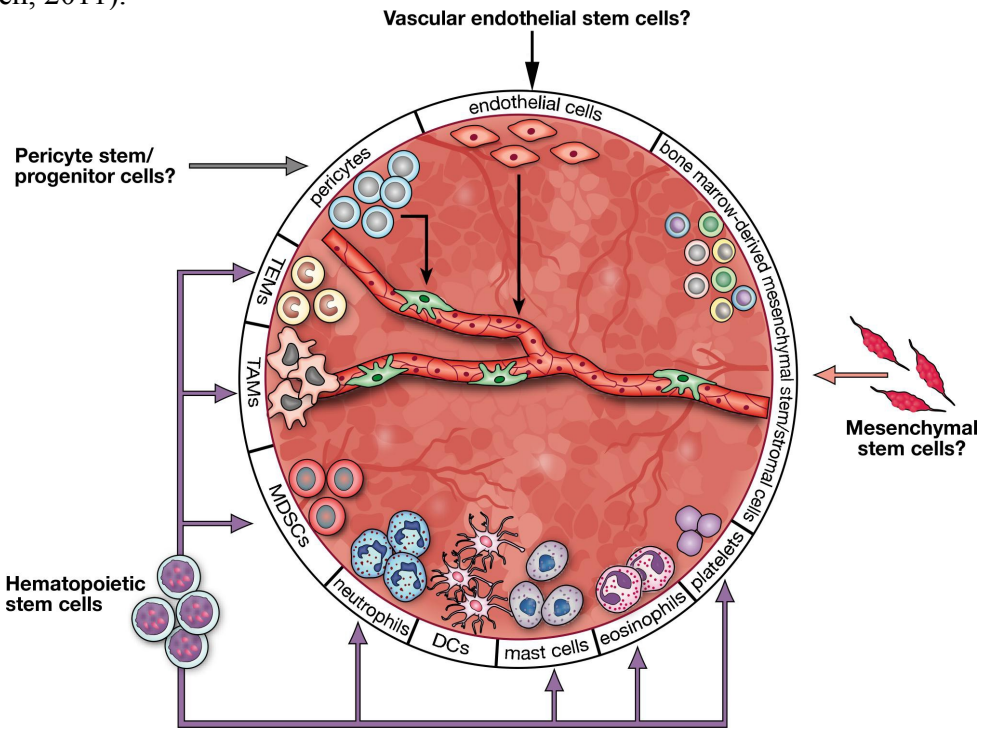

Fig. 3: Stem cells and their daughter cells that participate in angiogenesis. Various tissue infiltrating hematopoietic cells derived from BM are involved in angiogenesis in a paracrine manner. Furthermore, tissue resident stem cells may have a direct contribution to the de novo endothelium by generating endothelial daughter cells (TEM: Tie-2 expressing monocyte; TAM: tumor associated macrophage; MDSC: myeloid derived suppressor cell; DC: dendritic cell) (edited from Fang and Salven, 2011).

2.1 ENDOTHELIAL PROGENITOR CELLs (EPC) AND VASCULAR ENDOTHELIAL STEM CELLs (VESC)

The angiogenic sprouting of fully differentiated ECs from pre-existing vessels is not the only mechanism contribute to adult physiological and pathological vascular growth. Similar to vasculogenesis in embryonic development, endothelial progenitor cells were found to contribute to the adult vascular growth and participate in angiogenesis (Asahara et al., 1997). This is the first evidence of progenitors on the endothelial cell hierarchy in adult. The recruitment of EPCs to growing endothelial sprouts may combine vasculogenic

and angiogenic mechanisms (Adams and Alitalo, 2007). The paradigm of endothelial stem cells and progenitors in adult was previously dominated by studies on circulating BM-derived EPCs (Asahara et al., 1997). Recent studies have provided solid evidence on the existence of resident stem cells on the vascular wall (Goligorsky and Salven, 2013; Purhonen et al., 2008; Rinkevich et al., 2011).

Asahara et al. (1997) first described a population of putative EPCs from human peripheral blood using CD34, an antigen expressed by HSCs and human endothelial cells. These circulating progenitors express CD45, the leukocyte common antigen. This piece of finding has indicated that putative EPCs are originated from the BM (Asahara et al., 1997). It is further shown by Peichev et al. (2000) that $AC133^{+}VEGFR\text{-}2^{+}CD34^{+}$ hematopoietic cells have the ability to differentiate into mature ECs which are $AC133^{-}$ and $VEGFR\text{-}2^{+}$ in vitro. The EPC hypothesis is then further investigated by others (Garcia-Barros et al., 2003; Hur et al., 2004). In 2004, two different EPC populations, early outgrowth EPCs (CFU-EPC) and late outgrowth endothelial cells (EOC), have been described by Hur et al. (2004).

It has been found that peripheral EPCs are derived from circulating monocytes (Rehman et al., 2003). Later, Rohde et al. (2006) have found that circulating monocytes mimic vascular ECs by sharing vascular endothelial surface markers. More importantly, in in vitro models, blood mononuclear cells (MNC) are also capable to generate both types of EPCs, CFU-EPCs and EOCs (Ingram et al., 2005). Yoder and Colleagues later have analyzed the morphology of the two types of EPCs and surface antigen expressions on CFU-EPCs and EOCs in detail. Yoder et al. (2007) have pointed out that CFU-EPCs are hematopoietic derived monocyte/macrophage cell colonies. Accordingly, Rohde et al. (2007; 2006) have also shown that CFU-EPCs possess hematopoietic cell characteristics. Later, Case et al. (2007) have studied the functional properties of isolated circulating $CD34^{+}VEGFR\text{-}2^{+}AC133^{+}$ cells. Negative late outgrowth endothelial cell colony forming ability and an enriched hematopoietic progenitor activity have been observed from the isolated circulating $CD34^{+}VEGFR\text{-}2^{+}AC133^{+}$ cells (Case et al., 2007). Desai et al. (2009) have analyzed CFU-EPCs using microarray and have found that CFU-EPCs possess a predominant gene expression of T lymphocytes rather than endothelial cells. Various secretory factors are identified from CFU-EPCs, including many proangiogenic factors such as interleukin-8 (IL-8), MMP-9, thymidine phosphorylase (TP) and cathepsins (Pula et al., 2009). Most importantly, although these generated spindle shaped cells in the CFU-EPCs display endothelial lineage features in vitro, such as endothelial antigen expression and endothelial nitric oxide production, they do not form blood vessels in vivo which is an functional evaluation of the endothelial lineage (Yoder et al., 2007). Yoder et al. (2007) have also stated that CFU-Hill or CFU-EPC assays, which are modified from Asahara et al. (1997), do not truly evaluate the cellular potential of giving rise to endothelial lineage. Taken together, the CFU-EPCs described earlier are pro-angiogenic hematopoietic cells (Pula et al., 2009; Rehman et al., 2003). The CFU-EPCs are more likely to participate in neo-vessel formation in a paracrine manner but not incorporate into vessel lumens (Goligorsky and Salven, 2013; Richardson and Yoder, 2011).

Endothelial outgrowth cell colonies are formed later in culture from adherent cells. These later outgrowth cells, called EOCs or endothelial colony forming cells (ECFC), are considered clearly as endothelial cells with endothelial characteristics and vessel forming ability (Yoder et al., 2007). Ingram et al. (2004) have developed a novel method to enumerate ECFCs where they have found ECFCs with high proliferative potential (HPP-ECFC) in human umbilical cord blood. Yoder et al. (2007) have reported that ECFCs from

human umbilical cord blood have a clonogenic expansion ability and display a proliferative hierarchy. Further, the generated ECFCs form chimeric blood vessels in immunocompromised mice (Yoder et al., 2007). Interestingly, Lin et al. (2000) have isolated mononuclear cells from patients who received an sex-mismatched bone marrow transplantation and have found that the endothelial cell colonies that appeared early are host-derived with limited proliferative potential while high proliferative endothelial colonies emerge later and are of donor origin. However, the origin of the EOCs remained to be further studied.

Various in vivo models have been set up to study the angiogenic processes, such as the corneal micropocket, subcutaneous sponge/matrix microbead, subcutaneous Matrigel plug, injuries and various tumor angiogenesis models (Norrby, 2006). Many studies to date have used these models to test whether EPCs have the capacity to home to the injury site, incorporate into pre-existing vessels or form new blood vessels. The ability of in vivo new vessel formation is one of the most important characteristics in defining endothelial progenitors.

Meanwhile, endothelial lineage specific genetic markers combined with bone marrow transplantation or parabiosis are used in order to trace cells of origin in angiogenesis. More importantly, it is now possible to use an EC-specific promoter, such as VE-cadherin-Cre/Rosa26R, to drive gene expression in an endothelium restricted manner. These methods have enabled cellular fate mapping and tracking in a time and spatially controlled way in the vasculature (Eklund et al., 2013).

For instance, Tie-2 is a receptor tyrosine kinase expressed by both endothelial cells and hematopoietic cells (Hamaguchi et al., 1999). In 2003, De Palma and colleagues have performed Tie2p/e-GFP tagged bone marrow transplantation to mice. Numerous Tie-2^{+} monocytes (TEM) are recruited in the tumor perivascular area. They hasn't observed any incorporation of bone marrow derived cells into the blood vessel wall in all three tumor models including LLC, B16 melanoma and mammary carcinoma (De Palma et al., 2003). Later, Gothert et al. (2004) have used a transgenic mouse with a tamoxifen-inducible reporter system driven by stem cell leukemia-1 (SCL-1) endothelial enhancer to study the contribution of BMDCs in tumor angiogenesis. No bone marrow derived cells have been found in the newly formed blood vessels (Gothert et al., 2004). Purhonen et al. (2008) also have further studied the question whether BM-derived EPCs is able to incorporate into newly-formed blood vessels in various models including adenoviral VEGF induced angiogenesis, subcutaneous Matrigel plugs, subcutaneous B16 melanomas and APCmin adenomas. To trace the bone marrow derived cells, a non-pathological model of parabiosis which connects the circulation of transgenic mice expressing GFP with wild type mice has been used in the study, as well as the bone marrow reconstitution using transgenic mice expressing GFP or DsRed (Purhonen et al., 2008). Various time points, from one day to six months, have been studied. However, BM-derived ECs have not been identified on the vascular wall (Purhonen et al., 2008). On the other hand, using a parabiosis model, Kim et al. (2009) have shown that $CD31^{+}F4/80^{+}$ monocytes, instead of circulating EPCs, contribute to angiogenesis upon an ischemic recruitment, which are probably first recruited to the angiogenic site to guide the vascular growth. Besides these tumor angiogenic models or neo-angiogenic models, the possible incorporation of BM-EPCs in compensatory lung growth or unilateral femoral artery occlusion models has also been investigated (Voswinckel et al., 2003; Ziegelhoeffer et al., 2004). BM-derived EPCs do not incorporate into the vasculature in either model (Voswinckel et al., 2003;

Ziegelhoeffer et al., 2004). Over the time, using different angiogenic models, time points and detection methods, various researchers have shown an undetectable or negative incorporation of BM-derived cells to blood vascular wall (Ahn and Brown, 2008; Beck et al., 2003; De Palma et al., 2005; De Palma et al., 2003; Dudley et al., 2010; Gothert et al., 2004; Hagensen et al., 2010; Machein et al., 2003; Okuno et al., 2011; Perry et al., 2009; Purhonen et al., 2008; Rajantie et al., 2004; Shinde Patil et al., 2005; Voswinckel et al., 2003; Wickersheim et al., 2009; Ziegelhoeffer et al., 2004).

Collectively, Richardson and Yoder have suggested that the terms already existent for each cell subsets should be used in referring to the circulating cells in angiogenesis and the term of EPC should retire (Richardson and Yoder, 2011).

Although rare circulating endothelial progenitor cells may exist (Bertolini et al., 2006; Lin et al., 2000), they most likely derive from resident blood vessels. Human umbilical vein endothelial cells (HUVEC), umbilical cord blood derived EPCs and human aortic endothelial cells (HAEC) are all differentiated ECs derived from vessel wall and are able to be passaged for over 40 population doublings (Bompais et al., 2004; Dernbach et al., 2004; Ingram et al., 2005; Schniedermann et al., 2010). Very interestingly, Ingram et al. (2005) have identified a subset of cultured vessel wall derived ECs with highly proliferative ability and in vivo vessel formation capacity. More recently, Weissman's group has used fate mapping and clonal analysis methods to study tissue of origin that is involved digit tip regeneration (Rinkevich et al., 2011). Rinkevich et al. (2011) have shown that circulating progenitors do not contribute to the vessel wall in digit tip regeneration. From their results, they have concluded that it is local non-circulating tissue resident cells that are responsible for generating new BECs for adult vascular growth.

These accumulating findings hint for a possible existence of tissue resident VESCs that respond to angiogenic stimulus and are capable of generating endothelial cells during vascular growth in adults (Fang and Salven, 2011). New findings have now shifted the paradigm in endothelial cell hierarchy to local stem and progenitor cells (Goligorsky and Salven, 2013; Purhonen et al., 2008; Rinkevich et al., 2011). However, the identification of local stem cells for endothelium is still unknown.

Over the years, much effort has also been endeavored to identify and characterize the cellular origin of ECs using cell surface markers. Identifying EPCs/VESCs from mature ECs or other progenitors based solely on surface antigen expression is challenging because many cell types share the same surface antigens. Originally, Asahara et al. (1997) have postulated the usage of available known antigens expressed on angioblasts and HSCs to identify putative EPCs. On the hunt for the surface marker identities of putative endothelial progenitors, flow cytometry is a very useful and indispensable tool. Cell surface markers that identify stem/progenitor cells and endothelial cells can be used in combination to detect, quantify and isolate putative endothelial progenitors from cell sources for further functional analyses (Barber and Iruela-Arispe, 2006). However, this may still include side populations that do not directly contribute to the adult vascular growth. With the limitation of cell surface marker characterization, genetic fate mapping and other lineage tracking techniques are needed for the identification of VESCs (Barber and Iruela-Arispe, 2006). It is very important to demonstrate the putative VESCs have the ability to give rise to mature functional ECs both in vitro and in vivo.

2.2 HSCs CONTRIBUTE TO ANGIOGENESIS IN A PARACRINE MANNER

Pluripotent hematopoietic stem cells (HSC) are the cellular progenitor pool of all lineages of blood cells. HSCs give rise to myeloid cells and lymphoid cells through lineage-restricted differentiation. Compelling data have shown that bone marrow derived cells are recruited to the local environment and play important roles in local angiogenesis process (Fig. 3).

Studies have supported that BMDCs presumably function as angiogenic effector cells in angiogenesis. BMDCs promote angiogenesis mostly by expressing various cytokines. Currently, TEMs, mast cells, and myeloid derived suppressor cells (MDSC) have been extensively studied on their roles in angiogenesis (reviewed in Fang and Salven, 2011; Jain and Duda, 2003; Murdoch et al., 2008).

2.2.1 MYELOID DERIVED SUPPRESSOR CELLs (MDSC)

MDSCs are one heterogeneous cell population, including myeloid precursors and immature myeloid cells, regulating immune response and angiogenesis. These immature myeloid cells from the bone marrow are progenitors of neutrophils, monocytes, granulocytes and dendritic cells (DC). Their activation and proliferation are influenced by factors produced by cells in the microenvironment. MDSCs are characterized by the expression of CD11b, widely expressed by myeloid lineage, and Gr1, markers for neutrophils. In mouse, MDSCs are termed as $CD11b^{+}Gr1^{+}$ cells and are broadly separated into 2 groups which are $CD11b^{+}Gr1^{+high}$ and $CD11b^{+}Gr1^{+low}$ respectively (Yang et al., 2004). MDSCs secrete proangiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), protease such as MMP-9, monocyte chemotactic protein-1 (MCP-1) and IL-8, and accelerate vessel remodeling (Boelte et al., 2011; Ferrara, 2010; Finke et al., 2011; Fridlender et al., 2012; Guedez et al., 2012).

2.2.2 MACROPHAGEs AND DENDRITIC CELLs (DC)

Infiltrating macrophages are heterogeneous and are able to polarize into two different activation states upon recruitment, M1 and M2 macrophages. M1 macrophages are pro-inflammatory and promote T-helper 1 (Th1) activation by IFN-γ. They are protectors to defend the host from infections and tumors by activating immune responses. M2 macrophages, on the contrary, exhibit an immunosuppressive phenotype and induce T cell destruction (Orlikowsky et al., 1999). Tumor associated macrophages (TAM) have been extensively studied. It has been postulated that M2-TAMs are pro-tumor and proangiogenic, while M1-TAMs are pro-inflammatory and anti-tumor (reviewed in Sica et al., 2008). TAMs provide a plethora of angiogenic factors, such as VEGF, bFGF, IL-1β, ECM-degrading proteases and matrix remodeling enzymes (Baer et al., 2013; Carmi et al., 2009; Coffelt et al., 2009; Giraudo et al., 2004; Lewis et al., 2000; Ribatti et al., 2007). These macrophage-secreted factors can enhance the proliferation and survival of ECs and promote ECM remodeling for vascular sprouts (Bergers et al., 2000; Sierra et al., 2008; Squadrito and De Palma, 2011). Macrophages have also been found to modulate vasculature via direct transfer of exosomes by microvesicles (Simons and Raposo, 2009). Furthermore, macrophages can act as a cellular chaperon by direct cell-cell contacts with ECs to facilitate sprouting angiogenesis. Fantin and co-workers have demonstrated that macrophages facilitate endothelial tip cell fusion by cell-to-cell interaction (Fantin et al., 2010). However, macrophages also negatively regulate the vasculature. In hyaloid vessels,

Wnt7b expressing macrophages stimulate ECs to enter into cell cycle via cell-cell contracts which leads to scheduled vascular regression (Lobov et al., 2005). In conclusion, the angiogenic activity of TAMs and their subsets is most likely to rely on their abilities to secrete angiogenic factors, their close contacts to blood vessels and interactions with endothelial cells (Carmi et al., 2009; Coussens et al., 2000; De Palma et al., 2005; Lewis et al., 2000; Lin et al., 2006; Ribatti, 2009).

DCs are an important cell population mediating innate and adaptive immune responses. They are the major representatives of antigen presenting cells (APC) and induce T cell and B cell responses along with immune tolerance (reviewed in Colonna et al., 2004). DCs modulate angiogenic response by secreting vasoactive mediators (Riboldi et al., 2005). Tumor associated plasma cytoid DCs secrete tumor necrosis factor-α (TNFα) and IL-8 to support angiogenesis (Curiel et al., 2004). Immature DCs have been shown to promote tumor vascularization via β-defensin and VEGF-A (Conejo-Garcia et al., 2004). Furthermore, the ablation of DCs results in an impaired angiogenesis and an inhibited tumor growth in mice (Fainaru et al., 2010). Very recently, $CXCR4^+$ DCs have been found to be localized in close contact to the decidual vasculature and produce proangiogenic factors (Barrientos et al., 2013).

2.2.3 NEUTROPHILs, EOSINOPHILs AND MAST CELLs

Neutrophils, differentiated from the myeloid progenitor cells in the BM, are the most plentiful leukocytes in the bloodstream (reviewed in Murdoch et al., 2008; Tazzyman et al., 2009). Similar to TAMs, tumor associated neutrophils (TAN) are also categorized into two distinct subpopulations, called N1 and N2 (Fridlender et al., 2009). Increasing infiltrating neutrophils are found in many human tumors including melanomas, gastric and colon cancer (Eck et al., 2003; Mhawech-Fauceglia et al., 2006; Nielsen et al., 1996). Neutrophils are crucial to physiological angiogenesis where they secrete VEGF to regulate vascular proliferation (Mueller et al., 2000). Benelli et al. (2002) have observed that angiogenesis in Matrigel is significantly inhibited when neutrophils are depleted from mice, indicating a positive role of neutrophils in angiogenesis. Further support to elucidate the role of neutrophils in angiogenesis has been provided by other studies (reviewed in Murdoch et al., 2008; Tazzyman et al., 2009). Nozawa et al. (2006) have found that TANs produce an abundant amount of MMP-9 during the neo-vascularization in a pancreatic islet carcinogenesis model. Nozawa and colleagues have depleted neutrophils transiently and have found that the association between VEGF and VEGFR is suppressed (Nozawa et al., 2006). As a result, the frequency of angiogenic switch on-set is also largely reduced during early carcinogenesis process (Nozawa et al., 2006). Later, Ardi et al. (2007) have shown that human TANs release TIMP-free MMP-9 as a catalytic angiogenic stimulator. However, the relationship between infiltrating neutrophils and angiogenesis is a "friend or foe" situation. Data have also shown that neutrophils regulate angiogenesis negatively. Neutrophils could produce proteases and elastase to cleave plasminogen into small anti-angiogenic molecules (Scapini et al., 2002).

Eosinophils are a small subpopulation of white blood cells. They constitute only 1-3% of peripheral nucleated cells (Iwasaki et al., 2005). Eosinophils have a crucial role in immune defense and allergic reactions (Legrand et al., 2008; Rothenberg and Hogan, 2006). Eosinophils are most likely to contribute to angiogenesis by a paracrine manner. VEGF is released from eosinophils upon stimulation by GM-CSF or IL-5 (Horiuchi and

Weller, 1997). Furthermore, it has been proposed that eosinophils may induce angiogenesis by secreting tissue factors (TFs) (Moosbauer et al., 2007).

Mast cells are bone marrow derived leukocytes. Mast cells are crucial to many pathological and physiological processes, such as inflammation, angiogenesis and cancer. They are often found in the tumor perivascular area, indicating a potential function in tumor angiogenesis (reviewed in Maltby et al., 2009). An elevated mast cell level is observed in numerous tumors such as breast tumor, myeloma and oral squamous cell carcinoma (Conti et al., 2007; Fisher et al., 1985; Iamaroon et al., 2003; Ribatti et al., 1999; Theoharides and Conti, 2004). During vascular growth, mast cells secrete various angiogenic factors, such as VEGF, bFGF, IL-8, TGF-β and TNFα, heparin and MMPs (Coussens et al., 1999; Coussens et al., 2000; Grützkau et al., 1998; Halova et al., 2012; Möller et al., 1998; Qu et al., 1998; Walsh et al., 1991).

2.2.4 PLATELETs

The hemostasis on vasculature is important during the lifetime. A normal platelet level in the blood stream is crucial for vascular hemostasis. Platelets originate from megakaryocytes in the BM. Platelets serve as a cellular reservoir of various growth factors, cytokines, chemo-attractants, and many other molecules involved in angiogenesis. There are three types of secretory vesicles in platelets: α-granules, dense granules and lysosomes, containing different factors and cytokines. Thus, upon activation platelets could release many pro-angiogenic or anti-angiogenic factors (reviewed in Jain and Duda, 2003). In the tumor microenvironment, platelets release many angiogenic regulatory proteins (Klement et al., 2004). Platelets take up angiogenic factors and sequester them in α-granules (Klement et al., 2009). More recently, Feng et al. (2011) have shown that platelets facilitate the recruitment of proangiogenic cells from BM to local tissues and promote angiogenesis. Besides the secretion of angiogenic regulatory factors, platelets are able to affect angiogenesis by the shedding of microparticles. Platelet microparticles act as a delivery machinery to transport proteins such as angiogenic regulatory factors and other bioactive molecules to other cells (Italiano et al., 2010; Janowska-Wieczorek et al., 2006).

3. TRANSFORMING GROWTH FACTOR-β (TGF-β) AND ANGIOGENESIS

TGF-β superfamily consists of many cytokines, such as TGF-βs and bone morphogenetic proteins (BMP). There are three isoforms of the TGF-β ligand, TGF-β1, TGF-β2 and TGF-β3. TGF-β dimers induce a heteromeric complex formed by type I (activin receptor-like kinases (ALK)) and type II receptors (including activin receptor type IIA (ActRIIA), ActRIIB, BMP type II receptor, TGF-β type II receptor and type II anti-Müllerian hormone receptor). Once the heteromeric complex is formed, the type II receptor phosphorylates the type I receptor. Signals then propagate into cells via downstream molecules. There are two TGF-β signaling pathways, canonical and non-canonical. The canonical pathway is Smad-dependent. In the non-canonical TGF-β pathway, the signals are transmitted through TNF receptor associated factor 4, p38, JUN N-terminal kinase (JNK) or some other factors (reviewed in Akhurst and Hata, 2012; Massagué, 2012).

TGF-βs are involved in many vital biological processes including embryonic development. TGF-βs serve as an immune-regulatory mediator because of their pro-and anti-inflammatory properties. TGF-β and its isoforms are required for hematopoiesis and

endothelial differentiation in the embryonic development (reviewed in Piek et al., 1999). The role of TGF-β in angiogenesis, vascular homeostasis and vascular disease has been extensively investigated (Pardali et al., 2010; reviewed in ten Dijke and Arthur, 2007; Walshe, 2010). TGF-βs have the capability to modulate vascular development and remodeling (Gorden et al., 1997; Mandriota and Pepper, 1997; Pepper et al., 1993). Targeted disruption of TGF-β1 gene in mice results in mortality during mid-gestation or at an age of 3 weeks (Pepper, 1997). The targeted disruption in TGF-β1 causes defects in yolk sac vasculature and hematopoiesis. Thus 50% of TGF-$\beta1^{-/-}$ embryos are mortal during mid-gestation. Others have completed embryogenesis but exhibit dysfunction in their immune system after weaning. One possible explanation is that the maternal TGF-β1 may compensate the lack of TGF-β1 in the offspring (Pepper, 1997). It has also been demonstrated that mutations of proteins in the TGF-β pathway in mice result in defective vascular development and thus lead to embryonic lethality (Choi and Ballermann, 1995; Dunker and Krieglstein, 2000). A few decades ago, it has been shown that subcutaneous injection of TGF-β in newborn mice evokes a prominent angiogenic response in vivo (Roberts et al., 1986).

Although the importance of TGF-β in angiogenesis is apparent, its detailed mechanism is less clear, very complicated and with controversial. Firstly, an inhibitory effect caused by TGFs on the proliferation of cultured endothelial cells has been observed (Baird and Durkin, 1986; Fràter-Schröder et al., 1986). The inhibitory effect of TGF- β is supported by further studies. TGF-β also inhibits angiogenesis and induces EC apoptosis (Lu et al., 2009; Walshe et al., 2009a; Walshe et al., 2009b). However, conflicting reports demonstrate that TGF-β also has a mitogenic and proliferative effect (Iruela-Arispe and Sage, 1993; RayChaudhury and D'Amore, 1991; Sutton et al., 1991). Studies have shown that TGF-β induces tube formation (Gajdusek et al., 1993; Kuzuya and Kinsella, 1994). These controversial results of TGF-β regulating angiogenesis indicate a complex mechanism by which TGF-β modulates angiogenesis (Fierlbeck et al., 2003; Pepper et al., 1993). Pepper et al. (1993) have shown that TGF-β stimulates cell proliferation, tube formation and migration at low concentrations (100 pg/ml - 1 ng/ml) but inhibits these processes with a higher concentration (5-10 ng/ml). TGF-β induced endothelial cell apoptosis contributes to the formation of glomerular capillary lumen (Fierlbeck et al., 2003). Interestingly, TGF-β can activate EC proliferation and migration via TGF-β/ALK1 axis, whereas it inhibits the proliferation and migration of ECs through the TGF-β/ALK5 pathway (Goumans et al., 2003; reviewed in Lebrin et al., 2005).

In most tissues, including the endothelium, TGF-β binds to the type II receptor, then recruits and phosphorylates ALK5 (Franzén et al., 1993). Signals then transduce to Smad proteins (Smad 2, 3 and 4) (Chen et al., 1998; reviewed in Jakobsson and van Meeteren, 2013). Besides ALK5, ECs specifically express ALK1, another type I receptor (Panchenko et al., 1996; Roelen et al., 1997). Upon ligand activation, signals further propagate to Smad 1, 5 and 4 (reviewed in Jakobsson and van Meeteren, 2013). In addition, endoglin, a type III receptor, is also expressed on ECs. It has shown that endoglin can reduce the activation of TGF-β signaling (Barbara et al., 1999; Letamendía et al., 1998). An study on ALK1 mutant embryo implies a balance between ALK1 and ALK5 signaling pathway in ECs may be critical to the interplay of TGF-β on endothelium (Oh et al., 2000). However, to date, the mechanism of action of TGF-β in angiogenesis is still not clear.

In addition to the direct effect on ECs, TGF-β has a chemotactic effect on pericytes and fibroblasts (Antonelli-Orlidge et al., 1989; Joseph-Silverstein and Rifkin, 1987;

Postlethwaite et al., 1987). Furthermore, TGF-β also affects vascular mural cells and immune cells by its interaction with receptors. TGF-β has been shown to affect VSMCs via ALK5/TβR-II complex (Carvalho et al., 2007). The downstream signaling then involves Smad2/3 and p38, p42/44 and JNK (Feinberg et al., 2004a; Feinberg et al., 2004b; Hocevar et al., 2005; Seay et al., 2005). It has been speculated that TGF-β may serve as a potential vascular smooth muscle differentiating factor (Seay et al., 2005). Wang et al. (2008) have shown that TGF-β stimulates MSCs to differentiate into pericytes and thus promotes vascular maturation. Many immune effective cells such as macrophages are responsive to TGF-β. The TGF-β1 null mice have a multifocal immune response (Kulkarni et al., 1993; Shull et al., 1992). Similarly, in vivo TGF-β deficiency leads to an inhibited inflammatory response (Wang et al., 2010b).

Taken together, the mechanism of TGF-β in adult angiogenesis is not very clear. It is not well understood how TGF-β affects adult vasculature in vivo and contributes to angiogenesis because of the complex interactions among infiltrating cells, circulating blood cells and resident blood vessels and the complexity that how TGF-β signaling pathway is transduced.

4. ADULT STEM CELLs AND THEIR NICHEs

A stem cell niche is not considered to be simply a physical location or structure, but rather an environment where extrinsic signals interact and integrate to affect the behavior of stem cells. It is an interactive in vivo microenvironment where stem cells reside and are nurtured to maintain tissue homeostasis and generate progenitors in a proper manner (Scadden, 2006; Schofield, 1978). The general niche is composed of resident stem cells and niche cells. Niche cells are cells from heterogeneous cell populations. They provide a supporting environment for stem cells to maintain a proper stem cell capacity and protect stem cells from any challenge from outside stimuli (Crittenden et al., 2002; Kiger et al., 2000; Xie and Spradling, 2000). Within the niche, stem cells maintain the ability to be activated to produce progenitors or transit amplifying cells and self-renew. The stem cell niche has the function to assure a balance of activation and quiescence in stem cells. The niche possesses not only anatomic but also functional dimensions (Scadden, 2006).

The idea that heterologous cell populations compose the niche was first shown in the gonads of Drosophila melanogaster and C. elegans (Kiger et al., 2000; Xie and Spradling, 2000). This idea is then further supported by the identification of osteoblast in the bone marrow (Calvi et al., 2003; Kiel et al., 2005; Zhang et al., 2003). In mammals, established adult stem cell niches are found in bone marrow, hair follicle, and intestine (reviewed in Li and Xie, 2005; Moore and Lemischka, 2006).

4.1 THE HEMATOPOIETIC STEM CELL NICHE IN THE BONE MARROW

The hematopoietic stem cell niche had not been identified for many years. HSCs are not only located in BM but also are found in other tissues including adult liver (Taniguchi et al., 1996). Much effort has been put to investigate HSCs in the BM and their anatomical locations (Calvi et al., 2003; Heissig et al., 2002; Kiel et al., 2007; Kiel et al., 2005; Kimura et al., 2011; Zhang et al., 2003). The HSC niche is now identified in the bone as a microenvironment where groups of cells locate in the specific tissue location for stem cell maintenance (Fig. 4). Recent findings has shown that the niche in the bone marrow is perivascular, partly composed by endothelial cells and mesenchymal stromal cells (reviewed in Morrison and Scadden, 2014). The niche expresses a series of molecules

important for tissue specific stem cell homing, proliferation, differentiation and self-renewal (Li and Xie, 2005; Scadden, 2006; Yin and Li, 2006). The niche also signals to control the fate and amount of stem cells (reviewed in Li and Xie, 2005; Moore, 2004; Moore and Lemischka, 2004, 2006). HSC maintenance and hematopoiesis is primarily happened in the bone marrow in adult, whereas in some occasions it is also found in extramedullary sites (Kim, 2010; Morrison and Scadden, 2014).

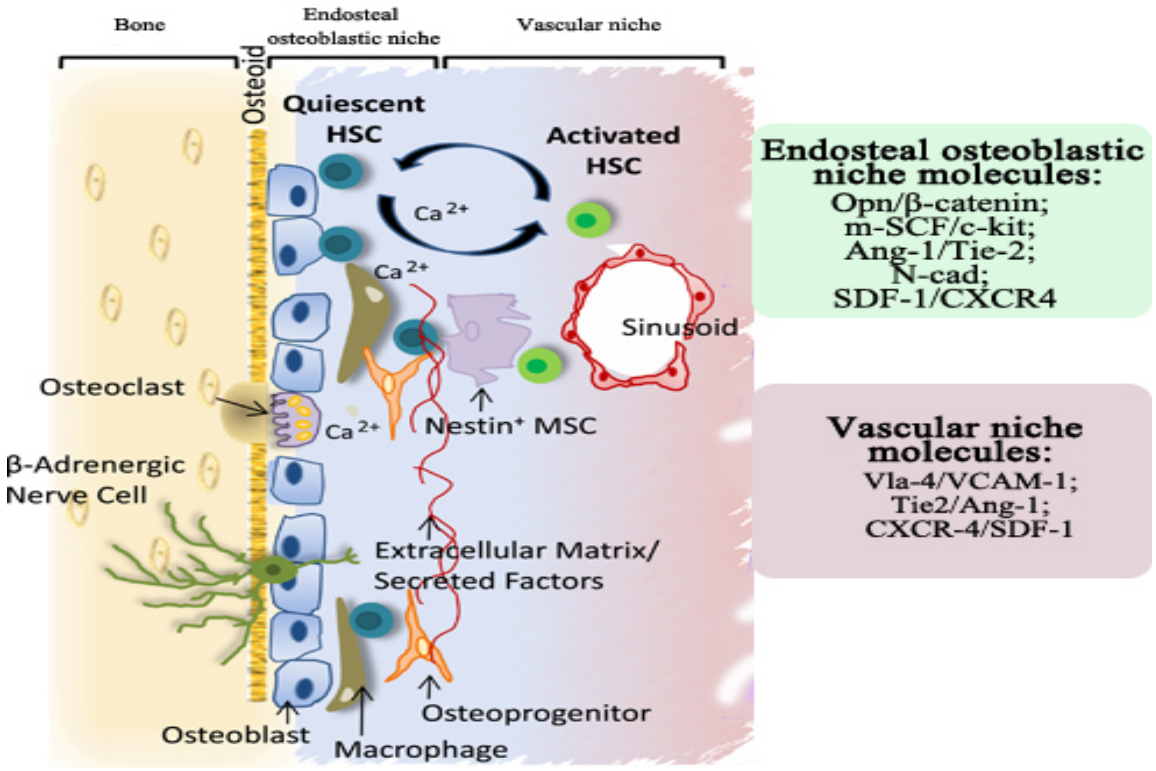

Fig. 4. The HSC niche in the bone marrow. Schematic graph illustrates interactions among HSCs, endosteal osteoblastic niches and vascular niches. HSCs dock to osteoblasts and recruit to the vascular niche close to sinusoidal endothelium (edited from Levesque et al., 2010).

Currently, HSC niche in the bone is composed of two niches, endosteal osteoblastic niche and vascular niche. Osteoblasts (OB) compose the endosteal osteoblastic niche and protect HSCs in the BM (reviewed in Taichman, 2005). In 2002, Heissig et al. (2002) have found that HSCs and hematopoietic cell clusters are recruited to the so-called "vascular zone"-a vascular niche in the BM. Osteoblastic and vascular niches are crucial in HSC maintenance and mobilization. Once HSCs are mobilized, HSCs leave the osteoblastic niche, are recruited to the vascular niche, and then enter the blood vessel (Yin and Li, 2006).

4.1.1 ENDOSTEAL OSTEOBLASTIC NICHE

In the BM, HSCs are identified in close contact with osteoblasts within the endosteal region in steady state or upon homing after BM transplantation (Nilsson et al., 1997; Xie et al., 2009; Zhang et al., 2003). It has been reported that osteoblasts' quantity in the BM affects HSC number (Calvi et al., 2003; Zhang et al., 2003). Later, the osteolineage cells have been proven to be involved in HSC maintenance (Visnjic et al., 2004). Indeed, OBs express many molecules important for HSC maintaining, self-renewal and survival, such as Ang-1 (Arai et al., 2004).

There are several signaling axes important to osteoblastic niche function, such as N-cadherin/β-catenin, Tie-2/Ang-1, osteopontin/β-integrin and stem cell factor (SCF)/c-kit (reviewed in Yin and Li, 2006). N-cadherin and β-catenin are adherens junction molecules. N-cadherin is expressed by osteoblasts. Both molecules could be used as ectopic markers of osteoblastic niche (Zhang et al., 2003). HSC anchorage and quiescence are regulated through Tie-2/Ang-1 signaling (Arai et al., 2004). Enhanced Ang-1/Tie-2 signaling has been shown to improve the long term repopulation capacity in HSCs in vivo (Ikushima et al., 2013). OBs also produce osteopontin, an extracellular matrix molecule. Osteopontin is able to retain HSCs in endosteal region and enhance the primitive HSCs in quiescent status through β1-integrin (Nilsson et al., 1997). Osteopontin expression on the osteoblasts has been found to inhibit HSC proliferation (Stier et al., 2005). SCF, also known as steel (Sl) factor or KIT ligand, regulates HSC activity in vivo and self-renewal in vitro (Fleming et al., 1993). The Sl/Sl mice have a defective niche in supporting HSCs; however SCF/c-kit interaction may not be the key element to initiate hematopoiesis and self-renewal of HSCs at least in fetus (Ikuta et al., 1991; Ikuta and Weissman, 1992). Furthermore, significant amount of SCF secretion by OBs can increase the adherence of HSC to stromal cells (reviewed in Yin and Li, 2006). However, OBs are not the only cell type residing in the niche area and affecting the function (Scadden, 2006). Current evidence indicates that the effect of osteoblastic cells on HSCs may be indirect. In vivo imaging studies have shown that very few HSCs are in direct contact with osteoblasts (Kiel et al., 2009; Kiel et al., 2005; Lo Celso et al., 2009; Nombela-Arrieta et al., 2013; Sugiyama et al., 2006). Furthermore, genetic modification of primitive osteolineage cells or mature osteoblasts has little effect on HSC proliferation (Raaijmakers et al., 2010). Many other types of cells including stromal cells or perivascular cells may take part in the hematopoietic niche, which requires further study.

4.1.2 VASCULAR NICHE

Investigations have later characterized the existence of the vascular niches, where HSCs reside near sinusoids within the BM (Heissig et al., 2002; Kiel et al., 2005). The vascular niche is believed to promote the proliferation and differentiation of actively cycling or transient HSCs (Heissig et al., 2002; Kiel et al., 2005). $Lin^-CD41^-CD48^-CD150^+$ HSCs are found largely to be in contact with sinusoidal endothelium (Kiel et al., 2005). The BM sinusoidal endothelial cells (BMSEC) possess distinct phenotypic and morphologic characteristics compared to other vascular ECs (Aird, 2007a, 2012; Nolan et al., 2013). BMSECs express Notch ligands, Jagged-1 and Jagged-2, suggesting a possible interaction of Notch signaling with HSCs (Butler et al., 2010). Furthermore, the deletion of SCF on ECs causes a lower long-term-HSC frequencies and repopulation capacity (Broudy, 1997; Ding et al., 2012).

Besides BM vasculature, cells in close contact with the vasculature also have been considered as cellular niche components. CXCL12 (SDF-1) expressing cells have been found to be adjacent to the sinusoids in the BM. SDF-1 is important for HSC mobilization, homing and engraftment (Lapidot and Kollet, 2002; Sugiyama et al., 2006). In summary, endothelial cells in the vascular niche enhance and maintain the HSC primitive property and are crucial for the maintenance of the HSC pool.

5. ROLE OF C-KIT IN STEM CELLs

5.1 C-KIT AND W LOCI

C-kit, a type III receptor protein-tyrosine kinase, is the receptor for SCF. C-kit is the cellular counterpart of v-kit oncogene, encoded by the W locus in mouse where many mutations are known (Lev et al., 1994). The W locus encoding KIT, also known as the dominant white spotting locus, was first identified in mice with defects in mast cell development and hematopoiesis in the early 90s (Lev et al., 1994). C-kit and its interaction with SCF are crucial for many physiological processes such as hematopoiesis, pigmentation and fertility (Ikuta et al., 1991; Koshimizu et al., 1991; Lennartsson and Rönnstrand, 2012; Parrott and Skinner, 1999). C-kit is widely expressed on hematopoietic stem cells/progenitors as well as many other differentiated cell types including mast cells, melanocytes, endothelial cells, endocrine cells and epithelial cells (Ashman, 1999; Broudy et al., 1994; Lammie et al., 1994; Majumder et al., 1988; Natali et al., 1992; Nocka et al., 1989). Importantly, c-kit mRNA is also found in embryonic brain in mice (Keshet et al., 1991; Qiu et al., 1988).

5.2 FUNCTION OF C-KIT

The KIT-null mutations result in embryonic lethality with severe anemia (Russell, 1979). Some other homozygous KIT mutations, such as W-sash mutation, W41 mutation and Wv mutation are viable but have effects on fertility, pigmentations and mast cells development (Chabot et al., 1988; Duttlinger et al., 1993; Reith et al., 1990).

C-kit expression is detectable on early hematopoietic cells and is lost gradually during differentiation (Broudy, 1997). Thus, c-kit is usually used as a progenitor cell/stem cell surface marker to identify putative progenitors. SCF/c-kit axis is important for the proliferation and survival of HSCs (Fleming et al., 1993; Ikuta et al., 1991; Ikuta and Weissman, 1992). It is also involved in the migration, differentiation as well as proliferation of germ cells and melanoblast lineages (Keshet et al., 1991; Matsui et al., 1991; Orr-Urtreger et al., 1990). Conditional KIT knockout in hematopoietic stem and progenitor cells (HSPC) within the BM in adult mice leads to hematopoietic failures (Kimura et al., 2011).

Besides its role in hematopoiesis, c-kit also has an important role in the cardiovascular systems. C-kit dysfunction results in a defect in cardiac stem cell differentiation (Li et al., 2008). It has been shown that mice with impaired function in c-kit carry cardiac failure after myocardial infarction (Li et al., 2008).

The SCF/c-kit axis is also crucial for many other tissues during development such as lung, heart and nervous system (Bernex et al., 1996). KIT, on the chromosome 5, has been shown to associate with airspace enlargement, which may protect the integrity of lung tissue by regulating the proliferation of local resident progenitors (Lindsey et al., 2011; Reinhard et al., 2005).

In addition, SCF stimulates the survival and the ability of capillary formation in HUVECs indicating a positive role of c-kit/SCF in angiogenesis (Matsui et al., 2004). SCF/c-kit signaling also interacts with VEGF signaling. VEGF expression is suppressed upon KIT inactivation using imatinib mesylate/STI-571, a tyrosine kinase inhibitor, in human cancer cells (Ebos et al., 2002). The expression of VEGF is mediated by SCF/c-kit signaling via PI3K/Akt pathway (Litz and Krystal, 2006; Matsui et al., 2004). Furthermore,

in a murine tumor models, imatinib mesylate disrupts lymphoma angiogenesis by targeting PDGF-β+ VSMCs (Ruan et al., 2013).

6. TARGETING ANGIOGENESIS IN HUMAN DISEASE

Blood vessel maintenance and growth regulation are vital for human health. Insufficient angiogenesis or excessive vascular growth is involved in many human diseases, such as coronary artery disease, stroke, ischemic disease, and tumor growth and metastasis.

6.1 ANGIOPREVENTION IN HUMAN CANCER

During cancer growth, angiogenic switch is turned on to promote vascular sprouting. Angiogenesis has long been a therapeutic target in cancer treatment, based on the concept raised by Folkman in the 1970s (Folkman, 1971; Hanahan and Folkman, 1996; Hanahan and Weinberg, 2011; Potente et al., 2011). Preventing vascular ECs from proliferating and migrating upon stimulation is one approach to disrupt angiogenesis. Another possible way is to inhibit angiogenic proteins/molecules in the microenvironment (reviewed in Kerbel and Folkman, 2002). Numerous studies have revealed various growth factors and signaling pathways involved in angiogenesis that provide potential targets for anti-angiogenic therapies (Davis and Yancopoulos, 1999; O'Reilly et al., 1994a; O'Reilly et al., 1994b; Tolsma et al., 1993; Weis and Cheresh, 2011; Zhai et al., 1999a; Zhai et al., 1999b).

Among these various angiogenic growth factors, the VEGF family has been studied with most extensive effort. In preclinical models, tumor growth is inhibited by blocking VEGF (Chung and Ferrara, 2011; Gerber and Ferrara, 2005; Presta et al., 1997; Saharinen et al., 2011; Warren et al., 1995; Wildiers et al., 2003). For instance, bevacizumab inhibits VEGF binding to VEGF receptors, VEGFR-1 and VEGFR-2 (reviewed in Jain, 2005a). Bevacizumab has been approved to treat patients suffering from late stage colon cancer and non-small-cell lung cancer, kidney and brain cancers (Bergers and Hanahan, 2008). Molecule inhibitors of VEGFR tyrosine kinase (TKI) are also developed as anti-angiogenic agents. Sunitinib and sorafenib, small molecular tyrosine kinase inhibitors, have been demonstrated to block the activation of VEGFRs and other kinases and thus to inhibit cancer growth (Demetri et al., 2006; Escudier et al., 2007; Motzer et al., 2007).

Various angiogenesis inhibitors have been shown to suppress tumor growth and metastases, and to improve chemotherapy efficacy in certain combination (Bagri et al., 2010; Gerber and Ferrara, 2005). However, the lengthening of progression-free survival (PFS) of angiogenesis inhibitors in clinics is not always sustained (reviewed in Sennino and McDonald, 2012). The overall survival improvement is modest.

Notably, VEGF signaling inhibitors are also possibly able to promote tumor escape and progression (reviewed in Ebos and Kerbel, 2011). One possible mechanism of the resistance to anti-angiogenic therapies is that alternative pro-angiogenic pathways are activated or up-regulated in the tumor (Bergers and Hanahan, 2008). Many other growth

factors, such as Ang-1/2, FGF and PDGF, can compensate the inhibitory effect caused by blocking VEGF signaling and promote angiogenesis (Ebos and Kerbel, 2011). For instance, AMG386, an Ang 1 and 2 neutralizing peptibody, has shown a PFS extension in a randomized phase II trial in patients with recurrent ovarian cancer (Karlan et al., 2012). In addition, some factors are able to recruit myeloid cells and other BMDCs to contribute to angiogenic re-initiation indirectly (reviewed in Ribatti and Crivellato, 2009). More interestingly, imatinib, a tyrosine kinase inhibitor of PDGFR-β, causes an impaired tumor growth and vascular SMCs proliferation as well as a decreased microvascular density (Ruan et al., 2013).

6.2 THERAPEUTIC ANGIOGENESIS IN ISCHEMIC DISEASE

Tissues suffering from inadequate blood supply can lead to illness and even mortality. Therapeutic angiogenesis aims at inducing neovascular growth to treat ischemic disease. There are at least four therapeutic approaches to induce angiogenesis; increasing the expression of angiogenic genes, supplying extrinsic angiogenic factors, delivery of angiogenic effector cells and delivery of progenitors/stem cells.

Angiogenic gene deliveries by viral vectors or non-viral vectors, especially VEGF delivery, have been developed to induce angiogenesis. However, overexpression of VEGF in the myocardium leads to heart failure and vascular tumorigenesis (Lee et al., 2000). Furthermore, the adenoviral delivery of FGF4 fails to reach a therapeutic efficacy in a Phase III trial to treat chronic angina pectoris (Henry et al., 2007).

Peripheral blood mononuclear cells (PBMNC) have been isolated and studied in clinical trials to treat patients with limb ischemia and peripheral arterial disease (reviewed in Lawall et al., 2010, 2011; Losordo and Dimmeler, 2004). Meta-analyses of clinical trials on intracoronary autologous BM cells have shown a 3-4% increase in ejection fraction (EF). However, the outcome improvement mostly persists for one year without long-term benefits (reviewed in Rangappa et al., 2010).

Cardiac stem cells, c-kit$^+$ cells residing within the myocardium, have been isolated and utilized to regenerate myocardium (Beltrami et al., 2001; Urbanek et al., 2005). Human cardiosphere derived stem cells have been shown to have an increase in EF as well as viable myocardium within the infarct zone in acute myocardial infarction models in immunodeficient mice (Messina et al., 2004; Nelson et al., 2009).

In stem cell therapy, sufficient numbers of progenitors and their viability after transplantation are the two most crucial challenges in this application. The long-term clinical benefit failure with cellular therapies indicates that investigations on a more precise stem cell type and interactions of putative stem cells with local environment are needed.

AIMS OF THE STUDY

This study was undertaken to investigate the role of hematopoietic cells, hematopoietic progenitors and local tissue resident stem cells in angiogenic situations, as well as to elucidate the mechanism of TGF-β on regulating angiogenesis in vivo.

The specific aims were:

I: To identify the cellular origin of ECs in adult angiogenesis
II: To elucidate the mechanism of TGF-β on modulating angiogenesis in vivo
III: To study how hematopoietic cells and progenitors in the tumor microenvironment support tumor angiogenesis

MATERIALS AND METHODS

The materials and methods are described in detail the original publications. Lists of materials and methods used in the studies are summarized below.

1. MATERIALS

Table 1. Mouse line used in this study

Mouse Line	Description	Source	Used in
C57bl/6J	Wild type (wt) inbred strain	ScanburAB, Sweden	I, II, III
C57bl/6J-Tg(ACTB-EGFP)1Osb/J	"Enhanced" GFP (EGFP) expressed in all of the tissues, with the exception of erythrocytes and hair, under the control of β-actin promoter.	The Jackson Laboratory, Bar Harbor, ME	I, II, III
C57bl/6J-Kdrm1Jrt;lacZ	Transgenic mice carry a β-galactosidase (β-gal) reporter gene under the control of the VEGFR-2 promoter	The Jackson Laboratory, Bar Harbor, ME	I
FVB/N-Tg(TIE2-lacZ)182Sato/J	Transgenic mice carry a β-galactosidase reporter gene under the control of the murine Tie-2 promoter.	The Jackson Laboratory, Bar Harbor, ME	I
C57bl/6J-Kit^{W-sh}/BsmJ	Transgenic mice carry the Kit^{W-sh} mutation is an inversion spanning a 2.8 Mb segment proximal to the KIT locus that disrupts 5' regulatory sequences.	The Jackson Laboratory, Bar Harbor, ME	I

Table 2. Cell lines and Primary Cells used in this study

Cell lines/Primary Cells	Description	Source	Used in
MS-1	Mile SVEN 1, a pancreatic islet endothelial cell line from C57BL/6 mouse	ATCC	I
B16-F1	Murine melanoma cell line	ATCC	I, III
Human PBMNC	Peripheral blood mononuclear cell from donors separated by density gradient centrifugation	Peripheral blood from healthy donors	II
Mouse primary lung ECs	Primary endothelial cells isolated from adult mouse tissues by FACS or magnetic separation, including $CD31^{+}CD105^{+}$ cells, Lin^{-} $CD31^{+}CD105^{+}$ cells, $Lin^{-}CD31^{+}CD105^{+}Sca\text{-}1^{+}CD117^{+}$ cells and Lin^{-} $CD31^{+}CD105^{+}Sca\text{-}1^{+}CD117^{-}$ cells	Mouse	I
Leukocyte ($CD45^{+}$)	Separated using Magnetic beads from mouse peripheral blood or bone marrow	Mouse	I
BM	Isolated mouse bone marrow cells	Mouse	I, II, III

Table 3. Antigen used in this study

Antigen	Fluorescent conjugated Antibody	Source	Used
CD31	APC conjugated rat anti mouse CD31	BD	I
CD105	PE conjugated rat anti mouse CD105	eBioscience	I
Sca-1	V450 conjugated rat anti mouse Sca-1	BD	I, III
CD117	PE-Cy7 conjugated rat anti mouse CD117	eBioscience	I, III
VEGFR-2	Alexa Fluor 700 anti-mouse VEGFR-2	eBioscience	I
CD150	PerCP-eFluro 710 anti-mouse CD150	eBioscience	III
CD48	FITC anti-mouse CD48	eBioscience	III
CD45	FITC anti-mouse CD45	eBioscience	I, III
CD115	FITC anti-mouse CD115	eBioscience	I
α-smooth muscle actin	FITC anti-mouse α-smooth muscle actin	Abcam	I
CD11b	FITC anti mouse CD11b	BD	I
CD34	Alexa Fluor 700 anti-mouse CD34	eBioscience	I
CD14	FITC anti-mouse CD14	eBioscience	I
CD106	FITC anti-mouse CD106	BD	I
VEGFR-1	Alexa Fluor 488 anti-mouse	R&D	I
CD184	FITC anti-mouse CD184	BD	I
CD144	Alexa Fluor 488 anti-mouse CD144	eBioscience	I
CD104	FITC anti mouse CD104	Abcam	I

Table 4. Primary Antibodies used in this study

Antigen	Description Clone	Source	Used
CD31	PECAM-1, rat anti-mouse CD31	BD	I, II, III
CD105	Endoglin, rat anti-mouse CD105	BD	I
Sca-1	Rat anti-mouse Sca-1	BD	I
VEGFR-2	Rat anti-mouse VEGFR-2	BD	I
F4/80	Rat anti-mouse F4/80	Abcam	I, III
Actin,α-smooth muscle	Monoclonal anti-actin, α-smooth muscle, Cy3 conjugated	Sigma	I, II
CD34	Rat anti-mouse CD34	BD	I
vWF	Rabbit anti-mouse/human von Willebrand Factor	DAKO	I, II, III
VE-Cadherin	Rabbit polyclonal anti-mouse VE-cadherin	Abcam	I, III
ZO-1	Rabbit polyclonal anti-mouse anti-ZO-1 (N-term)	Invitrogen	I
CD117	Goat anti-mouse CD117 & Rat anti mouse CD117	R&D Systems	I
CD117	Mouse anti human CD117 Mab (Clone MS-289-P)	Neomarkers	I
CD16/CD32	Rat anti-mouse CD16/32	BD	I
CD115	FITC conjugated rat anti-mouse CD115	BD	I
CD14	FITC conjugated rat anti mouse CD14	BD	I
CD45	Monoclonal mouse anti human CD45 (Clone 2B11+PD7/26),	Dako	III
CD45	Rat anti-mouse CD45	BD	I, II, III
β-galactosidase	Rabbit polyclonal anti β-galactosidase	Chemicon International	I
VEGF	Polyclonal rabbit anti-VEGF Ab-1	NeoMarkers	I, II, III
VEGF	Rabbit polyclonal anti-mouse VEGF	Abcam	III
VEGF	Neutralizing anti-VEGF mouse monoclonal IgG (Clone AVA)	Genentech	II
phospho-p38 MAP Kinase	Polyclonal rabbit anti-mouse antibodies against phospho-p38 MAP kinase (Thr180/Thr182)	Cell Signaling Technology	II
phospho-p44	Phospho-p44/p42 MAP kinase (Thr202/Th204)	Cell Signaling Technology	II
Ki67	Rat anti-mouse Ki67	Dako	III
Ki67	Rabbit polyclonal anti-mouse Ki67 (SP6)	Abcam	I, III
VCAM-1	Mouse monoclonal (6G9) anti-human VCAM-1	Abcam	III
CD45	Rat anti-mouse CD45	BD	I,II, III

Antigen	Description Clone	Source	Used
N-cadherin	Rabbit polyclonal anti-mouse/human N cadherin	Abcam	III
SDF-1	Mouse monoclonal anti-human SDF-1 (Clone: MM0211-9N26)	Abcam	III
CD11b	Rat anti-mouse CD11b	BD	I, III
SDF-1	Rabbit anti mouse SDF-1α	Abcam	III
Jagged-1	Rabbit polyclonal anti-human Jagged-1	Abcam	III
β-catenin	Rabbit monoclonal anti-human β-catenin E247	Abcam	III
osteopontin	Rabbit anti-osteopontin	Millipore	III
Angiopoietin-1	Rabbit anti-mouse Angiopoietin-1	Abcam	III
CD150	Rabbit anti-mouse CD150	Abcam	III
MMP-9	Rabbit anti-mouse MMP-9	Abcam	III

Table 5. Proteins used in this study

Protein/Peptide	Description	Source	Used in
bFGF	Recombinant mouse bFGF	Invitrogen	I
EGF	Recombinant mouse EGF	Invitrogen	I
VEGF	Recombinant mouse VEGF	Invitrogen	I
$VEGF_{164}$	Recombinant mouse $VEGF_{164}$	R&D systems	II
TGF-β1	Recombinant mouse TGF-β1	R&D systems	II

2. METHODS USED IN THIS STUDY

Table 6. Methods used in this study

Methods	Used
Cell Isolation	I, III
Bone marrow transplantation	I, II
Flow Cytometry	I, III
Immunofluorescence	I, II, III
Immunohistochemistry	I, II, III
In vivo animal model	I, II, III
RNA extraction	I, II
Real time-PCR	I, II
Colony assay	I, III
Cell proliferation assay	III
Cell culture	I, II, III
Northern blot	II
Bone marrow transplantation	I, II, III

Cell Isolation

Mouse lung endothelial cells were isolated from lungs dissected from adult C57BL/6J mice (Scanbur AB), C57BL/6-Tg(ACTB-EGFP)1Osb/J mice, C57BL/6J-Kdrtm1Jrt; lacZ mice, FVB/N-Tg(TIE2-lacZ)182Sato/J mice, or C57BL/6J-KitW-sh mice (all from The Jackson Laboratory). Mice were first anesthetized with Rompun vet (Bayer) and Ketaminol vet (Intervet) and were then perfused with 20 ml of PBS. The lung tissue was collected. After removing fat tissue from the lung, the tissue was minced manually and was then digested with DMEM medium (Invitrogen) containing 0.8 U/ml of Dispase II (Roche), 1 mg/ml of Collagenase H (Roche), 100 U/100 µg/ml of Pen/Strep (Invitrogen), 2% FCS and 2 mM L-glutamine at 37° C for 1 h. The homogenate was filtered through a 100 µm nylon mesh falcon (BD Biosciences), followed by a 30 µm nylon mesh falcon (BD Biosciences). Red blood cells were subsequently lysed in the buffer containing 10 mM $KHCO_3$, 155 mM NH_4Cl, 0.1 mM EDTA (pH 7.5) at room temperature for 2 minutes. The cell pellets were collected by centrifugation at 300g for 6 minutes and resuspended in DMEM medium containing 100 U/100 µg/ml of Pen/Strep, 2% FCS, and 2 mM L-glutamine.

For lineage depletion, Mouse Lineage Cell Depletion Kit (Miltenyi Biotec) was used according to manufacturer's instructions. Subsequently, the cells were incubated first with anti-mouse CD16/CD32 blocker (BD Biosciences) for 5 minutes at 4°C and with a cocktail of fluorochrome-conjugated antibodies for 30 minutes at 4°C.

$Lin^-CD31^+CD105^+Sca\text{-}1^+CD117^+$ cells and $Lin^-CD31^+CD105^+Sca\text{-}1^+CD117^-$ cells were sorted using BD FACSAria flow cytometer (BD Biosciences) and collected into 5 ml Falcon tubes continuously or into a 96 multi-well plate at a rate of one cell/well using an automated cell deposition unit. Compensation adjustments were performed with single color positive controls and were calculated automatically with FACSDiva software version 4.1.2 (BD Biosciences). Dead cells and cell debris were excluded by gating according to the forward and side light scatters. The positive cells were gated by comparing to isotype controls (BD Biosciences).

BM cells from adult C57BL/6J mice, C57BL/6-Tg(ACTB-EGFP)1Osb/J mice, C57BL/6J-KitW-sh mice and adult wt C57BL/6J mice bearing B16-F1 melanomas were isolated by flushing femurs and tibias of the donor mice with 29-gauge needle using DMEM supplemented with 2 mM L-glutamine and 100 U/100 µg/ml of Pen/Strep. $CD45^+$ BM, lin^- BM cells or lin^+ BM cells were then processed using CD45 microbeads (Miltenyi Biotec) or Mouse Lineage Cell Depletion Kit according to manufacturer's instructions.

Subcutaneous B16-F1 melanoma tumors were dissected from wt C57BL/6J mice and digested with DMEM medium containing 0.8U/ml of Dispase II, 1 mg/ml of Collagenase H, 100 U/100 µg/ml of Pen/Strep, 2% FCS and 2 mM L-glutamine at 37° C for 1 h. Red blood cells were subsequently lysed in the buffer containing 10 mM $KHCO_3$, 155 mM NH_4Cl, 0.1 mM EDTA (pH 7.5) at room temperature for 2 minutes. Lin^+ cells were then separated with mouse Mouse Lineage Cell Depletion Kit according to manufacturer's instructions. Cells were counted automatically with Nexcelom cell counter (Nexelom Biosciences). Lin^- cells were further separated using using CD45 microbeads.

In vitro EC colony assays and hematopoietic cell colony assays

Freshly isolated ECs were plated in duplicate in 1 ml of 0.8% methylcellulose containing 15% FCS, 1% L-glutamine, 1% BSA, 10^{-4} mM 2-Mercaptoethanol, 0.2 mg/ml human transferrin, 0.01 mg/ml recombinant human insulin (all from Stemcell Technologies) and 100 ng/ml recombinant murine VEGF (Invitrogen). Colonies

containing 15 or more cells at day 7 were counted manually using an inverted microscope. In preliminary experiments the isolated cell populations were plated at various plating densities and scored at different time points, and an ideal plating density for each population and scoring time point were determined.

Freshly isolated $CD45^+$ BM cells, lin^-BM cells, lin^-CD45^+ cells from tumors and $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^+$ ECs were plated in duplicated in 1ml of MethoCult GF M3534 supplemented with 10 ng/ml GM-CSF (Invitrogen) and 10 ng/ml M-CSF (Invitrogen) or M3434 medium (Stemcell technologies). Cell densities ranging from 500 to 100 000 cells per plate were studied.

Cell culture

Freshly isolated lung ECs and picked-up EC colonies were cultured on 1% gelatin coated plates or T-75 flasks in IMDM medium containing 15% FCS, 1% L-glutamine, 1% BSA, 10^{-4} mM 2-mercaptoethanol, 0.01 mg/ml recombinant human insulin, 100 ng/ml recombinant murine VEGF, 100 ng/ml recombinant mouse bFGF, and 100 ng/ml recombinant mouse EGF (all from Invitrogen) and 0.2 mg/ml human transferrin (Sigma–Aldrich) at 37°C in a humidified 5% CO_2 atmosphere.

The B16-F1 melanoma cell line (ATCC no. 6323) was maintained in DMEM supplemented with 2 mM L-glutamine, 100 U/100 μg/ml of Pen/Strep, and 10% FCS at 37°C in a humidified 5% CO_2 atmosphere.

The MS-1 endothelial cell line (ATCC no.2279) was maintained in DMEM supplemented with 2 mM L-glutamine, 100 U/100 μg/ml of Pen/Strep, and 5% FCS at 37°C in a humidified 5% CO_2 atmosphere.

FACS analysis

MS-1 endothelial cell line and cultured passage 1 and 24 lung ECs were first washed with PBS and then treat by 1 mM EDTA at 37 °C for 10 minutes. Cell pellets were subsequently collected by centrifugation and resuspended in DMEM containing 2% FCS. Cells were incubated with anti-mouse CD16/CD32 blocker to reduce Fcγ II/III receptor-mediated antibody binding at 4 °C for 5 minutes, followed by 30-mintue fluorochrome-conjugated antibody incubation at 4 °C in dark. After antibody incubation, cells were washed twice in PBS and resuspended.

Isolated tumor associated lin^- hematopoietic cells or lin^- BM cells were incubated with Anti-biotin-Vioblue (Miltenyi Biotec), APC conjugated anti-mouse CD45, PE conjugated anti-mouse Sca-1 (all from BD), PE-Cy7 conjugated anti-mouse CD117, PerCP-eFluro710 conjugated anti-mouse CD150, and FITC conjugated anti-mouse CD48 (all fromeBioscience).

Total RNA purification

Total RNA from freshly isolated mouse lung $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^+$ ECs or PBMNCs was extracted using RNeasy Kit (Qiagen) according to manufacturer's instruction.

Real-time quantitative RT-PCR

The quality of the total RNA from isolated lung ECs was analyzed with Agilent 2100 BioAnalyzer. Real-time quantitative RT-PCR analyses were performed by SABiosciences - QIAGEN using the SYBR Green real-time PCR detection method. The Ct value of the housekeeping gene GAPDH was subtracted from the value of the gene of interest for normalization. The 2^(-delta Ct) was used to calculate the normalized relative quantity of the gene of interest.

RNAseq expression data analysis

RNAseq data (level 3) for 375 skin cutaneous melanoma samples were downloaded from the Cancer Genome Atlas (TCGQ) database (Network, 2008). The analysis was limited to samples with less than 100% of tumor cell content in order to capture as much as possible from the gene expression of the host stromal cells within the tumor mass. A total of 54 primary melanoma tumor samples were further analyzed by TCGA with the MapSplice pipeline (MapSpliceRSEM V2) under this criterion (Wang et al., 2010a). The gene-level expression is quantified with RSEM and upper-quartile normalized after alignment (Li et al., 2010). RSEM quantified gene-level gene expression was transformed to base 2 log scale. The RNAseq data were analyzed using the Anduril data analysis framework (Ovaska et al., 2010).

In vivo angiogenesis and tumor growth

For transplanting the progeny of a single EC, GFP-tagged $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^+$ ECs were isolated from C57BL/6-Tg(ACTB-EGFP)1Osb/J mice, and were plated in adherent endothelial colony forming assays at a frequency of one CFC per plate. The colonies were expanded for twelve days and subsequently studied under Axiovert 200 inverted epifluorescence microscope (Carl Zeiss) using both bright field and eGFP channels. The colonies were manually picked up by scraping and pipetting up using a micropipette under Olympus CKX31 inverted microscope (Olympus). The cells were resuspended in PBS, and mixed with 200 µl of Matrigel (Basement Membrane Matrix; BD Bioscience) supplemented with VEGF (100 ng/ml) and bFGF (10 ng/ml), and injected subcutaneously into wt C57BL/6J mice. After 2-3 weeks, the mice were killed and the vasculature of the plugs was analyzed. In some experiments, freshly isolated $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^+$ ECs or $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^-$ ECs from transgenic C57BL/6-Tg(ACTB-EGFP)1Osb/J mice were injected subcutaneously into wt C57BL/6J mice together with 200 µl of Matrigel supplemented with VEGF (100 ng/ml) and bFGF (10 ng/ml). 10000 to 100000 ECs were transplanted. After 2 weeks, the mice were sacrificed and the vasculature of the plugs was analyzed.

In the serial transplantation experiment, C57BL/6J mice were first inoculated with syngeneic B16-F1 melanoma cells (2×10^6 B16-F1 cells in 200 µl of PBS) mixed with 15 CFUs of GFP-tagged $CD31^+CD105^+$ ECs. After two weeks, individual tumor was collected and digested with DMEM medium containing 0.8 U/ml of Dispase II, 1 mg/ml of Collagenase H, 100 U/100 µg/ml of Pen/Strep, 2% FCS and 2 mM L-glutamine at 37° C for 1 h. Mouse Lineage Cell Depletion Kit was used to deplete lineage cells in the single cell suspension according to manufacturer's instructions. The lin^- cells (containing B16-F1 cells and 10000 GFP-tagged ECs per tumor) were subsequently transplanted subcutaneously to a new wt host. This isolation and re-transplantation procedure was repeated every two weeks. Part of the tumors in each generation was processed for tissue analyses while others were used for re-transplantation.

For TGF-β induced angiogenesis, equal volumes of TGF-β1 (60 ng/injection/mouse; R&D Systems), $VEGF_{164}$ (60 ng/injection/mouse; R&D Systems) or PBS buffer were injected subcutaneously every other day at the same injection site at the ear of into C57BL/6J mice. After 14 days, the mice were sacrificed and the ears were photographed and processed for tissue analyses.

To study the mechanism of the proangiogenic effect of TGF-β1, equal volumes of 60 ng of TGF-β1 or PBS buffer were injected subcutaneously at the same injection site at the

ear of C57BL/6J mice every other day for two weeks. An immunoneutralizing anti-VEGF mouse monoclonal IgG antibody (clone AVA, Genentech) or isotype control was given intra-peritoneally to the TGF-β1 treated mice once per week at a dose of 5 mg/kg.

In the AMD3100 in vivo experiment, Alzet #1002 osmotic minipumps (Alza) were implanted subcutaneously in the dorsolateral area of wt C57BL/6J mice. The mice were treated with either AMD3100 (Sigma-Aldrich; delivery of the drug at a dose of 120 μg/day corresponding to a dose of 5 mg/kg/d and a rate of 2.5 μl/h) or equal volumes of PBS.

In experiments where tumor growth rate was studied, 2×10^6 B16 cells were suspended in 200 μl of growth factor reduced Matrigel and then injected subcutaneously. The tumors were measured every other day using a vernier caliper. Tumor volume was calculated using the Pi/6 x L x W x W formula with L as the longest diameter and W the diameter at the position perpendicular to L.

Syngeneic bone marrow transplantations

Unselected BM cells (7×10^6) from C57BL/6-Tg(ACTB-EGFP)1Osb/J mice , C57BL/6J mice or C57bl/6J-KitW-sh/BsmJ mice were transplanted into corresponding syngeneic wild type mice via tail vein injection. One day before the BM transplantation, the recipient mice received a total myeloablation by irradiating at a lethal dosage of 9.1 Gy. The mice were subjected to TGF-β1 induced in vivo angiogenesis assay or tumor implantation 5-8 weeks after the BM reconstitution. All animal experiments were approved by the Provincial State Office of Southern Finland.

Immunohistochemistry, immunofluorescence and whole mounts

For whole mounts, all samples were first fixed in 4% PFA, blocked with PBS buffer containing 5% normal goat serum (Vector Laboratories), 0.2% BSA, 0.09% Na-Azide, and 0.3% Triton-X (Sigma–Aldrich). Cartilage was removed from the ears before fixing. Fat tissue and connective tissue were removed from the plugs before fixation. The samples were carefully washed after fixation and incubated with the primary antibodies for 2 days at room temperature. Subsequently, samples were washed and incubated with fluorochrome conjugated secondary antibodies overnight at room temperature. Finally, the samples were mounted with antifading medium (Vectashield; Vector Laboratories).

For cryosections, samples were fixed with 2% PFA for 1 hour at room temperature and incubated in PBS contacting 20% sucrose overnight at 4°C for cryopreservation. Samples were later embedded in OCT compound (Tissue-Tek) and frozen at ‐70°C. 8–80 μm sections were stained with the primary antibodies overnight at 4°C and subsequently incubated with fluorochrome-conjugated secondary antibodies for 30 min at room temperature in dark. The sections were then mounted with antifading medium.

For colonies in colony assays, methylcellulose medium was carefully removed by washing with PBS. Cells were then fixed in 4% PFA. Subsequently, the cells were washed, blocked with PBS buffer containing 5% normal goat serum, 0.2% BSA, 0.09% Na-Azide, and 0.3% Triton-X, and incubated with the primary antibodies for overnight at 4°C followed by 1-hour fluorochrome-conjugated secondary antibody incubation at room temperature.

For immunohistochemistry of cryosections, 7-μm cryosections were air-dried and fixed in cold acetone for 10 minutes. For blocking endogenous peroxidase, the sections were treated with 0.3% H_2O_2 in methanol for 30 minutes. The sections were then rehydrated in PBS for 5 minutes, blocked with 5% normal serum in PBS at room

temperature for 30 minutes, and incubated with primary antibodies at 4 °C overnight. Subsequently, the sections were treated with Vectastain Elite ABC anti-rabbit or anti-rat kits (Vector Laboratories) for primary antibody detection according to manufacturers' instructions followed by 10-minute incubation with Chromogen Immpact DAB or AEC kit (Vector Laboratories). Finally, the slides were mounted using VectaMount AQ medium (Vector Laboratories).

For immunohistochemistry of paraffin sections, the sample sections were first deparaffinized and rehydrated by incubating with xylene, graded washes of ethanol and finally distilled water. After deparaffinizing and rehydrating, the sections were treated using the heat-induced epitope retrieval method with a buffered citric acid solution (Dako). The sections were then incubated for 30 minutes in 0.3% H_2O_2 in methanol at room temperature to reduce endogenous peroxidase and blocked with 5% normal serum for 30 minutes at room temperature. Subsequently, the sections were incubated with primary antibodies at 4°C overnight followed by using Vectastain Elite ABC kits for primary antibody detection according to manufacturers' instructions. The peroxidase activity was detected by 10-minute incubation with Chromogen Immpact DAB or AEC. Finally, the slides were mounted using VectaMount AQ medium.

Endochondral ossification was analyzed using Russel-Movat's Pentachrome Stain Kit (American MasterTech) or Safranin-O (Merkck KGaA) according to manufacturers' instruction.

Samples were analyzed and photographed with an Axioplan 2 upright epifluorescence microscope, a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss; LSM 5 Software version 3.2), Zeiss LSM780 laser scanning confocal microscope (Carl Zeiss; Zen Software) or a Leica DM LB microscope (Leica Microsystems GmbH).

Human tumor tissue samples

Paraffin-embedded human tumor samples from patients with histologically diagnosed malignant melanoma and malignant breast cancer were from the archives of the Department of Pathology, University of Helsinki.

RESULTS AND DISCUSSION

The main results of the study are summarized and are referred to the original publications by using Roman numerals.

1. IDENTIFICATION OF VASCULAR ENDOTHELIAL STEM CELLs ON THE BLOOD VESSEL WALL (I)

1.1 RARE ENDOTHELIAL COLONY FORMING CELLs WITH HIGH PROLIFERATIVE ABILITY IN VITRO EXIST WITHIN ADULT ECs

Theoretically, there are two possible ways in the creation of new ECs. It is possible that all differentiated ECs may have the similar ability to produce daughter cells, as pancreatic beta cells do (Dor, 2006; Dor et al., 2004). On the other hand, unidentified resident tissue stem cells may differentiate to de novo ECs, as is the case of the epithelial stem cells in skin (Tumbar et al., 2004). HUVECs and HAECs are differentiated mature ECs with high proliferative ability in vitro (Bompais et al., 2004). Ingram et al. (2005) have identified a subset of cultured vessel wall derived ECs with highly proliferative ability in vitro and vessel formation capability in vivo. Furthermore, in human proepicardium, vascular progenitor cells (VPC) expressing VEGFR-2 and c-kit have been identified in the vascular niches and distributed through the coronary circulation (Bearzi et al., 2009). Additionally, the genetic fate mapping and clonal analysis suggest that it should be local tissue resident cells that contribute to de novo ECs in adult angiogenesis (Rinkevich et al., 2011). However, cells that give rise to vascular endothelium in adult are still not known.

We first assessed if all adult endothelial cells had the similar ability to produce progeny. Thus we isolated $lin^-CD31^+CD105^+$ ECs from adult mouse lung tissues and examined endothelial colony forming ability of these isolated ECs. Colony-forming cells (CFC) were consistently detected at approximately 1.5 colony-forming units per every thousand $lin^-CD31^+CD105^+$ ECs (Fig.1A in I). These EC colonies adhered to the culture dish in the methylcellulose medium (Fig.1A in I). Later we confirmed that these formed colonies were EC colonies with the phenotypic expression of various EC markers CD31, VE-cadherin, CD105 and vWF (Fig.1C in I).

Subsequently, we isolated $CD31^+CD105^+$ ECs from transgenic C57bl/6-Tg(ACTB-EGFP)1Osb/6J and wt C57BL/6J mouse lung tissues respectively. The isolated CD31+CD105+ ECs from transgenic C57bl/6-Tg(ACTB-EGFP)1Osb/6J mouse are GFP-positive and ECs from wt mice are GFP-negative. We mixed one colony-forming unit of the $GFP^+CD31^+CD105^+$ ECs with twenty colony-forming units of wt $CD31^+CD105^+$ ECs and plated on a 2-D culture. A clonal growth pattern of the GFP^+ ECs was observed after 12-day culture (Fig.1B in I). These results indicated that isolated adult lung $lin^-CD31^+CD105^+$ ECs contains rare endothelial CFCs.

To further study the proliferative potential of these endothelial CFCs, individual colonies were picked up and expanded in EC growth medium in vitro. Five colonies out of 148 separate colonies were found to have a high proliferative ability to produce tens of millions descendants (Fig.2A in I). These long-term expanded ECs express endothelial markers CD105 and VEGFR-2 (Fig.2B in I). Furthermore, these long-term cultured ECs also expressed stem cell marker c-kit (Fig.2B, Table S1 in I), indicating a potential role of

c-kit in these highly proliferative CFCs. Thus, a subpopulation of ECs with a high proliferation ability and clonal growth pattern in vitro must exist.

1.2 CFCs ARE ENRICHED WITHIN C-KIT$^+$ POPULATION

Next, we analyzed the frequency of CFCs in various subpopulations in lin$^-$CD31$^+$ECs from mouse lung using CD105, Sca-1 and c-kit by FACS sorting. C-kit and Sca-1 are surface marker molecules expressed by many adult stem cells (Broudy, 1997; Matsui et al., 2004; Shinde Patil et al., 2005). We observed that CD105 and Sca-1 were largely expressed by lin$^-$CD31$^+$ ECs from the adult lung vasculature, while about 39% of the population was c-kit-expressing cells (Fig.3A in I). Therefore, we compared the colony forming ability of c-kit-enriched and c-kit-depleted subpopulations of isolated lin$^-$CD31$^+$CD105$^+$ECs. CFCs were enriched in c-kit-expressing ECs whereas c-kit-depleted ECs hardly formed any EC colonies. A 10-folder higher colony forming frequency was observed in c-kit-enriched ECs compared to c-kit-depleted ECs (Fig.3B in I).

Moreover, a similar CFC frequency of c-kit$^+$ ECs was observed using a different method. A single GFP$^+$ lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ cell from transgenic C57bl/6-Tg(ACTB-EGFP)1Osb/6J mice was sorted into individual wells containing 2500 GFP-negative lin$^-$CD31$^+$CD105$^+$ ECs from wt C57BL/6J mice of 96-well plates using FACS. After a 7-day culture, 0.6% of all 960 wells contained a GFP$^+$ clonal area (Fig.3C in I).

1.3 EC COLONIEs ARE NOT FORMED BY HEMATOPOIETIC CELLs

Further cell-surface antigen expression of c-kit-expressing EC population was analyzed using flow cytometry. Isolated adult lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ ECs also expressed various endothelial-lineage cell markers, such as VE-cadherin, vascular cell adhesion molecule 1 (VCAM-1), VEGFR-2, VEGFR-1, integrin beta 4 and CD34 (Fig.3D in I). Meanwhile, hematopoietic lineage markers including CD45, CD11b, CD115, CXCR4, and F4/80 were not expressed by adult lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ ECs (Fig.3D in I). Moreover, mRNA levels of hematopoietic lineage markers including CD45, CD14, CD16, F4/80 and CD115 were not detected from c-kit expressing ECs (Fig.S2 in I).

To further exclude the possibility that hematopoietic lineage cells might be responsible for the EC colony formation in vitro, we compared the colony forming capacity of lin$^-$ BM or CD45$^+$ BM cells with isolated adult lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ ECs. The BM cells produced typical hematopoietic colonies in standard murine hematopoietic colony forming assays, while c-kit$^+$ ECs from adult mouse lung tissues generated only endothelial cell colonies with the same assay settings. Importantly, in endothelial colony assay system, neither lin$^-$ BM nor CD45$^+$ BM cells form any colonies (Fig.4D in I).

In addition, lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ ECs from C57bl/6J-Kdrtm1Jrt;lacZ mice and FVB/N-Tg(Tie2-lacZ)182Sato/J mice were isolated and studied in endothelial colony assays in vitro respectively. A lacZ-beta-gal reporter is inserted under Tie-2 promoter or VEGFR-2 promoter, which are expressed primarily by vascular ECs. The positive expression of lacZ in these colonies further confirmed that these colonies were EC colonies (Fig.4E and S4C in I).

Taken together, the EC colonies are not derived from hematopoietic cells. There is a rare population of c-kit$^+$ ECs that has high proliferative ability in vitro while other ECs have limited expansion capacity.

1.4 C-KIT$^+$ ECs RESIDE ON QUIESCENT AND NEO-ANGIOGENIC VASCULAR VESSEL WALL

Quiescent vasculature from other mouse tissues, such as subcutaneous tissues, liver and kidney, was studied to verify the existence of c-kit-expressing ECs. Lin$^-$ CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ ECs were observed in all these tissues but its frequency varied among tissues. 18% of lin$^-$CD31$^+$ ECs in the liver was c-kit$^+$ while 2% in the kidney was c-kit expressing ECs. C-kit-expressing ECs were observed on the capillary walls as well as on the arterial walls and in veins (Fig.4A and S3 in I).

In addition, plentiful c-kit$^+$ ECs were observed in subcutaneous B16 melanoma tumor vasculature, as well as in the blood vessels in subcutaneous Matrigel plugs (Fig.4B in I). C-kit$^+$ ECs were also found in the blood vessels of human malignant melanoma and in invasive breast cancer samples (Fig.4C in I).

It has been reported that juvenile nasopharyngeal angiofibroma and a subset of angiosarcoma express c-kit and resemble fetal ECs (Invernici et al., 2005; Miettinen et al., 2000). C-kit expression is also identified on other tumor endothelium, including glioblastoma, renal clear cell carcinoma and Kaposi's sarcoma-associated herpes virus (Miettinen and Lasota, 2005; Miettinen et al., 2000; Moses et al., 2002a, b; Sihto et al., 2009). C-kit expression on the quiescent blood vasculature was quite low whereas neo-angiogenic blood vessels contained various c-kit$^+$ ECs (Fig.4 in I). This indicated a possible role of c-kit in regulating endothelial proliferation. End differentiated ECs have very limited proliferation rate during homeostatic turnover. During angiogenesis processes such as tumor angiogenesis, mature vascular ECs might also acquire more stem-ness characteristics in vivo and turn into a transient state, for instance, by turning on c-kit expression.

1.5 C-KIT$^+$ ECs ENCLOSE VESCs

1.5.1 FUNCTIONAL BLOOD VESSELs GENERATED FROM A SINGLE C-KIT EXPRESSING CELL IN VIVO

The ability to generate descendants is one of the properties possessed by stem/progenitor cells. Thus, GFP$^+$ ECs originating from a single GFP$^+$ lin$^-$ CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ CFC were transplanted with Matrigel subcutaneously into syngeneic wt C57bl/6J mice. First, GFP$^+$ lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ ECs from the lung tissues of adult C57bl/6-Tg(ACTB-EGFP)1Osb/J mice were isolated and seeded at a density of one CFC per plate in endothelial colony assays. After 12 to 14 days, individual colonies were picked up and transplanted with Matrigel to wt mice. GFP-positive tube formations were observed in these Matrigel after 2 weeks (Fig. 5). Furthermore, to learn if these GFP+ tubes are functional blood vessels and connected to the host circulation, we performed blood perfusion on the host mice with red fluorescein-labeled microspheres (Fig.5B in I). GFP$^+$ vessels were labeled with these microspheres indicating the functionality of the vasculature formed by descendants of a single lin$^-$CD31$^+$CD105$^+$Sca-1$^+$c-kit$^+$ CFC.

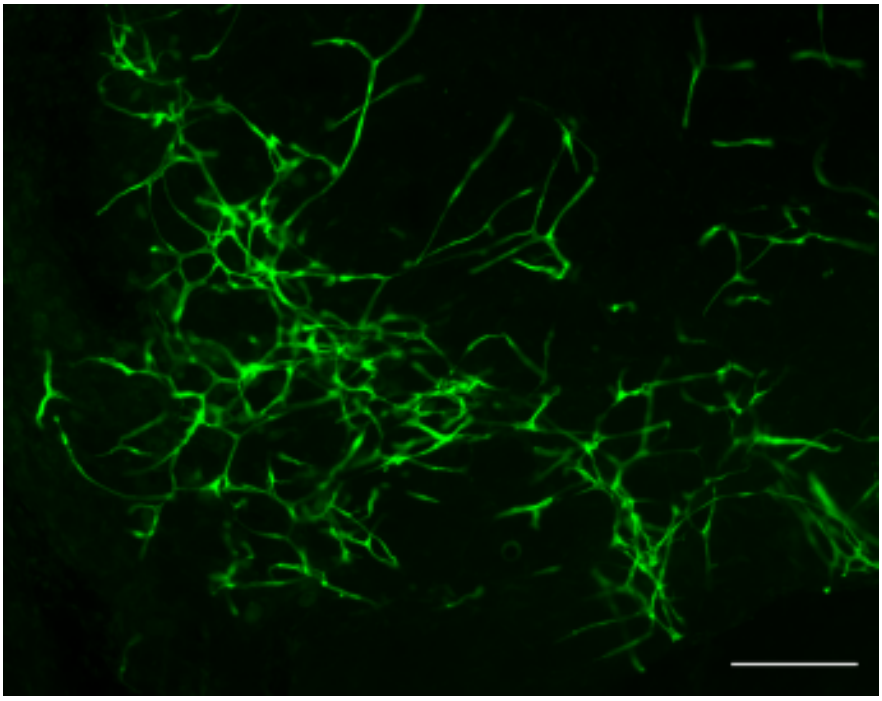

Fig. 5 A single c-kit expressing colony forming cell generates functional blood vessels in Matrigel. This is a macroscopic graph of the whole Matrigel illustrating GFP^+ blood vessels formed from one single GFP-tagged c-kit^+ VESC. Scale bar: 200 μm

1.5.2 IN VIVO SELF-RENEWAL

The other functional characteristic of adult stem cells is the self-renewal ability. This means that they are capable of generating numerous proliferative progenitors repeatedly under stimulation. Thus we designed a serial transplantation experiment using subcutaneous B16 melanoma models with adult GFP^+ $CD31^+CD105^+$ECs isolated from C57bl/6-Tg(ACTB-EGFP)1Osb/J mice (Fig. 6). GFP^+ vessels were identified in tumor vasculature from the primary transplants to the tertiary transplants (Fig.5C in I). These results proved that transplanted GFP+ ECs contained VESCs with self-renewal ability.

1.5.3 IN VIVO BLOOD VESSEL FORMATION OF C-KIT^+ ECs

To confirm in vivo whether $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^+$ ECs are enriched for VESCs with the ability to form blood vessels, we transplanted freshly isolated adult $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^+$ ECs or $lin^-CD31^+CD105^+Sca\text{-}1^+c\text{-}kit^-$ ECs isolated from C57bl/6-Tg(ACTB-EGFP)1Osb/J mice with Matrigel subcutaneously into syngeneic wt C57bl/6J mice. After 14 days, only occasional GFP+ ECs, not GFP^+ vessels, from c-kit-depleted transplants containing high numbers of c-kit-depleted ECs were observed. In contrast, abundant GFP^+ vessels were found in all twelve c-kit-enriched transplants (Fig.6 in I). Thus, we confirmed that c-kit^+ ECs were enriched with VESCs capable of generating new blood vessels in vivo.

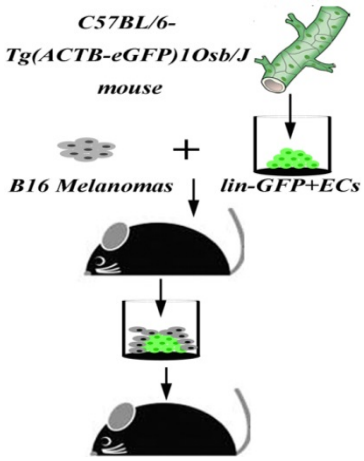

Fig. 6: Schematic illustration of serial transplantation. This is an illustration of a serial transplantation flow chart. GFP^+ ECs isolated from the transgenic mice were co-transplanted with B16 melanoma to a syngeneic wt mouse. After 14 days, the tumor was collected and lin^+ cells were depleted. Lin^- fraction containing GFP^+ ECs and B16 melanoma cells were then transplanted to a secondary recipient.

1.6 THE FUNCTIONAL ROLE OF C-KIT IN ECs

C-kit, a tyrosine kinase growth factor receptor, is important in stem cell survival and proliferation (Fleming et al., 1993; Ikuta et al., 1991; Ikuta and Weissman, 1992). The KIT null mutation leads to an embryonic death in mice (Russell, 1979). $Kit^{W\text{-}sh}$ mutation is an inversion in 5' regulatory sequences of the c-kit gene resulting in defective c-kit expression (Grimbaldeston et al., 2005). The $Kit^{W\text{-}sh}$ homozygote mice are healthy and fertile but have tissue-specific defects in c-kit expression and a deficiency in mast cells (Duttlinger et al., 1993; Grimbaldeston et al., 2005). Immunohistological and RNA expression studies on the $Kit^{W\text{-}sh}$ homozygotes have shown that c-kit expression is normal in the testis and brain, while absent in mast cells and lung (Duttlinger et al., 1993; Grimbaldeston et al., 2005). Accordingly, we found that c-kit expression in $Kit^{W\text{-}sh}$ homozygotes was low or undetectable in lung tissues including lung vasculature (Fig.7A in I). In the isolated adult ECs from $Kit^{W\text{-}sh}$ homozygote mice, significantly fewer endothelial CFCs were found (Fig.7B in I), indicating a possible role of c-kit in endothelial colony forming and endothelial proliferation. Impaired tumor angiogenesis and retardation of tumor growth were observed in $Kit^{W\text{-}sh}$ homozygote mice (Fig.7C and 7D in I). It has been shown that SCF stimulates the survival and the ability of capillary formation in HUVECs (Matsui et al., 2004). Together with our results, SCF/c-kit signaling is important for proliferative capacity of ECs. Moreover, there were a significantly decreased number of proliferating ECs in the tumors from $Kit^{W\text{-}sh}$ homozygote mice. Taking into account the possible effect of c-kit deficient hematopoietic system on tumor growth, we also did a total myeloablation to wt C57bl/6J mice. We then reconstituted these mice with BM from $Kit^{W\text{-}sh}$ mutant and from healthy wt C57bl/6J mice. No difference in tumor growth was observed between these two groups (Fig.S5 in I). Thus we concluded that the retardation in tumor angiogenesis and growth was an effect of endothelial c-kit deficiency and did not result from hematopoietic c-kit deficiency.

The current results provide solid evidence on adult endothelial lineage hierarchy (Fig.8 in I). Considering the role of VESCs in cellular turnover and angiogenesis, these cells may

be useful in cellular therapies to restore blood vessels in ischemic diseases. Thus, more detailed phenotypic characterization on these cells is required in order to identify and isolate VESCs more efficiently. Solely depending on cell surface antigen characterization on VESCs is not enough since side populations/other cells expressing the same markers may be still included. Clonal analysis and cell lineage tracking are needed for a further understating of these cells.

2. MECHANISMs OF TGF-β IN INDUCING ANGIOGENESIS (II)

The role of TGF-β in regulating vascular endothelium is complicated. It has been shown that TGF-β may promote angiogenesis in vivo (Madri et al., 1988; Roberts et al., 1986; Yang and Moses, 1990). On the other hand, TGF-β induces EC apoptosis in vitro and potentially inhibits vascular growth (reviewed in Pardali et al., 2010).

2.1 ANGIOGENIC EFFECT OF TGF-β IN VIVO

In our studies, in line with other reports (Madri et al., 1988; Roberts et al., 1986; Yang and Moses, 1990), we confirmed that TGF-β1 induced angiogenesis. Mice that received TGF-β1 or $VEGF_{164}$ injections subcutaneously in the ear showed elevated vascular density (Fig.1 in II). Notably, the ears of mice injected with TGF-β1 were also thicker than mice treated with PBS buffer or $VEGF_{164}$ (Fig. 1B in II). In the ears of TGF-β treated mice, some vascular mural cells were loosely associated to the blood vessels (Fig.2 in II). The irregular pattern of pericytes in these TGF-β treated mice is in agreement with earlier findings on the effect of TGF-β on mural cells (Carvalho et al., 2007). In addition, the vasculature in TGF-β treated mice displayed an abnormal and disorganized pattern including the enlargement of vascular lumen (Fig.2 in II). In chronic inflammation, enlarged vascular lumen is one parameter in the vascular remodeling, which is similar to the TGF-β induced angiogenesis (Alagappan et al., 2013; Siafakas et al., 2007). It has been long known that TGF-β has a chemotactic effect. Many immune effective cells such as macrophages are responsive to TGF-β. Similarly, in vivo TGF-β deficiency leads to an inhibited inflammatory response (Wang et al., 2010b). It is possible that TGF-β may recruit hematopoietic cells to the microenvironment and induce angiogenesis.

2.2 ANGIOGENIC HEMATOPOIETIC EFFECTORs IN ANGIOGENESIS (II &III)

To test our hypothesis, we transplanted GFP-tagged BM from C57bl/6-Tg(ACTB-EGFP)1Osb/J mice to wt C57BL/6J mice in order to trace the hematopoietic cells. When treated with TGF-β in the ears of these GFP-BM reconstituted mice, a massive recruitment of GFP^+ BMDCs was observed (Fig.3A in II). Furthermore, we confirmed this observation by detecting $CD45^+$ cells in the TGF-β treated ears of wt C57BL/6J mice. CD45 is a pan hematopoietic cell marker. Concurrently, VEGF secretion in the TGF-β treated mice was also increased when comparing to mice treated with buffer (Fig.3B in II). Many of these infiltrated hematopoietic cells were in close contact to the vasculature (Fig.3D in II). Besides the close spatial association with vasculature, abundant evidence have shown that hematopoietic cells contribute to angiogenesis mostly by secreting angiogenic factors (reviewed in Fang and Salven, 2011). It is highly possible that the recruited hematopoietic cells are strong producers of VEGF. Co-localization of VEGF and $CD45^+$ hematopoietic cells were subsequently shown by immunostaining (Fig.3C in II). These VEGF-expressing hematopoietic effectors recruited by TGF-β1 may play a central role in the in vivo

angiogenic effect of TGF-β1. It is possible that TGF-β1 acts as a chemotactic molecule to recruit VEGF-expressing hematopoietic cells to induce angiogenesis. The other scenario is that TGF-β1 acts as a chemotactic molecule to recruit hematopoietic effectors and also directly induces the expression of VEGF in these hematopoietic effectors.

Considering the massive recruitment of BM-derived cells, many inflammatory cells may constitute a major population in those cells. Moreover, in tumorigenesis, where angiogenesis is active, chronic inflammation is also evoked (Balkwill and Mantovani, 2012; Candido and Hagemann, 2013). More and more attention has been drawn to the complicated interactions among inflammation, angiogenesis and tumorigenesis.

2.3 TGF-β INDUCED ANGIOGENESIS IS MODULATED VIA VEGF

To further elucidate the mechanism of TGF-β in regulating angiogenesis, we first treated mice with TGF-β in combination with immune-neutralizing anti-mouse VEGF antibodies or control IgG. The recruitment of infiltrating hematopoietic cells was not affected (Fig.4 in II) in mice that received VEGF neutralizing antibodies, but angiogenesis in these mice was strongly inhibited (Fig.4C in II). This indicated that the VEGF-expressing hematopoietic effector cells could regulate TGF-β induced angiogenesis.

To understand the mechanisms of action of TGF-β, we then treated human PBMNCs in vitro with TGF-β. mRNA levels of VEGF in human PBMNCs were increased by TGF-β stimulation on a dose dependent manner (Fig.5A in II). TGF-β also affected VEGF protein secretion. Stimulating PBMNCs with 2 ng/ml of TGF-β in serum free medium resulted in a significant increase in VEGF secretion dependent on time course (Fig.5C in II). Furthermore, VEGF secretion of PBMNCs was blocked when PBMNCs were treated with 2 ng/ml of TGF-β and 1 mmol/L of cycloheximide (Fig.5C in II). Taken together, the VEGF protein production and its mRNA synthesis were induced by TGF-β in PBMNCs in vitro. These in vitro results indicated that TGF-β1 not only acted as a chemotactic molecule to recruit hematopoietic effectors but also a direct inducer of VEGF production in these hematopoietic effectors.

2.4 TGF-β INDUCED HEMATOPOIETIC VEGF SECRETION IS REGULATED BY p38 AND p44/p42 MAPK PATHWAYs

Next, we studied the signaling pathways involved in TGF-β induced hematopoietic VEGF secretion. p38 and p44/p42 MAPK pathways were found to be involved in the TGF-β induced VEGF secretion. Blocking these pathways with inhibitors resulted in the suppression of VEGF secretion by the human PBMNCs and also prevented the secretion of VEGF induced by TGF-β (Fig. 6 in II). In vivo, in line with our in vitro findings, activated p38 or p44/42 was observed in infiltrating hematopoietic cells in TGF-β treated mice (Fig. 6 in II).

2.5 DUAL ACTION MECHANISMs OF TGF-β IN INDUCING ANGIOGENESIS

Bone marrow derived cells have been shown to be involved in many angiogenic situations, including tumor angiogenesis. These infiltrating cells contribute to angiogenesis in a paracrine manner and function as a rich source of angiogenic factors (Fang and Salven, 2011). Although the in vitro models in some studies show an inhibitory effect of TGF-β on EC growth (Pepper et al., 1993), TGF-β induces angiogenesis in vivo. The balance between ALK1 and ALK5 signaling pathway in ECs may be one mechanism elucidating the actions of TGF-β (Oh et al., 2000). Interactions of infiltrating cells and

stromal cells in the microenvironment provide an alternative mechanism of TGF-β induced angiogenesis. We found that TGF-β1 had a chemotactic effect on hematopoietic cells and directly induced the VEGF production of these hematopoietic effectors. In general, TGF-β recruits a massive amount of hematopoietic cells to infiltrate to the local environment and directly induce infiltrating hematopoietic cells to secrete VEGF. The dual actions of TGF-β then lead to induced angiogenesis and vascular remodeling.

3. ECTOPIC HEMATOPOIETIC MICROENVIRONMENT IN TUMORs (III)

3.1 PROLIFERATING HEMATOPOIETIC CELLs IN TUMOR

Hematopoietic infiltration is important for tumorigenesis (Murdoch et al., 2008; Shojaei et al., 2008; Wu et al., 2007). Many others have observed a massive recruitment of hematopoietic cells in the tumor microenvironment (Coffelt et al., 2010; De Palma et al., 2005; De Palma et al., 2003; Nozawa et al., 2006). Accordingly, we quantified the amount of mature hematopoietic cells and their committed progenitors residing in the tumor environment (0.86 ± 0.21 cm^3, mean ± SE) to that of hematopoietic stem cell niche in the bone. The lineage markers are an antibody cocktail (including Ter-119, CD5, B220, CD11b and Gr-1) that is targeted to mature hematopoietic cells, such as B cells, T cells, monocytes/macrophages, granulocytes, erythrocytes and their committed precursors. A comparable number of lin^+ cells (mature hematopoietic cells and their committed precursors) were enclosed in the tumor microenvironment as in the BM of one tibia (Fig.1A in III). We also found that B16 melanoma tumor microenvironment enclosed large numbers of proliferating hematopoietic cells (ki-67^+CD45^+) as well as human primary tumors (Fig.1 in III). Additionally, histone H3 is one of the histone proteins in the core chromatin in eukaryotic cells. Serine 10 and 28 on histone H3 are only phosphorylated during mitosis and histones are not phosphorylated during apoptosis (Goto et al., 1999; Gurley et al., 1978; Paulson and Taylor, 1982; Wei et al., 1998). By using an antibody against phosphorylated histone H3 (Ser10), we also observed tumor infiltrating hematopoietic cells undergoing mitoses (Fig.1A in III). These proliferating hematopoietic cells in murine melanomas were heterogeneous populations expressing common progenitor markers and also myeloid markers, Sca-1, F4/80 and CD11b (Supplemental Fig.1 in III). Importantly, the proliferating hematopoietic cells in situ were able to secrete angiogenic factors such as VEGF and MMP-9, indicating their paracrine roles in supporting tumor angiogenesis (Fig.1A in III). Hematopoietic cells have been found to induce tumor angiogenesis by secreting angiogenic factors such as VEGF and MMP-9 (Bergers et al., 2000; Fang and Salven, 2011; Murdoch et al., 2008; Nozawa et al., 2006). Monocytes and MDSCs have been found to contribute to tumor growth and tumor angiogenesis (De Palma et al., 2005; Dolcetti et al., 2008; Squadrito and De Palma, 2011; Venneri et al., 2007; Yang et al., 2004). Local proliferation of macrophages has been found during inflammation and atherosclerosis (Jenkins et al., 2011; Robbins et al., 2013). The prominent proliferating hematopoietic cells we observed may constitute largely by these proliferating myeloid cells. However, extramedullary hematopoiesis has been found in yolk sac, fetal liver and spleen (Kim, 2010). It would be interesting to investigate whether a more primitive hematopoietic progenitor population capable of producing progeny exists inside tumors.

3.2 HEMATOPOIETIC PROGENITORs RESIDE IN THE TUMOR MICROENVIRONMENT

In adult mouse bone marrow, $lin^{-/low}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) cells are a heterogeneous population of multipotent cells (Manz et al., 2002; Morrison et al., 1997; Uchida et al., 1994). Later, SLAM families (CD48, CD150, CD229, and CD41) are applied to distinguish HSCs, HPCs and MPPs (Kiel et al., 2005; Oguro et al., 2013). To further study these amplifying hematopoietic cells, we analyzed the heterogeneity of $lin^{-/low}$ $CD45^{+}$ cells in tumors using FACS and investigated progenitor numbers in these cells. CFU-GM, CFU-GEMM and BFU-E were identified in the assay (Fig.2A in III), indicating $lin^{-/low}$ $CD45^{+}$ tumor associated hematopoietic cells enclosed progenitors with the capacity to produce multiple linages. We found LSK cells were 4.9% ± 1.3% (mean ± SE) of tumor associated $lin^{-/low}$ hematopoietic cells. HSPC composition of LSK cells using CD48 and CD150 from tumor had a similar rate in MPPs as from BM while HSCs and HPCs are derivate (Fig.2B in III). However, much fewer progenitors were enclosed in 500 of lin^{-} tumor associated hematopoietic cells compared 500 of lin^{-} BM hematopoietic cells (3.4± 1.3 vs 37.2 ±6.2, total CFUs, mean ± SE, Fig. 7A). HSCs have been found in BM and other tissues including adult liver (Taniguchi et al., 1996). However, they are considered to have very little function outside specific anatomic locations (Scadden, 2006). It was possible that tumor-associated primitive hematopoietic progenitors might express progenitor markers but lack HSPC activity. This may possibly explain that tumor associated lin^{-} hematopoietic cells contains much fewer progenitors than BM hematopoietic cells whereas phenotypic composition were similar. Phenotypic marker expressions of HSPCs in tumors may also be affected by the tumor microenvironment. This is another possible explanation. Considering the limited amount of these populations in the BM and possibly even less amount in tumors, it is very challenging to identify their existence in a solid and repeatable manner using current detection method such as FACS as tumor cells represent the major population of the tumor mass. Thus, lineage depletion and CD45 enrichment using MACS of tumor samples are very important to exclude as many melanoma cells as possible. $Lin^{-/low}CD45^{+}$ cells from tumor may be then further purified using FACS. However, the contamination of tumor cells is inevitable. In colony assays where tumor associated $lin^{-/low}CD45^{+}$ cells are assessed, contaminating tumor cells also grow and form tumor spheroids. Thus, to avoid or to eliminate the effect of the inevitable contaminating tumor cells as much as possible, it is very essential to optimize the seeding density in the assay. This also may result in an underestimated low progenitor numbers from colony assays as tumor cells compete with HSPCs for nutrients. It will be very interesting to know whether other progenitor populations, such as CLPs, CMPs, MEP and GMPs (using lineage, Sca-1, c-Kit, CD16/32 and CD34) (Akashi et al., 2000), can be identified in the tumor mass. Whether functional HSCs truly exist inside tumors also need to be further investigated, for instance by isolating highly-purified HSCs (e.g. LSK, $CD48^{-}$ $CD150^{+}$) from tumor and analyzing their repopulation ability by transplanting into syngeneic irradiated mice in vivo and colony-forming ability in vitro in a single-cell culture.

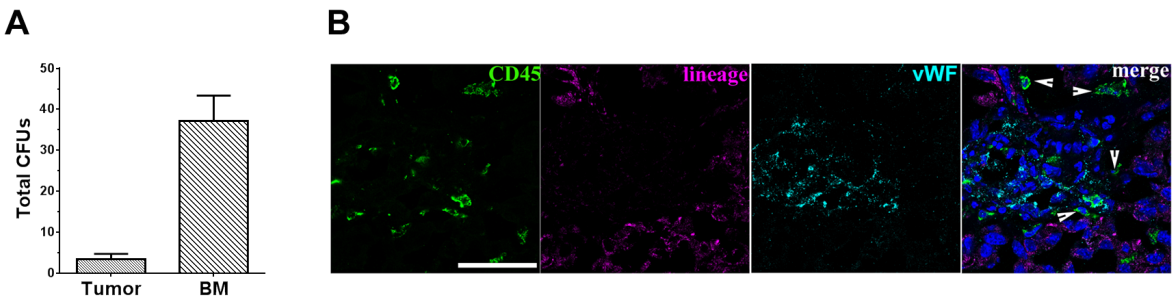

Fig.7. Hematopoietic Progenitors in Tumors. (A) Hematopoietic colony assays showed that tumor microenvironment encloses hematopoietic progenitors whereas the frequency was much lower than in the BM. Total colony units were counted under an inverted microscope after 8 days. (B) Lin^- hematopoietic cells (lin^-CD45^+) were found in proximity to tumor vasculature (vWF). Cells that were in proximity to the vessels (white arrow) were found. A representative confocal scan shows their spatial relation. CD45: green, Lineage: Magneta, vWF: Cyan. Scale bar: 50 μm

In preliminary studies, we studied lin^-CD45^+ cells in tumors. Lin^-CD45^+ cells in close contact to vasculature were identified. However, lin^-CD45^+ cells near but not in close contact to the vasculature were also identified (Fig. 7B). This result is still quite circumstantial, since lin^-CD45^+ cells only enclosed a small number of progenitors. In the bone, quiescent HSCs are found to be close to osteoblast while activated HSCs are located near sinusoid vessels (Ding et al., 2012). $Lin^-CD48^-CD150^+$ cells have been used to detect HSCs in immunofluorescence. In fact, it has been found that 38% of HSCs in spleen and 26% of HSCs in the bone marrow are not visibly in close contact to endothelium (Kiel et al., 2005). More recently, it has been identified that 36.8% and 67% of HSCs were located near arterioles and sinusoids while 14.9% and 32.9% are found to be adjacent (Kunisaki et al., 2013). On the other hand, HSCs have been found in liver and spleen and extramedullary hematopoiesis is observed in these tissues with the absence of osteoblastic niche (Kim, 2010). In tumors, we found that $lin^-CD48^-CD150^+$ cells locate in proximity to tumor vasculature (Fig.2C in III). However, unlike in the BM where $lin^-CD48^-CD150^+$ cells are exclusively $CD45^+$ (Kiel et.al, 2005), tumor blood vessels express also CD150. To be sure that the detected cells are of hematopoietic origin, in additional to markers, such as CD45 or fluorescence-tagged BM, a cocktail of lineage markers are required to trace tumor associated hematopoietic cells are required in order to identify genuine HSPCs in tumors. $Lin^-c\text{-}kit^+$ hematopoietic cells ($CD45^+$ or GFP-tagged) in tumors were studied in our preliminary experiments to examine their spatial interaction between tumor vasculature (using CD31, vWF or VE-Cadherin). Combinations of Alexa 405, Alexa 488, Alexa 594 and Alexa 647 were used. However, at least in our hands, such fluorescent combination and detection of surface markers were not stable and provided good signals on the blue fluorescent. A few conjugated antibodies were also tested in order to optimize the detection in a stable and repeatable manner. Unfortunately and similarly, those combinations neither were detected in a repeatable manner nor provided good signals. It remains unclear, even in the BM HSC niches, whether these HSCs and progenitors were migrating or whether they locate in other possible niches that remain unrecognized. Whether HSPCs in tumors are associated with tumor vasculature in a similar pattern as in the bone is not known yet. To further study HSPCs (e.g. LSK or HSCs) enclosed in tumor and elucidate the possible niche structure, a more powerful and repeatable four-five-color immunofluorescence detection method is needed to elucidate the possible niche structure in tumors. HSCs and progenitors circulate freely in the circulation system. Tumors are

highly vascularized and a vascular niche has been recently described in the bone marrow (Ding et al., 2012). Whether there is a possible existence of an extramedullary vascular niche in tumors needs further detailed investigation.

3.3 HEMATOPOIETIC MICROENVIRONMENT IN TUMOR

We also studied whether tumor microenvironment had some common features of the BM HSC niches. Adhesion molecules, paracrine signaling molecules of osteoblastic and vascular niche and signs of endochondral ossification were identified in all murine melanomas and human tumors samples we examined (Fig.3 in III). Since our immunohistological studies showed a potential for osteoblast or osteoblast development, it would be interesting to find out in future if maturation of a specific cell type could be possible in tumors and further determine the cellular origin of the potential osteoblast or osteoclast development. However, current studies have shown controversial issues on the function molecules that defines HSC niche. For instance, in the osteoblastic HSC niche in the bone marrow, there are controversial studies on the role of N-cadherin in the osteoblastic niche (Zhang et al., 2003; Kiel et al., 2009). Moreover, although quiescent HSCs locate near osteoblasts and osteopontin is expressed by these osteoblasts, study has shown that osteopontin may negatively control the HSC pool size (Stier et al., 2005). These results may indicate, on the other hand, the importance of the vascular niche.

Genomic profiling on melanoma has shown that melanoma cells express osteopontin, N-cadherin, Angiopoietin-1, Jagged-1, SDF-1 and β-catenin (Alonso et al., 2007; Blackburn et al., 2009; Chien et al., 2009; Haqq et al., 2005; Harlin et al., 2009; Kashani-Sabet, 2004; Kashani-Sabet et al., 2009; Ramgolam et al., 2011; Shoo et al., 2010). RNAseq data (level 3) for 375 skin cutaneous melanoma samples were downloaded from the Cancer Genome Atlas (TCGA) database (Network, 2008). Duplicated and metastasis samples were then removed from the sample set. Analysis to samples were limited to samples with less than 100% of tumor cell content in order to capture as much as possible from the gene expression of normal and stromal cells within the tumor. By this criterion, 54 primary tumor melanoma samples were yielded from the 375-sample set. We then analyzed these eight gene expressions (ANGPT1, CDH2, CTNNB1, CXCL12, JAG1, SPP1, VCAM1 and VEGFA) in 54 human primary melanomas samples. According to their logarithmic RSEM expression level in the primary melanomas, the values were classified into high (>10), intermediate (5-10) and low (<5) (Fig.3 in III). The cutoff value of 5 is a conservative estimate representing about the lowest 40% of expression values over the entire 375-sample set. We considered that low expression values (<5) to be insubstantially expressed in the tumor samples. In accordance with our immunohistochemistry analysis, all these eight genes were expressed in these 54 samples. Six of these eight genes were simultaneously expressed in 96% of the sample set, while 30% of the primary melanoma samples concurrently express all eight genes. In addition, 93% of the primary melanoma samples expressed PTPRC (also known as CD45, a hematopoietic cell surface marker) and 89% of them expressed ITGAM (also known as CD11b, expressed by monocytes, granulocytes, macrophages and nature killer cells (Solovjov et al., 2005)). In addition, all 54 melanoma samples concurrently expressed CXCR4, receptor for SDF-1 (CXCL12), at a high or intermediate level.

3.4 IN VIVO EVACUATION OF HEMATOPOIETIC CELLs FROM TUMORs BY AMD3100

AMD 3100, a non-peptide antagonist of CXCR4, is used in clinics to mobilize hematopoietic stem/progenitors from HSC niches to peripheral circulation (Broxmeyer et al., 2005; De Clercq, 2003; De Clercq, 2009; Liles et al., 2003). AMD3100 specifically antagonizes the interaction between SDF-1 and CXCR4 and thus HSCs, instead of homing to BM, are mobilized into circulation (De Clercq, 2009). Earlier studies have shown that AMD3100 mobilizes HSCs to the circulation in ischemia and inhibit BMDCs recruitment (especially $CXCR4^+$ hemangiocytes) in brain tumor (Capoccia et al., 2006; Kioi et al., 2010). In agreement, we showed that the both hematopoietic cell density and vascular density were decreased by AMD3100 blocking in murine melanomas in vivo whereas AMD3100 does not affect the proliferation of melanoma tumor cells in vitro (Fig.4 in III). Moreover, proliferating hematopoietic cells in tumors was also decreased by AMD3100 blocking, while the colony forming ability of BM cells was not affected by AMD3100 (Fig.4 in III). Thus this indicated that AMD3100 not only inhibited total hematopoietic cell recruitment but also proliferating hematopoietic cells and thus resulted in inhibited tumor growth and tumor angiogenesis. Future studies on analysis of HSPC compartment changes in tumors, BM, and peripheral blood caused by AMD3100 is needed to clearly elucidate the mechanism of AMD3100 on HSPC in tumors.

Nevertheless, the effect of AMD3100 on inhibiting tumor growth and tumor angiogenesis may not be elucidated as a single mechanism of action on one cell type. Tumors interact with the stromal microenvironment during growth and development. It has been shown that tumor cells preferably metastasize to CXCL12-rich niches in several mouse models and also are able to compete with HSCs for endosteal niche when homing to BM through CXCR4/CXCL12 axis (Hassan et al., 2011; Kim et al., 2010; Shiozawa et al., 2011). It is possible that AMD3100 not only affect the recruitment of hematopoietic cells including HSPCs but also affect their secretion of angiogenic factors. It is also possible that AMD3100 treated tumor cells exert a trophic effect on infiltrated hematopoietic cells and subsequently affect the whole tumor microenvironment leading to inhibited tumor growth. Genomic profiling or proteomic analysis of the effect of AMD3100 on tumor cells and BMDCs will be helpful to understand the mechanism of action of AMD3100 on tumor growth.

In conclusion, during tumorigenesis, the malignant tumors initiated an ectopic microenvironment that shares the common features possessed by osteoblastic and vascular niche in the bone (Fig.5 in III). The tumor environment enclosed hematopoietic progenitors with multilineage potential. Phenotypic HSPC compartment analysis of tumor associated hematopoietic cells showed deviated expression from that of BM. The therapeutic ablation of proliferating hematopoietic cells from the ectopic niche would lead to an inhibition on tumor growth and tumor angiogenesis. These hematopoietic effectors support angiogenesis in a paracrine manner.

It is very attempting to hypothesize that tumor cells, together with tumor ECs, initiate a niche environment that mimics the HSC niches to support HSPCs in the tumor microenvironment. The idea of the possible existence that an extramedullary vascular niche may contribute to the initiation and creation of HSC niche in these tissues and many other cell types may also be capable to create a HSC niche environment is also very interesting. Additional work will be required to fully prove this hypothesis. Whether these

adhesive and secretive molecules that we observed in the tumor microenvironment are indispensable for the HSPCs in the tumor also needs further specific signaling studies on each of these axes. Future studies demonstrating local inhibition of niche signals that affect niche functions in tumors shall be conducted to fully understand the interactions among tumor growth, HSPCs and niche functions.

CONCLUSION AND FUTURE PROSPECTIVES

Most tissues contain tissue-specific stem cells that allow them to undergo tissue maintenance and regeneration. Various tissue-specific stem cells and progenitors are actively participating in angiogenesis by generating daughter cells composing blood vessels or by secreting various angiogenic factors.

The cellular origin of de novo endothelial cells during adult vascular growth is a fundamental question in vascular biology. A rare VESC population with high proliferative potential is present somewhere, for example, in existing blood vessel in situ, and responds to neo-vascularization by generating endothelial cells (Rinkevich et al., 2011). Notably, during early embryonic development, aorta-gonad-mesonephric region (AGM) contains HSCs that arise from aortic endothelium, which indicates an embryonic endothelial-hematopoietic transition (Kissa and Herbomel, 2010). The vascular wall is also considered to harbor various progenitor cells including MSCs, HSCs, and smooth muscle progenitor cells (Hu et al., 2004). Taken together, the vascular wall may serve as a reservoir of progenitors of many lineages. Our results showed that rare self-renewing c-kit$^+$ adult VESCs residing on the vascular wall were involved in adult angiogenesis by generating functional blood vessels in vivo. With an endothelial c-kit defect, the colony-forming ability of VESCs and in vivo tumor growth and angiogenesis were abolished.

C-kit may regulate the stem cell-like characteristics and proliferative potential in ECs. By targeting c-kit/SCF axis in ECs may result in potential therapeutic benefits in patients. Additionally, c-kit expression is identified along the endothelial tubes within the endometrium assigned to pericytes. Similarly, c-kit positive pericytary networks have been identified along the endothelial tubes within the uterine tube wall (Rusu et al., 2013). The capability of these c-kit$^+$ VESCs to differentiate/transdifferentiate into mural cells needs to be further studied.

In many clinical trials, chemotherapies are frequently combined with anti-angiogenic drugs to treat cancer. It would also be interesting and potentially therapeutically useful to know how VESCs react to chemotherapies and anti-angiogenesis drugs and subsequently affect angiogenesis. VESCs, responsible for the generation of de novo blood vessels, would be a more ideal and precise therapeutic target to inhibit angiogenesis.

On the other hand, abundant hematopoietic cells are recruited to local microenvironment in angiogenesis, especially during tumor angiogenesis (De Palma and Naldini, 2006; De Palma et al., 2005; De Palma et al., 2003; Rajantie et al., 2004). Similarly, in TGF-β induced angiogenesis, we also observed a massive recruitment of infiltrating hematopoietic cells. These infiltrating hematopoietic cells were in close connection to the blood vessels (Fig. 3D in II). TGF-β caused a prominent hematopoietic cell infiltration in mice and induced them to secrete VEGF through p44/p42 and p38 MAPK signaling pathway in vivo. The results elucidated the dual action mechanism by which TGF-β induces angiogenesis in vivo.

We also found an abnormal morphology of the vasculature, including an irregular pericyte pattern and enlarged vascular lumen. The enlarged vascular lumen indicated that an inflammation might be induced by TGF-β. Angiogenesis and inflammation are connected in many pathological conditions, such as tumorigenesis (reviewed in Balkwill and Mantovani, 2012; Candido and Hagemann, 2013; Hanahan and Weinberg, 2000, 2011). Inflammation may contribute to the microenvironment by supplying proangiogenic

factors and extracellular matrix-modifying enzymes (DeNardo et al., 2010; Grivennikov and Karin, 2010a, b; Karnoub and Weinberg, 2006). Further studies on the interactions among hematopoietic cells, blood vessels and local microenvironment will provide more information on the complexity of tumorigenesis.

In the thesis work, we also showed that it is possible that malignant tumor initiated an ectopic hematopoietic microenvironment by possessing common signatures of osteoblastic and vascular HSC niches. We found that tumor microenvironment contained hematopoietic progenitors with the ability to produce BFU-E, CFU-GM and CFU-GEMM in vitro. The frequencies on phenotypic expression detected of HSPCs using SLAM family (HSC, MPP and HPC) were deviated from HSPCs in the BM. Multipotent hematopoietic progenitors resided within the tumor and generated hematopoietic cells and secreted angiogenic factors to support tumor growth and angiogenesis. $Lin^{-}CD48^{-}CD150^{+}$ cells were found in proximity to tumor vasculature. Whether the identified HSCs are functional still need further investigation. Our immunohistochemical and RNAseq analysis showed that tumor microenvironment shared common features of the microenvironment in the bone marrow. However, whether an extramedullary perivascular niche exists inside tumor is not fully elucidated. Enlightening from the evolving definition on BM niche components and their regulatory mechanisms on hematopoiesis are needed to further clarify the possible extramedullary perivascular niche. Evacuation of hematopoietic cells using AMD3100 in tumor-bearing mice resulted in a decreased level of proliferating hematopoietic cells in tumors accompanied by inhibited tumor growth and angiogenesis (Fig.4 in III). The newly discovered tumor hematopoietic progenitors and their residing microenvironment in our results provided evidence on the new components of tumor microenvironment. Tumor, considering as a developing organ entity, encloses a heterogeneous population of cells and requires crosstalk between the tumor entity and the tumor vasculature. Tumor vasculature may also provide angiocrine signals that affect cell differentiation in the developing tumors as it is in other developing organ such as liver and bone (Ding et al., 2014; Ding et al., 2011). Understanding how HSPCs and their progeny, tumor cells, blood vessels and other cells in the microenvironment work in concert to regulate tumor growth and development will provide opportunities to improve current cancer therapy.

As such, the identification and characterization of c-kit-expressing VESCs by phenotype $lin^{-}CD31^{+}CD105^{+}Sca\text{-}1^{+}c\text{-}kit^{+}$ on the vascular wall in this thesis work provided important evidence on the cellular origin of de novo ECs in adult vascular growth. Further identification of additional and more powerful cell-surface markers is needed for more efficient isolation of VESCs. With a more detailed understanding of VESCs, potential cellular therapeutics using these cells may be applied to patients. On the other hand, in various angiogenic conditions, proliferating hematopoietic progenitors and infiltrating hematopoietic cells secrete angiogenic factors such as VEGF to support angiogenesis. Further studies on the interactions among hematopoietic progenitors, hematopoietic effector cells, blood vessels and other cellular components of the local microenvironment would provide more potential therapeutic targets to affect tumor growth.

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