Vascular development and safeguard mechanisms against tumorigenesis: Oncogene-induced apoptosis and cellular senescence

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Doctoral Thesis
Karolinska Institute
Stockholm 2012

Published by Karolinska Institutet.

ISBN 978-91-7457-867-6

Printed by
REPROPRINT AB
Stockholm 2012
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

Abstract

Angiogenesis, apoptosis and senescence are all cellular processes that have an impact on tumor development. Angiogenesis or the new vessel formation from pre-existing ones is known to be required for invasive tumor growth and metastasis. Apoptosis and cellular senescence are both considered crucial safeguards mechanisms against neoplastic transformation. The *MYC* oncogene plays an important role in the regulation of all three of these as well as many other fundamental processes crucial for cell growth and tumorigenesis.

In the first part of this thesis we explored endothelial cell migration by the exposure of human vein endothelial cells (HUVECs) and human umbilical artery endothelial cells (HUAECs) to stable hill-shaped gradients of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2). Time-lapse analysis showed that a gradient of VEGFA165 efficiently induced chemotaxis of endothelial cells of different vascular origin. Stable gradients of FGF2 were able to attract venular but no arterial endothelial cells. In addition to the directed migration of endothelial cells, we also investigated the lymphatic vessel formation in the developing mice kidney. Immunohistochemical analysis of kidney explants and whole mount of dissected kidney suggested that renal lymphatic vessel formation predominately occurs via invasive sprouting from surrounding lymphatic plexus.

In the second part of this thesis, we first aimed to clarify the relative importance of the intrinsic (mitochondrial) and extrinsic (death receptor) anti apoptotic pathways in the *in vivo* MYC driven transformation of hematopoietic stem cells. Expression of MYC alone resulted in the development of both myeloid and T-lymphoid tumors within two months after transplantation of HSCs. Expression of MYC together with BCL-X_L or BCL-2 (inhibiting the intrinsic pathway) resulted in almost immediate development of AML like disease. In contrast, expression of MYC together with $FLIP_L$ (inhibiting the extrinsic pathway) did not accelerate tumorigenesis. These results suggest that MYC-induced transformation of HSC accelerates and polarizes hematopoietic tumor development towards aggressive AML by co-expression of inhibitors of the intrinsic but not the extrinsic pathway of apoptosis. Secondly, we aimed to determine whether pharmacological inhibition of cyclin dependent kinase 2 (CDK2) interferes with MYC-driven tumor development *in vivo* trough senescence. Mice transplanted with HSCs expressing MYC and BCL-X_L as briefly described above were treated with a specific CDK2 inhibitor on daily basis via intraperitoneal injections or osmotic minipumps. Despite the very aggressive AML development in this model, CDK2 targeting significantly delayed the onset of disease and improved mice survival by restoring senescence. The senescence induction correlated with induction of $p19^{ARF}$, $p21^{CIP1}$ and activation of pRb. This suggests that pro-senescence therapy via CDK2 inhibition should be further evaluated as a new potential strategy to combat MYC-driven AML and possibly other MYC-related tumors.

Key words: Angiogenesis, apoptosis, senescence, vascular growth factors (VEGFs), cyclin dependent kinase 2 (CDK2), MYC, BCL-X_L, $FLIP_L$

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Contents

This doctoral thesis is based on the following papers:

I. Endothelial cell migration in stable gradients of vascular endothelial growth factor A and fibroblast growth factor 2: effects on chemotaxis and chemokinesis. Irmeli Barkefors, Sébastien Le Jan, Lars Jakobsson, **Eduar Hejll**, Gustav Carlson, Henrik Johansson, Jonas Jarvius, Jeong Won Park, Noo Li Jeon and Johan Kreuger. *J Biol Chem, 2008, 283,13905-13912*

II. *In vivo* and *ex vivo* studies of lymphatic vessel formation in the developing mouse kidneys. **Eduar Hejll**, Fredrik Lanner, Filip Farnebo, Lars-Gunnar Larsson and Leif Oxburgh (*Manuscript*)

III. Inhibition of the intrinsic but not the extrinsic apoptosis pathway accelerates and drives Myc-driven tumorigenesis towards acute myeloid leukemia. Högstrand K, **Hejll E**, Sander B, Rozell B, Larsson LG, Grandien A. PLoS One. 2012;7(2):e31366. Epub 2012 Feb 29

IV. Restoration of senescence upon Cdk2 inactivation delays MYC-driven acute myeloblastic leukemia. **Eduar Hejll**, Matteo Bocci, Vedrana Tabor, Per hydbring, Alf Grandien and Lars-Gunnar Larsson (*masnuscript*)

Abbreviations

Arf	Alternative reading frame
ATM	Ataxiatelangiectaxia mutated
BEC	Blood endothelial cell
bHLHLZ	Basic-helix-loop-helix leucine zipper
Bim	Bcl-2 interacting mediator of cell death
BMP4	Bone morphogenic protein 4
Cdc	Cell division cycle
Cdk	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitor
CML	Chronic myeloid leukemia
CSC	Cancer stem cell
DBD	DNA binding domain
E	Embryonic day
EC	Endothelial cell
EGFR	Epidermal growth factor receptor
ES	Embryonic stem cell
FGF2	Basic fibroblast growth factor
GSK	Glycogen synthase kinase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HSC	Hematopoietic stem cell
Ink4	Inhibitor of Cdk4
LEC	Lymphatic endothelial cell
MAPK	Mitogen activated protein kinase
Max	MYC associated protein x
MEF	Mouse embryonic fibroblast
PDGRF	Platelet derived growth factor receptor
PI3-K	Phosphatidylinositol-3 kinase
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
REF	Rat embryonic fibroblast
RTK	Receptor tyrosine kinase
SA-B-Gal	Senescence-associated B-galactosidase
Skp2	S-phase associated kinase associated protein 2
TERT	Telomerase reverse transcriptase
TNF-α	Tumor necrosis factor- α
TNF-R	Tumor necrosis receptor
Ub	Ubiquitin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Background

Over the past century we have gained crucial knowledge about the biological processes that govern life. These complex processes have become clearer for us but still there is more to understand. Our need to cure diseases has led us to discover new ways to combat illness that killed thousands before. For instance, diseases such as diabetes lead most certainly to death and after several efforts made by scientists in 1922 the first patient with diabetes was cured. Thanks to the discovery of insulin by Frederick G. Bating and Jonh Macleod, now thousands of people can survive and have a normal life. However, we are still fighting against devastating diseases including cancer. According to the World Health Organization (WHO), cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2008 and deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030.

The discovery of the double helix structure and function of the DNA has tremendously impacted our deep understanding of life and it is probably the most important discovery among all other crucial findings that have help us to decipher life. The DNA structure and function discovery has led us to the dissection of crucial molecular mechanisms behind tumor progression and other human diseases. It has helped us to improve the ability to diagnose diseases and the production of pharmaceuticals to treat them. Now we know that cancer arises from one single cell, and that sequential accumulation of genetic lesions and epigenetic changes lead to cell transformation. These genetic lesions are influenced by different factors such as physical, chemical and biological carcinogens. In addition, cancer development is influenced by the genetic inherited cancer predisposition of each person. The gained advances in genetics and molecular biology aided us to identify and understand that the imbalance of oncogene and tumor suppressor genes plays a crucial role in tumor development. The majority of known oncogenes are mutations of certain normal "good" genes named proto-oncogenes [1]. These "good" genes are normally involved in the control of cell growth and division. In the other hand tumor suppressor genes encode for proteins that control cell growth, repair DNA damages and dictate to cells when to die [2]. Tumor cells with disturbed oncogenes and tumor suppressors take over crucial mechanism that guaranties uncontrolled cell survival. The intense research efforts over the past decades have led to the conclusion that the complexity of cancer can be reduced to a number of underlying principals.

It is believe that cancer share six common traits which dictates neoplastic transformation. These complex traits are called the hallmarks of cancer and include sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [3]. Lately and after new gained insights in cancer biology, several additional hallmarks have been added to the list of complex traits shared by cancer cells. The new include traits are the deregulation of metabolism, evasion of the immune system, genomic instability and tumor-promoting inflammation [4]. Moreover, it has become clearer that the tumor microenvironment also plays a key role in tumor development. The understanding of the underlying molecular mechanisms behind these special and complex traits of cancer can help us to develop new novel strategies against human cancer.

In this doctoral thesis, angiogenesis (Part I), and oncogene related apoptosis and senescence are explored (Part II). Each one of these processes is believed to take part in the different aspects of tumor development. For instance, angiogenesis or the new blood vessel formation from preexisting ones is known to be required for invasive tumor growth and metastasis. Avascular tumors are often restricted in growth due to the lack of a blood supply. In order to enhance their growth and metastatic potential, tumors make an "angiogenesis switch" through perturbation of the local balance of proangiogenic and antiangiogenic factors [5]. The second vascular organ or the lymphatic system is also highly involved in tumor development. It is supposed that the lymphatic system is essential to the body´s surveillance against cancer. In fact the lymphatic system is used as escape routes for cancer cells during metastasis. The MYC oncoprotein regulates several cellular processes of importance for cell growth and division, and it is often deregulated in cancer. MYC is also an inducer of intrinsic safeguard mechanism against cell transformation, including apoptosis/programmed cell death and cellular senescence, which needs to be overcome by genetic lesions and epigenetic alterations during cellular transformation.

Part I

1.1 Vascular Development

1.1.1 Blood and lymphatic vascular system

The lymphatic and cardiovascular systems are two major components of the vertebrate body and are highly involved in different critical physiological processes. The cardiovascular system transports nutrients, oxygen and other molecules to different tissues. During this transport fluid and macromolecules are extravasated to the interstitial space and drained back to the venous system by the lymphatic system. Both blood and lymph vessels also play key roles in cancer progression. Tumor vessel formation guaranties nutrient supply and also provides routes for cancer cells during metastasis. The blunt ended and uni-directional lymphatic system also fulfills other physiological functions such as lipid absorption in the intestinal tract, as well as transport of immune cells to lymphoid organs [6]. Blood and lymph vessels differ in their anatomy. The major blood vessels, the veins and arteries, are composed of a single layer of blood endothelium (tunica intima) surrounded by an internal elastic lamina, which in turn is enclosed by smooth muscle cells (tunica media). The third layer, the tunica adventitia, consists of connective tissue. Capillaries and post-capillary venules are supported by a layer of smooth muscle actin (SMA) positive pericytes (Figure 1).

The lymphatic vascular bed is composed of a one-way network of thin-walled capillaries, which are found in the skin and most vascularized organs except for the central nervous system, bone marrow, cartilage, cornea and epidermis. The lymph capillaries and smaller lymphatic vessels are composed of single layer of lymphatic endothelial cells and are not covered with pericytes or smooth muscle cells. Large collecting lymphatic vessels have a smooth muscle cell layer, basement membrane and valves which prevent back flow of lymph [6] (Figure 1B and C).

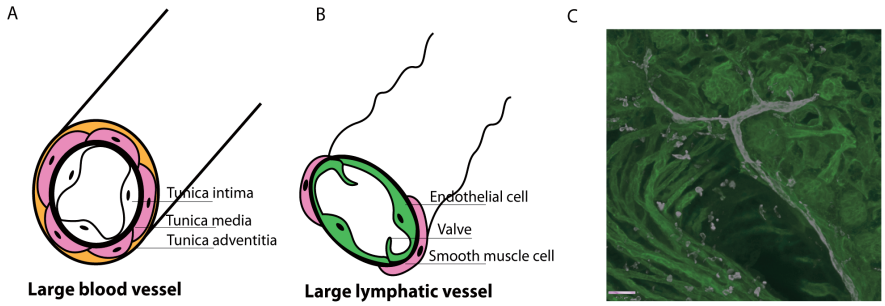

Figure 1. Blood and lymphatic vessel morphology A) large blood vessel B) Large lymphatic vessel structure C) Renal lymph (white) and blood vessels (green), unpublished data.

1.1.2 Vasculogenesis

Vasculogenesis is the process of *de novo* vessel formation during embryonic development from endothelial precursor cells, angioblasts [7]. Vasculogenesis starts in the primitive streak where vascular endothelial growth factor (VEGF) receptor -2 (also denoted Flk-1) positive cells appear in response to basic fibroblast growth factor (bFGF) and bone morphogenic protein 4 (BMP4). These cells are believed to be the progenitors for both blood cells and endothelium (haemangioblast). The progenitors migrate into the extra-embryonic yolk sac and on embryonic day (E) 7-E7.5 form blood island structures, which will contain an outer layer of endothelial precursor cells termed angioblasts and an inner mass of hematopoietic precursor cells. The outer cells of the blood islands, the angioblasts, undergo further differentiation and form a primary vascular plexus of blood vessels [8]. Inside the embryo mesodermal derived angioblasts differentiate into endothelial cells and form a primitive vascular plexus at E8-8.5. Blood circulation in the primitive plexus is dependent of the connection of the vascular channels to the developing heart tube at E9.0.

1.1.3 Angiogenesis

In order to achieve circulation, the vascular plexus is remodeled gradually by different processes where angiogenesis, the formation of new vessel from pre-existing ones, is involved [9]. Differentiation, regression, migration and branching are some of the events leading to vascular plexus maturation. An orchestra of precisely synchronized growth factors, receptors and other crucial molecular players are implicated in these events (7). Angiogenesis is taking place during embryonic development as well as during normal physiological conditions such as aerobic and endurance exercise, wound healing, ovulation, inflammation.

In addition, angiogenesis occurs during pathological conditions such as tumor growth and chronic inflammation. Angiogenesis is a complex process, which is regulated by interactions between endothelial cells and other cell types such as fibroblasts, macrophages and smooth muscle cells. Cellular compounds including cytokines, growth factors and extracellular matrix molecules mediate intercellular interactions between these cells [10].

In the adult angiogenesis can occur either through sprouting or intussusception [11]. In sprouting angiogenesis, vessel formation is achieved by a well-defined program where growth factors that trigger the angiogenic responses are released at the site of angiogenesis. Endothelial cells need to detect and respond to extracellular chemical gradients by direct or negative migration, a process called chemotaxis. Directed cell migration is governed by a well-synchronized orchestra of secreted and ECM bounded growth factors including VEFG, bFGF and angiopoetins [12].

These growth factors bind to specific receptors on endothelial cells in the blood vessels. Growth factor binding activates different intracellular pathways, which lead to production of specific proteins involved in angiogenesis [13]. Enzymes such as proteases are then released to the extra-cellular matrix and digest basement membrane components thus allowing selected blood endothelial cells (ECs) to sprout from the vessel (figure 2).

Sprouting of the ECs is followed by the formation of a stalk of proliferating cells in the surrounding matrix. The formed stalk is headed by a non-dividing tip cell, which prevents adjacent cells from converting into tip cells (figure 2) [14]. The tip cell and cells in the stalk contains several actin rich membrane protrusions called filopodia, which express different receptors for sensing secreted and cell-bound guidance cues provided by surrounding cells [15]. Polymerized actin bundles form the sensing filopodia and are induced by VGFR-2 activation [15].

After these events the ECs form solids sprouts that connect to a neighboring vessel and restructure into a lumen lined by endothelial cells. Finally the newly formed vessel integrates in the vascular network and in order to make a more stable blood vessel supporting circulation, pericytes and smooth cells are recruited to the newly formed vessels. In intussusception or splitting angiogenesis, pre-existing larger capillaries are divided into two new smaller vessels by ingrowth of tissue pillars [11].

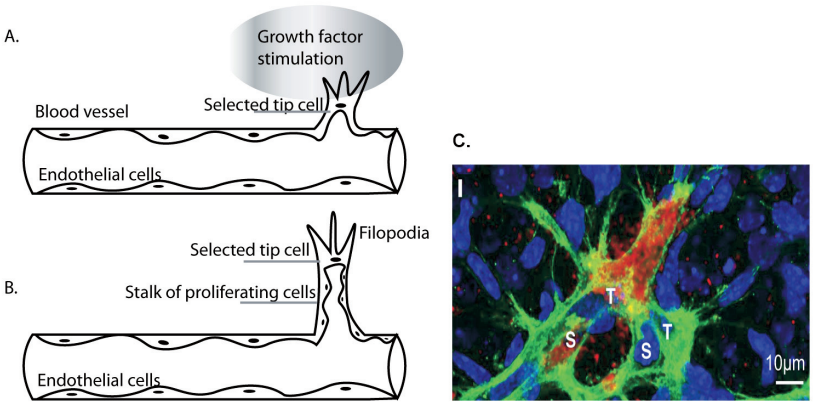

Figure 2. Sprouting mechanisms in angiogenesis. A) Drawing of the cell tip formation B) stalk formation C) Experimental evidence for the formation of tip cells (T) and stalk cells (S)[14].

1.1.4 Vascular endothelial growth factors (VEGFs) and their receptors

VEGFs stimulate blood endothelial angiogenesis as well as lymphangiogenesis. The VEGFs are related to platelet-derived growth factors and bind as dimers to VEGF-receptors (VEGFRs). Ligand binding enhances receptor dimerization or heterodimerization, which in turn promotes autophosphorylation of intracellular domains. Phosphorylation activates specific downstream signals leading to cell responses such as migration, survival and proliferation. VEGFRs belong to the receptor tyrosine kinase (RTK) family. RTKs are composed of an extracellular domain where growth factors bind, a polypeptide chain transversing the cell membrane, and an intracellular polypeptide chain where tyrosine kinase activity is found. Five VEGFs have been described; VEGF-A, -B, - C, -D and placental growth factor (PlGF). Beside these known factors, there are other proteins structurally related to VEGF; parapoxvirus VEGF-E and snake venom VEGF-F. VEGF-A, -B and PlGF are ligands for VEGFR-1, VEGF-A and -E are ligands for VEGFR-2, and VEGF-C and D are ligands for VEGFR-3 (figure 3). Further, proteolytic processing of VEGF-C and D allows them to bind VEGFR-2 albeit with a lower affinity than to VEGFR-3 [16]. In addition, VEGF-C stimulation of lymphatic endothelial cells also induced the formation and activation of VEGFR-3/VEGFR-2 heterodimers [17].

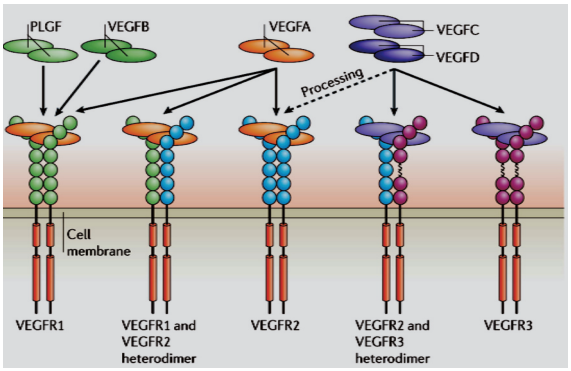

Figure 3. VEGFRs / VEGFs and their interactions [17].

VEGFA is a mitogen and a survival factor that specifically acts on endothelial cells and its expression is upregulated by hypoxia. VEGFA deficient embryos show abnormal blood vessels and die at midgestation [18],[19]. Disruption of the gene encoding for VEGF-C leads to severe edema at E12.0 and the embryos die at E15.5. The lymphatic vasculature in these embryos failed to develop although PROX-1 positive cells were observed in the cardinal veins. However, the PROX-1 positive cells failed to sprout and form lymphatic sacs indicating that VEGFC paracrine function is essential for the migration and survival of the PROX-1 committed vein endothelial cells [20]. In conclusion, of these growth factors, VEGFA and VEGF-C seem to be essential for embryonic survival, angiogenesis and lymphatic vessel formation or lymphangiogenesis.

The VEGF-A receptors, VEGFR2 and 1 are suggested to be highly involved in embryonic vascular development. Transgenic mouse embryos carrying a disrupted VEGFR-2 gene die in uterus between 8.5 and 9.5 days post-coitum, as a result of an early defect in the development of haematopoietic and endothelial cells[21]. VEGFR-1 signaling seems to be essential for endothelial cell- cell or cell matrix interactions required for vascular organization but not necessary for endothelial cell differentiation[22].

VEGFR-3 is an important player in lymphangiogenesis and a key regulator in embryonic vessel development; it is expressed in embryonic blood vessels while it becomes restricted to the lymphatic vasculature in the adult[23]. Targeted inactivation of VEGFR-3 leads to defective blood vessel development in early embryos causing fluid accumulation in the pericardial cavity and cardiovascular failure at E 9.5[24]. Nevertheless recently studies have also implicated VEGFR-3 in angiogenic sprouts. These studies suggested that VEGFR-3 is highly expressed in areas of active angiogenesis in the intersomites and also in tip cells of

angiogenic sprouts in mouse retina and melanomas models[25]. The understanding of VEGFR dynamics in pathologies such as cancer is of great importance for the development of new treatments. Targeting tumor angiogenesis and lymphangiogenesis with new strategies will hopefully impair tumor growth and reduce the formation of metastasis.

1.1.5 The origin of the lymphatic endothelial cell

The 'second circulation system' of the body, the lymphatic vascular system was first described around 400 BC by Hippocrates, who saw vessels containing "white blood". In 1627 Gasparo Aselli, an Italian anatomist, described the lymphatic system in well fed dog guts as milky ways (Figure 5A). Later on in the beginning of the last century the anatomist Florence R. Sabin (1902-1904) injected ink in pig embryos and observed a subcutaneous network of lymphatic vessels. Through a long series of these ink injections, Florence R. Sabin traced back the lymphatic vessels to tiny buds close to the veins [26,27](Figure 5B). Based on these observations she laid down the first theory for lymph vessel development[28,29]. According to Sabin, the lymphatic system develops from lymphatic primitive sacs, which are formed by sprouts from the venous system. In this centrifugal theory, the neck and the head lymphatic endothelial cells sprout from the jugular sac and spread centrifugally into the surrounding tissues and organs where local capillaries form. Another theory (termed centripetal) proposed by McClure and Huntington (1908-1910) claims that lymphatic endothelial cells arise de novo from undifferentiated mesenchymal cells or circulating precursor cells[30]. According to this theory, initial lymphatic sacs arise in the mesenchyme independent of the veins and secondarily establish venous connections (3). Most of the experimental evidence for the non-endothelial theory derives from experimental studies using *xenopus* and chick models[31]. However, recent studies in mice by Wilting et al have shown that non-endothelial precursor cells contribute to lymphatic sac formation[32]. Despite these findings it is still unknown to what extent the non-endothelial precursors contribute to lymphatic vessel development. Thus, embryonic lymphatic vessel might have a dual origin: endothelial and mesenchymal (figure 4).

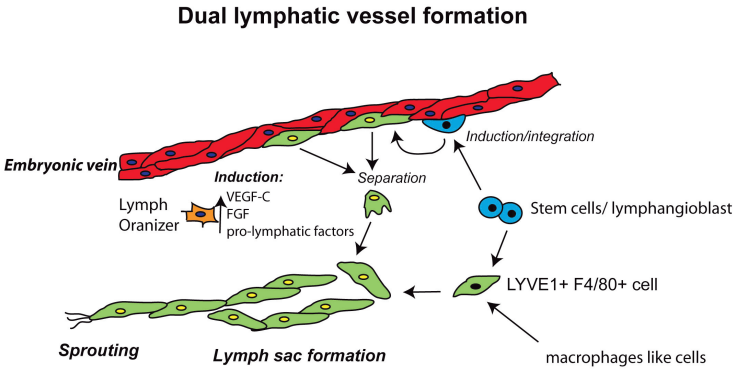

Figure 4. Large embryonic veins are suggested to be a mayor source for lymphatic endothelial cells. Vein endothelial cells commit into lymphatic cells and form embryonic lymphatic sacs, which sprout and form primitive lymphatic plexus. Other non-endothelial sources such as mesenchymal cells have been also suggested to contribute to lymphatic vessel formation.

In 1966, Lark and Burke determined the morphological difference between lymphatics and blood vessels and the first successful attempt to isolate lymphatic endothelial cells was carried out[33]. It was a hard task since no specific lymphatic endothelial markers were available at that time. Decades passed and specific endothelial markers such as prospero homeo domain -1 (PROX-1), lymphatic endothelial hyaluronan receptor (LYVE-1), Podoplanin, VEGFR-3 were discovered. The discovery of these specific lymphatic endothelial markers has contributed to the elucidation of some of the mechanisms behind lymph vessel formation and their implication in pathological conditions such as cancer, inflammation, psoriasis and asthma[34].

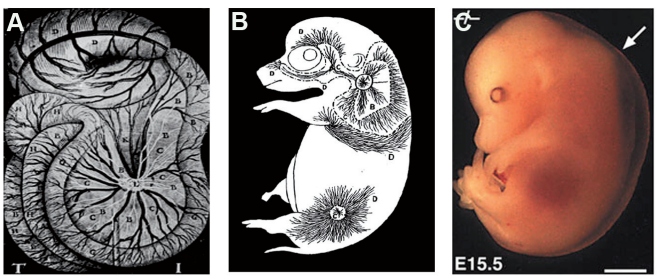

Figure 5. A)'Milky veins in dogs', drawing by Gasparo Aselli (1627), cartoon adopted from Nature. .**B)** Florence R. Sabin (1904), drawing of proposed lymphatic vessel formation in pig embryos **C)** Experimental studies of VEGF-C-/- deficient mice and its role in lymphatic vessel formation.

1.1.6 Lymphangiogenesis, molecular view of the centrifugal theory

Lymphatic vessel formation is believed to start in mid-gestation, after the establishment of the cardiovascular system. On approximately E9.5-10.0 venous endothelial cells from the anterior cardinal vein start to express LYVE-1 [35]. Following this initial step, a polarized subset of LYVE-1 positive competent venous endothelial cells commence to express PROX-1 [36,37]. The induction of these lymphatic markers is believed to be triggered by unidentified signals from the surrounding mesenchyme. PROX-1 is considered to be a key component in the commitment program into lymphatic endothelial cells. Forced expression of this transcription factor in BECs leads to up-regulation of LEC specific markers[38]. PROX-1 induction is also speculated to be initiated by the large vein remodelling events that take place between E9.5 and E13.5[39]. The vascular endothelial growth factor receptor 3 (VEGFR-3) is expressed after E8.5 in developing venous endothelial cells. As development progresses, VEGFR-3 expression becomes primary confined to the lymphatic endothelium [23]. At E10-E11 the VEGFR-3 ligand, vascular endothelial growth factor C (VEGF-C), is released by nearby mesenchyme cells and induces budding off from the cardinal vein, migration and proliferation of the committed LECs [20]. In PROX-1 null embryos the budding off is arrested, resulting in embryos lacking lymphatic vasculature [37]. The induced migration of the committed PROX-1 positive cells is believed to be mediated by VEGFR-3. During the migration of the committed cells, expression of other LECs marker such as Podoplanin and neurophilin-2 is upregulated. PDPN-deficient mice die at birth due to respiratory failure and showed groove defects in the lymphatic vasculature [40]. The migration of the committed LEC leads to the formation of primitive lymphatic sacs. During the lymph sac formation processes, BECs markers such as C34 and laminin are down regulated[35]. Further sprouting at E11.5-E14.5 from the primitive sacs leads to the formation of the primary lymphatic plexus, which spreads throughout the head, neck, thorax and forelimbs. Later on after the formation of the jugular lymphatic sacs, lymphatic sac development also occurs in the posterior part of the embryo from local abdominal veins. These sacs sprout and subsequently penetrate surrounding interstitial tissues.

1.1.7 Kidney vascular development

The mammalian kidney is a highly intricate organ involved in many important physiological functions such as waste clearance, pH regulation, blood pressure regulation, and hormone production. It is also affected by a variety of live threatening pathologies such as diabetic

nephropathy and renal cell carcinoma. This fascinating organ is highly vascularized and receives a significant portion of the cardiac input. The permanent mammalian kidney, the metanephros is formed by delicate interactions of three different cells lineages: the epithelium of Wolffian-duct derived ureter, the mesenchyme of the nephric blastema giving rise to the secretory nephron and the endothelial cell lineage providing the vascular supply of the kidney[41]. These cell interactions are modulated by a fine tuned orchestra of growth factors. Among these factors VEGFA seems to play a critical role in renal development by promoting endothelial cell differentiation, capillary formation and proliferation of tubular epithelial cells[42].

The murine kidney blood vasculature development begins right after the initiation of metanephroe formation at approximately E11.5, when endothelial cells from the embryonic aorta sprout and invade the developing kidney (angiogenesis) [31]. However it has been shown that developing kidneys also contain endogenous blood endothelial cells progenitors, which contribute to kidney microvasculature formation (vasculogenesis). These progenitors are observed in avascular areas of the developing kidney and are speculated to be of mesenchymal and epithelial origin [43]. In summary and according to experimental evidence, vasculogenesis and angiogenesis are potential mechanisms for local vessel formation in the developing kidney.

During embryonic development, organ lymphatic vasculature is believed to originate from LECs sprouting from nearby extra lymphatic plexus[44], [29]. Lymphatic vessel formation and function in highly vascularized organs such as the kidneys has received little attention and the mechanisms behind lymphatic vessel formation and its implication in kidney function are still obscure. It is also unclear if individual organs like the kidneys contain other non-endothelial precursor cells that may contribute to in-situ lymphatic formation. Knowledge of renal lymphatic function and its implication in renal diseases is limited. The understanding of renal lymphatic formation will hopefully give us new and valuable insights, which may help us to develop new strategies to combat kidney diseases.

Part II

1.2 Oncogene induced apoptosis and cellular senescence

1.2.1 The cell cycle

There are around 210 different cell types and about 75 trillion cells in the human body. All these cell types arise by cell division /multiplication from one single cell, the fertilized egg. Cell division is therefore a fundamental process for life. This process ensures the development and replacement of dead and old cells in every organ of the human body. From skin to liver tissues, cell division guaranties the wellness of each different organ of the body. Cell division is governed by the cell cycle, also named the cell division cycle. The cell cycle is well conserved among eukaryotes and it is composed by a serial of events leading to DNA replication and cell division. The mammalian cell cycle is activated by mitogenic signals and is composed of different and well-defined phases: the G_1 (gap 1), S (synthesis), G_2 (gap 2) and M (mitosis) phases of the cell cycle. In addition, the resting, quiescent state of a cell is referred to as G_0. The interphase is a common name for G_1, S and G_2. In this phase the cell is growing and preparing for mitosis by the accumulation of essential cell constituents and the replication of the DNA. In the G1 phase external mitogenic and anti mitogenic signals dictate whether the cell should proceed to S phase, enter quiescence, go into a state of cellular senescence (see further section 1.3.4 on cellular senescence) and/or differentiate. Upon mitogenic signals the cell cycle starts by the initiation of synthesis of macromolecules such as RNA and proteins. If the cell decides to pursue with the cycle, in the next step, the S phase, the DNA is replicated. The main goal of this process is to create an identical copy of the entire genome. After the S phase the cell enters into the following second gap G2 phase during which growth and preparation for cell division occurs. In the following mitosis phase (M), nuclear division takes place followed by the division of the cytoplasm, which allows the formation of two new cells [45].

The cell cycle is controlled by different checkpoints that ensure the fidelity of cell division. One of the major functions of the cell division checkpoints is to assess DNA damage. When DNA damage is found, the cell cycle is arrested or delayed and an attempt to repair the damage is initiated. If the DNA damage is irreparable the cell is target for destruction via apoptosis. There are at least 3 main cell division checkpoints, G1 (restriction)

check point, G2 checkpoint and the metaphase checkpoint. The G1 check point is found at the end of the G1 phase of the cell cycle, right before the entry into S phase. In this checkpoint growth factor/anti-growth signals decide whether the cell should continue cell cycle progression, delay division or enter the G0 phase. The G2 checkpoint is located at the end of G2 phase and controls the start of the M phase. In the M phase, the proper chromosome segregation is controlled by the spindle assembly checkpoint (SAC). There are other less unknown check points found in the cell cycle and these cover other aspects in the complex progression of the cell cycle. The cell cycle checkpoints are of great importance for cell function and therefore highly relevant for tumor development [45].

1.2.2 Cyclin dependent kinases and the cell cycle

The cell cycle is driven by oscillations in the activities of cyclin-dependent kinases (CDKs), which are activated by protein subunits called cyclins. CDK activity is tightly regulated by periodic synthesis and degradation of cyclins, cyclin kinase inhibitors and by reversible phosphorylation. There are at least 13 loci in humans encoding for CDKs and 25 loci encoding for cyclins respectively [46]. Among the different CDKs there are only 4 CDKs that actively take part in the cell cycle, CDK1 or CDC2, CDK2, CDK4 and CDK6 and ten cyclins belonging to four different groups (A, B, D and E) [47]. These different cyclins are specific for each specific phase of the cell cycle, where they accumulate and activate their CDK interacting partner. Different CDK-cyclin complexes are involved in the regulation of the different cell cycle transitions: D type cyclins (D1, D2 and D3) together with CDK4 and CDK6 for G1 phase progression, E type cyclins (E1 and E2) with CDK2 for G1-S-transitions, A type cyclins (A1 and A2) with CDK2 for S phase progression, and B type cyclins (B1 and B2) with CDK1 complexes for entry into M phase[47]. Upon mitogenic signals mediated by different signaling pathways the cell cycle machinery is stimulated to start cell division by leaving the G0 resting phase. In early G1 phase and upon stimulation by several mitogenic cascades including the Ras/Raf-1/Mek/ERK and β-catenin-Tcf/LEF pathways, D type cyclins (D1, D2 and D3) are induced and associate with CDK4 and CDK6 to form cyclin D/CDK4 or 6 complexes. The formed CDK4/6-cyclin-D complexes partially phosphorylate and contribute to inactivation of the pocket proteins Retinoblastoma (Rb), p107 and p103. The partial inactivation of Rb allows the expression of E type cyclins (E1and E2), which in turn associates with CDK2. The formed CDK2-cyclin E complexes contribute further to the phosphorylation and inactivation of Rb. Normally in its hypo-phosphorylated (active) form, pRb acts a tumor suppressor by inhibiting cell cycle progression. Inactive Rb

dissociates from the E2F transcription factor thus allowing the activation of the E2F-1-mediated transcription of genes involved in nucleotide metabolism, DNA synthesis and additional processes required for the S phase. Cyclin A is expressed in the S phase and associates with CDK2 to form cyclin-A-CDK2 complexes which continues to phosphorylate Rb. Cyclin B is induced during the G2 and the M phase of the cell cycle where it binds to CDK1. The formed CDK1-cyclin B complex mediates the onset of mitosis by inducing the nuclear envelope breakdown and the initiation of the prophase. Cyclin B is degraded by the APC E3 ubiquitin ligase complex at the metaphase-anaphase transition, which is required for exit from the M phase[45,47].

Mouse genetic studies have shown that CDK2, CDK4 and CDK6 are non-essential for the mammalian cell cycle [46,48,49]. Instead it seems that only CDK1 is crucial for proper cell cycle progression[50,51]. Embryos lacking all the interphase CDKs had a normal organogenesis and developed to midgestation. Interestingly, CDK1 interacts with all cyclins resulting in Rb hyperphosphorylation and the subsequent expression of genes that are regulated nu E2F transcription factors. Mice lacking CDK2 are viable and showed a survival of 2 years of age, thus indicating that CDK2 is not essential for proliferation and survival of most type of cells. However, CDK2 seems to be required for the completion of prophase I during meiotic cell division in male and female germ cells [52].

The activity of the formed CDK/cyclin complexes is tightly regulated during the cell cycle progression. Beside the control of the cell cycle by the fluctuation of cyclin levels, there are other several layers of control that modulates the activity of cyclin-CDKs complexes. There are seven known proteins that prevent cell cycle progression by inhibition of the cyclin-CDK complexes. The INK4 proteins (Inhibitors of CDK4), $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{IN4D}$ specifically targets CDK4 and CDK6 complexes. These CKIs have no effect on CDK2 and CDK1 complexes. The other CKIs, $p21^{Cip1}$, $p27^{Kip1}$ and $p27^{Kip2}$ primarily inhibit CDK2 and CDK1 complexes, but are able to bind and regulate all cell cycle CDK complexes[45].

Cellular stress originated from DNA damage results in the induction and activation of p53, which in turn activates p21. Growth inhibitory signals derived from anti-mitogenic factors such as TGFβ induces the activation of p27 and the subsequent arrest of the cell cycle.

The second family of CKIs is the INK4a/ARF family and includes the gene products p16INK4a and p19ARF. Activated p16 binds to CDK4 and arrest the cell cycle in G1 phase. p19ARF activation results in the stabilization of the tumor suppressor p53[45].

Extracellular anti-proliferative signals in the form of TGF beta can arrest the cell cycle by a rapid and strong transcriptional induction of p15 INK4B, which in turn blocks the formation and the already formed cyclin D-CDK4 complexes. p15INKB1 mRNA levels in human keratinocytes are drastically elevated after exposure to TGFβ. TGFβ is also a weak inducer of the more widely acting p21 which can block the action of the remaining cyclin-CDK complexes. Stress signals such as DNA damage are potent inducers of p21 but in a p53 dependent manner. Mitogenic signals such as those mediated by P13K lead to the phosphorylation of the nuclear p21Cip1. Akt/PKB phosphorylates p21 in the nucleus causing it translocation to the cytoplasm where p21 cannot not longer block cyclin-CDK complexes [45].

Cyclins are tightly regulated at different levels, and deregulated cyclins are often observed in cancer. For example, cyclin E, the crucial activator of CDK2, is often deregulated in carcinomas, lymphomas and sarcomas [53]. D type cyclins are also deregulated in several cancers including carcinomas, melanomas and hepatomas [45,54]. CDKs are highly involved in tumor progression and emerging evidence from different studies suggest that depending on the cellular context tumor cells have specific requirements for individual CDKs. CDK4 has been implicated in Ras-mediated transformation. CDK4-null mouse embryonic fibroblasts showed resistance to response to Ras activation and the disruption of CDK4 in this setting led to senescence that was ARF/p53 independent [55]. CDK4 has been shown to be dispensable for mammary gland development, but is needed for the development of mammary gland tumors initiated by different oncogenes including HRAS and MYC [56]. Recently, CDK2 was implicated MYC repression of Ras induced senescence in rat embryonic fibroblast [57]. Additionally, CDK2 was also involved in MYC induction of cellular senescence. Loss of CDK2 enhances MYC induced senescence in pancreatic B cell tumors and improves the survival of mice bearing lymphoma [58]. The specific requirement for the different CDKs in tumor cells gives an excellent opportunity to elaborate novel strategies against cancer.

1.3 Safe guard mechanisms

1.3.1 Apoptosis

Apoptosis or programmed cell dead is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis. Apoptosis plays an important role in development and in several pathologies including cancer. Apoptosis is often deregulated in incipient cancer cells. During normal morphogenesis apoptosis sculpts the developing organs by removing cells that are no longer biologically necessary. Genetic modified mice lacking key components of apoptosis showed developmental defects such as excess of neurons in the brain, facial abnormalities, delayed destruction of the webbing between the fingers and abnormalities in the palate and lens (ref biology of cancer) Apoptosis also is a crucial event in normal tissue physiology. For example apoptosis continually eliminates the epithelial cells in the villi of the small intestine. These cells are eliminate after four to five day journey from the bottom to the intestinal crypts to the tips of the villi [59].

The apoptosis program guaranties the death of the cells by a cascade of well-coordinated events. The cellular events leading to cell death start with the formation of blebs in the plasma membrane followed by the collapse of the nucleus into a dense structure. This dense structure is then fragmented as the chromosomal DNA is cleaved into small fragments. Ultimately the apoptotic cell breaks up into small fragments called the apoptotic bodies. These bodies are often removed by neighboring cells and circulating macrophages [59].

Apoptosis can be triggered by several stimuli including growth factor deprivation, hormones and cytokines, radiation, toxins, hypoxia, etc. Moreover, aberrant oncogene expression is also known to induce apoptosis [59]. Two main pathways trigger the complex apoptosis program: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Characteristic for these two apoptotic pathways is the activation of a family of cysteine proteases called caspases. In addition to these two pathways the perforin/granzyme pathway can also induce apoptosis. Each one of these pathways is triggered by specific signals that stimulate an energy dependent cascade of molecular events. All these 3 pathways converge in the same execution pathway in which caspase 3 is cleavage resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocyte cell receptors [60].

1.3.2 The extrinsic pathway or death receptor mediated pathway

The extrinsic pathway conveys apoptotic messages via transmembrane receptor-mediated interactions [61]. The transmembrane receptors mediating these interactions are members of the tumor necrosis factor (TNF) receptor gene superfamily and are usually called the death receptors (i.e., Fas, also called CD95/Apo-1; TNF receptors; TRAIL receptors) [62,63]. These members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain called the death domain. The best characterized ligands for these death receptors are FasL, TNF-A, Apo3L, Apo2L. Upon ligand binding the intracellular domains of the death receptors aggregate and cytoplasmic adapter proteins are recruited [64,65,66,67]. For example, FasL binding to its receptor Fas results in the binding of the adapter protein Fas associated dead domain (FADD) and binding of the TNF ligands to the TNF receptor triggers the binding of the adapter protein TNF associated dead domain (TRADD) with the recruitment of FADD and TRIP[68,69]. Procaspase -8 then binds to FADD and form the death inducing signaling complex (DISC) that in turn activates the autocatalytic function of procaspase 8 [70]. The activation of procaspase-8 in the DISC triggers a cascade of caspase activity that leads to cell death. The extrinsic pathway is inhibited by a protease-deficient caspase homolog, FLICE-inhibitory protein (FLIP). This protease-deficient caspase is highly homologous with procaspase 8 and is also recruited to the DISC but his role is controversial. FLIP is known to bind and inhibit FADD and caspase-8 [71,72,73]. However, it has been suggested that physiological levels of FLIP might induce apoptosis.

1.3.3 The intrinsic or mitochondrial pathway

The intrinsic pathway for programmed cell death is characterized by non-receptor mediated intracellular signaling that perturbs the status of the mitochondria and initiates apoptosis. This signaling pathway is induced by different stress stimuli including DNA damage and oncogene activation [61]. The different stress stimuli cause changes in the inner mitochondrial membrane resulting in loss of the mitochondrial transmembrane potential and release of two groups of sequestered pro-apoptotic proteins into the cytosol. The first released group of sequestered proteins is composed by cytochrome c, Smac/Diablo, and the serine protease HtrA/Omi [74,75]. These released proteins induce the caspase-dependent mitochondrial pathway. Cytochrome c binds to the cytosolic protein Apaf-1 as well as

procaspase-9 facilitating the formation of the apoptosome [76,77]. The formation of this complex is believed to be the point of no return, and apoptosis will occur. Smac and/DIABLO and HtrA2/Omi are believed to contribute to apoptosis by the inhibition of IAP (inhibitors of apoptosis proteins) activity [78,79]. The second pool of pro-apoptotic proteins being released into the cytosol are AIF, endonuclease G and CAD. The release of this group of pro-apoptotic proteins is a late event and occurs when the cell is committed to die. AIF, endonuclease G (endoG) and CAD are translocated to the cell nucleus where they aid DNA fragmentation and condensation of nuclear chromatin[80]. Once in the nucleus AIF causes DNA fragmentation into approximately 50 to 300 kb pieces and condensation of peripheral chromatin (Stage I condensation)[81]. Nuclear translocated endoG cleaves chromatin DNA into nucleosomal fragments [82]. Both IAF and endoG cleaves chromatin independently of caspases. Therefore these nucleases represent a caspase-independent apoptotic pathway initiated from the mitochondria. CAD in turn is cleaved by caspase -3 in the nucleus and induces oligonucleosomal DNA fragmentation and more advanced chromatin condensation (Stage II condensation) [81].

Members of the Bcl-2 family of proteins and the tumor suppressor protein p53 regulate the intrinsic pathway of apoptosis [59]. Pro and anti- apoptotic members compose the Bcl-1 family. The anti apoptotic protein members include the Bcl-2, Bcl-x_l, Bcl-xs, Bcl-w and BAG. The pro-apoptotic members include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk. p53 is a critical regulator of these proteins and its suggested that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria by alteration of mitochondrial membrane permeability [61].

1.3.4 Cellular senescence

Cellular senescence is a mechanism that prevents cells to divide indefinitely. This phenomenon was first described decades a go by Hayflick and colleagues in human fibroblasts. Hayflick cultured these cells under conditions that enhanced a continuous cell replication. After a number of finite cellular divisions (known as the Hayflick effect) these cells lost their replicative potential (replicative senescence) [83,84]. Senescent cell are characterized by the inability to progress though the cell cycle despite mitogenic stimulation. In contrast to quiescence, the observed growth arrest in senescent cells is persistent. In spite the fact that these cells are growth arrested, senescent cells remain metabolically active. It has been suggested that senescence plays an important role in regulating aging and tissue

homeostasis. In addition, several data have pinpointed cellular senescence as another important barrier against tumor cell transformation [85]. Apart from irreversible cell cycle arrest, senescent cell are characterized by altered morphology. Senescent cells are often large and flattened out. Further, cells undergoing cellular senescence exhibit senescence-associated beta-galactosidase (SA-b-gal) activity due to increased lysosomal activity and often display so called senescence-associated heterochromatin foci (SAHF) characterized by for instance increased trimethylation of histone 3 at lysine 9 ($H3K9me^3$)[86,87]. Senescent cells also display a specific gene expression patterns, including senescence-associated secretory phenotype (SASP) which involves the production of factors that reinforce the senescence arrest, alter the microenvironment, and trigger immune surveillance of the senescent cells [88,89,90,91].

1.3.5 Replicative senescence

The main cause of the replicative senescence described by Hayflick et al is attributed to telomere dysfunction. Telomeres consist of tandem non-coding DNA repeats (TTAGGG) and the principal function of these repeats is to cap the chromosomes. After each cell division telomeres in human cells are shortened due to the end replication problem of DNA polymerase and the suppression of telomerase expression. For example normal human diploid fibroblast shorten their telomeres with 50-100 base pairs after each cell division. In order to compensate for telomere shortening, mammalian cells express an enzyme called telomerase. This ribonucleoprotein has two major components: an enzymatic telomerase reverse transcriptase catalytic subunit, hTERT and an RNA component (hTR or hTERC) [92]. After reaching a critical telomeric length and loosing the cap, the telomere ends are recognized as DNA damage and induces DNA damage checkpoints. Two different check points have been identified that limits proliferation in response to telomere dysfunction: the first mortality stage (M1) and the second mortality stage (M2) also called crisis stage. The M1 is characterized by permanent cell cycle arrest and depends on p53 activation. Normally human fibroblasts undergo replicative senescence after 50-70 divisions. Cells with dysfunctional p53 bypass the senescence barrier and continue to proliferate independent of the critical length of the telomeres. Further shortening of the telomeres induces the M2 checkpoint, which is p53 independent and is characterized by extensive chromosomal instability and cell death [93]. In contrast to normal human cells where telomere shorting occurs as an anti proliferative mechanism, cancer cells maintain their telomere length by

overexpression of telomerase or by a mechanism known as alternative lengthening of telomeres.

1.3.6 Premature and oncogene-induced senescence

Experimental evidence from *in vitro* and *in vivo* studies have suggested that in addition to replicative senescence, cellular senescence can be induced prematurely by diverse stimuli including dysfunctional non-telomeric DNA damage, activated oncogenes, (oncogene induced senescence, OIS), oxidative stress and different agent causing DNA damage[85]. This is an important mechanism to suppress tumorigenesis by preventing proliferation of cells at risk for neoplastic transformation. For instance in primary cells, potent oncogenes such as RAS initially acts as strong mitogenic factor via the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol kinase (P13K) cascade. However RAS eventually induces premature senescence via the p53 and P16 (INK4A) pathways [94]. These two pathways are believed to establish and maintain the growth arrest caused by senescent signals. Activation of the MAPK kinase pathway by RAS ultimately leads to the induction of expression of the alternate-reading-frame protein ARF, which stabilizes p53 by antagonizing the activity of the E3 ubiquitin-protein ligase HDM2 (MDM2 in mice) [95]. Stabilized p53 establish the senescence growth arrest in part by inducing the CDK inhibitor p21. This kinase inhibitor in turn prevents CDK2-mediated phosphorylation and inactivation of pRB. Senescence signaling also engages the p16-pRb pathway. This pathway is characterized by the induction of the CDK inhibitor p16, which also prevents pRB phosphorylation and inactivation by inhibiting CDK4/6. pRB in turn stops the cell cycle and promotes senescence by suppressing the activity of the E2F transcription factor. As discussed in the cell cycle section, E2F is required for the stimulation of genes needed for cell cycle progression. It has been shown that E2F it self induces ARF expression and the subsequent p53 activation [96].

The early stages of tumor progression are characterized by unbalanced cell cycle and deregulated oncogenes. The combination of loss control of the cell cycle and oncogene stress results in aberrant DNA replication with stalled replication forks, which in turn triggers the DNA damage response (DDR). This response includes the activation of ATM and CHK2 kinases, which phosphorylate and augmentation the activity of the tumor suppressor p53. DNA damage induced by carcinogens, cancer drugs, irradiation or oncogene-induced replication stress or oxidative damage thereby at least in part induce senescence via accumulation and activation of p53, which promotes cellular senescence or apoptosis, as

mentioned above [59].

1.3.7 The *MYC* proto-oncogene

The *MYC* oncogene family, including *MYC* (c-*myc*), MYCN (N-*myc*) and MYCL (L-*myc*) regulates several normal cellular processes such as cell growth, cell division, metabolism, differentiation, stem cell function, apoptosis, senescence and angiogenesis. This family of potent proto-oncogenes is also highly involved in the development of many human cancers. Deregulated MYC expression caused by various types of genetic insults leads to constitutive MYC activity in a variety of cancers and promotes oncogenesis [97]. For instance rearrangements of the *MYC* family loci by amplifications or translocations are often observed in several types of cancers. The most well known examples are the translocations of *MYC* located on the human chromosome 8 into the immunoglobulin loci in 100% of Burkitt´s lymphoma cases and the amplification of MYCN in 40% of advanced neuroblastomas. These genomic rearrangements are strongly connected to poor prognosis [98]. In fact MYC was first discovered in Burkitt's lymphoma patients. The *MYC* proto-oncogenes encode DNA binding transcription factors that belong to the basic helix-loop-helix leucine zipper (bHLHZip) family. MYC heterodimerizes with its interaction partner Max and together they bind to specific DNA sequences termed E-boxes in promoters of the target genes. The binding of the MYC-MAX heterodimer at E boxes activates transcription of target genes [99]. MYC is also in known to repress transcription by binging to other interaction partners including MYC interacting zinger protein 1 (Miz-1) [100]. MYC gene regulation is mainly achieved by recruiting transcriptional cofactors involved in modulation of RNA polymerase II function and of chromatin structure, including histone acetyl transferase (HAT) complexes [97]. The human MYC protein contains 439 amino acids with 150 amino-terminal residues comprising the transactivation domain (TD) that contains two conserved MYC family boxes I and II (MBI and MBII). In MBI two crucial residues for MYC function and stabilization are found, threonine 58 (T58) and serine 62 (S62). Phosphorylation of serine 62 by different kinases stabilizes MYC, while threonine 58 phosphorylation destabilizes MYC [101]. These two residues seem to be phosphorylated in an interdependent manner; phosphorylation of S62 catalyzed by ERK kinases is a prerequisite for phosphorylation of T58 by GSK3β [102]. The carboxy-terminal 90 amino acids constitute the DNA binding and helix-loop-helix-leucine zipper dimerization domain (HLH-LZ) (Figure 6). This domain is essential for the binding to MYC obligate partner Max and for the transformation of primary and immortal cells [97].

Figure 6. MYC oncoprotein domains. MBI: MYC box I, MBII: MYC box 2, NLS: nuclear localization signal, B: basic region, HLH: helix-loop-helix motif, LZ: leucine zipper motif.

The MYC protein is believed to regulate about 10-15% of the genes in the genome and like other crucial key regulators of the cell the expression and activity of MYC are tightly regulated at multiple levels. In fact MYC has a very short half-life and it's directed to proteasomal degradation through the ubiquitin pathway. In this regulatory pathway, the small and highly conserved polypeptide ubiquitin is covalently conjugated to lysine residues of the target proteins by the combined action of at least three enzymes: the ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin-protein ligase (E3). Attachment of poly-ubiquitin chains to target proteins leads to recognition by the activated 26S proteasome and rapid degradation. MYC ubiquitylation is mediated by at least three different E3 ligases, SCF^{Fbw7}, SCF^{Skp2} and HectH9 [103]. Fbw7 a component of the SCF^{Fbw7} ubiquitin ligase and tumor suppressor associates with MYC and promotes its turnover by proteasomal degradation. Phosphorylation of MYC at T58 by glycogen synthase kinase 3β (GSK3β) seems to be required for Fbw7 recognition and ubiquitylation of MYC [104,105,106]. The importance of the T58 residue in regulating MYC stability is highlighted by the fact that the T58 residue is often mutated in a subset of Burkitt's lymphomas [105].

1.3.8 MYC cellular function

Cellular growth is prerequisite for subsequent cell division and in order to achieve cell division, the cell has to be stimulated by mitogenic signals followed by cellular growth through the synthesis of new cell components. MYC is known to be a master regulator of cell growth and proliferation. Endogenous MYC is activated trough different cellular stimuli and leads to the transcription of a spectrum of target genes that regulate the cell cycle and carbohydrate and nucleotide metabolism, as well as ribosomal and mitochondrial biogenesis. MYC expression is induced by several mitogenic pathways including Wnt, Notch, STAT, receptor tyrosine kinases (RTKs) such as platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR), as

well as hormone receptor pathways [97] (Larsson). These pathways are often upregulated during cell transformation and leads to aberrant MYC activation. By stimulating the transcription of all three RNA polymerases (RNA Pol I, II and III), MYC coordinates the process of rRNA transcription and processing, ribosomal protein transcription and translation, and translation initiation [97]. MYC has also been linked to energy metabolism through its regulation of glycolysis. Under normal condition mammalian cells uses oxygen to convert glucose energy via oxidative phosphorylation in the Krebs cycle, unlike cancer cells that rely on glycolysis for the generation of energy and are less dependent on oxygen, a phenomenon called the Warburg effect [107]. MYC stimulates glucose uptake and glycolysis by regulation of genes encoding GLUT1, phosphofructokinase and enolase [108]. The increase of glucose uptake and the upregulation of other glycolytic genes lead to the formation of lactate even under aerobic conditions. Nevertheless, MYC is also involved in mitochondrial biogenesis and energy production by oxidative phosphorylation. Activated MYC is involved in the direct regulation of nuclear genes controlling mitochondrial mass, structure and function [97,109].

In addition to regulating cell growth, metabolism and protein synthesis, MYC plays an important role in cell cycle regulation by regulating genes involved in cell cycle progression, such as cyclins, cyclin-dependent kinases (CDK), Cdk inhibitors (CKI), E3 ubiquitin ligase components targeting CKIs and replication proteins [97]. Moreover, MYC also has been directly implicated in the control of DNA replication. Over expression of MYC in mammalian cells causes increased replication origin activity subsequent DNA damage and check point activation [110]. MYC is also highly involved in stem cell self renewal and differentiation. Recently MYC was shown together with OCT4, SOX2, NANOG to be required for reprogramming of primary somatic fibroblast (iPS) [111,112].

MYC is a strong inducer of apoptosis and its up-regulation induces apoptosis by induction of p19Arf, which in turn stabilizes the tumor suppressor p53 [113], and by suppressing the anti-apoptotic genes *BCL2* and *BCL-x*$_L$ and genes involved in the main apoptotic pathways. The tumor suppressor p53 is often mutated during the progression of MYC induced tumors and targeted deletion of p53 in the Eμ-myc transgenic mice model hastens MYC induced B lymphoma development [114].

Recently different reports have shown that MYC overexpression not only triggers apoptosis but can also induce cellular senescence under certain conditions. Loss of CDK2 together with ectopic MYC expression sensitized mouse embryonic fibroblast (MEFs) to senescence, increased senescence in pancreatic B-cell tumors and delayed the onset of MYC-driven lymphoma [58]. Similarly, loss of the Werner gene (*WRN*) also induces senescence under conditions of MYC overexpression [115]. However, in contrast to many other oncogenes such as RAS and BRAF, MYC usually acts as a suppressor of senescence [116,117,118]. We recently demonstrated that suppression of RAS-induced senescence by MYC in rat embryonic fibroblasts (REFs) is associated with CDK2 activity and requires phosphorylation of MYC at Ser- 62 by CDK2 [57]. Loss of CDK2 therefore seems to switch MYC from a suppressor to an inducer of senescence (Figure 7) [119,120]

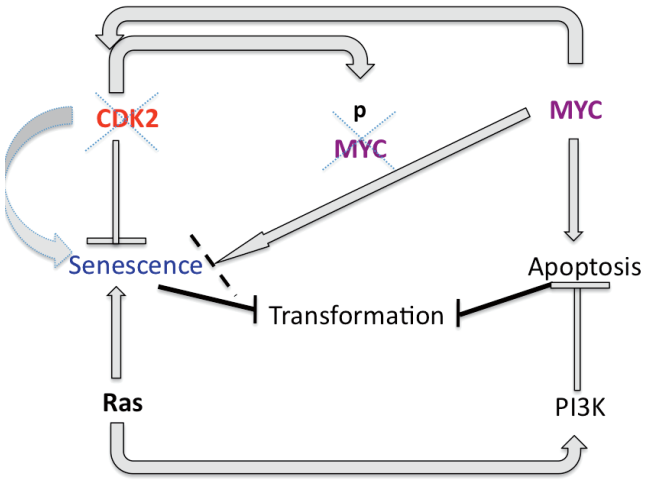

Figure 7. MYC induction/repression of senescence. Loss of CDK2 and overexpression of MYC induce senescence. MYC´s phosphorylation by CDK2 is crucial for MYC repression of Ras induced senescence

2. AIMS of this study

The content of this doctoral thesis work is divided into two parts. In the first part (paper I) we examined endothelial cell migration in response on stable gradients of VEGFA and the formation of lymphatic vessels in the developing mouse kidneys. Directed endothelial cell migration plays a central role in physiological and pathological angiogenesis. This complex process is governed by the interplay between gradients of growth factors and their receptors. The mechanisms behind the cell response to graded distributions of chemotactic factors are still poorly understood. In order to clarify the response of endothelial cells to stable chemotactic gradients, human umbilical vein endothelial cells (HUVECs) and human umbilical artery endothelial cells (HUAECs) were expose to stable hill- shaped gradients of VEGF and FGF2 using a microfluidic chemotaxis chamber (MCC) and time lapse microscopy.

Lymphatic vessels are believed to develop from large embryonic veins and mesodermal cells. Lymphatic vasculature in the different organs is suggested to form via sprouting processes during development. Lymphatic vessel formation and function in highly vascularized organs such as the kidneys has received little attention and the mechanisms behind lymphatic vessel formation and its implication in kidney function are still obscure. Intrarenal lymphatics have been postulated to play an important role in the progression of renal diseases and transplantation. We therefore aimed to investigate lymphatic formation in the mouse embryonic kidney (paper II) .

The general aim of the second part of this thesis was to clarify MYCs role in the induction of the safe guards mechanisms against tumor transformation. Deregulated MYC induces apoptosis, which is often overcome during tumor transformation. Moreover MYC has been proposed as a new regulator of oncogene-induced senescence. In the first part of the second part (paper III) of this thesis, we aimed to address the importance of the extrinsic (death receptor-mediated) versus the intrinsic (mitochondrial) pathways of apoptosis in MYC driven transformation of HSCs. In the second part (paper IV) we aimed to investigate whether pharmacological inhibition of CDK2 interferes with MYC-driven tumor development *in vivo* trough senescence.

3. Results and discussion

3.1.1 Endothelial cell migration in stable gradients of growth factors (Paper I)

In paper I, we used a microfluidic chemotaxis chamber (MCC) in order to clarify the response of HUVECs and HUAECs to shape graded chemotactic gradients. MCC were made of transparent PDMS in order to allow live imaging of the tested endothelial cell. The MCC was attached to culture dishes containing the mentioned endothelial cells. We first tested the shape profiles of VEGFA165 by adding it together with FITC-dextran in the middle of the MCC reservoir. The shape profiles along the length axis of the chamber were tested by the measuring the fluorescence emitted by FITC-dextran. The simulation of the shape profile with FICT-dextran and VEFGA165 showed that FITC-dextran profiles serve as a good approximation for the distribution of VEGFA165 in the MCC. Additional experiments were performed in order to test the effect of FITC dextran on the function of the tested VEGF165. These analyses showed that FITC-dextran did not interfere with the function of VEFG165.

3.1.2 Migration of endothelial cells towards stable hill shapes of VEGFA165 (Paper I)

We next tested primary endothelial cells isolated from artery (HUAECs) or vein (HUVECs) for migration. These cells attached well to gelatin-coated culture dishes after attachment of the MMC and exposure to flow rates between 60 and 2000 μm/s. For the initial and subsequent measurements of migration, cells located in the middle and in the edges of the MCC were not include in the analysis. Individual endothelial cell migration was recorded by time lapse microscopy during 200 min. Cell migration activity was more efficient during the first hour of the running experiment and about 85% of the HUVECS located in the left of the CMM migrated toward the middle of the chamber and in higher concentration of VEGF165. Individual cell migration was scored using a polar plot where the starting point of all cells is assigned to the middle of the plot. In control experiments HUVECs migration towards the middle of the chamber was not observed in gradients devoid of VEFGF165. In addition to HUVECS, arterial endothelial cells were also exposed to hill-shaped VEGF165 gradients. These cells also responded to the VEGF gradients and were attracted towards an increase in concentration of VEGF165. The above results suggest that endothelial cells from different vascular beds can migrate towards stable hill shaped VEGF65 gradients in the MCC. We also modulated the VEGFA-induced chemotaxis by gradually lowering the pump flow speed. By stepwise lowering the pump shallower gradients were produced due to the increased diffusion

time of the growth factor molecules in the MCC. HUVECs were exposed to hill-shape VGFE165 gradients (0-50ng/ml at the inlet) and to flow speeds between 60 and 200 um/s. Exposure to the these different created gradients reveled that the chemotactic response decline together with decreasing gradient steepness until it resembled random migration at 60 um/s.

3.1.3 Chemotactic response of endothelial cells to stable gradients of the VEGFA121 isoform (Paper I)

VEGFA co-receptor binding also plays a crucial role in receptor activation and signaling. It has been shown that in developing tissues and in different cell signaling assays that proteoglycans co-receptors are needed for the proper distribution and activation of several VEGFA splice forms (ref). It is still unclear if VEGFA binding to HSPGs is strictly required for the target endothelial cell to interpret positional information provided by the gradient. In order to clarify this, we generated gradients with VEGFA121 isoform. This VEGF isoform is unable to interact with proteoglycans and neurophilin co-receptors. HUVEC exposed to stable hill-shaped gradients of VEGF121 (0-50 ng/ml) in the MCC showed increased migration and the positive chemotaxis induced by VEGFA121 was 22 ± 10 μm as compared with 45 ± 10 μm for VEGFA16

3.1.4 Endothelial cell response to a gradient of FGF2 (Paper I)

Many different cytokines are involved in the regulation of chemotactic endothelial cell migration during angiogenesis. Together with VEGFA and the angiopoietins, FGF2 have been considered the major promoters of chemotactic endothelial cell migration. Nearly all the experimental data concerning the chemotactic potential of FG2 on endothelial cells derive from studies using the Boyden chamber. Using our chamber and exposing the cell to a hill-shape FGF2 gradient profile (0-50 ng/ml) equivalent to with the one tested for VEGFA did not significantly induce chemotaxis of HUAECS but had a small effect in HUVECs. Cell migration in response to FGF2 was also tested at different flow rates and the highest chemotactic response exhibited by HUVECs was seen in gradients ranging from 0 to 50 ng/ml of FGF2 generated at a flow speed of 200 μm/s.

3.1.5 Endothelial chemotactic responses in different regions of the gradients (Paper I)

The increased non-directional (chemokinesis) activity of HUVEC migration due to the effects of VEGFA and FGF2 were evaluated by analyzing total cell migration distances in the absence of growth factor, comparing with growth factor given either at constant concentration

of 50 ng/ml or in the form of a gradient (0-50ng/ml). Total migration of cell exposed to constant levels of VEGFA165 was considerably reduced compared to non-stimulated cells. Cell migration was promoted in cell exposed to a gradient of VEGF165 and the speed of migration was comparable with unstimulated cell. Constant concentration of VEGF165 together with the absent of flow was shown to reduce chemokinesis. The VEGF121 isoform reduced chemokinesis and constants concentration of FGF2 (50ng/ml) gave a small increase in chemokinesis. Different doses of VEGF and FGF2 effects on chemokinesis were analyzed and VEGFA but not FGF2 was shown to reduce chemokinesis at all tested concentrations.

3.2 Lymphatic vessel formation in the developing mouse kidneys (Paper II)

In order to explore renal lymphatic formation in a developing organ, embryonic kidneys were dissected from wild type mouse embryos between E.13.5 and E17.5 and immunostained for lymphatic and blood endothelial markers. Whole mounted kidney immunostainings revealed a well organized network of CD31 -positive blood vessels together with scattered cells expressing LYVE-1 in all analyzed time points. At E16.5, lymphatic vessel-like structures of variable size positive for podoplanin, PROX-1, LYVE-1 and VEGFR-3 were observed adjacent to the renal ureteric pelvis. At the following time point e.g. E17.5; two large LYVE-1 positive vessels emerging from the ureteric bud region were found in renal pelvis and in the ventral renal cortex region. These LYVE-1 positive vessels followed large CD31 positive renal veins and branched in the renal cortex.

3.2.1 Contribution of lymphatic precursors in the formation of renal lymphatic vasculature (PaperII)

Recent studies have shown that during embryonic development PROX-1 positive macrophage like cells are involved in lymphatic sac formation[32]. Other studies also have implicated F4/80 positive macrophages in lymphatic vessel formation during wound healing processes[121]. Aiming to explore the contribution of lymphatic precursor cells in organ lymphatic vessel formation, we next analyzed the observed scattered single LYVE-1 positive cells for the expression of the pan macrophage marker F4/80 and PROX-1. A large fraction of the single cells expressing LYVE-1 was also shown to co-express F4/80. However these double positive cells were never found to express PROX-1 or to be part of clearly identifiable lymphatic structures. The observed LYVE-1 and F4/80 positive cells in E15.5 and E17.5 time points were flow sorted and analyzed for the expression of VEGFC, VEGFR2, VEGFR3 and PROX-1 transcripts. The flow sorted analysis showed subpopulations of cells expressing

both LYVE-1 and F4/80, these cell fractions also were relatively constant and around 0.5-1% of the total cells of embryonic kidney at E13.5 and E15.5. However at E7.5 the double positive population of cells increases to 3.5 % of all cells. At this time point of kidney development the fraction of LYVE-1 positive cells also increased and it may reflect the observed invasion of lymphatic vessels into the kidney around E16.5-17.5. Transcript analysis of the flow-sorted fractions of kidney cells revealed that VEGFC expression was lower in LYVE-1 and F4/80 positive cells and in addition these cells were not expressing VEGFR-2 and VEGFR-3. Thus suggesting that these cells may not respond to VEGFA and the pro-lymphatic factor VEGFC. PROX-1 transcript levels were highest in LYVE-1 positive cells at E15.5 and no significant up regulation of PROX-1 message was seen in F4/80 positive cells. LYVE-1 positive cells also expressed VEGFR-2, thus indicating their endothelial origin.

3.2.2 Lymphatic vessel formation in embryonic renal explants (Paper II)

We next explored *in-situ* lymphatic vessel formation during kidney development. E13.5 kidney explants were grown on filter discs and in collagen I, followed by VEGF-C and fibroblast growth factor (FGF-2) stimulation. *In-situ* lymphatic vessel formation was accessed by immunostaining for the lymphatic markers PROX-1, LYVE-1, podoplanin and CD31. The immunostainings revealed PROX-1 positive lymphatic vessel-like structure formation in the cortex of 3 days un-stimulated explants (figure 6A). However treatment with VEGFC and FGF2 increased the formation of lymphatic structures (Figure 6B and D). These lymphatic structures showed high expression of LYVE, podoplanin and PROX-1 and low expression of CD31 (Figure 6 B-C). In some areas of the kidneys, formed sprouts showed signs of BEC to LEC trans-differentiation. Further analysis of the kidney explants after 3-4 days of culture, revealed the formation of PROX-1 clusters in the extended CD31 positive network.

In conclusion lymphatic vessel formation during embryonic murine kidney development is mainly achieved by invasion of LECs. These cells might sprout from pre-existing lymphatic plexus and invade the kidney following the ureteric bud. Once in the kidney cortex these large lymphatic vessels tend to branch into small vessel of different caliber. However when embryonic kidneys were dissected and cultured for three days in the presence of pro-lymphatic factors, renal lymphatic precursor cells seem to be stimulated to transdifferentiate into LECS. These stimulated cells form lymphatic like structures that are positive for the commonly used lymphatic markers. Further studies are required to firmly establish whether transdifferentiation from BECs to LECs take place in the developing kidneys.

Result and Discussion part II

3.3 *In vivo* impact of the intrinsic and the extrinsic pathways of apoptosis on MYC induced malignant transformation of HSCs (Paper III)

MYC is known to be involved in the development of multiple human cancers including acute myeloid leukemia. Deregulated MYC is known to be a strong effector of the two main apoptotic pathways (intrinsic and extrinsic). This anti-tumorigenic capacity of MYC is often overcome during tumor progression by genetic lesions. Mutations and deregulation of both the intrinsic and extrinsic apoptotic pathways have been found in AML. However, the relative contribution of these two pathways for the AML disease has not been investigated methodically. Aiming to study the effect of the anti-apoptotic genes products regulating the two apoptotic pathways on MYC driven transformation, HSCs were isolated from bone marrow and transduced with combinations of two different retroviruses: one containing MYC-YFP and the other either BCL-X_L (regulating the intrinsic apoptotic pathway) or $FLIP_L$ (regulating the extrinsic pathway) linked to GFP. The transduced HSCs were then transplanted into lethally irradiated syngeneic recipient mice.

3.3.1 Blockade of the intrinsic but not of the extrinsic pathways of apoptosis hastens MYC driven tumorigenesis (Paper III).

The myeloid and lymphoid cell populations of mice transplanted with HSCs transduced with MYC-YPF were efficiently reconstituted. However, 7-9 weeks after transplantation the mice started to display early sings of the disease. These included ruffed fur, wasting and slow movements that rapidly developed into hind limb paralysis within two days. The mice rapidly became terminally ill and were subsequently sacrificed. Necropsy analysis of these mice showed that the animals had enlarged spleen and liver, non-coagulating blood, spotted lungs, and in many animals also enlarged lymph nodes and thymus were observed. On the contrary to the MYC-YFP mice, control mice transplanted with HSCs transduced with Mock-YFP and Mock-GFP remained healthy during the whole duration of the study. Mice transplanted with MYC-YFP showed a median survival of 56 days after HSCs transplantation. We next examine the influence of the intrinsic pathway of apoptosis on MYC driven neoplasia. Isolated HSCs were transduced with a combination of MYC and BCL-$X_{L,}$ which blocks the intrinsic apoptosis pathway. These transduced cells were then transplanted into lethally irradiated mice. The MYC/BCL-X_L transplanted mice became moribund 2 weeks after transplantation and had a median survival of 17 days. The mice also displayed similar sings of

the disease as the MYC-YFP mice; necropsy analysis of moribund mice showed that tumors were disseminated throughout the hematopoietic tissues with extensive involvement of spleen and bone marrow and tumor infiltration of the liver. These results clearly showed that inhibition of the intrinsic pathway of apoptosis accelerates MYC driven transformation.. We next tested if $FLIP_L$, an inhibitor of the extrinsic apoptotic pathway, affects MYC driven tumorigenesis in HSCs. Bone marrow isolated HSc were co-transduced with retroviruses containing MYC-YFP and $FLIP_L$-GFP and transplanted into irradiated mice. These MYC-YPF/$FLIP_L$-GFP mice showed similar kinetics and signs of the disease as the Mock-MYC mice. All the MYC/$FLIP_L$ mice became terminally ill within 9 weeks and had medial survival of 58 days. Autopsy of the MYC/$FLIP_L$ mice showed that these mice had similar pathological features as the Mock/MYC mice and these include enlarged spleen and liver, non-coagulating blood, spotted lungs and in many animals also enlarged lymph nodes and thymus. In conclusion these results suggested that inhibition of the intrinsic but not the extrinsic pathways of apoptosis synergized with MYC to promote tumorigenesis of HSC.

3.3.2 Expression of BCL-X_L but not of the inhibitor of the receptor-mediated anti-apoptotic pathway $FLIP_L$ drives MYC-driven tumorigenesis towards acute myeloid leukemia (Paper III).

Acute myeloid leukemia is characterized by a clonal expansion of HSCs and myeloid progenitors cells that have undergone malignant transformation. MYC is often overexpressed in AML due to mutations and translocations events. Aiming to determine the spectrum of tumors produced in mice transduced with Mock/MYC, MYC/BCL-X_L and MYC/$FLIP_L$, cell suspensions from hematopoietic tissues were analyzed by flow cytometry using myeloid, T-cell, B-cell and erythroid markers. Mice reconstituted with HSCs expressing Mock/MYC developed myeloid leukemia and T-cell lymphoma, in agreement with previous reports [122]. The two tumor types localized to different hematopoietic compartments; the bone marrow displayed tumors of myeloid origin and the thymus and enlarged lymph nodes displayed T-cell tumors. Spleen and the infiltrated liver often showed a mixed population of both myeloid and lymphoid origin. In contrast, the vast majority of cells dominating the hematopoietic compartments of MYC/BC-LX_L mice were myeloid ($CD11b^+$, $Gr1^+$) AML-like cells. In addition, the MYC/BCL-X_L mice had a pronounced increase in the total number of white blood cells compare to Mock/Mock transplanted mice. Differential count analysis confirmed that approximately 50% of cells in MYC/BCL-X_L transplanted mice were blasts. These results suggest that the anti-apoptotic protein BCL-X_L drives MYC-driven tumorigenesis towards acute myeloid leukemia. Our results differ from a study performed by Luo et al.

[123]. Their results indicated that expression of MYC alone in bone marrow cells that were transplanted into recipient mice solely gave rise to acute myeloid leukemia. They also argued that co-expression of MYC and BCL-2 gave rise to a mixture of AML and pre-B acute lymphoid leukemia in BALB/c mice. One explanation to this discrepancy in the two studies results could be that tumor initiating MYC target cells differs between the systems. Another possibility is that Luo et al used unfractionated mononuclear bone marrow cells, whereas we transduced Lin- bone marrow cells. Besides the paper by Luo et al., another study reported that overexpression of MYC results in development of aggressive pre-B lymphomas with low penetrance [124]. One possible explanation why their results differ from ours relies on the fact that they used retrovirally transduced fetal liver cells (E14.5), and therefor may hit another tumor-initiating cell.

We next generalized our results to other apoptotic member of the BCL-2 family and to other strains of mice. Isolated HSCs were transduced with MYC together with BCL-X_L or BCL-2. These HSCs were then transplanted in to BALB/c recipient mice. The transplanted mice rapidly developed similar AML-like disease as the DBA/2 mice transplanted with HSCs over expressing BCL-X_L and MYC. The MYC/BCL-X_L BALB/c mice had a mean survival of 16 days while the MYC/BCL-2 BALB/c mice had a mean survival of 19 days. The noticed minor difference in survival between these mice was statistically significant and might be explained by the lower proportion of BCL-2/MYC double expressing cells among the transplanted cells. Both group of animals also showed grossly enlarged spleens with means values of 757 mg for BCL-X_L/MYC BALB/c and 899 mg for BCL-2/MYC BALB/c. As in the analysis of the DBA/2 mice, the tumors found in the BCL-X_L/MYC predominantly expressed myeloid markers (CD11b and Gr1). In contrast, the FLIPL/MYC BALB/c mice developed myeloid tumors ($Gr1^+$ $CD11b^+$ and/or $Gr1^+$) or $CD4^+$ T-cell lymphoma. Infiltrated liver and spleen of these animals often showed a mixed population of both myeloid and lymphoid origin similar to Mock/MYC mice. Interestingly, the $CD4^+$CD8+ lymphoid tumor cells population was diminished among $FLIP_L$/MYC-induced T-cell lymphoma cells compared to the lymphoma cells expressing only MYC. This was accompanied by an increased $CD4^+$ population of cells, thus suggesting that $FLIP_L$ is involved in the selection of MYC-induced lymphoid but not of myeloid tumors. We also confirmed the flow cytometric results by immunohistochemical analysis. Spleen and liver tissue sections were stained with antibodies against T-cell markers (CD45R, CD3) and myeloid marker (myeloid peroxidase, MPO). Mock/MYC spleen and liver tissues were extensively positive for CD3 and MPO

markers. $CD3^+$ cells were mostly distributed as perivascular cuffs in liver tissues whereas the myeloid (MPO^+) cells showed a more diffuse distribution. Moreover, the spleen architecture was grossly distorted and the tissue was predominately positive for CD3 and MPO markers. Mock/Mock spleen tissue showed normal organ architecture and the liver did not show any sign of tumor infiltration. MYC/BCL-X_L and MYC/BCL2 spleen and liver tissues only expressed the myeloid marker MPO. Immunohistochemical analysis of tumor cells presented in all analyzed tissues showed similar staining patters as Mock/MYC. These results suggest that Mock/MYC mice mainly developed myeloid leukemia and lymphoblastic T-cell lymphoma. Coexpression of the anti apoptotic family members (BCL-X_L and BCL-2) and MYC strongly favored tumor development towards myeloid leukemia. Further, coexpression of MYC and $FLIP_L$ did not affect the distribution of myeloid versus lymphoid tumors cells, but changed the ratio between single CD4+ and double positive $CD4^+CD8^+$ T cell lymphomas.

3.3.3 Expansion of myeloid leukemic cells in MYC and MYC/BCL-X_L expressing mice at the expense of other hematopoietic cell populations (Paper III)

We next investigated the kinetics behind lymphoma/leukemia development and the effect of MYC and MYC/BCL-X_L expression on the different blood cell populations. Hematopoietic development was studied at different time points (7, 14 and 35 days) after transplantation of Mock/MYC or MYC/BCL-X_L transduced cells in recipient mice. The percentage of cells expressing MYC-YFP was almost constant (about 50% of all cells) at day 7 and 35 after transplantation, but this population underwent a rapid expansion after 35 days. In the other hand Mock-GFP/Mock-YFP-expressing cells did not expand but stayed at a constant level. In contrast, BCL-X_L-GFP/MYC-YFP-expressing cells rapidly expanded from day 7 to day 14 after transplantation. BCL-X_L-GFP/MYC-YFP expressing cells in the bone marrow expanded from 20% at day 7 to 75% at day 14 and in the spleen these cells expanded from about 20% at day 7 to 40% at day 14. The phenotype of the cells expressing Mock-GFP, MYC-YFP and BCL-X_L-GFP/MYC-YFP were also analyzed at different time points. MYC expressing myeloid cells accounted for around 60% of the cells in the bone marrow and 20-30% of the cell in the spleen at day 7. MYC expression was also detected in pre-B, B, $CD4^+$ and $CD8^+$ T cells and in erythroid cells. However, these populations did no undergo expansion days 7-14, but there was a tendency towards a B-cell or T-cell expansion at day 35. BCL-X_L-GFP/MYC-YFP expressing pre-B, B, $CD4^+$ and $CD8^+$ T cells as well as erythroid $Ter119+CD71^{hi}$ and $Ter119+CD71^{low}$ cells could be found at day 14. However the vast

majority of cells expressing BCL-X_L-GFP/MYC-YFP were found among myeloid $CD11b^+$ $Gr1^+$ cells. In summary the above results suggest that the myeloid $CD11^+$ $Gr1^+$ population dominated at early time pints both of mice transplanted with MTC-YFP and BCL-X_L-GFP/MYC-YFP expressing HSCs.

3.3.4 Blast transformation of myeloid and lymphoid cells in MYC and MYC/BCL-X_L (Paper III).

Activation and blast transformation were investigated by the analysis of the mean fluorescence intensity in forward scatter (Δmfi) in $CD11b^+Gr1^+$, $CD19^+IgM^-$ and $CD19^+IgM$ cells from bone marrow and spleen from 7, 14 and 35 days after transplantation. MYC-YFP/BCL-X_L-GFP AND MYC expressing cells were shown to be blast transformed compared to non-transduced cells, Mock-GFP cells or cells expressing only BCL-X_L-GFP in the same animal. MYC/BCL-X_L cells expressing $CD19^+Ig^+B$ cells markers showed an increased cells size compared to cells expressing MYC alone. The noticed cell size might suggest that these cells also had an increase cell survival due to the expression of BCL-XL. In any case it was mainly MYC and MYC/BCL-X_L expressing myeloid CD11+Gr-1+ cells that expanded rapidly and reached elevated steady-state levels. Our results suggest that mice transplanted with HSC overexpressing MYC or MYC/BCL-X_L contained high numbers of pre-malignant MYC-expressing cells of different lineages. The populations remained at constant steady-state levels until 6-7 weeks after transplantation in MYC expressing mice.

Recently, different reports have implicated MYC in the regulation of oncogene-induced senescence. For instance, Campaner et al reported that loss of CDK2 together with ectopic MYC expression sensitized MEFS to senescence, increase senescence in pancreatic B-cell tumors and delayed the onset of MYC-driven lymphoma. Moreover, Hydbring et al demonstrated that suppression of RAS-induced senescence by MYC in REFs is associated with CDK2 activity. The authors demonstrated that phosphorylation of MYC at Ser 62 by CDK2 plays a crucial role in MYC repression of cellular senescence. We first aimed to clarify and to corroborate if specific CDK2 inhibition promotes MYC induction of senesce in HSCs transduced with MYC and BCL-X_L. The transduced HSCs were treated for five days with the CDK2 inhibitor CVT2584 and scored for senescence. The selective inhibition of CDK2 reduced the proliferation of the transduced HSCs. In addition CDK2 inhibition induced cellular senescence in the MYC/BCL-X_L transduced cells (paper IV).

3.4 Pharmacological inhibition of CDK2 improves the survival of MYC/BCL-X_L mice (Paper IV)

The *in vitro* results showed that pharmacological inhibition of CDK2 lead to the induction of senescence in MYC/BCL-X_L transduced HSCs. Based on these results we next aimed to inhibit CDK2 in an *in vivo* mouse model. For this purpose we used a bone marrow transplantation mouse model described in paper III [122]. In this setting isolated bone marrow HSCs were retrovirally transduced with MYC and BCL-X_L and transplanted into lethally irradiated mice. As described above the mice started to show sings of the disease 2 weeks after transplantation and rapidly become terminally ill due to the development of acute myeloid leukemia. Three to six days after HSC reconstitution the mice were treated with different concentrations of the CDK2 inhibitor CVT2584 and DMSO respectively. Two modalities of administration were used; either via IP injections on a daily basis or continuous supply via osmotic minipumps. Control mice became moribund 2-3 weeks after transplantation as expected and pharmacological inhibition of CDK2 improved mice survival with 3-4 days regardless modality of treatment. The mice were also treated with the topoisomerase inhibitor adriamycin (ADR) as a reference. Treatment with this well-known anticancer drug also gave same survival as the CDK2 inhibitor. However, benefits with CVT2584 are that it is more selective than ADR and, in contrast to ADR, does not seem to cause DNA damage (data not shown). Furthermore, one advantage in this type of treatment approach is that CDK2 is a non-essential gene; pharmacological inhibition of CDK2 is likely to give less side effects in a clinical setting.

3.4.1 CDK2 inhibition delays the onset of MYC driven leukemia by the induction of cellular senescence (Paper IV)

We next explored the nature of the delayed onset of MYC driven leukemia observed in the CVT2584 treated mice. Our studies showed that this was primarily due to the induction of cellular senescence as demonstrated by increased SA-beta-gal activity and H3K9me^3 heterochromatin foci formation, the strongly reduced proliferation rates and cellularity in analyzed spleen, liver and bone marrow tissues and lack of apoptosis in CVT2584 treated MYC/BCL-X_L mice. Interestingly, we also detected the presence of F4/80 activated macrophages in spleen tissues of CT2584 treated animals. Taken together these results suggest that the pharmacological inhibition of CDK2 forced parts of the leukemic population into senescence and activated circulating/resident macrophages that may take part in the clearance of senescent AML cells.

3.4.2 Pharmacological inhibition of CDK2 in MYC/BCL-X_L mice reduces MYC phosphorylation and induces the senescence program by engaging the p53/p19Arf/p21 and pRb pathways (Paper IV)

Previous results have shown that MYC phosphorylation by CDK2 seems to play an important role in the MYC-mediated suppression of oncogene induced senescence [117]. Our results show that MYC phosphorylation was significantly reduced in all mice administered with the CDK2 inhibitor CVT2584. Protein analysis also revealed that pharmacological inhibition of CDK2 in the MYC/BCL-X_L mice led to the up-regulation of p19ARF and the CDKI p21, as well as activation of pRb, which all are important effectors of senescence. The observed up-regulation of these proteins and activation of pRb indicate that the selective inhibition of CDK2 in the MYC/BCL-X_L leukemic mice induced the senescence program trough the activation of p53/p19Arf/p21 and pRb pathways. These results are in agreement with previous results published by Campaner et al, where a similar senescence program is induced in cultured MEFS lacking CDK2.

In summary our results suggest that the pharmacological inhibition of CDK2 improved mice survival with 3-4 days and delayed the onset of MYC driven leukemia by the induction of cellular senescence. However, the CDK2 inhibition in this model only gave moderate mice survival. One should bear in mind that this is a very aggressive tumor model with massive and extremely rapid expansion of MYC/BCL-X_L leukemic cells leading to death in 2-3 weeks. The effectiveness of the treatment might be masked by tumor aggressiveness in this mouse model. Therefore other less aggressive models could be used in other to further evaluate the efficacy of CDK2 inhibition for treatment of MYC driven tumorigenesis. Nevertheless, our result showed that CDK2 inhibition led to a significant improvement of survival in a very aggressive tumor model, and the concept of CDK2 inhibition as a potential new pro-senescence treatment strategy against tumors driven by MYC should be evaluated further.

4. Conclusions

- Stable gradients of VEGFA165 efficiently induced chemotaxis but not chemokinesis of endothelial cells
- A gradient of VEGFA121 was shown to guide endothelial cells, suggesting that VEGFA does not need to interact directly with proteoglycan and neuropilin co-receptors to induce chemotaxis
- Gradients of FGF2 weakly attract venular cells but not arterial cells
- Lymph vessel formation in the embryonic mouse kidney predominantly occurs via invasive sprouting from the surrounding tissue
- Cells present within the developing kidney were shown to occasionally express lymphatic markers
- The anti-apoptotic proteins of the intrinsic pathway accelerate the development of MYC-driven hematopoietic tumors to a larger extent of those that the extrinsic pathway of apoptosis
- Pharmacological inhibition of CDK2 improved mice survival with 3-4 days and delayed the onset of MYC driven leukemia by the induction of cellular senescence.

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6. Acknowledgements

This doctoral thesis would not have been possible without the support and help of many people that in one way or another contributed in the preparation and completion of my doctoral studies:

First and foremost my head supervisor **Lars-Gunnar**, it is with great gratitude that I acknowledge you for your excellent scientific guidance, patient and help during my PhD studies. Thank you for believing in me and supporting me despite the turbulence I had in my first years of my PhD studies at Uppsala University.

My co-supervisor Alf and Kari for all the help with the mice and FAC analysis.

The red haired lady (**Susanna**) for your support and for all protein rich cakes. You always bring a good balance to the lab. **Natalie** my very first supervisor, for introducing me to the MYC world and for teaching me how to deal with SUMO. **Per** for introducing me to senescence and the vivid scientific discussions. **Hamid**, for being such a good co-worker and being so happy most of the time. I know, I was a bit hard with you but it was my way to show you that I really like you. **Qinzi**, I never saw you angry, thank you for being so positive. **Mariam**, I guess we are very different but you remind me how to deal with differences. **Alina**, the hard rock lady, for the positivism and discussions about life. **Sara**, the second red haired lady, for being so nice with me. I never got the cake you were supposed to bake for me. **Helene**, for making the lab such a nice place to work.

The members of Marie´s group, Hanna, Anna, Margaretta , Inga for all the support and shared moments in the kitchen.

All people at MTC for helping me with all the things necessary for my research.

My friends : Olle, Victor, Bengt for being a great support outside the lab.

Finally, I thank my family for supporting me throughout all my PhD studies.