Development Of A Fish Melanoma Angiogenesis Model

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SUMMARY

Malignant melanoma is the most severe form of all skin cancers with a particular poor prognosis once metastases have developed. Angiogenesis, the formation of new blood vessels, is a prominent feature of human melanoma, which have angiogenic activity already early in development. This is at least partly ascribed to the action of MAPK- and PI3K pathways which are hyperactivated in most melanoma. Animal models which combine in depth in vivo examinations with the opportunity to perform small molecular screens are well suited to gain a more detailed insight into how this type of cancer modulates its angiogenic program. Here, a first transgenic melanoma angiogenesis model was established in the fish species *Oryzias latipes* (Japanese medaka). In this model, tumors are generated by the pigment cell-specific expression of the oncogenic receptor tyrosine kinase Xmrk. Xmrk is a mutated version of the fish Egfp. Furthermore, to get an angiogenesis model, a medaka line with endothelial cell specific GFP expression was used. By using crosses between these Xmrk- and GFP transgenic fishes, it was shown that angiogenesis occurs in a reactive oxygen species- and NF-κB-dependent manner, but was hypoxia-independent. It was observed that blood vessel sprouting and branch point formation was elevated in this model and furthermore that sprouting could even be induced by single transformed cells. The mouse melanocytes expressing the oncogenic receptor tyrosine kinase Xmrk as well human melanoma cells, which display various oncogenic alterations, produced pro-angiogenic factors, most prominently angiogenin, via NF-κB signaling. Furthermore, inhibiting NF-κB action prevented tumor angiogenesis and even led to the regression of existing tumor blood vessels. In summary, the present medaka melanoma angiogenesis model displays a high sensitivity for angiogenesis detection and is perfectly suited as *in vivo* model for the testing of anti-angiogenesis inhibitors, as exemplified by the NF-κB inhibitor.

Furthermore, results indicate that it might be a promising anti-tumor strategy to target signaling pathways such as the NF-κB pathway which are able to induce angiogenesis-dependent as well as -independent pro-tumorigenic effects.

ZUSAMMENFASSUNG

Das maligne Melanom ist die schwerste Form aller Hautkrebsarten und hat eine besonders schlechte Prognose, sobald sich Metastasen gebildet haben. Angiogenese, die Bildung von neuen Blutgefäßen aus bestehenden Gefäßen, ist bei humanen Melanomen häufig zu beobachten. Es wurde gezeigt, dass diese Tumore eine hohe angiogene Aktivität besitzen. Diese wird zumindest teilweise der Aktivität der MAKP und PI3K Signalwege zugeschrieben, welche in den meisten Melanomen hyperaktivert sind. Tiermodelle, die sowohl *in vivo* Untersuchungen als auch „small molecular screens“ erlauben, sind gut geeignet, um detaillierte Kenntnisse über pro-angiogene Programme zu erlangen. In dieser Arbeit wurde ein erstes transgenes Melanom-Angiogenese-Model in der Fischspezies *Oryzias latipes* (Medaka) etabliert. In diesem Model werden Tumore durch eine pigmentzell-spezifische Expression der onkogenen Tyrosinkinase Xmrk erzeugt. Xmrk ist eine mutierte Version des Egfp im Fisch. Weiter gibt es in diesem Modellorganismus eine endothelzell-spezifische GFP-Linie. Durch Kreuzen der Xmrk- und GFP-transgenen Linien konnte das beschriebene Tumorangiogenese-Modell generiert werden. An Hand dieses Models konnte gezeigt werden, dass Angiogenese in einer reaktiven Sauerstoffspezies- und NF-κB-abhängigen Weise auftrat, jedoch unabhängig von Hypoxie stattfand. Es wurde beobachtet, dass die Sprossung- und Verzweigungsvorgänge der Blutgefäße sogar durch einzelne transformierte Pigmentzellen induziert wurden. Sowohl Mausmelanozyten, die die onkogene Rezeptortyrosinkinase Xmrk expriemieren, als auch humane Melanomzellen produzieren pro-angiogene Faktoren, wie zum Beispiel Angiogenin, über den NF-κB-Signalweg. Eine Hemmung von NF-κB hatte eine Verminderung der Tumorangiogenese zur Folge und führte sogar zur Rückbildung von bestehenden tumorösen Blutgefäßen. Zusammenfassend zeigt das Medaka-Melanom-Angiogenese-Model eine hohe Sensitivität für den Nachweis von Angiogeneseprozessen und ist vortrefflich geeignet als *in vivo* Modell zur Durchführung von Angiogenese-Inhibitor-Tests, wie beispielsweise mit dem NF-κB-Inhibitor gezeigt wurde. Eine pharmakologische Hemmung multi-potenter Signalwege mit Angiogenese-abhängigen und –unabhängigen pro-tumorigenen Effekten wie beispielsweise den NF-κB-Signalweg könnte eine erfolgsversprechende Antitumorstrategie darstellen.

1 INTRODUCTION

1.1 Malignant melanoma

Malignant melanoma is the most severe form of all skin cancers. It is responsible for about 90% of all skin cancer-related deaths. Malignant melanoma arises from pigment-producing cells, melanocytes, which transform into malignant cancer cells with high metastasizing and proliferative abilities. Melanocytes are located at the basal layer of the epidermis. They originate from neural-crest progenitors and their homeostasis is regulated by adjacent keratinocytes [1].

1.1.1 Incidence

Worldwide the incidence of melanoma is rising annually among Caucasian populations[2][3]. In central Europe 10 to 12 new cases, in the U.S. 10 to 25 cases and in Australia 50 up to 60 cases per 100000 individuals per year are recorded. The incidence is influenced by pigmentation of the population and geographical parameters like altitude and latitude [2].

1.1.2 Types of malignant melanoma

There are at least four distinct types of cutaneous malignant melanoma. The superficial spreading malignant melanoma (SSM) accounts for 57.4 percent, the nodular melanoma (NM) accounts for 21.4 percent, the lentigo maligna melanoma (LMM) accounts for 8.8 percent and acral lentiginous melanoma (ALM) accounts for 4 percent of all melanomas among the German population (n=30.015) [4].

1.1.3 Melanoma progression

Under normal conditions melanocytes are tightly controlled by adjacent keratinocytes [5]. Both cell types are located in a cell layer between the dermis and the epidermis of the skin. Through mutations in growth regulating genes, production

of autocrine growth factors, and loss of adhesion receptors melanocytes are able to spread and proliferate. This can lead to the formation of a nevus and is considered one of the first steps in melanoma progression although the majority of nevi are of a benign phenotype. Still, the appearance of atypical nevus cells, which are able to spread in a radial manner, can change the situation. This is considered to be the primary malignant stage of melanoma progression and is termed radial growth phase (RGP) melanoma. Further progression leads to the vertical growth phase (VGP) melanoma. In this stage of melanoma progression the transformed cells show strong metastatic and proliferative potential. The transformed cells are able to invade the dermis and the subcutaneous tissue. Once they have reached the subcutaneous tissue the malignant cells are able to infiltrate the vascular and lymphatic system and are thereby able to spread their metastatic potential throughout the body [6][7].

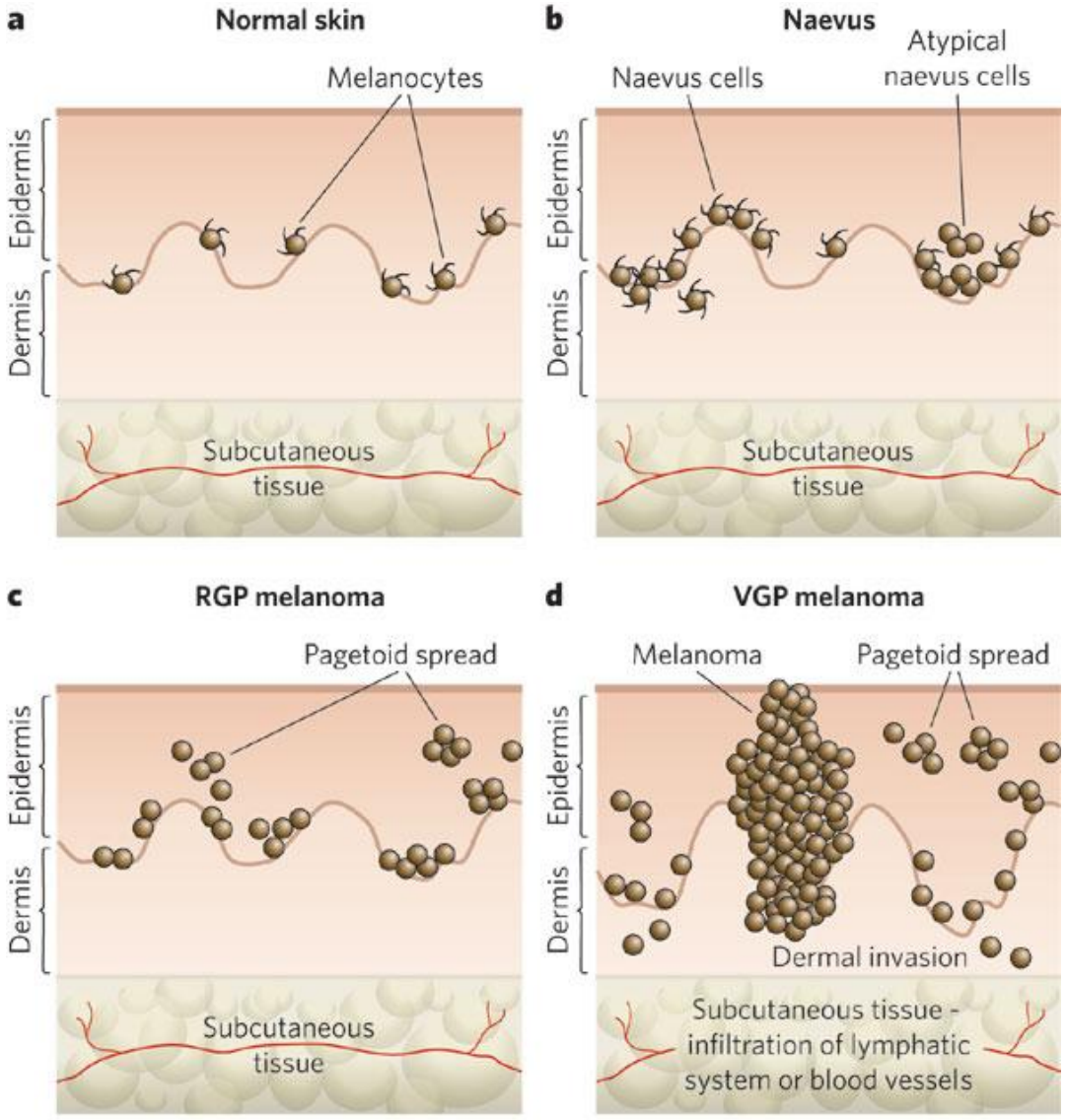


Figure 1: Melanoma progression stages.
(a) Normal skin stage: Here melanocytes are located at the border of dermis and epidermis. (b) Naevus stage: Here, benign melanocytic nevi occur with increased numbers of melanocytes. Some nevi are dysplastic, with morphologically atypical melanocytes. (c) Radial-growth-phase (RGP) melanoma stage is considered to be the primary malignant stage: Here an increased radial distribution of atypical melanocytes can be observed. (d) Vertical-growth-phase (VGP) melanoma stage is the first stage that is considered to have malignant potential and leads directly to metastatic malignant melanoma: Here, infiltration of the vascular and lymphatic systems can occur.

1.1.4 Melanoma pathogenesis

The pathogenesis of melanoma is influenced by genetic and environmental risk factors. The major environmental risk factor is UV light. The current rise in melanoma cases per year can be partly explained by increased popularity of sun-tanning or sunbed tanning [8]. Beside this melanoma risk has been also associated with somatic

or germ cell gene mutations which lead to familial or sporadic forms of the disease. Sporadic melanomas represent approximately about 90% of all melanoma cases whereas familial melanomas appear to approximately about 10%. Numerous genes and pathways have been identified which are involved in melanoma formation.

Among the genes which play a crucial role in melanoma development and progression are components of the *CDKN2A* locus. Mutations in genes of this locus were associated with familial melanomas [9], [10]. *CDKN2A* encodes two distinct tumor suppressor genes: inhibitor of cyclin–dependent kinase (*INK4A* or *p16*) and *ARF* (*p14*). INK4A inhibits cyclin dependent kinase (CDK) 4/6 -mediated phosphorylation and inactivation of RB (retinoblastoma protein), another tumor suppressor protein [11]. ARF inhibits MDM-2, an important negative regulator of the p53 tumor suppressor protein. MDM-2-mediated ubiquitination leads to subsequent degradation of p53 [12–15]. Therefore, INK4A and ARF negatively regulate the RB as well as p53 tumor suppressor pathway which lead to a predisposition to melanoma development in case of their loss.

Another pathway which plays an important role in melanoma development is the PI3K/AKT pathway. Here PI3K (phosphoinositid-3 kinase) gets activated upon binding to an activated RTK (receptor tyrosine kinase) like EGFR or a G-protein coupled receptor (GCR) [16]. In response to PI3K activation, PIP2 (phosphatidylinositol 4, 5-bisphosphate) is phosphorylated to PIP3 (phosphatidylinositol (3, 4, 5)-triphosphate). In the following, protein kinase B, also called AKT, is recruited to the cell membrane and is subsequently phosphorylated by adapter kinases such as phosphoinositide-dependent-kinases (PDK-1) [17]. Activation of AKT results in the regulation of several cellular processes like glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. A component which plays an important role in this pathway is the phosphatase and tensin homologue PTEN. PTEN is known to negatively regulate the PI3K/AKT pathway by dephosphorylating PIP3 to PIP2 and thereby blocking AKT membrane localization [18]. It has been described that PTEN deletions or mutations are present in 5% up to 20% of human melanomas [19], [20].

The RAS/RAF/MEK mitogen activated protein kinase (MAPK) pathway is one of the main pathways which are involved in melanoma initiation, progression and maintenance. This pathway is activated in melanocytes by growth factors like stem-cell factor (SCF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) which can bind to their respective RTK [21]. However, if they appear individually these growth factors can induce only weak or transient MAPK (mitogene activated protein kinase) activation. Only through the combined action of several stimuli a strong and sustained MAPK activity in melanocytes is achieved [21], [22]. Activated MAPK is able to induce several cellular responses like survival, proliferation by activation several transcription factors like MITF (microphthalmia- associated transcription factor) [23] or several cell cycle regulators like cyclin D1 [24] and also tumor maintenance enzymes like matrix metalloproteases [25]. MAPK is hyperactivated in up to 90% of human melanomas [26] indicating that this pathway plays a key role in the regulation of melanoma cells. One way by which MAPK hyperactivation is achieved is the accumulation of gain-of-function mutations in one of the three human *RAS* genes (*NRAS, HRAS* and *KRAS*). *NRAS* for example is mutated in 15% up to 30% of human melanomas [27]. However, the most commonly mutated gene in human melanoma is *BRAF* with 50% up to 70% [28]. Thereby the most common mutation is a substitution of a glutamic acid for valine at position 600 (V600E) [28]. It has been demonstrated that BRAFV600E is able to contribute to angiogenesis by activating vascular endothelial growth factor (VEGF) [29].

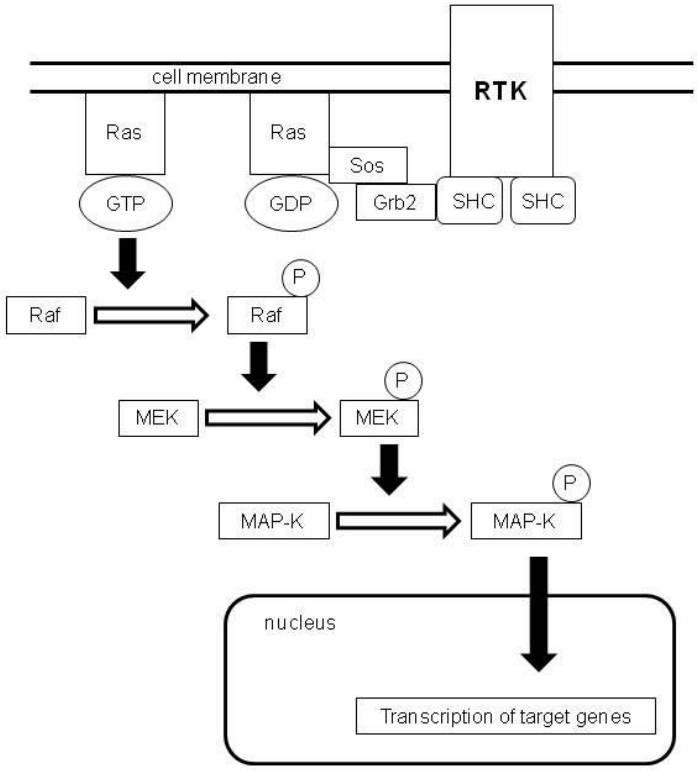


Figure 2: Activation of the RAS/RAF/MEK/MAPK-signaling pathway.

1.2 Fish melanoma models

1.2.1 The *Xiphophorus* melanoma model and Xmrk

One of the oldest animal models for cancer research in general and melanoma in particular are fishes of the genus *Xiphophorus*. At the end of the 20`s of the 20th century it was elucidated that genetic hybrids of certain strains of *Xiphophorus* can develop spontaneous melanomas. The crossing of platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus hellerii*) results in melanomas [30–33]. Responsible for the melanoma formation is the tumor locus *Tu* encoding for the oncogene *Xmrk* (*Xiphophorus* melanoma receptor kinase) which is present in the platyfish (*Xiphophorus maculatus*) but is absent in the swordtail (*Xiphophorus helleri*) [34]. The *Tu* locus is regulated by a yet unidentified tumor suppressor locus *R* in *Xiphophorus maculatus*. Vascularisation and growth of transplanted *Xiphophorus* melanomas in

nude mice showed no differences compared with transplanted human melanomas [35].

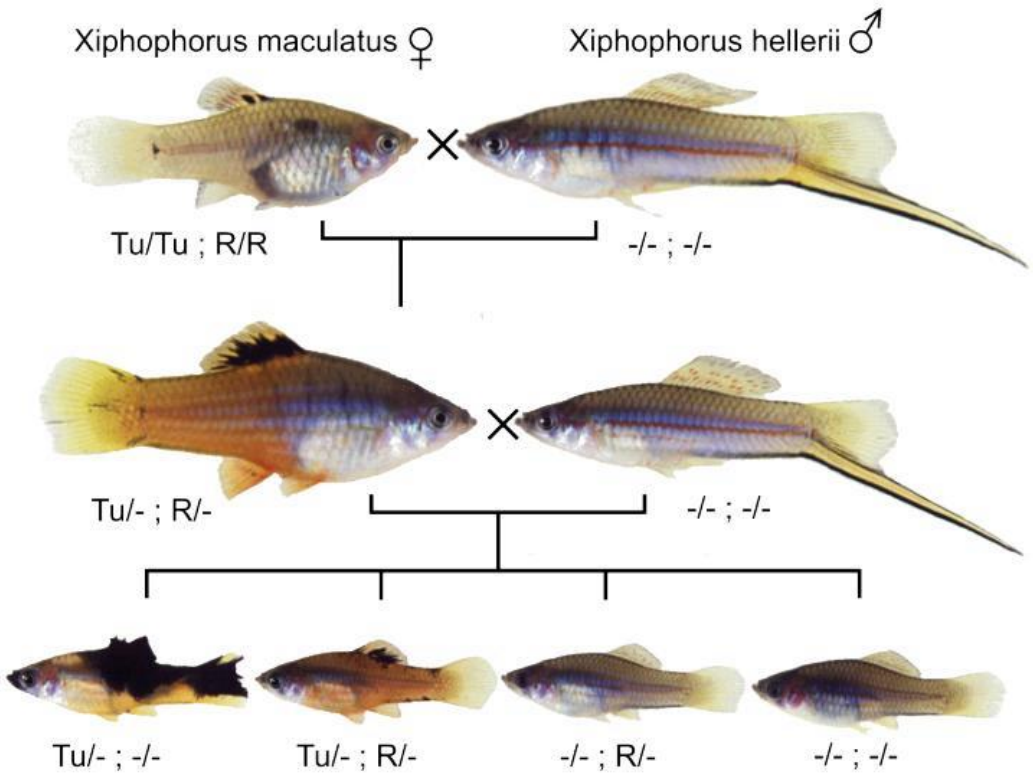


Figure 3: The classical Gordon-Kosswig-Anders cross.
A female platyfish (*X. maculatus*) exhibiting the tumor-bearing *Tu* allele and the tumor suppressor *R* allele is crossed to a swordtail male (*X. hellerii*) lacking both alleles *Tu* and *R*. The F1 offspring is heterozygous for *Tu* and *R* allele and develops large melanotic pigment spots at the fins which are non-malignant. Females of the F1 offspring are then backcrossed again to swordtail males (*X. hellerii*) lacking the *Tu* and *R* alleles. In the F2 offspring 25% of the animals develop melanoma due to absence of *R* and presence of *Tu*, 25% of the animals demonstrate the same genotype as F1 offspring and 50% of the animals lack the *Tu* allele resulting in healthy individuals without any pigmentation changes compared to founders.

The *Tu* locus was isolated by positional cloning and demonstrated a relationship with the epidermal growth factor receptor gene (*egfr*). It was later shown that this identified gene originates from a gene duplication of the *Xiphophorus egfr* gene, being the fish ortholog of human *EGFR* (*hEGFR*) and the protooncogene of the identified gene designated *xmrk* (*Xiphophorus* melanoma receptor kinase). The oncogenic potential of *xmrk* resides in two activating mutations in the extracellular domain of the growth factor receptor gene. These mutations lead to a constitutive signaling of the growth factor receptor which leads in turn to activation of a variety of cellular signal pathways. This activation of pathways results in several cellular

responses which participate in the formation of the neoplastic phenotype of melanoma cells [36].

The oncogenic protein Xmrk is able to recruit and activate several kinases, transcription factors and adapter proteins comparable to its ortholog, the human EGFR. Xmrk induces pathways like PI3K and STAT5 [37–39] which are required for transformation by suppressing apoptosis in case of STAT5 due to an increase in BCL-X, an antiapoptotic protein. Through the binding of adapter protein GRB2 (growth factor binding protein-2) the induction of the RAS-RAF-MAP kinase pathway is initiated, which result at the end in an increased rate of proliferation [40][41]. The same is true for the independent STAT5 pathway. But activated (phosphorylated) MAPK can additionally lead to the inhibition of differentiation via MITF (microphthalmia transcription factor) [42] and to survival of tumor cells at ectopic sites by inducing OPN (osteopontin). Osteopontin is secreted and can then bind to integrins like $\alpha_v\beta_3$-integrins to protect the cells from apoptosis [43]. The docking protein FYN, a member of the SRC kinase family, is involved in the maintenance of phosphorylated MAPK due to blocking of MAP kinase phosphatase 1 (MKP-1) [22], [44] and it is, together with the focal adhesion kinase (FAK) involved in Xmrk-mediated pigment cell migration, which is however MAPK-independent [45]. Furthermore, Xmrk was shown to induce the generation of reactive oxygen species (ROS), which can mediate the genesis of a melanocytic multinuclear phenotype and senescence [46].

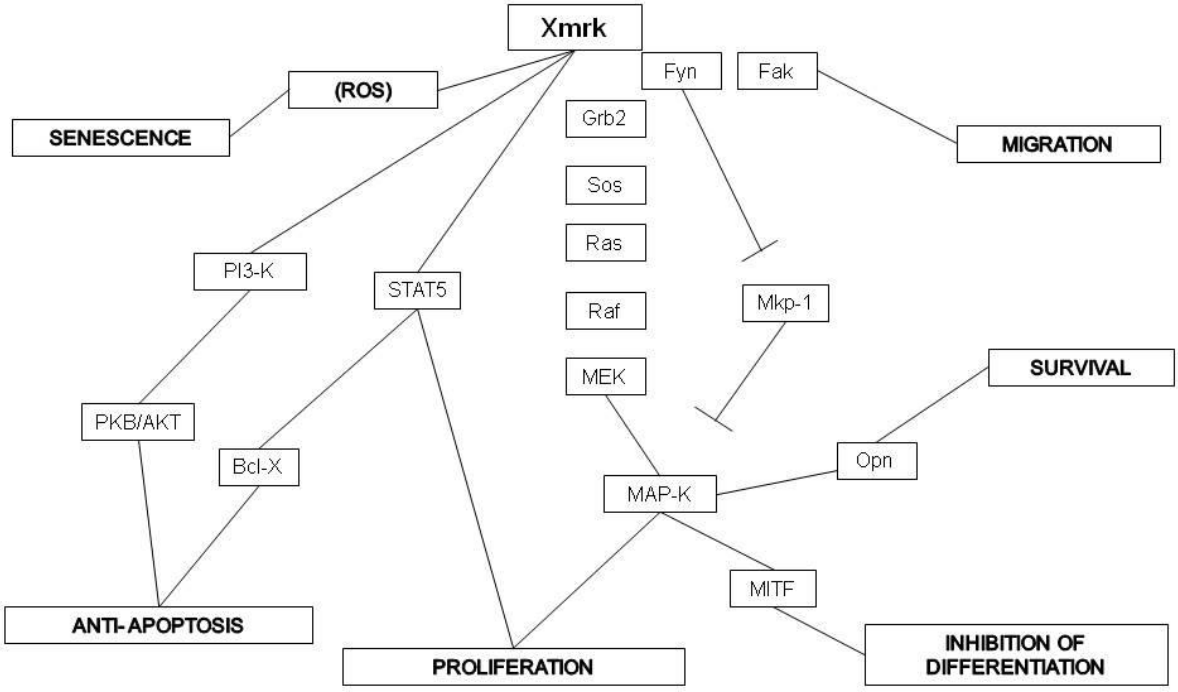


Figure 4: Xmrk-mediated cellular processes and corresponding activated molecular pathways.

The *Xiphophorus* melanoma model system offers the opportunity to understand one tumor on different organization levels. Here, molecules and their interactions with others as well as tumor mediated changes in tissues and organs can be examined in a whole organism. The formation of the tumor is mediated by a single oncogenic receptor which sits at the top of several contributing pathways.

1.2.2 Zebrafish melanoma models

In comparison to *Xiphophorus*, a livebearing fish, *Danio rerio* (zebrafish) is egg-laying and has an extracorporal embryonic development which allows for microinjection of transgene DNA which will randomly integrate in the genome. Furthermore the embryos are transparent making imaging and observation of embryonic development feasible.

It has been shown that zebrafish transgenic for activated Braf (V600E) under the control of the *mitfa* promoter lead to patches of ectopic melanocytes, termed fish (f)-nevi but fail to develop melanoma [47]. Notably, when crossed to a p53- deficient ($p53^{M214K}$) line in these fishes formation of invasive melanomas could be observed [47].

In another transgenic approach zebrafish had been generated, which express human $NRAS^{Q61K}$ under the control of the *mitfa* promoter [48]. Fish stably expressing the transgene show a hyperpigmentation and an anormal pigmentation pattern but fail to promote melanoma formation. Like in zebrafish transgenic for activated Braf, crossing to a p53- deficient fish line leads to the genesis of melanoma.

Furthermore it had been demonstrated that the expression of $hras^{V12}$ under pigment cell specific control of the *mitfa* [49], or *kita* promoter [50] or when expressed at low levels throughout the fish [51] can promote ectopic melanocyte generation and melanoma.

1.2.3 Medaka melanoma models

Medaka, like zebrafish, is egg-laying, exhibits an extracorporal development, translucent embryos and a short generation time. Therefore medaka is an excellent model to investigate tumor development. Transgenic approaches have been used to generate a medaka melanoma model system, like the Xmrk-mediated medaka melanoma model [52]. In this system the medaka fishes express the oncogene *xmrk* which was fused to the pigment cell specific promoter *mitfa* from medaka. It had been previously shown that Xmrk is able to transform a variety of other cell types [53], [54]. *xmrk*-transgenic juvenile or adult fishes developed large areas of hyperpigmentation or progressively growing pigment cell tumors [55]. In addition to black pigmented melanomas, other pigment cell tumors of red and yellow pigment-containing xanthophores and erythrophores were observed. As melano-, xantho- and erythrophores express the pigment cell marker Mitf-a, Xmrk is expressed in all these cell types. The red and yellow tumors were designated xantoerythrophoromas or XE

tumors. A highly invasive tumor growth with invasion of internal organs and muscles was observed for melanotic tumors (figure 5).

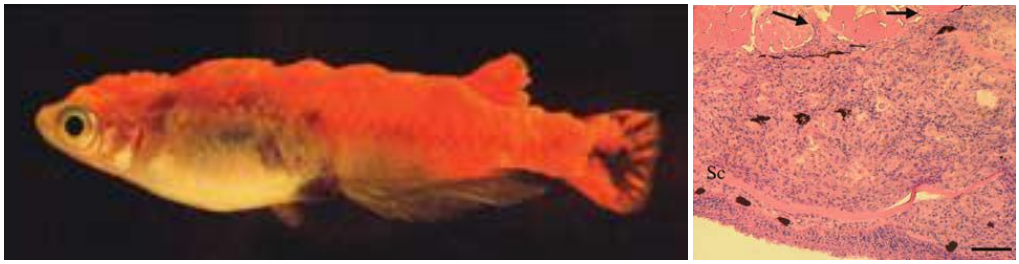


Figure 5: Histology of pigment cell tumors in mitf::xmrk transgenic medaka.
Display of macroscopic and microscopic appearance of exophytic xanthoerythrophoroma. Left image: 10-week-old female medaka (*Carbio*) with exophytically growing xanthoerythrophoroma. Right image: Xanthoerythrophoroma growing in the dermal compartment and locally invading the underlying trunk musculature (arrows). Images adapted from [55].

Similar to the Xiphophorus model, melanoma penetrance is 100% in the *xmrk*-trancgenic medaka model. In this model stable *xmrk*-transgenics show a very stereotype tumor development with an early onset. This makes this system suitable for the analysis of chemical compound testing and the examination of tumors from early to late stages of development.

1.3 Angiogenesis

The formation of new blood vessels from the pre-existing vasculature, e.g. due to wounding or the influence of hypoxia- or cancer-mediated factors, is called angiogenesis. Blood vessels are part of the circulatory system which is responsible for supplying the body with blood. This is important to supply distant tissues and organs with nutrients, electrolytes and oxygen. Furthermore blood vessels serve to take away waste products such as carbon dioxide from those regions.

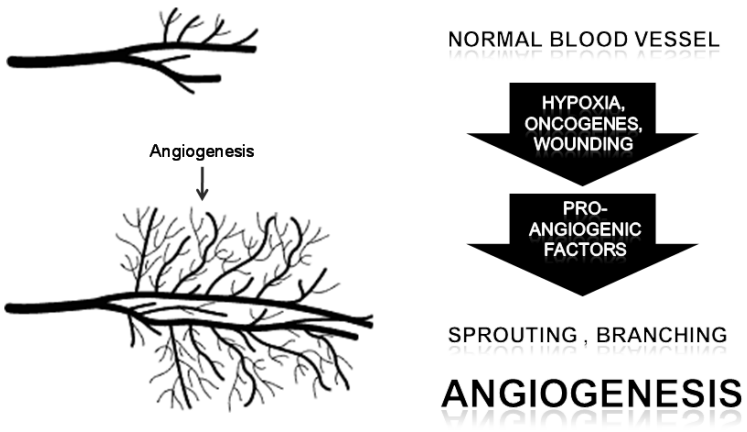


Figure 6: Angiogenesis.
Scheme of a formation of a new blood vessel from the pre-existing vasculature due to wounding or the influence of hypoxia- or cancer-mediated factors.

In contrast to angiogenesis, vasculogenesis describes the *de novo* generation of vessels from precursor cells such as angioblasts. An angioblast is an undifferentiated endothelial progenitor cell that has yet to integrate into a blood vessel [56].

Vasculogenesis takes place during embryonic and early development [57]. In both processes angiogenic signals play an important role. In the last decades a broad variety of pro- and anti-angiogenic factors have been discovered such as heparin binding peptide growth factors like VEGF [58], [59], PlGF, FGF-1 or FGF-2 [60]), non-heparin binding peptide growth factors like TGF-α [61], [62], TGF-β [63], EGF [61], [64] or IGF-I [64], [65], inflammatory mediators like TNFα [62], IL-8 [66] or IL-3 [67], enzymes like COX-2 [68] or angiogenin [69], hormones like oestrogens [70], oligosaccharides like hyaluronan oligosaccharides [71], [72] or gangliosides [73], hematopoietic factors like erythropoetin [74], cell adhesions molecules like VCAM-1 [75] or E-selectin [75], [76] and others like nitric oxide [62] or ANG-1 [77], [78].

1.3.1 Types of angiogenesis

Angiogenesis can be subdivided into two groups: physiological and pathological angiogenesis [79].

Usually the rate of proliferation of endothelial cells, in the adult, is very low compared to other cell types within the body [80]. Processes like wound healing [59], [81], endometrial growth during the menstrual cycle or reproduction [79] are an exception in which angiogenesis occurs. These processes are reported as physiological angiogiogenesis. Physiological angiogenic processes are highly regulated by a cascade of pro-angiogenic factors or angiogenesis inducing signals. These factors are turned on for a short period of time and then they are completely shut down. Furthermore pro-angiogenic factors are balanced by so called anti-angiogenic factors which play a counter part to the inducing molecules. An imbalanced or unregulated angiogenesis could result in a variety of diseases [79] like rheumatoid arthritis [82], diabetic retinopathy [83] or juvenile hemangliomas [84].

Unregulated or imbalanced angiogenesis is named pathological angiogenesis resulting in an aberrant growth of blood vessels (i.e. permanent maintained neovascularization). Pathological angiogenesis occurs also in tumor development. Tumor- or sustained angiogenesis was described by Hanahan and Weinberg as one of the hallmarks of a cancer [85].

Tumor development is usually a multi-step event. The start of uncontrolled growth of some tumor is often correlated with a loss of regulated or controlled cell proliferation. After the tumor mass has reached a certain size some tumors stop growing and reach a steady state due to the fact that the number of proliferating cells counterbalances the number of dying cells [80]. The growth stop is often the result of a lack of nutrients and oxygen [86]. It has been shown in several transplantation studies that an avascular tumor - a tumor that is not associated with or supplied by blood vessels - has a size limit of about 0.2 to 2.0 mm in diameter [87]. This observation correlates with the tissue oxygen diffusion limit of approximately 100 to 200 µm [86]. By reaching a size of about two millimeters, cells in the inner mass of a tumor suffer from low oxygen tension (hypoxia) and nutrients. This may lead to an upregulation and/or secretion of pro-angiogenic factors like FGF-2 or VEGF, sometimes together with an inhibition and/or downregulation of anti-angiogenic molecules. This event provokes an imbalance between pro- and anti-angogenic factors and is called “angiogenic switch“ [63], [88].

In physiological and pathological angiogenesis, hypoxia is one of the main forces which lead to the onset of the angiogenic process [80]. Hypoxia is usually initiated by stabilization of hypoxia inducible factor 1 alpha (HIF-1α). HIF-1α is then able to induce several other pro-angiogenic factors like VEGF or FGF's [89], [90]. Furthermore hypoxia is also attracting macrophages [80].

Finally the neovascularization of a tumor enhances the possibility that tumor cells may get into the blood stream leading to a spread of these cells in other organs or tissues to form metastases.

1.3.2 Angiogenic mechanisms

Angiogenesis usually occurs through two main mechanisms, known as sprouting angiogenesis and intussusceptive angiogenesis (alias non-sprouting or splitting angiogenesis). However, beside these, angiogenesis can take place through other mechanisms like recruitment of endothelial progenitor cells (EPC's), vessel co-option, vascular mimicry or lymphangiogenesis.

1.3.2.1 Intussuseptive angiogenesis

In intussuseptive angiogenesis the wall of a capillary expands into the lumen of the capillary and this in the end results in a split of the capillary in two new vessels. Intussusceptive angiogenesis can be separated into four phases. The typical feature of phase one is a direct cell contact of endothelial cells located opposite of each other in the capillary wall. This contact is achieved by the walls protruding into the vessel wall and these walls then establish a zone of contact. Then in phase two, the endothelial cell junctions are reorganized and the vessel bilayer is perforated to allow growth factors and cells to penetrate into the lumen. Phase three is characterized by a core formation between the two new vessels at the zone of contact that is filled with pericytes and myofibroblasts. These cells begin laying collagen fibers into the core to provide an extracellular matrix for growth of the vessel lumen. Finally in phase four the core is enlarged without alterations to the basic structure [91], [92].

Intussusception is an important mechanism to reorganize existing cells. Thereby an increase in the number of capillaries is not corresponding with an increase in the number of endothelial cells. This mechanism of angiogenesis is especially important in embryonic development. Here, insufficient resources are available to create a rich microvasculature with new cells.

1.3.2.2 Sprouting angiogenesis

Sprouting angiogenesis is determined as the sprouting of vascular endothelial cells from pre-existing capillaries into adjacent tissue. This process involves several steps. Sprouting angiogenesis usually starts with an activation of endothelial cells by specific growth factors which bind to receptors on the endothelial cell membrane. As a result, activated proteases degenerate locally the extracellular matrix and basement membrane surrounding the endothelial cells. Then endothelial cells are able to invade into and through the surrounding matrix by proliferation and migration [93]. Polarization of migrating endothelial cells create a lumen and lead to the formation of an immature new blood vessels [94]. Recruitment of mural cells and generation of an extracellular matrix lead to a stabilization of these immature vessels [95]. This process is usual tightly controlled by pro- and anti-angiogenic regulators, which determine the level of ongoing angiogenesis.

1.3.2.3 Recruitment of endothelial progenitor cells (EPC)

Today it is generally accepted the new blood vessel formation can also occur due to the recruitment of endothelial progenitor cells. The first in vivo observation of this process came from mouse and rabbit bone marrow transplantation models which show an incorporation of EPC in blood vessels [96–98]. This EPC recruitment is promoted by several growth factors like PLGF and/or VEGF [99], [100] as well as several chemokines and cytokines which are produced during processes such as physiological stress or tumor growth.

The recruitment and incorporation of EPC is a multistep process, including chemoattraction, active arrest and homing within angiogenic vasculature,

transmigration to the interstitial space, incorporation into the microvasculature and differentiation into mature endothelial cells [93]. Some researchers doubt that EPC play a leading role in tumor angiogenesis [101–103], but nonetheless the impact of EPC in tumor angiogenesis cannot be disregarded.

1.3.2.4 Vessel co-option

It has been shown that tumors, even in an avascular stage, are able to grow in well-vascularized tissues like brain or lung [104], [105]. This phenomenon is defined as vessel co-option. Here tumor cells can grow alongside existing vasculature without causing an angiogenic response. Today vessel co-option has been observed in several tumor types like murine Lewis lung carcinoma, murine ovarian cancer, human Kaposi sarcoma and human melanoma [106–109].

1.3.2.5 Vasculogenic mimicry

In another process described as “vasculogenic mimicry” tumor cells are able to transdifferentiate into an endothelial like phenotype. These transdifferentiated tumor cells provide the tumor with a secondary circulatory system of vasculogenic structures and has been observed mainly in aggressive tumors like melanoma [110], [111]. But still less is known about the exact mechanism underlying vasculogenic mimicry. Recently it was suggested that low levels of oxygen or hypoxia, which are known to promote cell invasion, metastases and transformation in melanoma [112], [113], can lead to a vasculogenic mimicry, as demonstrated by tube formation in matrigel assays [114], [115].

1.3.2.6 Lymphangiogenesis

The formation of new lymphatic vessels from pre-existing ones is defined as lymphangiogenesis. This process is not a direct mechanism of angiogenesis but it is assumed that it is triggered in a similar way as angiogenesis. The lymph system is part of the vascular circulatory system but in contrast to the blood vascular network it is an open ended, one way transport system that drains extravasated fluid, collects lymphocytes and returns it to circulation [116]. Factors which play an important role in

lymphangiogenesis are VEGFR-3 [117], VEGF-C [118], VEGF-D [119] and also bFGF [120].

1.3.3 Angiogenesis models

To date, a broad variety of *in vitro* or *ex vivo* angiogenesis tools and angiogenesis assays are available such as endothelial cell proliferation and migration assays (e.g. wound healing assay), endothelial cell differentiation assays (e.g. tube formation assays, sprouting assays [121], [122] as well as organ culture assays (e.g. rat/mouse aortic ring assay [123], [124], chick aortic arch model [125] and vena cava-aorta model [126]).

These are useful tools for screening potential inhibitors or inducers of angiogenesis and for testing or validating new drugs [127]. However, they can only give limited information about the pro- and anti-angiogenic interactions that take place in a natural physiologic microenvironment. In addition, dosage issues or side effects of new drugs cannot be addressed. Therefore, in vivo models are essential to study effects of angiogenesis in a whole organism, especially angiogenic effects of tumors in their natural environment.

Several *in vivo* models encompassing different species exist. In the chick chorioallantoic membrane assay (CAM), test substances or cross-species xenografts as well as cancer cells are placed on the CAM and angiogenic processes are recorded after a few days by microscopy [128], [129].

The corneal angiogenesis assay uses the cornea of rabbits, guinea pigs, rats or mice to investigate neovascularization in the corneal pocket in response to closely positioned substances like concentrated conditioned medium, growth factors, cytokines or cross-species tissues such as tumor tissues, tumor cells and other cells or tissues into the corneal pocket [130], [131].

The murine dorsal air sac model uses a chamber ring which is made up of nitrocellulose filters and is loaded with tumor tissue or tumor cells. This ring is then

implanted into the dorsal skin of mice by lifting it with injected air. After 5 days the ring is removed and angiogenesis is assessed by counting vessels or vessel density using microscopy [132].

The chamber assays, first used in rabbits, later adapted to mice, rats and hamsters, use two symmetrical frames which are implanted, in the majority of cases, into the dorsal skinfold of the animal [133–135]. The result is a sandwich of two skin layers. One of the skin layers is then removed and tumor tissue or tumor cells are placed onto the remaining layer. The investigated area is then covered with a glass cover slip. Neovascularisation is then studied with trans-illumination technique devices.

Most of these assays require transplantation or xenotransplantation of tumor cells and application of substances like growth factors, cytokines or conditioned media. However, the use of special imaging and preparation equipment is often necessary to monitor tumor angiogenesis. In most assays test animals can only be analyzed at the experimental end point, when they are dissected to investigate affected organs or tissues.

Transparent animals, like the zebrafish (*Danio rerio*) or the Japanese ricefish medaka (*Oryzias latipes*) are able to overcome some of these problems. They produce large numbers of embryos per day with optical clarity and *ex utero* development which facilitate monitoring. For zebrafish, several xenotransplantation angiogenesis models do exist [136–138]. But most of them describe the vascularisation after injection of human melanoma or other tumor cells into embryos or larvae.

1.4 Angiogenesis in human melanoma

Formation of new blood vessels is a prominent feature of human melanoma. It has been shown that these tumors have angiogenic activity [139]. The first observations about the ability of human melanoma cells to induce angiogenesis date back to 1966 when Warren and Shubick transplanted human melanoma cells into a hamster cheek pouch which led to neovascularization. The onset of angiogenesis was shown to

occur in the radial growth phase of cutaneous melanomas [140]. An increase in the mean vascular density has been shown to correlate with melanoma progression in subsequent histochemical studies of melanomas [141] and several studies have reported an inverse correlation between tumor microvessel density and disease-free and overall survival of melanoma patients [142–145].

1.4.1 NF-κB and cancer

Since its discovery in 1986, the nuclear factor kappa B (NF-κB) became one of the most investigated transcription factors [146], [147]. NF-κB plays a major role in multiple cellular processes such as inflammatory, innate and adaptive immune response, proliferation, apoptosis, development and angiogenesis. It was originally found in the nucleus of B cells bound to an enhancer element of the immunoglobulin kappa light chain gene and was then the termed NF-κB. It was assumed that it is B cell specific but was later shown to be ubiquitously expressed.

The NF-κB complex consists of different subunits which can form a variety of homo- as well as heterodimers with different cellular functions. All components of the NF-κB complex belong to the reticuloendotheliosis (REL) family. The fact that v-REL is an oncoprotein of the REL retrovirus (REV-T) provided one of the first links between NF-κB and cancer [147], [148].

Two classes of REL proteins are known to play a decisive role in human NF-κB complexes (Table 1). These two classes are distinguishable by their mode of synthesis and action.

Class I REL proteins consists of NF-κB1 (p105) and NF-κB2 (p100). P105 and p100 are large precursor proteins which exhibit an aminoterminal REL homology domain (RHD), required for DNA binding and dimerization, as well as a series of ankyryn repeats at the C-terminal side. This C-terminal domain is removed in response to ubiquitin-dependent proteolytic procession, resulting in the two mature DNA-binding proteins p50 (derived from p105) and p52 (derived from p100), which both lack the transcription-modulating domains.

Class II REL proteins consists of RELA (p65), RELB, and c-REL. These proteins exhibit also a RHD at the N-terminal side as well as several transactivation domains (TAD) at the carboxyterminus. These proteins are synthesized in their mature forms and are not further processed by proteolysis.

Class	Protein	Aliases
I	NFKB1	p105 > p50
	NFKB2	p100 > p52
II	RELA	p65
	RELB	
	c-REL	

Table 1: Class I and II of NF-κB complex proteins.

These two classes of REL proteins can form various NF-κB complexes of different homo- and heterodimers.

The activity of these homo – and hetero dimers are regulated by two main pathways, a canonical or classical pathway and a non-canonical or alternative pathway (figure 13).

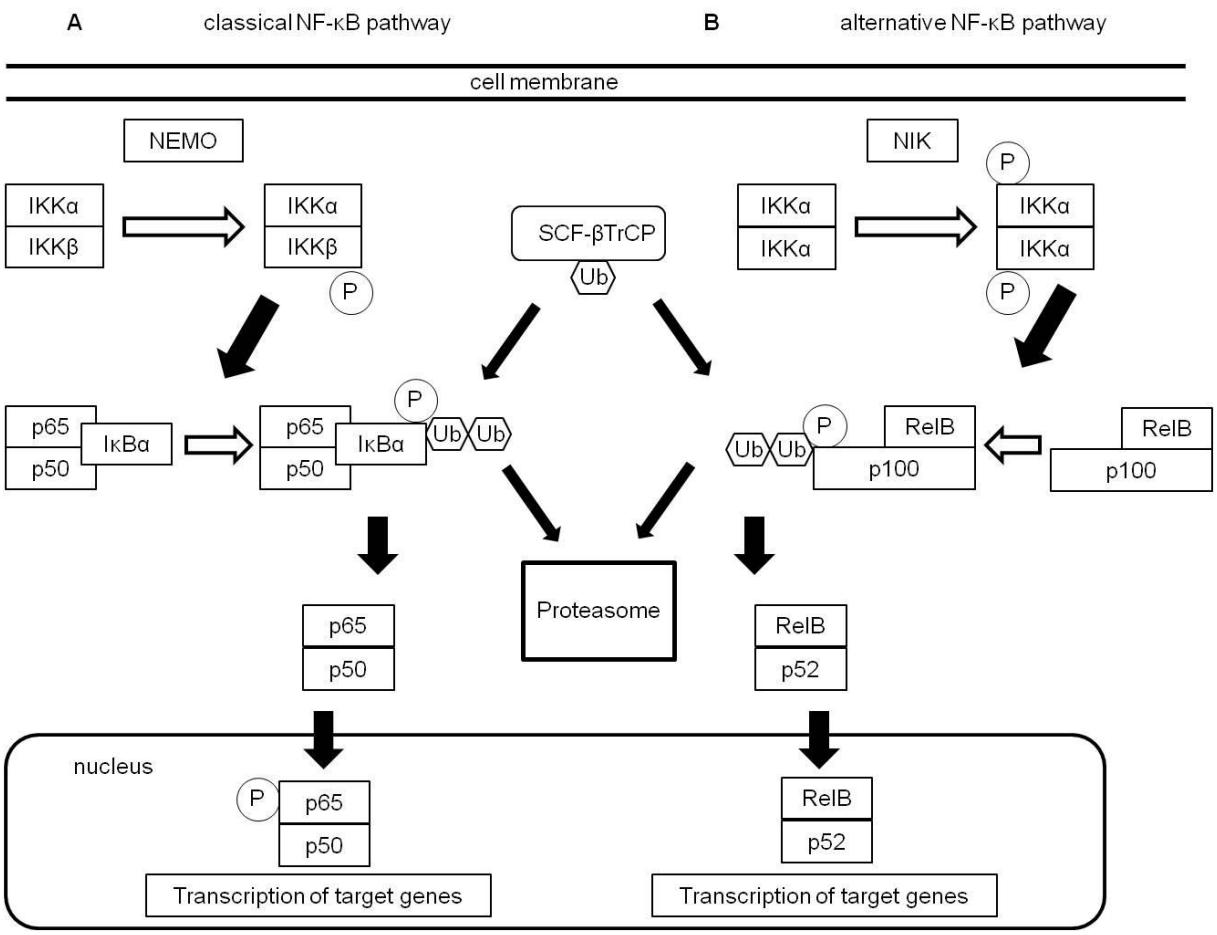


Figure 7: A model depicting the classical and alternative signaling pathway of NF-κB.
(*A*) Classical or canonical pathway is mediated by IKKβ and leading to phosphorylation of IκB. (*B*) The alternative or non-canonical pathway involves NIK activation of IKKα and leads to the phosphorylation and processing of p100, usually generating p52 (RELB) heterodimers.

In classical or canonical pathway regulation, dimers composed of RELA (p65), c-REL, and p50 were held captive in the cytoplasm by inhibitors of κB (IκB). There are seven IκB family members named IκBα, IκBβ, BCL-3, IκBε, IκBγ, as well as the precursor proteins p100 and p105. All these members exibit an N-terminal regulator domain and adjacent to this domain a series of five to seven of ankyrin repeats which can bind to the dimerization domain of NF-κB dimers [149].

This classical pathway can be induced in response to microbial and viral infections or activated by mitogens, growth factors, hormones, exposure to proinflammatory cytokines, several stresses (physiological, oxidative, physical), chemical agents, environmental toxins and medical drugs. These NF-κB-inducible factors lead to activation of the IκB kinase (IKK) complex. This 700-900-kDa IKK complex contain two kinase subunits, IKKα (IKK1) and IKKβ (IKK2) [150], and a regulatory subunit

termed NEMO (NF-κB essential modifier) or IKKγ. In case of the activation of the classical pathway the IKKβ subunit phosphorylates NF-κB-bound IκB's on two conserved serine residues (Ser 32 and Ser 36 of IκBα and Ser 19 and Ser 23 of IκBβ), within the N-terminal regulatory domain. Phosphorylated IκB's are detected by the ubiquitin ligase machinery. This leads to polyubiquitination of IκB's and their subsequent degradation by the 26S proteasome [151], or processing in case of p100 respectively p105 yielding active forms p52 respectively p50 [152]. The alternative pathway usually generates p52-RELB heterodimers while the classical pathway usually generates p52-RELA heterodimers [151], [153].

The alternative or non-canonical pathway can be induced by activation of the NF-κB-inducing kinase (NIK) through different members of the TNF-receptor superfamily, like CD40R [154], B-cell activating factor receptor (BAFFR) [155] and lymphohotoxin-β receptor (LTβR) or tumor necrosis factor receptor-associated factor 3 (TRAF3) . NIK then activates IKKα which phosphorylates p100 [156], [157].

After degradation of the IκB's the released NF-κB dimers are able to translocate to the nucleus. Phosphorylation of RELA (p65) by a several kinases during the phosphorylation and degeneration process of IκB's enhances the nuclear translocation of p65 [158], [159]. In the nucleus the NF-κB-dimers bind to specific sequences of promoter or enhancer regions of target genes.

IκBα is found amongst the first genes which are activated by NF-κB. IκBα can transport active NF-κB from the nucleus to the cytoplasm - a classic example for a negative feedback loop system. In normal cells, NF-κB activation is therefore transiently inducible. In tumor cells of different types, impaired regulation of NF-κB activation leads to a constitutively activation due to molecular alterations. This can result in an abnormal regulation or expression of different NF-κB-target genes. Several cellular alterations like evasion of apoptosis, sustained angiogenesis, self-sufficiency in growth signals, and metastasis may be the result of constitutive NF-κB activation in different types of cancers, including melanoma.

1.4.2 NF-κB in melanoma

Several in vitro studies have demonstrated that NF-κB binding is constitutively increased in human melanoma cell cultures compared to normal melanocytes [160], [161]. This correlates with elevated p65 expression levels, enhanced phosphorylation and nuclear translocation in human nevi and melanomas compared to normal skin p65 levels [161], [162].

Since melanoma progression is mediated through multiple genetic pathways, constitutive NF-κB activation can occur through several steps and in several mutated pathways, which are known to promote melanoma progression [163], [164].

1.4.3 Mechanisms of NF-κB activation in melanoma

Many typical mutations, which may occur in melanoma, are able to trigger the NF-κB pathway as discussed hereafter (see Figure 8 for an overview).

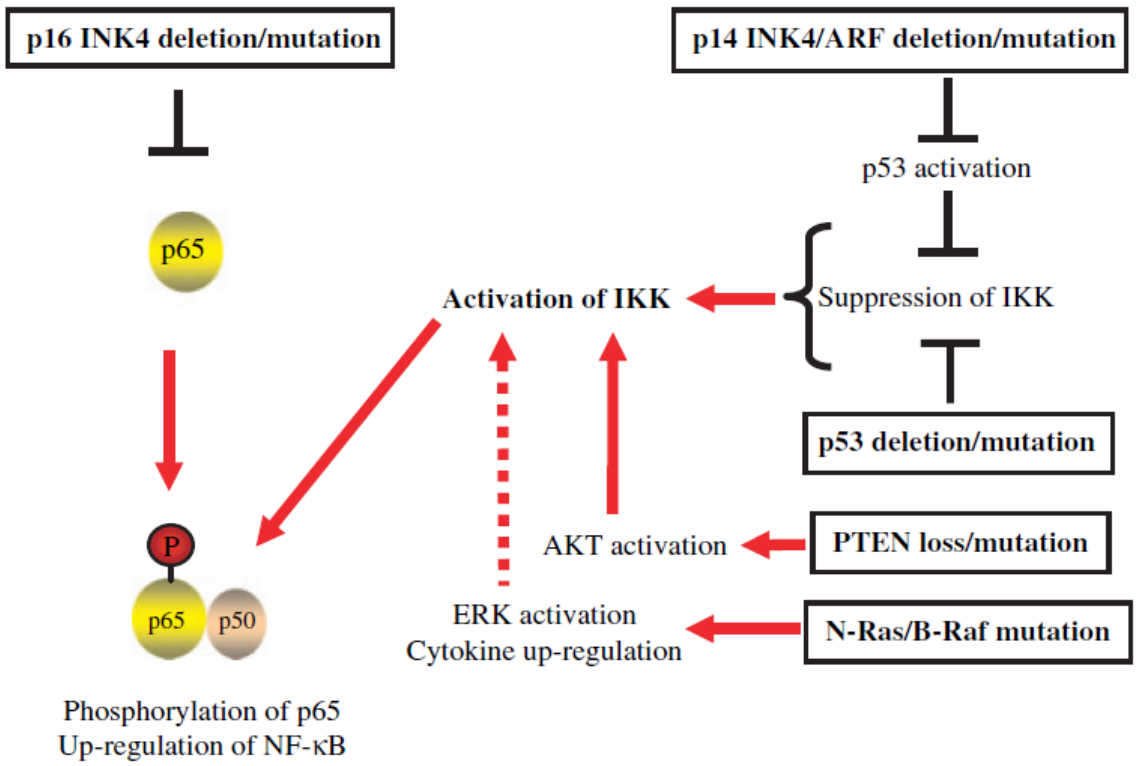


Figure 8: Gene mutations in sporadic melanoma and NF-κB upregulation.

These mutations directly/indirectly induce NF-κB upregulation. Red arrow (→) indicates direct positive regulation and dotted red arrow indicates indirect positive regulation. The black colored symbol (⊥ or ⊤) indicates negative regulation. This diagram was adapted from [165].

p16 INK4a is known to regulate the activity of the retinoblastoma (RB) protein family members and the regulation of cell proliferation [166], [167]. RB family members are known as tumor suppressor and proliferation inhibitors. RB or p16 INK4a mutations can lead to an enhanced activation of CDK4/6 followed by enhanced proliferation and immortalization [168–170]. It has been shown that wild-type p16 INK4a bind to p65 and inhibits NF-κB transcriptional activity [171], [172]. This suggests that mutated p16 INK4a leads to a reduced binding and thus to an upregulation of NF-κB [173].

A mutation which leads to a loss or inactivation of p14 ARF protein could directly result in an upregulation of NF-κB. p14 ARF is known as an activator of tumor suppressor gene p53 [174], [175] and a loss of p14 ARF results in a reduction of p53 activation [176], [177]. p53 is known to inhibit IKKα gene transcription due to specific p53 binding at the IKKα promoter [178]. Therefore an inhibition of p53 leads to an upregulation of IKKα, this in turn results in an activation of NF-κB.

The phosphatase and tensin homolog (PTEN) the regulatory molecule of the PI3K/AKT pathway is often mutated in melanoma [179], [180].

This leads to increased activity of AKT, which phosphorylates IKKα, resulting in the phosphorylation of p65 [181], [182]. It has also been reported that AKT itself can phosphorylate p65, resulting in an increased binding of the NF-κB complex to DNA [18].

Furthermore, NF-κB may be indirectly activated by oncogenic RAS and RAF (mainly through N-RAS and/or B-RAF mutations) due to constitutive activation of ERK [183]. Furthermore various cytokines, like TNF-α or IL-1α/β, as well as chemokines, which are regulated in their expression by constitutively activated ERK are known to be activators of NF-κB [184], [185].

1.5 Aims of the thesis

For the development of new drugs or treatment strategies it is necessary to understand the basic mechanisms and effects of malignancies like cancer, especially melanoma. Therefore, it is of great importance to have suitable models e.g. animal models to reveal on the one hand new factors which could play a role in the onset, development and progression of these malignancies and on the other hand to get more insights in the interactions of different malignant factors and their effects on the entire organism.

One aim of this thesis was to analyze if the oncogenic protein Xmrk alone - without any mutated other genes or exogenous treatments like radiation - is sufficient to induce angiogenesis in an organism like medaka or zebrafish. Furthermore, if Xmrk is able to induce angiogenesis, the second aim was to reveal the factors which are involved in this process due to their direct or indirect induction by Xmrk. Moreover, it was the aim to find out if the inhibition of these factors could decrease the potential angiogenic effects *in vivo*. Finaly, an aim was to analyze if there is a similar effect or stringent mechanism in human cells especially in melanoma cell lines.

2 MATERIALS, METHODS AND FISH MAINTENANCE

2.1 Fish strains

2.1.1 Zebrafish (Danio rerio)

The zebrafish TU-AB wildtype strain (ZFIN ID: ZDB-GENO- 010924-10) was used for injection experiments. This strain has been kept stock in the laboratory aquarium.

2.1.2 Medaka (*Oryzias latipes*)

Fish from the strain Carbio (outbred strain, mixed genetic background of southern medaka) were used. Transgenic fish lines *mitf::xmrk* [52] and *fli::egfp* were used to generate *fli::egfp;mitf::xmrk* double transgenic fishes.

2.2 Fish maintenance

Fishes were maintained under standard conditions with an artificial photoperiod (10 hours of darkness, 14 hours of light). For all assays, fishes were kept in a medium containing 17.4 mM NaCl, 210 µM KCl, 180 µM $Ca(NO_3)_2$, 120 µM $MgSO_4$, 1.5 mM HEPES.

2.2.1 Microinjection of zebrafish embryos

Prior to an injection experiment male and female zebrafishes were put in a spawning tank with a seperating insert and a sieve at the bottom. Prior to the start of the light-phase on the day designated for injection the male and female fishes were joined and subsequently spawning began. Laid eggs were transferred into a Petri dish and were washed with fresh medium.

For injection a dish was formed of 2% agarose in medium with “injection grooves” which held the eggs in position for injection. Prior to injection eggs were aligned inside the grooves and remaining liquid was almost completely removed with a 1ml plastic pipette.

The concentration of injected DNA was determined prior to injection by photometric measurements. The injection solution was aspirated into injection needles using Eppendorf microloaders. The needles were placed and fixed in a micromanipulator. Then the needle tip was carefully opened and for injection about 500 pl of liquid solution was injected into the cytoplasm of a one- or two-cell stage zebrafish embryo by appliance of a constant injection pressure.
The injected procedure was observed with a stereomicroscope. Applied injection pressure and injection time were adjusted to the size of the needle opening.

Embryos were kept in an incubator at 28°C after injection. A visual control for dead embryos and developmental malformations was carried out every day. Medium exchange was performed if necessary.

2.2.2 *In vivo* inhibitor treatment

For the *in vivo* treatment of transgenic *fli::egfp;mitf::xmrk* and *fli::egfp* medakas, 100 nM NF-κB activation inhibitor (Calbiochem) or 3 mM Tiron (dissolved in DMSO) were administered for the indicated timespan. The tank water including inhibitors was exchanged every second day. DMSO treatment served as control. Quantification of sprouts and branch points per mm inter-fin ray area was performed using 7 to 8 fishes from each group.

2.2.3 Microscopy of transgenic *fli::egfp;mitf::xmrk* and *fli::egfp* medakas

For imaging, fishes were anesthetized with a 1:2000 dilution of pure 2-phenoxyethanol in medium containing 17.4 mM NaCl, 210 µM KCl, 180 µM

$Ca(NO_3)_2$, 120 µM $MgSO_4$, 1.5 mM HEPES. Subsequently fishes were fixed with a tape stripe at the bottom of a petri dish. Then pictures were taken with a Leica DMI6000 B microscope. After pictures were taken fishes were immediately trensferet to fresh media to recover from anesthesia. To process the pictures, Leica Application Suite (LAS) Microscope Software and ImageJ software were used to analyze the raw data.

For confocal microscopy the caudal fins of transgenic *fli::egfp;mitf::xmrk* medakas were cut and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) over night. After three washing steps with PBS fins were gradually dehydrated with increasingly concentrated methanol solution (up to 100%) and incubated at room temperature overnight. Subsequently, fins were gradually hydrated with PBS and were incubated for one hour with the TO-PRO-3 DNA stain (1:1000, Invitrogen). After three washing steps with PBS, fins were incubated in PBS over night at room temperature. Fins were then transferred to glass slides and were covered with Mowiol® 4-88 (Roth). Images were then captured with a Nikon C2 confocal microscope. Volocity® 3D Image Analysis software from PerkinElmer (USA) and ImageJ software were used to process and analyze the raw data.

2.3 Cell culture

2.3.1 Murine cells

Mouse melanocytes transfected with HERmrk (Melan-a Hm) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal calf serum (FCS) in the presence of cholera toxin (12 nmol/L), and TPA (200 nmol/L) supplemented with penicillin (400 U/ml) and streptomycin (50 µg/ml).
For small molecule inhibitor experiments, Hm cells were starved for three days in 2.5% starving medium containing DMEM supplemented with penicillin (400 U/ml), streptomycin (50 µg/ml) and 2.5% dialyzed FCS (Invitrogen, Germany). Inhibitors were applied at indicated concentrations. One hour after inhibitor treatment, cells were stimulated with 100 nM human EGF (hEGF) from Tebu-bio, Germany.

2.3.2 Human cells

Human embryonic kidney cells HEK293 and adenocarcinoma-derived human alveolar basal epithelial cells (A549), either transfected with pRK5 or pRK5-xmrk [186] were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 µg/ml), and 10% fetal calf serum (FCS, Invitrogen).

Human melanoma cell lines Mel Im, Mel Wei, Mel Ho, UACC-257, SK-MEL-5, RPMI-7951, MEWO, M14, MALME-3, MDA-MB-435, SK-MEL-3, UACC-62
and A375 were maintained in DMEM Glutamax supplemented with penicillin (400 U/ml), streptomycin (50 µg/ml) and 10% FCS (Invitrogen).

2.4 Gene expression analysis

2.4.1 Starvation of cells

Hm cells were starved for three days in 2.5% starving medium and were subsequently stimulated with 100 ng/ml hEGF for indicated times.

2.4.2 RNA preparation

RNA extraction from stimulated Hm cells and human cell lines was done using Total RNA Isolation Reagent (ABGene) as recommended by the manufacturer.

2.4.3 Preparation of cDNA

cDNA was prepared from total RNA using the RevertAidTM First Strand cDNA Synthesis Kit with random hexamer primers (Fermentas). The synthesis was performed according to the manufacturer’s manual.

2.4.4 Real-time PCR

Real-time-PCR was carried out using the Eppendorf Mastercycler® ep realplex thermal cycler. Values for each gene were normalized to expression levels of β-actin. Relative expression levels were calculated applying REST software {Pfaffl, 2002 #33}. The data represent the results from at least two different biological replicates that were each analyzed at least by three independent real-time-PCR`s.

Table 2: Primer sequences

Gene name	Gene symbol	RefSeqID	Primer sequences	
ANG	ANG	NM_001145.4	Forward	5'AAGGACGCCAACCCCACCTAGA3'
			Reverse	5'ACAACAAAACGCCCAGGCCCAT3'
TIMP1	TIMP1	NM_003254.2	Forward	5'GCAGATCCAGCGCCCAGAGAG3'
			Reverse	5'GGTTGACTTCTGGTGTCCCCACG3'
TIMP2	TIMP2	NM_003255.4	Forward	5'GGCAGTGTGTGGGGTCTCGC3'
			Reverse	5'TGGGGCAGCGCGTGATCTTG3'
Ang	ANG	NM_007447.3	Forward	5'GGAACGCCCACCCTCCACTC3'
			Reverse	5'CCAACAGAGATTCCTGGACCCGG3'

PCR primers were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 3: 1x PCR mix for 5µl template of 5 ng/µl cDNA

Components	Volume [µl]
ddH_2O	14,00
10x reaction buffer	2,50
10 mM dNTPs	0,70
SYBR-Green 1:2000	1,00
Taq-Polymerase	0,30
5´Primer 10 pmol/µl	0,75
3´Primer 10 pmol/µl	0,75

template	5,00
sum	25,00

Table 4: Real time PCR program

Temperature [°C]	Time [min]	Repetitions
95	3:00	
95	0:15	40x
60	0:10	
60	0:20	
95	0:15	

Table 5: 10x Reaction buffer

Components	Amount
$(NH_4)_2SO_4$	100 mM
Tris-HCl pH 8.8 (at 25°C)	200 mM
KCl	100 mM
$MgSO_4$	20 mM
Triton X100	1%
BSA (nuclease free)	1%

2.5 Protein biochemical methods

2.5.1 Cell lysis and Western blotting

Cells were harvested and were then rinsed twice with PBS and lysed in lysis buffer containing 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (pH 7.8), 500mM NaCl, 5mM MgCl, 2,5mM KCl, 0.1% deoxycholate, 0.5% Nonidet-P40, 10mg/ml aprotinin, 10mg/ml leupeptin, 200mM Na_3VO_4, 1mM phenylmethanesulphonyl- fluoride and 100mM NaF for at least two hours on ice.
Shortly, 50 µg of each protein lysate was separated by SDS–polyacrylamide gel electrophoresis and analysed by immunoblotting according to standard protocols [187].

2.5.2 Antibodies

Polyclonal anti-mrk recognizing the C- terminal part of Xmrk ('pep-mrk') was generated by Biogenes (Berlin, Germany). Anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).
Anti-β-actin (C-4) and Anti-ANG-I (C-1) antibodies were purchased from Santa Cruz Biotechnology. Anti-P-NF-κB-p65 (Ser536) (93H1) antibody was purchased from Cell Signaling Technology (New England Biolabs). Secondary antibodies were conjugated to horseradish peroxidase and were directed against mouse (Pierce, Rockford, IL) and rabbit (Bio-Rad). Images were acquired with KODAK image station.

2.6 Pathway analysis

For the identification of pathways which regulate the expression of candidate genes *in vitro*, the following small molecule inhibitors in the indicated concentration were added to the cell culture media: AG1478 (inhibitor of EGFR and Xmrk), Tiron (*4,5*-dihydroxy-*1,3*-benzenedisulfonic acid disodium salt monohydrate; scavenger of reactive oxygen species), *N*-acetyl-*L*-cysteine, NF-κB activation inhibitor and Vitamin E (α-tocopherol), respectively. Cells without small molecule inhibitor treatment received the equivalent amount of solvent.

2.7 Immunofluorescence

Hm cells ($2x10^5$) were seeded on glass cover slips, starved for 3 days in DMEM with 2.5% dialyzed FCS (starving medium). Cells were then stimulated with 100ng/ml EGF for 24 h or remained unstimulated. Immunofluorescence was performed as described before by Meierjohann et al. [45]. The cells were fixed for 5 minutes in methanol (-20°C) and permeabilized for 2 minutes in acetone (-20°C). Subsequently the samples were blocked for 20 minutes with PBS/1% BSA and incubated with anti-

NF-κB p65 (E498) antibody (1:100; Cell Signalling) for 1 hour. After three washing steps with PBS, the coverslips were incubated with the second antibody Alexa Fluor 488 goat anti-rabbit IgG (1:1000; Invitrogen) for 1 hour in the dark. After at least three washing steps with PBS nuclear staining with 1 µg/ml of Hoechst 34580 (Invitrogen) was performed for 1 h in the dark. After at least three washing steps with PBS and H_2O, the cover slips were embedded with Mowiol® 4-88 (Roth) on object slides. The object slides were then kept in the dark at room temperature until microscopy was performed.

2.8 Angiogenesis array

Mouse and human angiogenesis arrays were purchased from RayBio®. All cell lines were seeded at a density of $1x10^6$ cells per 10 cm dish and were incubated in a total volume of 7 ml DMEM media with or without 10 µM of NF-κB activation inhibitor (Invitrogen). After 24 h, 2 ml of non-concentrated supernatant of each conditioned medium was used for the assay. Assays were done as recommended by the manufacturer`s protocol. Images of the provided probed membranes were acquired with KODAK image station. Statistical analysis of the raw data was performed with Microsoft Excel and Origin 8 software.

2.9 Microbacterial culture and Plasmid preparation

2.9.1 Bacterial culture media

Table 6: Luria-Bertani-medium (LB) (pH 7,5)

Components	weight [g]
trypton	10
yeast extract	5
sodium chloride	10
ddH_2O	ad 1l

Medium was sterilized by autoclaving.

Table 7: LB- Agar-medium (pH 7,5)

components	weight [g]
trypton	10
yeast extract	5
sodium chloride	10
agar	15
ddH_2O	ad 1l

Medium was sterilized by autoclaving and then distributed into petri dishes being still in hot condition.

2.9.2 Bacterial storage

For long term storage of bacteria suspension at –80°C, 700 µl of a freshly prepared bacteria suspension was mixed with 300 µl sterile glycerol in a vial and subsequently frozen in liquid nitrogen until being stored at -80°C.

2.9.3 Plasmid preparation

Plasmid preparation for transfection or injection was performed with PureYield™ Plasmid Mini/Midi/Maxiprep System form Promega (Mannheim, Germany) according to the manufacturer`s protocol.

3 RESULTS

3.1 Generation of a zebrafish Xmrk-melanoma model

The oncogene *xmrk* was used to generate a tool which allows in vivo investigations and observations of melanoma development in zebrafish like it was described previously for medaka. For zebrafish, a lot of mutant fish lines are available, therefore permitting investigations of different aspects of melanoma development such as metastasis, angiogenesis or senescence.

To generate the zebrafish Xmrk melanoma model a vector construct, which was kindly provided by Reinhard Köster (Prof. Dr. Reinhard Köster, Zoological Institute, TU Braunschweig, Braunschweig, Germany) was used (figure 9). Here, a Gal 4 PV16 element is driven by the pigment cell specific promotor *mitfa*. The translated Gal 4 protein can bind to a 14 fold upstream activating sequence (UAS) which results in the coexpression of two other genes with the help of an *E1b* promotor. The *E1b* promotor is only active when Gal 4 is bound to the UAS. The genes which are regulated upon Gal 4 binding are the oncogene *xmrk* and, in order to follow oncogene expression, GFP.

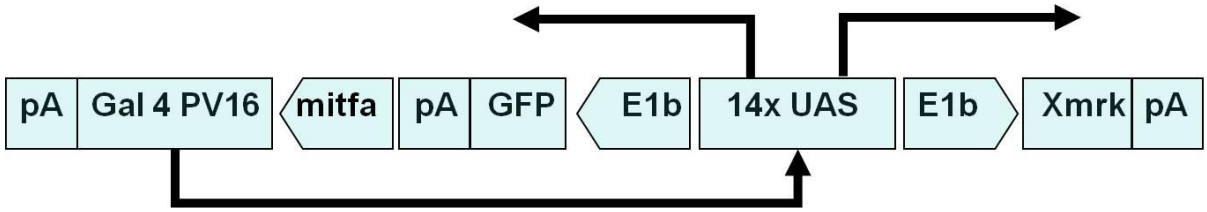


Figure 9: Zebrafish injection construct.
A Gal 4 PV16 element is driven by the pigment cell specific promoter mitfa. Gal 4 binds then to a series of upstream activator sequences (UAS) which allow bidirectional transcription of GFP and *xmrk* by the *E1b* promotor.

For the embryonic injections the injection plasmid was purified with a purification kit to get rid of bacterial toxins which could harm the development of the embryo. A concentration of 100nM plasmid was used for injection. 24h post injection GFP-positive pigment cells were observed in some embryos (figure 10).

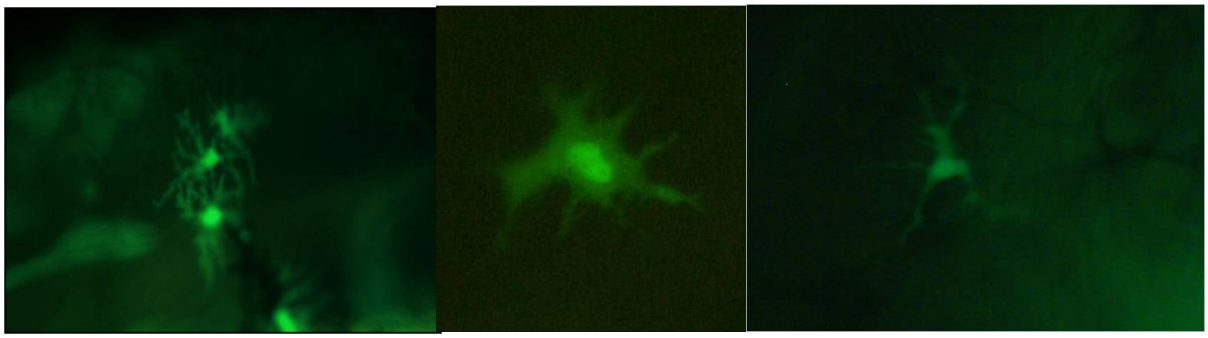


Figure 10: Green fluorescent pigment cells at different sites of the body of injected embryos.

Interestingly, eumelanin containing melanocytes showed no GFP-fluorescence (figure 11), probably due to quenching of the GFP-signals by eumelanin. Since mature pigment cells are of neural crest origin and nacre/mitfa mutants have fewer xanthophores and somewhat more iridophores, suggesting an overlap in genetic dependencies between melanophores and xanthophores and perhaps tradeoffs in allocation between these lineages and iridophores [188], it is assumed that GFP-fluorescence positive cells are either precursor pigment cell (chromoblasts, chromatophores) or precursor of melanocytes (melanoblasts, melanophores).

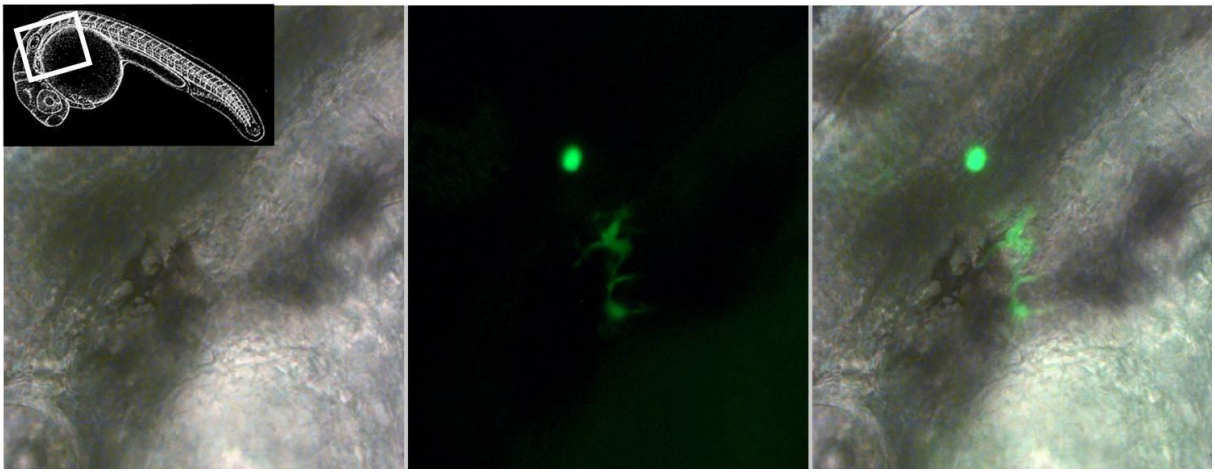


Figure 11: Eumelanin containing pigment cells and adjacent GFP-positive non-melanin containing pigment cells.
Left: bright field image including scheme (inset) to identify the position of the imaged cells. Middle: corresponding GFP fluorescence, Right: merge of bright field and GFP. Green fluorescence dot is unspecific fluorescence e.g. dead cell.

Surprisingly, some embryos showed abnormal features in early development. Several animals injected with the above mentioned construct developed an outgrowth of cells, most often at the dorsal side of the trunk of the animals

approximately 24 hpi (figure 12). Furthermore these cells were GFP-positive, suggesting the presence of the oncogene *xmrk*. Interestingly some of the injected animals displayed an even worse phenotype exhibiting a neoplasm at the dorsal trunk side at 24 – 48 hpi (figure 13). Here again GFP-positive cells can be clearly identified in the area of the malformation.

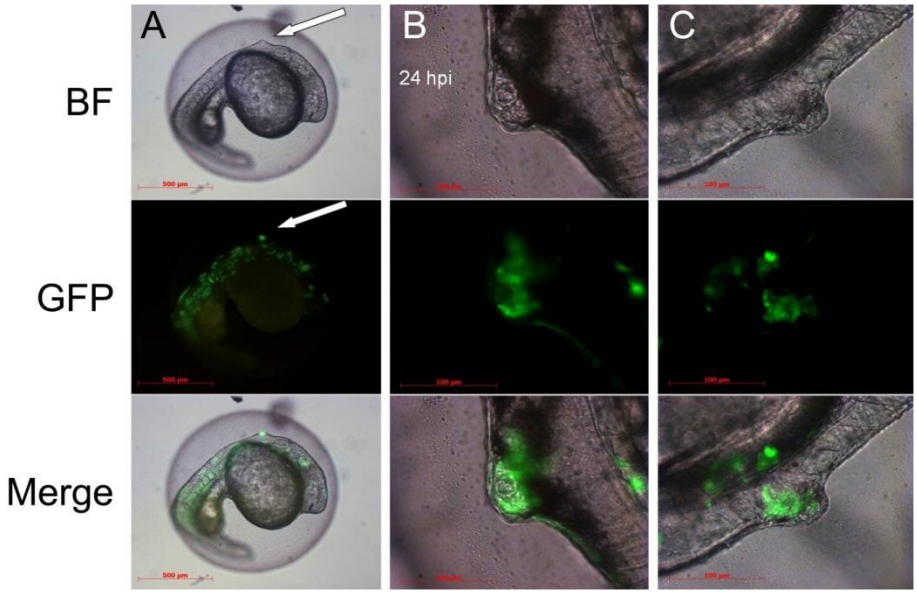


Figure 12: Representative images of a zebrafish embryo 24 hours post injection.
(A) A binocular image series of a whole zebrafish embryo 24 hours post injection (hpi) exhibiting cellular outgrowth at the dorsal body side indicated by a white arrow. The bars represent 500 µm. (B) and (C): magnification image series of lateral left (B) and lateral right (C) side of the cellular outgrowth structure of the embryo represented in column A. The bars represent 100 µm. Images are displayed from top row to bottom row in bright field (BF), corresponding GFP fluorescence (GFP) as well as a merge of BF/GFP.

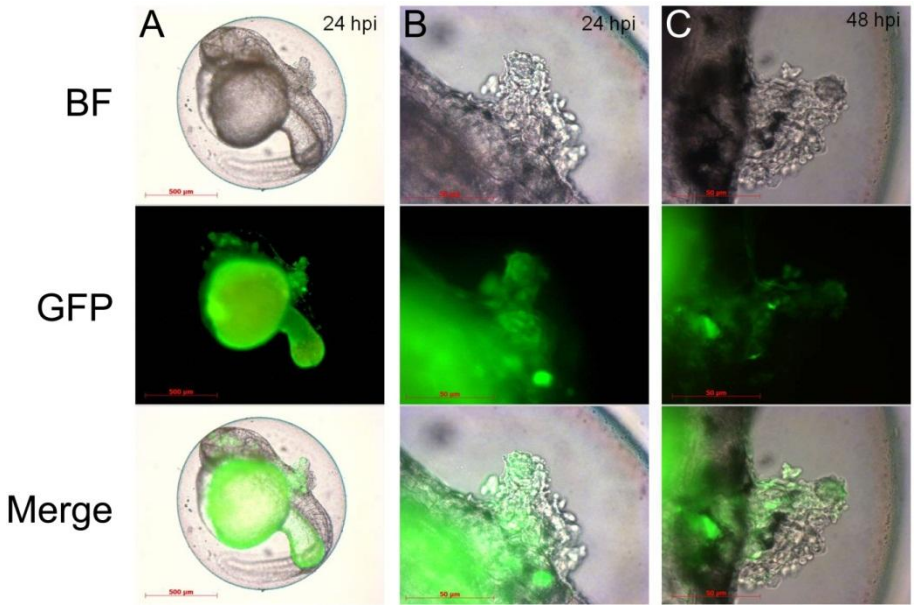


Figure 13: Representative images of a zebrafish embryo 24 and 48 hours post injection. (A) A binocular image series of a whole zebrafish embryo exhibiting a neoplasm at the dorsal trunk side at 24hpi. The bars represent 500 µm. (B) series of magnified images of the neoplasm of the embryo represented in column A at 24 hpi. The bars represent 100 µm. (C) series of magnified images of the neoplasm of the embryo represented in column A at 48 hpi. The bars represent 100 µm. Images are displayed from top row to bottom row in bright field (BF), corresponding GFP fluorescence (GFP) as well as a merge of BF/GFP.

The injected embryos (approx. 1000) were separated in GFP-positive and GFP-negative individuals after 3 days post injection. Surprisingly all injected animals of the GFP-positive cohort died approximately within one to four weeks after injection. In contrast, GFP-negative animals survived to adulthood and were confirmed to be *xmrk*-negative by PCR.

3.2 Medaka Melanoma Angiogenesis Model

3.2.1 *fli::egfp* medaka

The *fli::egfp* medaka, generated and kindly provided by Joachim Wittbrodt's Lab. (Prof. Dr. Joachim Wittbrodt, Centre for Organismal Studies, University of Heidelberg, Heidelberg, Germany), expresses *egfp* under the control of the hemiangioblast specific promotor *fli* [189]. To elucidate if the *fli::egfp* transgenic medaka is suited for imaging of blood vessels and for small molecule administration, a regeneration

experiment involving the caudal fin was performed. The fishes exhibit an outstanding regeneration capacity after wounding, tissue damage or fin amputation. Caudal fins of *fli::egfp* transgenic medaka were amputated, treated with a specific angiogenesis inhibitor and regeneration as well as the vascular pattern of the fin was observed over a period of 9 days. The VEGFR2-inhibitor ZM322881 was used to inhibit wound healing associated angiogenesis in the fin. This inhibitor was previously tested in zebrafish to investigate hypoxia mediated angiogenic effects in the fish retina [190]. Adult *fli::egfp* transgenic medaka fishes were separated into two groups with 10 animals each. Approximately two thirds of the caudal fin of each fish was amputated. Pictures were taken before and after amputation. One group was then treated with ZM322881 inhibitor, while the other group received a comparable volume of solvent. After nine days of continuous treatment the fins of the control group had reached almost the original size before amputation. However a growth delay was observed for the ZM322881 inhibitor treated group (figure 21, Δd).

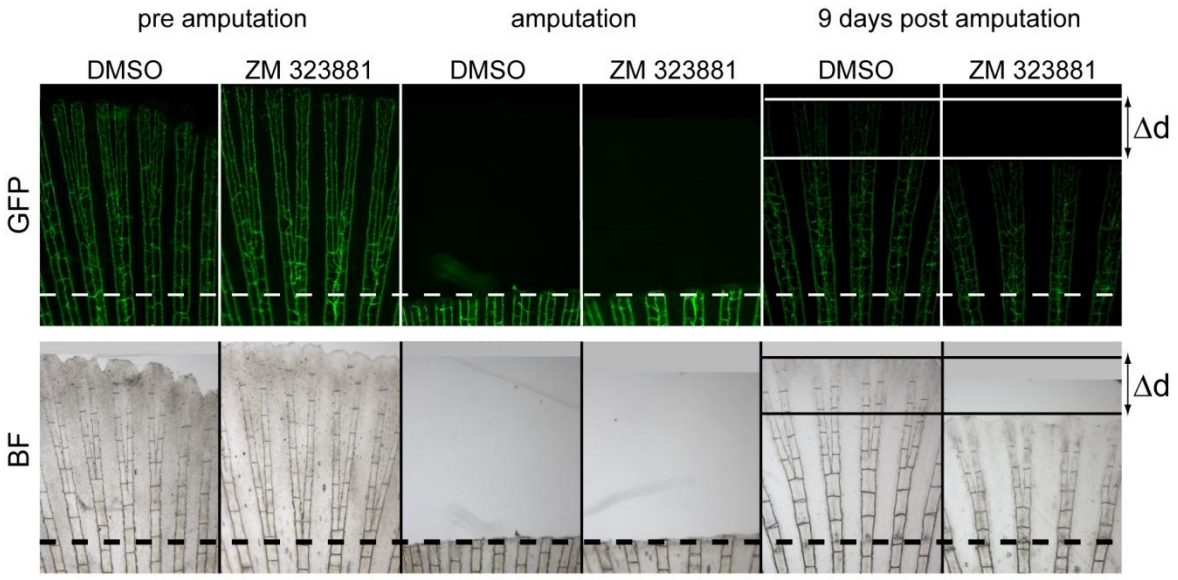


Figure 14: Representative images of the regeneration of the caudal fin of fli::egfp transgenic medakas treated with either an angiogenesis inhibitor or DMSO.

To quantify changes in the regenerative ability of ZM 323881-treated and control-treated *fli::egfp* transgenic medakas, the length of each fin was measured at amputation day and after 9 days. The result is displayed in figure 14. The fin regrowth of the control group was set to 100 %. As shown in figure 15 the regenerative capacity in presence of VEGFR2 inhibitor was about one third less

compared to the controls. This suggests that VEGFR2-inhibition leads to a delay in regeneration and that the medaka caudal fin is suitable for the administration of small molecular inhibitors.

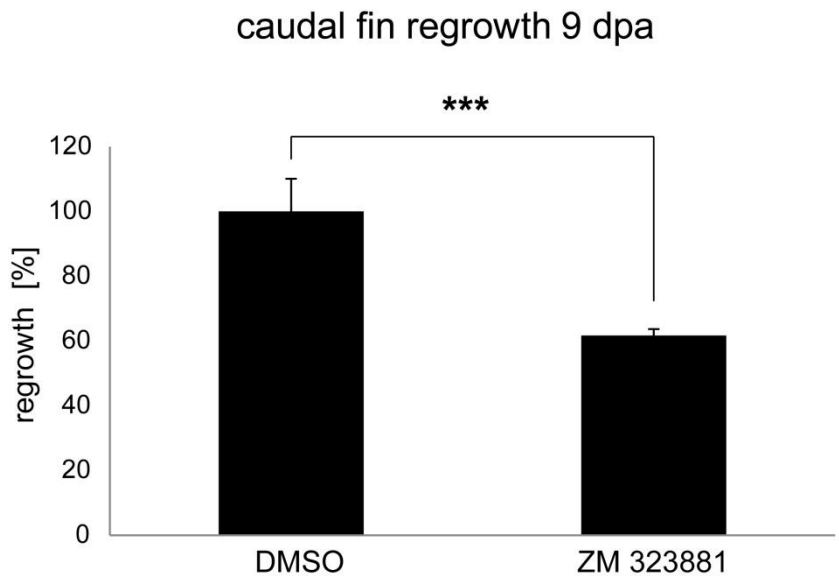


Figure 15: Regrowth in percentage of the caudal fin of a *fli::egfp* transgenic medakas treated either with VEGFR2-inhibitor ZM 323881 or an equivalent volume of solvent.
Statistical analysis was carried out with the Mann-Whiney U test (n=9 each).

3.2.2 Development of the *fli::egfp;mitf::xmrk* transgenic medaka as angiogenesis model

One of the hallmarks of cancer is the induction of angiogenesis. A main issue of this thesis was to find out if Xmrk is capable to induce angiogenesis in vivo. To elucidate this question, the medaka *mitf::xmrk* melanoma fish model was chosen. It expresses the oncogene *xmrk* under control of the pigment cell specific promotor *mitf* and was crossed with *fli::egfp* transgenic medaka.

The result of this crossing was a melanoma-prone medaka fish line with GFP-positive blood vessels. For the analysis of angiogenesis, the caudal fins were selected, as they display a well-structured vascular pattern (Figure 16, box magnification). Furthermore, the transparency of these fins is well-suited for imaging. The scheme (box magnification) of figure 16 shows a common vascular pattern of a caudal fin of a

medaka fish. The fin rays are formed by consecutive opposing hemispheres which create a tube like structure. Here blood, vessels are located in the center, pumping blood from the anterior to the posterior end of the fin rays. Once reaching the tip of the fin ray, the centered blood vessel subdivides into two new vessels, which then transport the blood from the fin ray tip back to the body of the fish, in a posterior to anterior manner. The subdivided two new blood vessels are located adjacent to the hemispheres. In addition, blood vessel sprouts and anastomoses, which are derived from the subdivided blood vessels, appear occasionally in the area between two fin rays (intra-fin ray area) (cf. figure 16 box magnification) in fli::egfp fishes

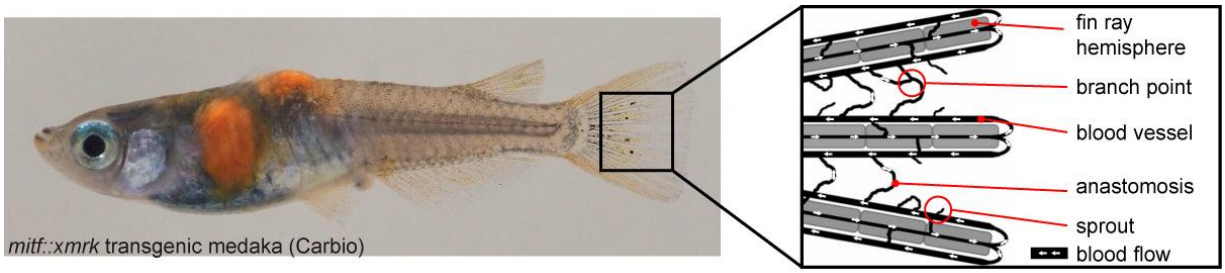


Figure 16: Phenotype of an adult mitf::xmrk transgenic medaka.
Lateral view of the transgenic mitf::xmrk medaka and scheme (box magnification) of the caudal fin vascular pattern.

Similar to *mitf::xmrk* fishes, the melanoma-bearing *fli::egfp;mitf::xmrk* animals display transformed pigment cells, visible as black (melanophore) and yellow or red-orange (xantho- or erythrophore) spots from which tumors can develop. These cells are not present in *fli::egfp* fishes (figure 17 and 18, images A and C). Interestingly, blood vessel density and enhanced angiogenic sprouting were strongly increased in *fli::egfp;mitf::xmrk* fishes pattern compared to *fli::egfp* animals (figure 17 and figure 18, images B and D).

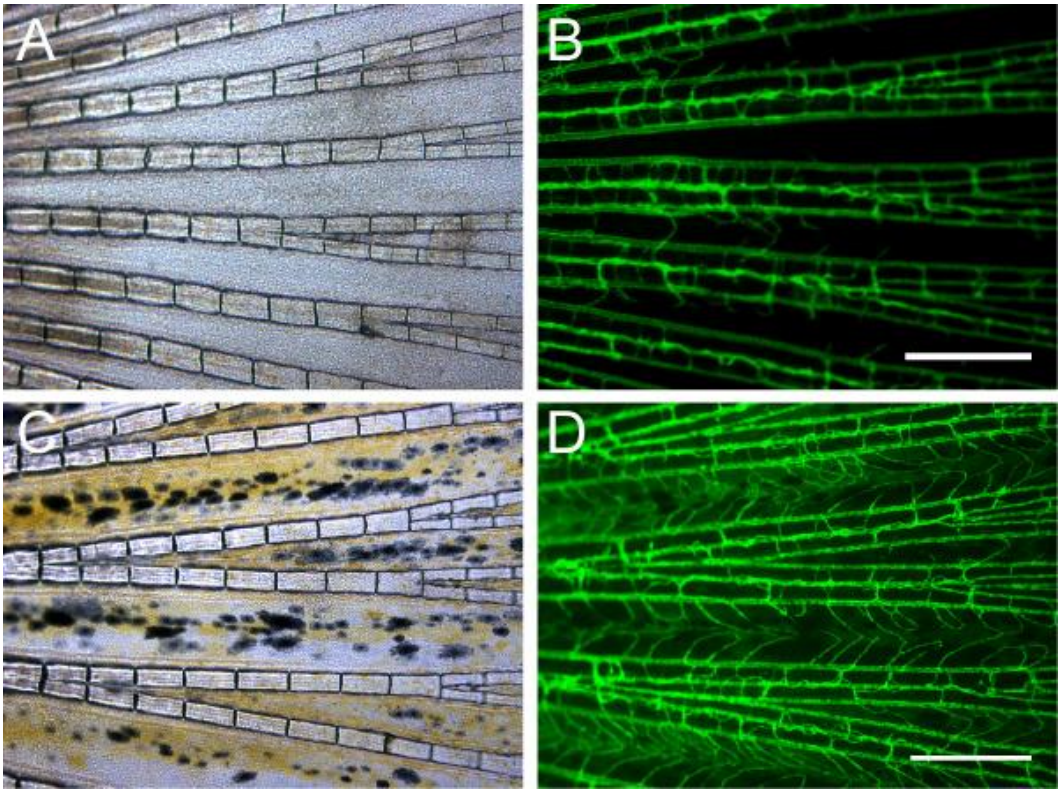


Figure 17: Comparison of patterns of pigment cells and blood vessel architecture between transgenic *fli::egfp*- and *fli::egfp;mitf::xmrk* medaka caudal fins at 50-fold magnification.
(A-B) Vessel architecture in caudal fins of *fli::egfp* transgenic medaka. (A) Bright field image and (B) corresponding GFP fluorescence image. (C-D) Vessel architecture in caudal fins of *fli::egfp;mitf::xmrk* medaka. (C) Bright field image and (D) corresponding GFP fluorescence image. All images were taken at 50-fold magnification. Bars represent 500 µm.

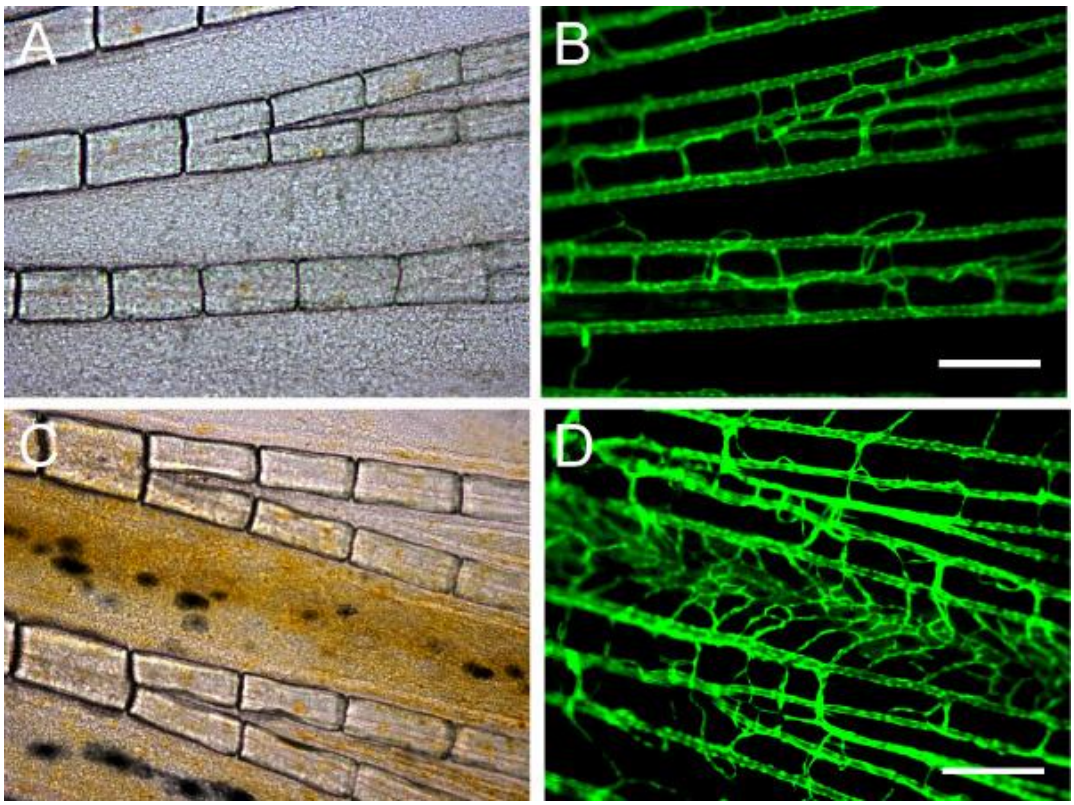


Figure 18: Comparison of patterns of pigment cells and blood vessel architecture between transgenic *fli::egfp*- and *fli::egfp;mitf::xmrk* medaka caudal fins at 100-fold magnification.
(A-B) Vessel architecture in caudal fins of *fli::egfp* transgenic medaka. (A) Bright field image and (B) corresponding GFP fluorescence image. (C-D) Vessel architecture in caudal fins of *fli::egfp;mitf::xmrk* medaka. (C) Bright field image and (D) corresponding GFP fluorescence image. All images were taken at 100-fold magnification. Bars represent 200 µm.

However, unfortunately the pigmented cells interfere with GFP-fluorescence. In some cases GFP-fluorescence was quenched (mainly in melanophores) or weakened (primarily in xantho- or erythrophores) (figure 19).

In order to minimize the quenching effect, only those fishes which displayed a moderate pigmentation pattern in the caudal fin were selected for analysis. As a consequence, experimental fishes were selected for age as well as pigment pattern.

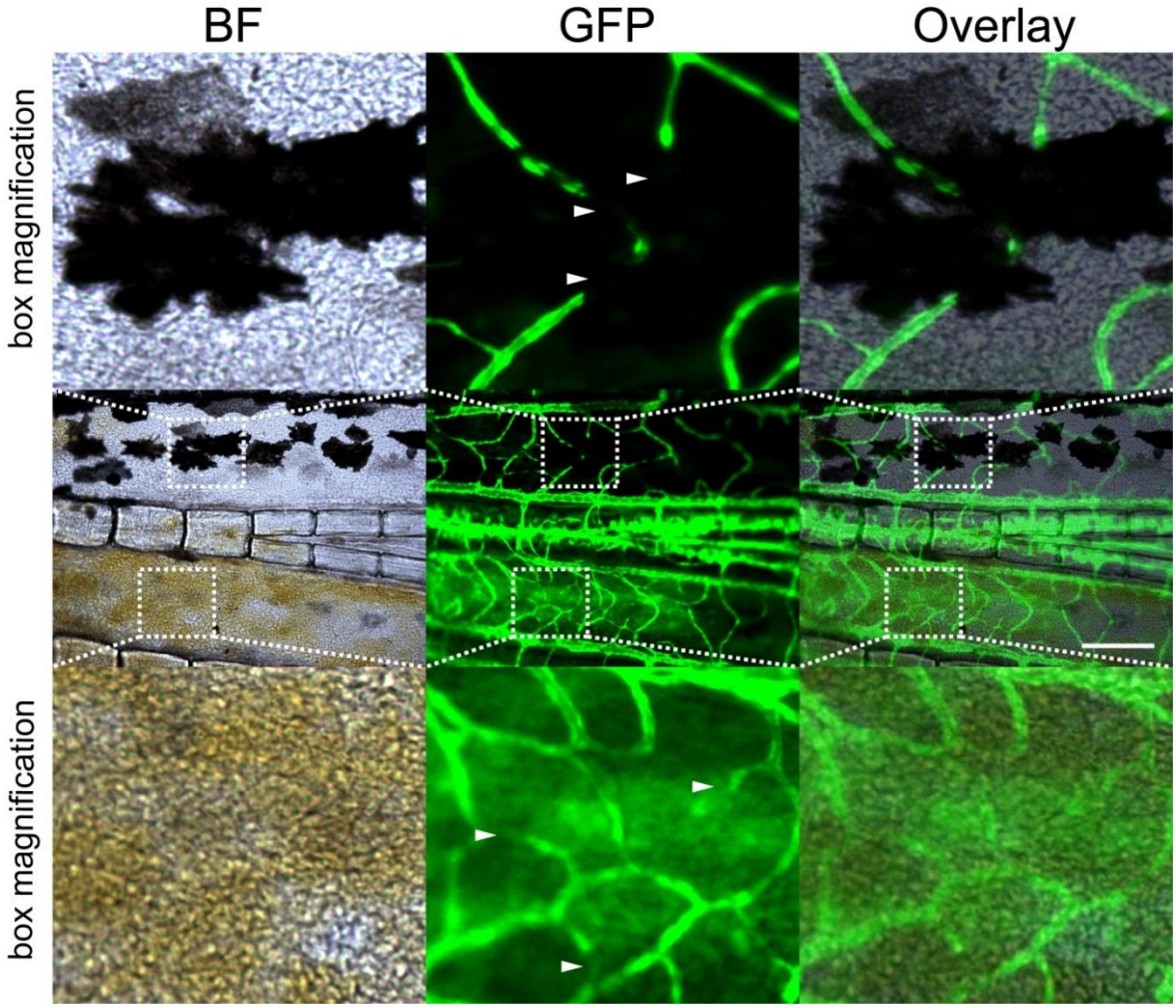


Figure 19: Interplay of pigments from melanophores and xantho- and erythrophores with GFP fluorescence signals of the blood vessels in the caudal fin of an adult *fli::egfp;mitf::xmrk* medaka.
The three columns represent from left to right: BF (Bright field), GFP and an overlay. The middle image panel represents the original image with an upper area containing melanophores and a lower area containing xantho- and erythrophores. The upper image panel is a box magnification of the upper area of the original images. Here GFP fluorescence is quenched by pigments of the melanophores indicated by white arrowheads. The lower image panel is a box magnification of the lower area of the original images. Here GFP fluorescence is interfered by pigments of the xantho- and erythrophores marked by the white arrowheads. Scale bar represents 200 µm.

To quantify the changes in vascular patterning among adult *fli::egfp;mitf::xmrk* and *fli::egfp* transgenic medaka fishes, the average number of sprouts and branch points appearing in intra-fin ray areas were examined and statistically analyzed using the Mann-Whitney U test (figures 20 and 21). Accordingly to figure 20 it is recognizable that the average number of sprouts in intra fin ray areas of *fli::egfp;mitf::xmrk* transgenic animals is strongly and significantly increased compared to control *fli::egfp*

transgenic animals. The same is true for the average number of branch points shown in figure 21.

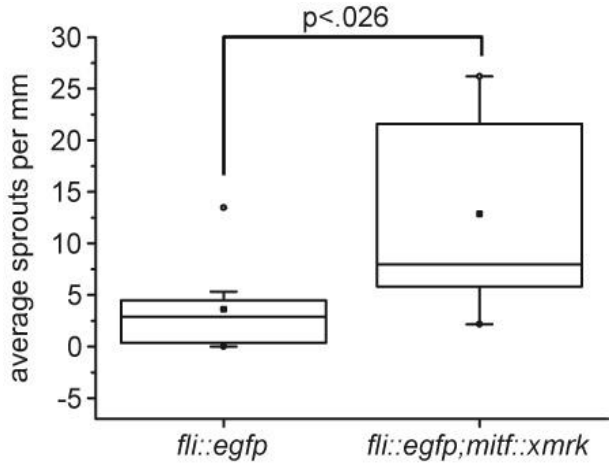


Figure 20: Average sprouts per mm.
Average number of inter-fin ray sprouts per mm caudal fin in *fli::egfp* and *fli::egfp;mitf::xmrk* transgenic medakas (n= 8 each). Statistical analysis was carried out with the Mann–Whitney U test.

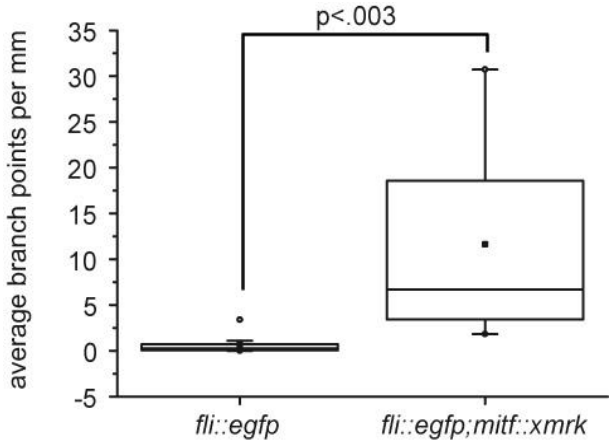


Figure 21: Average branch points per mm.
Average number of inter-fin ray vessel branch points per mm caudal fin in *fli::egfp* and *fli::egfp;mitf::xmrk* transgenic medakas (n=8 each). Statistical analysis was carried out with the Mann–Whitney U test.

Interestingly, in some medaka fishes transgenic for *fli::egfp;mitf::xmrk* a direct correlation between the number of transformed cells and the extent of vascular density was observed. Figure 22 displays an intra-fin ray area of a medaka transgenic for *fli::egfp;mitf::xmrk*, in which the area can be subdivided in two parts where one part contains many (indicated by an asterisk) and the other part contains

few pigmented *xmrk*-transgenic cells. The white dashed line in figure 22 indicates the border of the two areas. The area with the large amount of pigmented cells shows a higher vascular density compared to the other area.

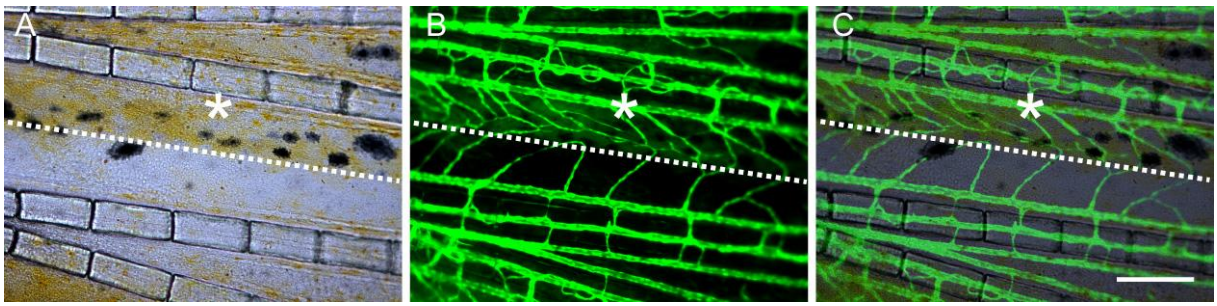


Figure 22: Caudal fin pigmentation and vascular pattern in *fli::egfp;mitf::xmrk* transgenic medaka.
(A) Bright field image. (B) Corresponding GFP fluorescence image. (C) Overlay. Bars represent 200 µm. White dashed line separates strongly pigmented area of inter fin ray tissue (asterisk), containing many *xmrk*-transgenic cells, from the less pigmented area, containing few *xmrk*-transgenic cells. Sprout density is much higher in areas containing a larger number of *xmrk*-transgenic pigments cells.

Furthermore, it was observed that even single pigmented *xmrk*-transgenic cells (figure 23, asterisks) are able to recruit endothelial sprouts or vessels.

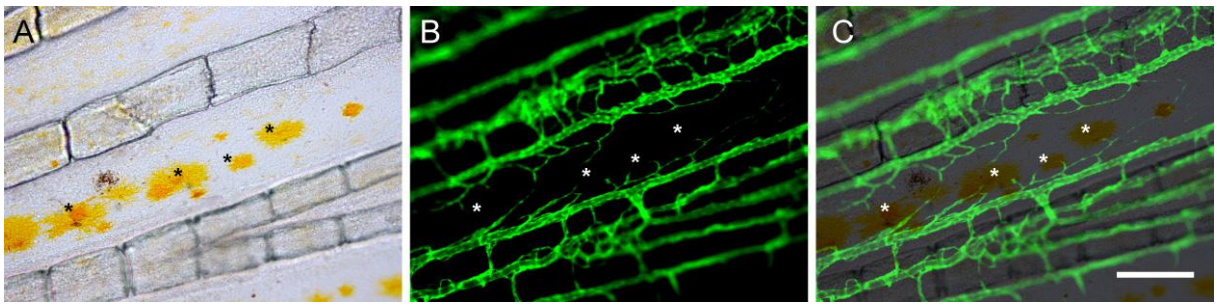


Figure 23: Sprout recruiting to transgenic pigment cells.
Representative image of an area containing few *xmrk*-transgenic cells, showing that sprouts are recruited to single transgenic pigment cells (asterisks). (A) Bright field image, (B) Corresponding GFP fluorescence image. (C) Overlay. Bars represent 200 µm.

To exclude that this angiogenic phenomenon was driven by hypoxia due to oxygen diffusion limitation of the tissue, a determination of the lateral thickness of the caudal fin was conducted. A caudal fin confocal microscopy of a representative *fli::egfp; mitf::xmrk* medaka was performed. Subsequently the received image stacks were calculated into 3D reconstruction. According to the figure 24, the lateral thickness of

the caudal fin across the fin rays (the thickest part of the fin), was determined to be approximately 100 µm. The intra fin ray areas, containing the pigment cells, are even less thick (figure 31A, B). As the oxygen tissue diffusion limit is approximately 1 mm [191][192] and hypoxia does not occur below this limit, it is concluded that lack of oxygen supply in the fin cannot be the driving force of the observed increased angiogenesis.

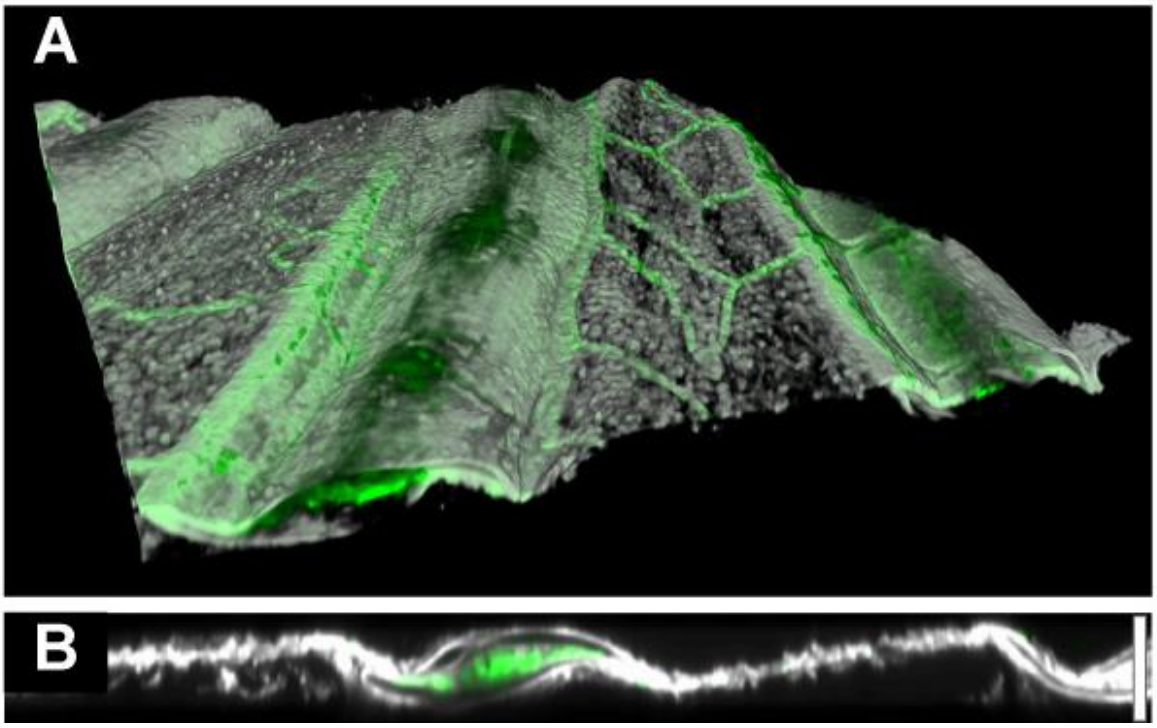


Figure 24: Confocal image of the caudal fin of a representative *fli::egfp;mitf::xmrk* medaka. The GFP-positive blood vessels are shown in green, nuclei are depicted in gray (TO-PRO-3 staining). (A) 3D reconstruction of the central part of the caudal fin. (B) 2D xz plane image, scale bar represents 100 µm.

3.3 NF-κB is activated by Xmrk

To get insight into the mechanism of Xmrk-dependent angiogenesis induction, I aimed at identifying angiogenic factors induced by Xmrk. For this purpose, I took advantage of various well-established and characterized *in vitro* systems.

In the HERmrk cell culture system, the extracellular part of human EGFR (HER) is fused to the intracellular part of Xmrk (mrk), giving rise to the chimeric protein HERmrk. HERmrk (or shortly “Hm”) was introduced into murine melanocytes (melan-a cells), which do not contain endogenous EGFR. In comparison to Xmrk, HERmrk is not constitutively active, but can be stimulated using human EGF [193]. The supernatants of unstimulated as well as EGF-treated HERmrk cells were examined in an ELISA-based angiogenesis array overview displayed in figure 25.

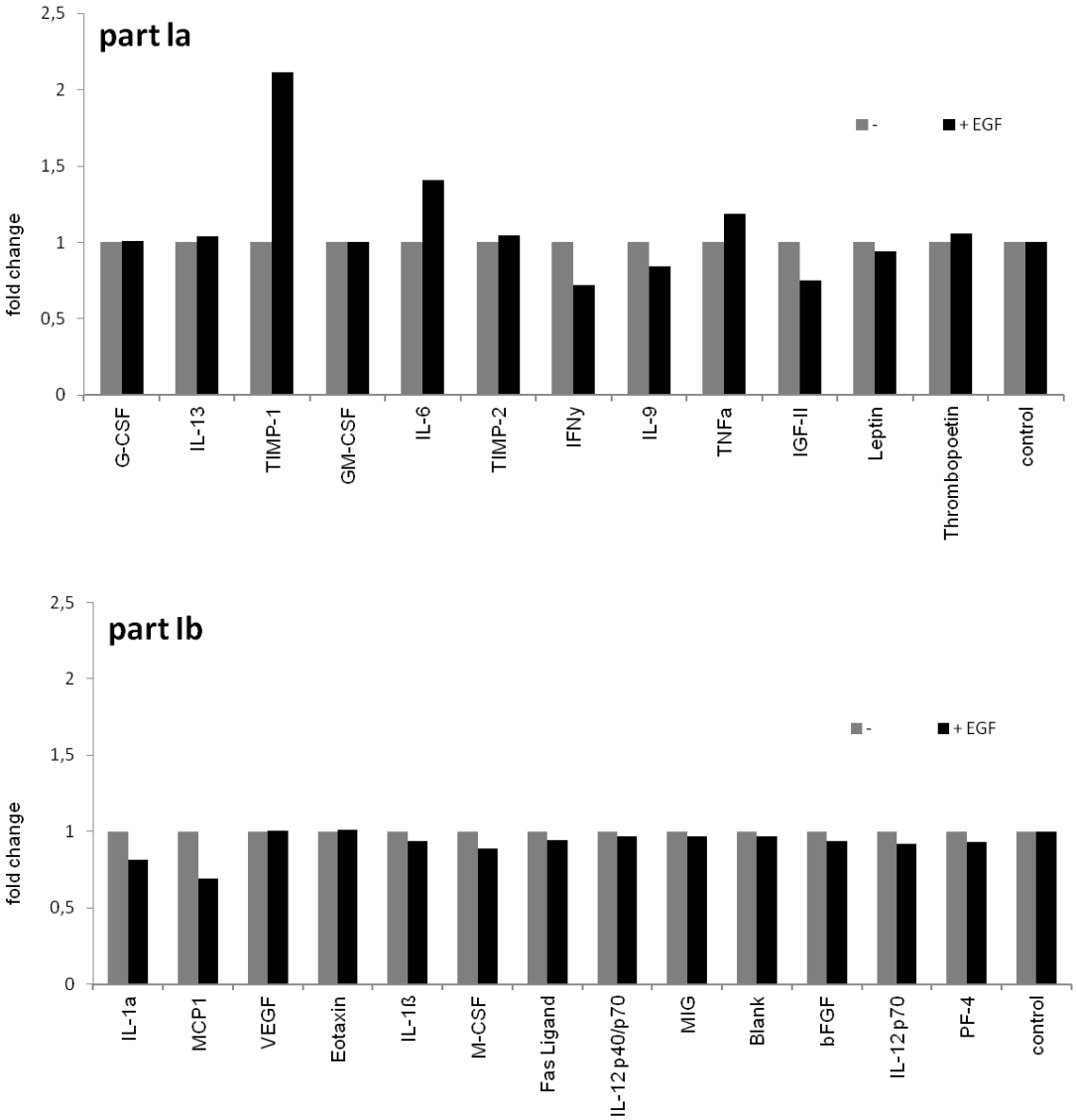


Figure 25: Mouse angiogenesis array (overview).
ELISA-based mouse angiogenesis array of the supernatant from unstimulated (grey bars) and EGF-stimulated (black bars) Hm transgenic melan-a cells.

No differences in expression of well-established angiogenesis inducers like bFGF and VEGF were observed. However, TNF-α and IL-6 were slightly induced and TIMP-1 was considerably induced after HERmrk stimulation (figure 26). All three factors are able to induce pro-angiogenic effects [194], [195]. Interestingly, they are known targets of NF-κB [196], [197].

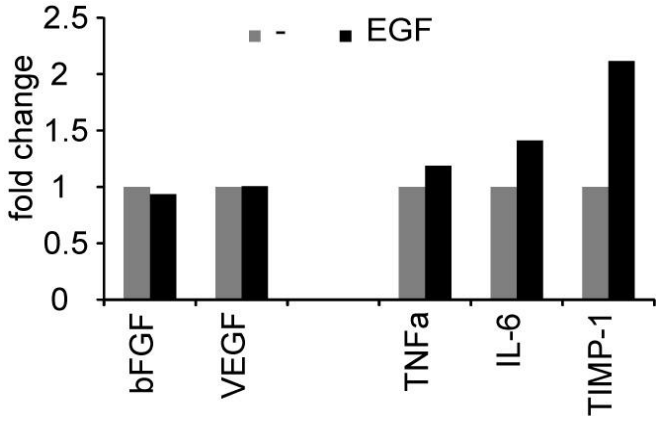


Figure 26: Mouse angiogenesis array (selected detail of figure 32).
ELISA-based mouse angiogenesis array of the supernatant from unstimulated (grey bars) and EGF-stimulated (black bars) Hm transgenic melan-a cells. In addition to the well-established angiogenesis inducers bFGF and VEGF, only those proteins that showed an induction after stimulation are displayed.

To elucidate if NF-κB is activated upon stimulation of HERmrk, the protein levels of the phosphorylated NF-κB subunit p65 (Ser536) in Hm cells at different time points upon EGF stimulation was analyzed. Phosphorylation of serine 536 in the transactivation domain 1 of p65 directly indicates DNA binding and transcriptional activity of the NF-κB complex [198]. After 3 and 4h of EGF stimulation a clear increase of phospho-NF-κB (Ser536) levels was detectable, as shown in figure 27.

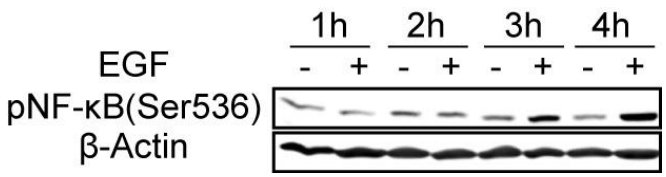


Figure 27: p65 P-NF-κB induction upon EGF-stimulation (Western blot).
Western blot of p65 P-NF-κB (Ser536) levels at indicated timepoints of Hm stimulation. β-Actin served as loading control.

Furthermore, an immunofluorescence performed with a NF-κB p65 antibody in Hm cells which were stimulated with EGF for 24 h or remained unstimulated showed a translocation of total p65 upon EGF stimulation (figure 28)

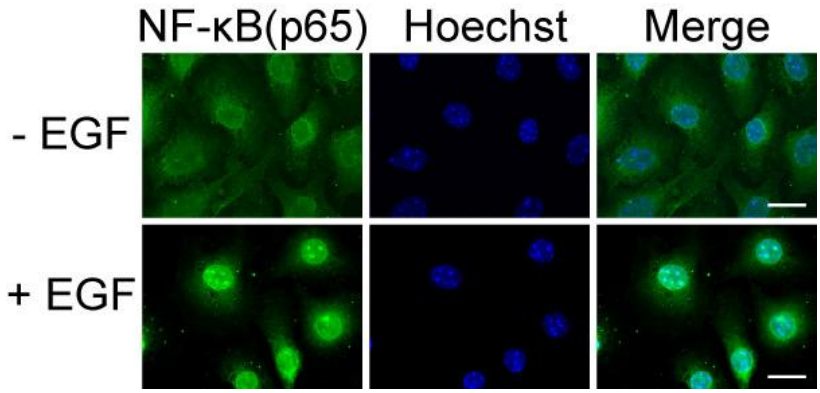


Figure 28: p65 NF-κB induction upon EGF-stimulation.
Immunofluorescence images illustrating nuclear translocation of NF-κB (p65) after Hm stimulation (shown in green). Second antibody is Alexa Fluor 488 goat anti-rabbit IgG. The nucleus is displayed in blue and nuclear staining was performed with Hoechst 34580.

As it was previously shown by Leikam et. al. [46] HERmrk can induce the generation of reactive oxygen species (ROS). ROS are known as potent activators of NF-κB [199].

To test the induction of NF-κB and angiogenic factors in a second, independent cell system, human HEK293 cells stably expressing the native oncogenic receptor Xmrk (HEK293-*xmrk* cells) were used. The human-specific angiogenesis array did not entirely overlap with the above used murine array, but instead it was able to detect additional factors such as VEGF-D, PDGF-BB and angiogenin.

Similarly to the murine angiogenesis assay results, no differences between HEK293 control cells and HEK293-*xmrk* cells were visible with respect to the well-established angiogenesis inducers bFGF, VEGF, VEGF-D, or PDGF-BB (figure 29, 30). In contrast to the Hm cell line (figure 25) an induction of TNF-α or IL-6 was not detectable in HEK293 cells.

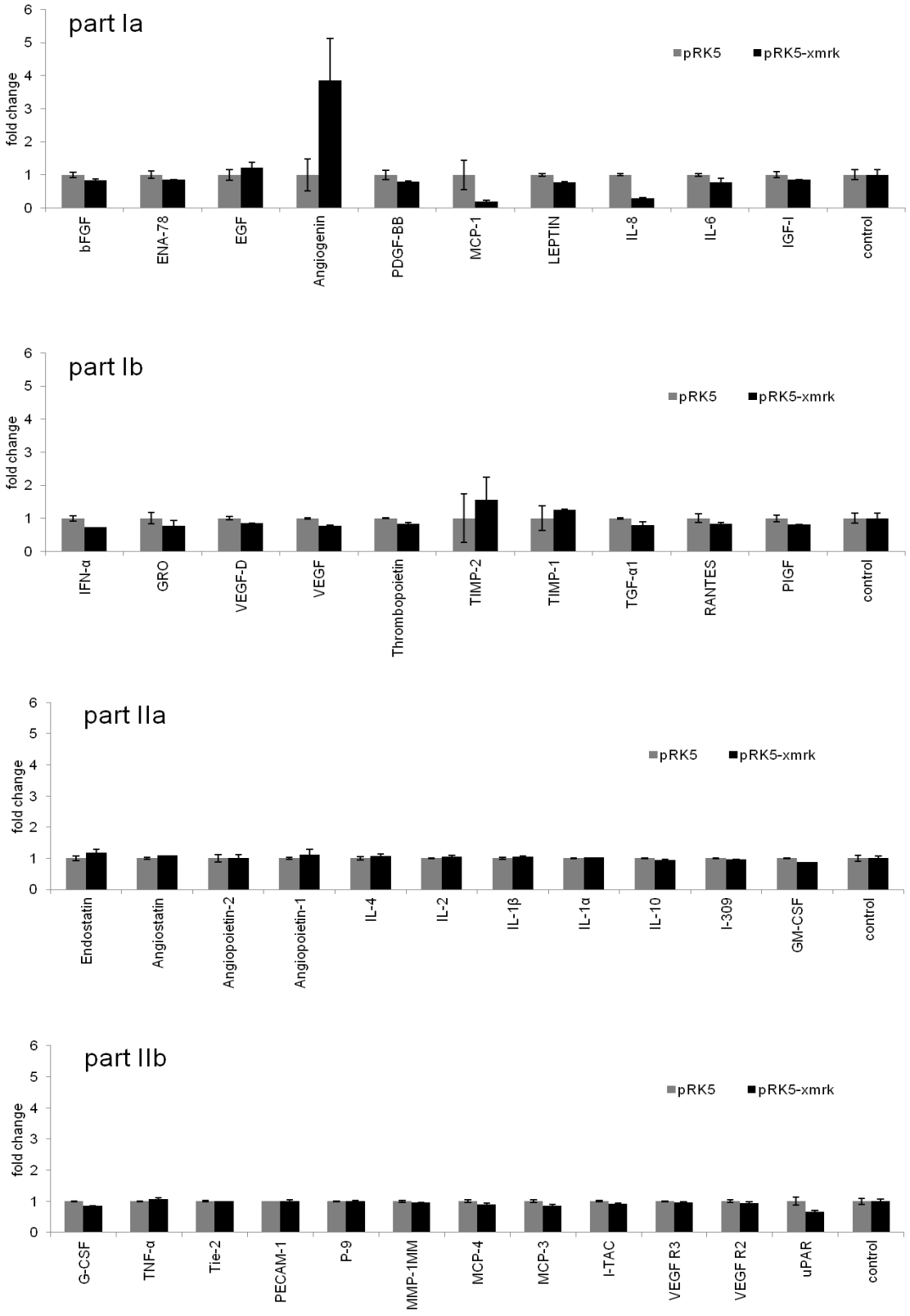


Figure 29: Human angiogenesis array (overview).
ELISA-based human angiogenesis array of 293T-pRK5 (grey bars) and 293T-pRK5-*xmrk* supernatant (black bars). In addition to the well-established angiogenesis inducers bFGF, PDBF-BB, VEGF-D and VEGF, only those proteins that showed an induction in response to Xmrk are displayed.

The only proteins which displayed an Xmrk-dependent induction were TIMP1, TIMP2 and, most prominently, angiogenin (figure 30). Like the TIMP proteins, angiogenin is also regulated by NF-κB [200].

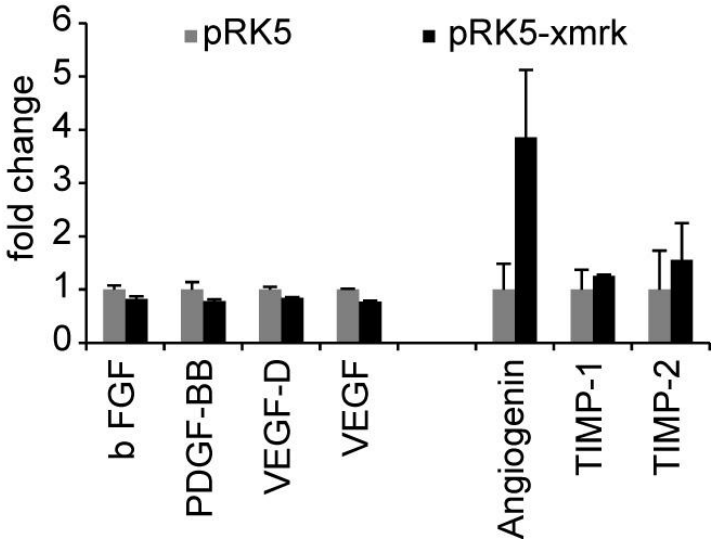


Figure 30: Human angiogenesis array (selected detail of figure 37).
ELISA-based human angiogenesis array of 293T-pRK5 (grey bars) and 293T-pRK5-*xmrk* supernatant (black bars). In addition to the well-established angiogenesis inducers bFGF, PDBF-BB, VEGF-D and VEGF, only those proteins that showed an induction in response to Xmrk are displayed.

By performing western-blot analysis it was found out that protein levels of both phospho-NF-κB (Ser536) and angiogenin were increased in Xmrk-expressing HEK293T cells in comparison to empty vector transfected control cells (figure 31).

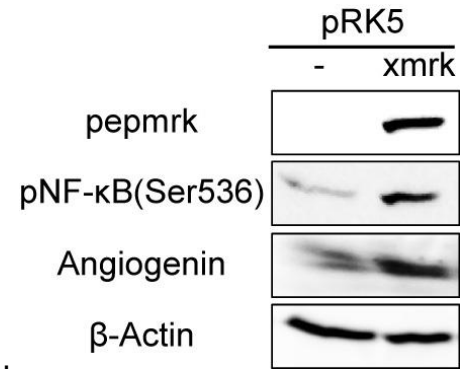


Figure 31: P-NF-κB and angiogenin protein level.
Western blot showing protein levels of Xmrk, P-NF-κB (Ser536), and angiogenin in 293T-pRK5 and 293T-pRK5-*xmrk* cells. β-Actin served as loading control.

Since angiogenin was not present in the murine angiogenesis assay, separate analyses were performed to investigate angiogenin regulation in the murine Hm cell system. Similarly to HEK293 system mRNA and protein (figure 32) expression levels of angiogenin were induced in response to HERmrk stimulation in the murine Hm cell system.

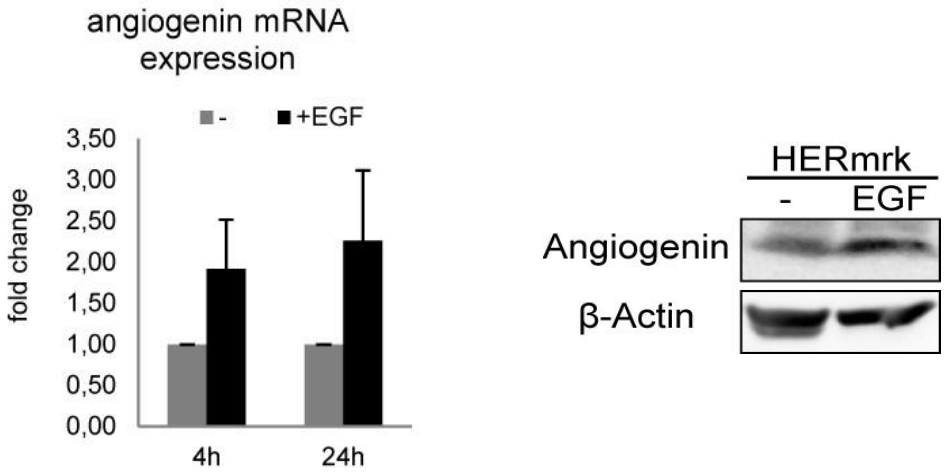


Figure 32: Angiogenin expression upon EGF-stimulation.
Realtime analysis showing angiogenin mRNA expression levels of untreated and EGF-stimulated Hmme cells after 4h and 24h of stimulation. Actin served as reference control. Western blot showing protein expression of angiogenin in Hmme cells left untreated or stimulated with EGF for 24 h. β-Actin served as loading control

3.4 NF-κB and ROS mediate angiogenesis *in vivo*

To address the question if NF-κB and ROS play a role in hypoxia-independent tumor angiogenesis *in vivo*, the *fli::egfp;mitf::xmrk* fish model was used.

Age-matched adult *fli::egfp;mitf::xmrk* medakas were separated into groups of 11-12 fishes. Groups were either treated with DMSO (control group), NF-κB inhibitor or Tiron for one week. Each fish was monitored before and after treatment. In many cases a decline in sprouts as well as branch points in the intra fin ray areas was observed after one week of continuously treatment with either NF-κB inhibitor or Tiron compared to controls (figure 33). Such a decline of sprouts is shown in figure 33 (arrowheads indicating pre- and post-treatment sprouts) for all experimental treatments including DMSO control.

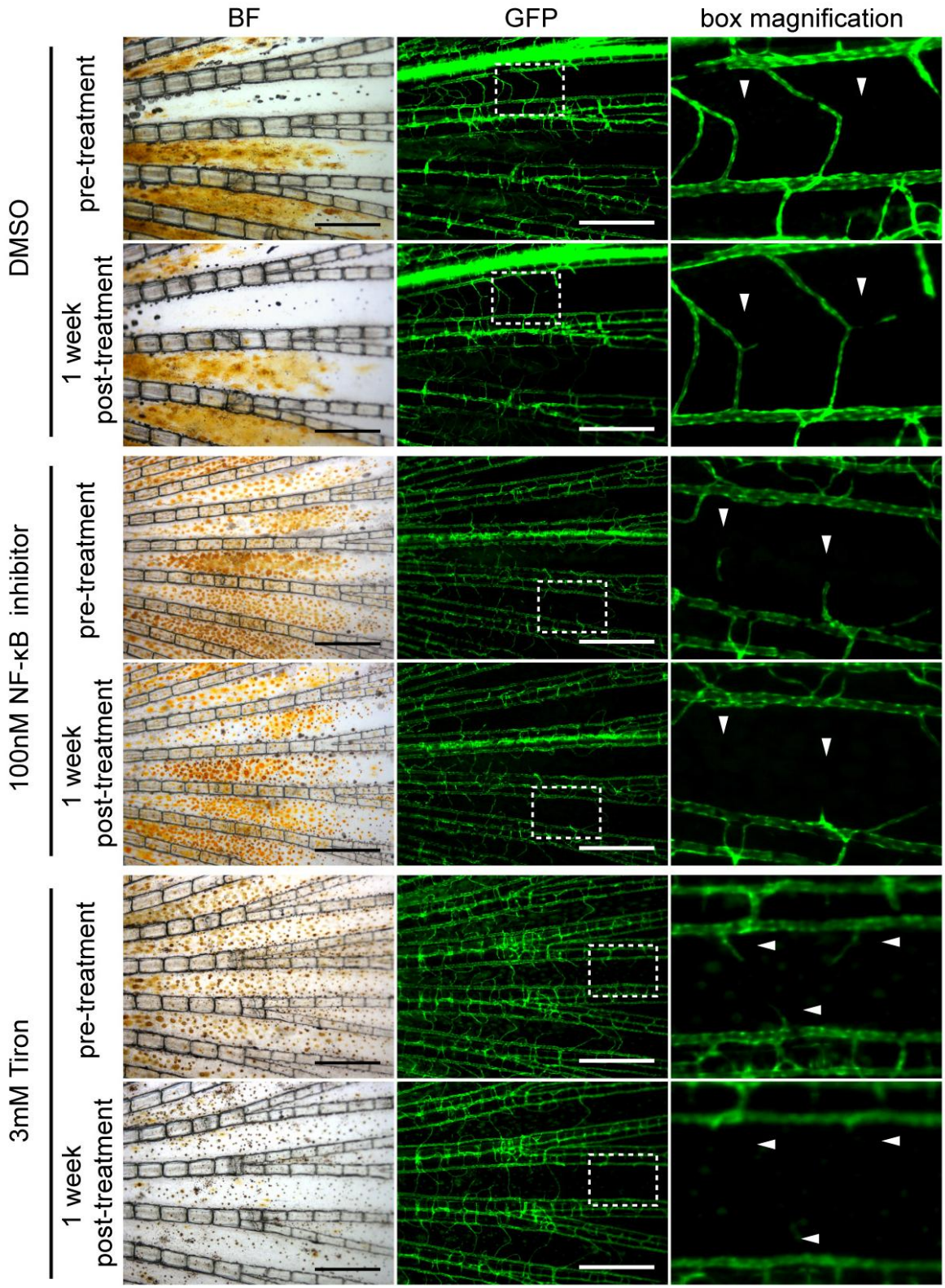


Figure 33: NF-κB and ROS mediate Xmrk-dependent angiogenesis in vivo.
Panels show caudal fins of transgenic *fli::egfp;mitf::xmrk* medaka fishes before treatment and after 1 week of continuous treatment with DMSO, NF-κB inhibitor (100nM) or Tiron (3mM). Left panel: bright

field images (BF); middle panel: corresponding GFP images (GFP); right panel: magnified view of the box indicated in the middle panel (box magnification). Scale bars represent 500 µm.

In single cases degeneration of established anastomoses upon NF-κB inhibitor treatment was observed (figure 34). Here after one week of continuous treatment with NF-κB inhibitor a disruption of pre-existing anastomoses was occasionally observed (figure 34, box magnification, indication by asterisks). However, this phenomenon has to be carefully interpreted due to the rare number of these observations which might be too low to be statistical relevant. Nevertheless, these observations show that the remodeling of the vascular pattern inside the intra fin ray area in this experimental setup is not only limited to the decrease of sprouts (figure 34 arrowhead) or branch points. In addition, a degeneration of pre-existing anastomosis or vessels can occur.

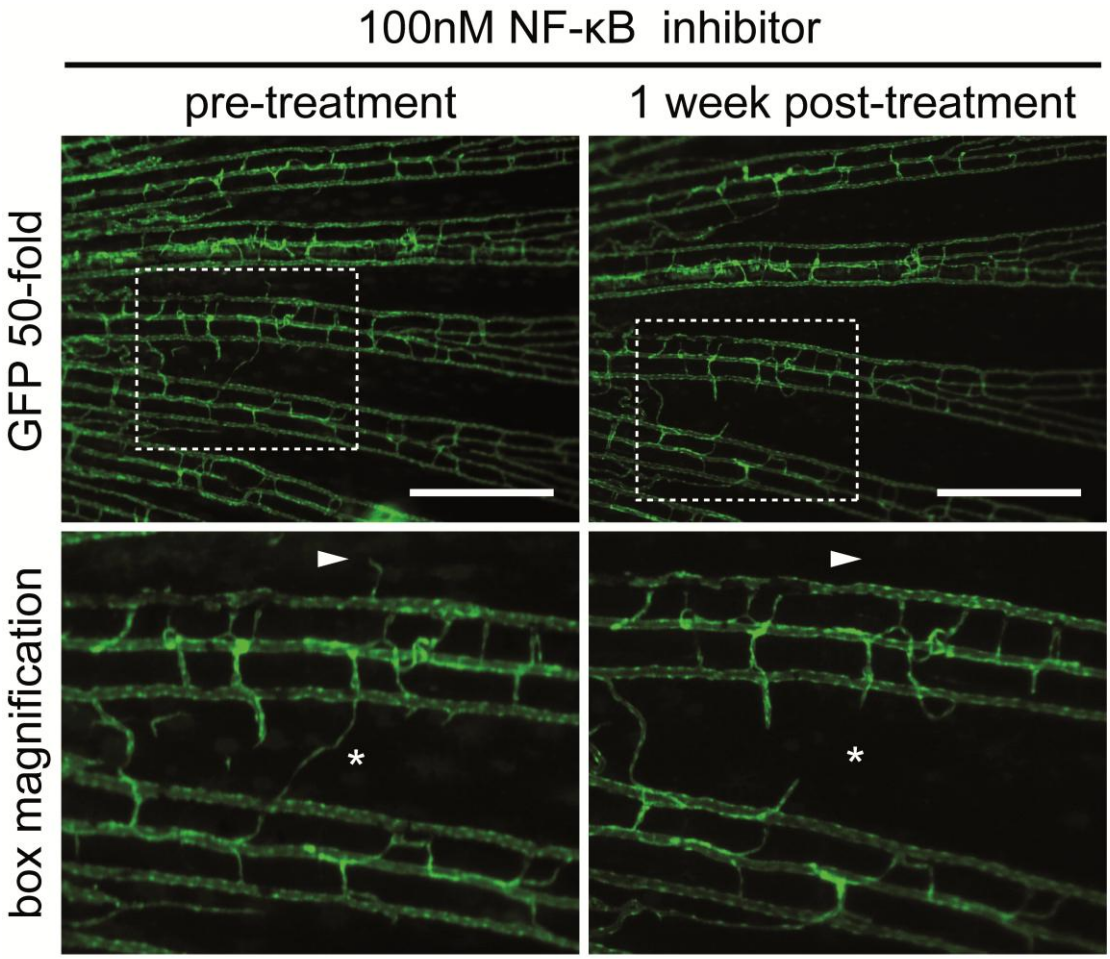
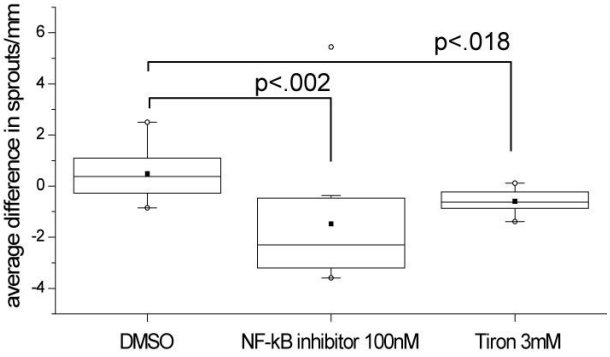


Figure 34: Degeneration of an anastomosis upon NF-κB inhibitor treatment.
GFP-positive blood vessel pattern of an adult *fli::egfp;mitf::xmrk* medaka before and after one week of treatment with 100nM of NF-κB activation inhibitor. Upper image panel shows GFP-channel at 50-fold

magnification. Lower image panel shows box magnification of the upper images. Degeneration of a sprout is marked by an arrowhead. Degeneration of an anastomosis is marked by an asterisk. Scale bars represent 500 µm.

To measure the statistical impact of changes in vascular patterning among adult *fli::egfp;mitf::xmrk* transgenic medaka fishes treated with DMSO (control group), NF-κB inhibitor or Tiron for one week, the average difference in the number of sprouts and branch points appearing in intra-fin ray areas were examined and analysed using the Mann-Whitney U test (figures 35 A, B). As shown in figure 35 A, DMSO-treated control fish displayed a slight increase in the number of blood vessel sprouts during one week, but almost no change of branch points during this time. The NF-κB inhibitor, however, led to a degeneration of preformed sprouts, resulting in a significant decrease of sprouts (figure 35 A) as well as a significant decrease in branch points (figure 35 B). The same trend was observed for Tiron treatment, but here only the decrease of the number of sprouts was found to be significant (figures 35 A, B).

A



B

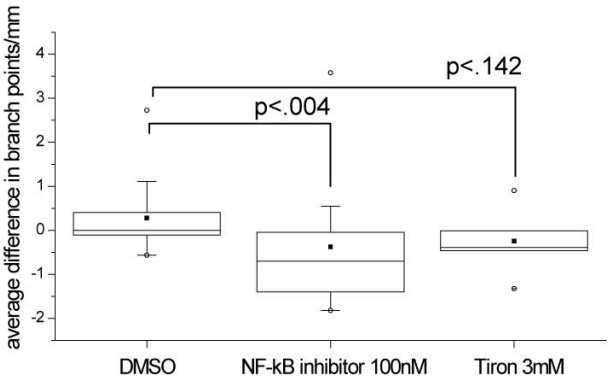


Figure 35: Average difference in the number of sprouts and average difference in the number of branch points.

Average difference in the number of sprouts (A) and average difference in the number of branch points (B) per mm caudal fin area after 1 week NF-κB inhibitor or Tiron treatment of transgenic *fli::egfp;mitf::xmrk medaka* fishes (n=12 each). DMSO treated control fish served as reference (n=12). Statistical analysis was carried out with the Mann–Whitney U test.

3.5 Role of NF-κB in human melanoma cells

Since NF-κB is often activated in human melanoma, where it facilitates tumor invasion and proliferation, prevents apoptosis and induces angiogenesis [201], [202], [203], [204]. To investigate a possible connection between NF-κB and the production of the angiogenesis inducer angiogenin in human melanoma cells, we treated the human melanoma cell line Mel Im with NF-κB inhibitor and performed a western blot analysis. Mel Im cells were chosen because of their high intrinsic NF-κB activity [203]. Pretreatment of Mel Im cells with NF-κB inhibitor strongly decreased phospho-NF-κB (Ser536) levels, which went along with a reduction of angiogenin protein (figure 36).

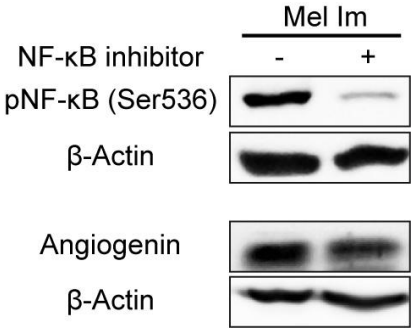


Figure 36: Angiogenin is induced upon NF-κB activation in human melanoma cell line Mel Im. Western blot of NF-κB-P-Ser536 and angiogenin protein levels of untreated or NF-κB inhibitor (10µM) treated Mel Im cells. β-actin served as reference.

Additionally, an ELISA-based human angiogenesis array was performed to detect the modulation of angiogenesis regulators in response to NF-κB inhibition. Interestingly, only angiogenin, TIMP1, TIMP2 and GRO were downregulated upon NF-κB inhibitor treatment, indicating that these factors are regulated by NF-κB in Mel Im cells (figure 37 for overview and figure 38 for specific factors in detail).

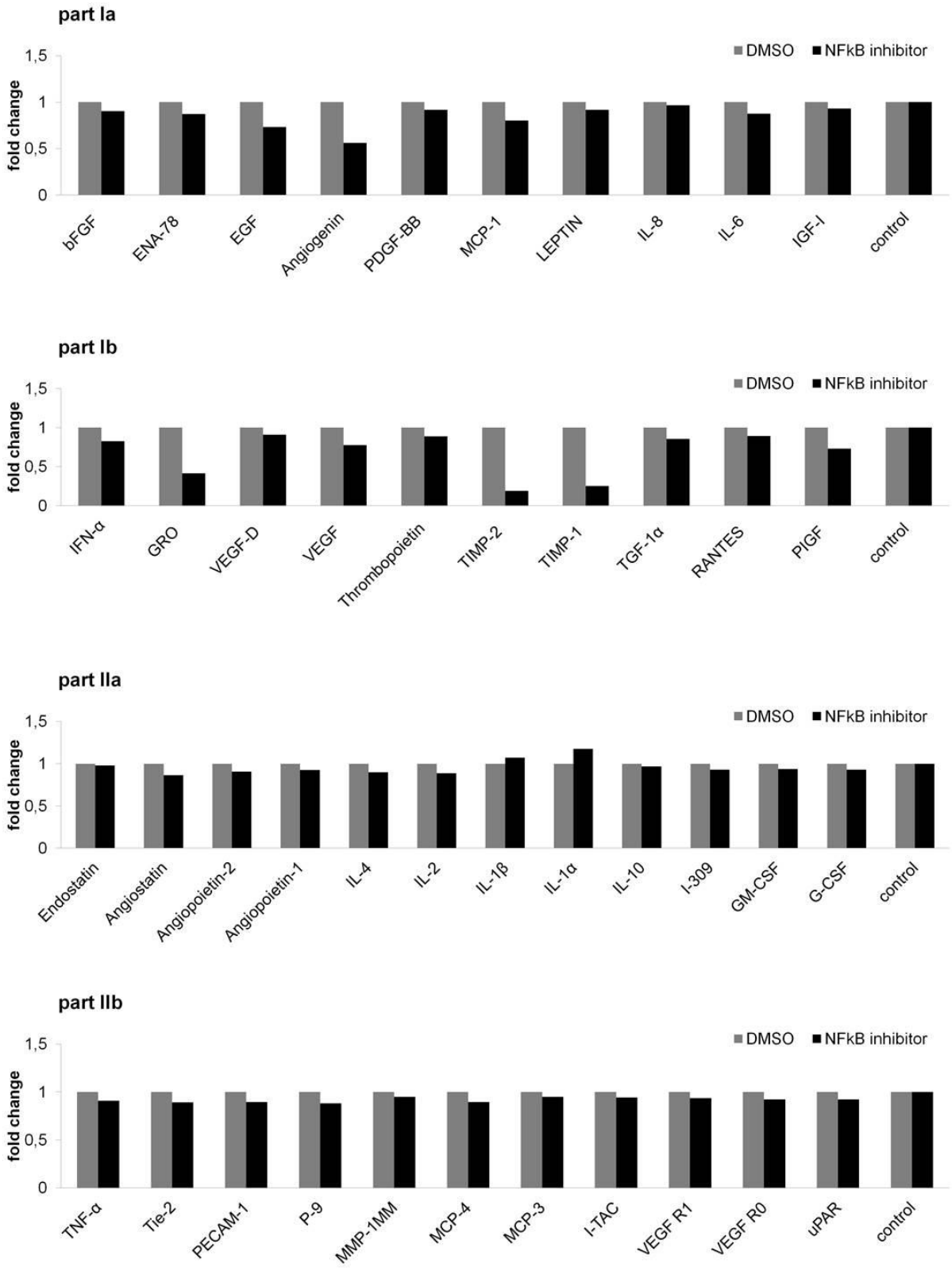


Figure 37: Human angiogenesis array of the supernatant from Mel Im cells (overview).
ELISA-based human angiogenesis array of the supernatant from Mel Im cells pretreated with DMSO (gray bars) or 10 µM of NF-κB inhibitor (black bars).

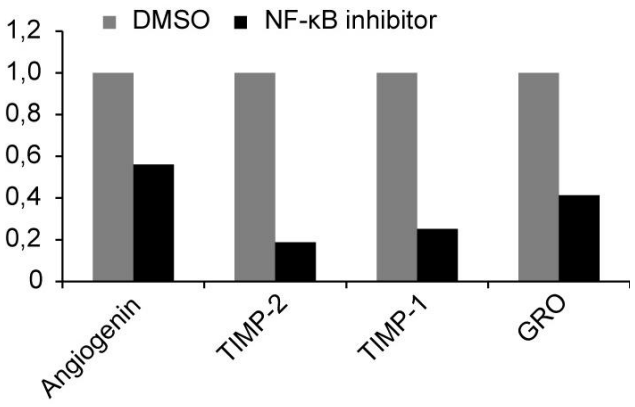


Figure 38: Human angiogenesis array of the supernatant from Mel Im cells (detail).
ELISA-based human angiogenesis array of the supernatant from Mel Im cells pretreated with DMSO (gray bars) or 10 µM of NF-κB inhibitor (black bars). The four most strongly regulated proteins are shown.

Realtime analysis of angiogenin, *TIMP-1 and TIMP*-2 mRNA expression levels of either DMSO or NF-κB inhibitor treated human melanoma cell lines Mel Im and Mel suggest that NF-κB inhibitor may reduce the mRNA amount of the genes (displayed in figure 39).

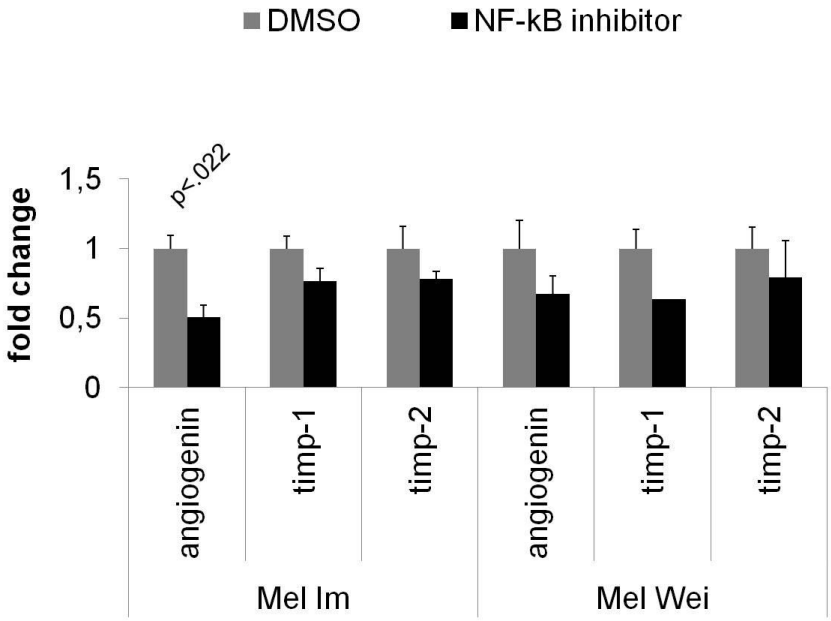


Figure 39*:* Angiogenin*, TIMP-1* and *TIMP-2* expression upon NF-κB inhibition in Mel Im and Mel Wei cells.
Realtime analysis showing angiogenin*, TIMP-1* and *TIMP-2* mRNA expression levels of DMSO treated and of NF-κB inhibitor (10 µM) cells after 24h of treatment. Actin served as reference control.

Altogether, angiogenin was consistently detected among all cell systems used for the described experiments. To test the dependence of angiogenin expression on NF-κB signaling in human melanoma cells, inhibitor experiments were performed. Pretreatment of melanoma cell lines Mel Wei, Mel Ho and A375 cells with NF-κB inhibitor strongly decreased phospho-NF-κB (Ser536) levels, which went along with a reduction of angiogenin protein shown in figure 40.

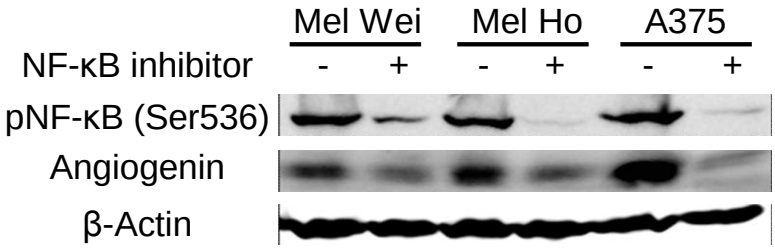


Figure 40: NF-κB-P-Ser536 and angiogenin protein levels upon NF-κB inhibition in human melanoma cell lines Mel Wei, Mel Ho and A375.
Western blot of NF-κB-P-Ser536 and angiogenin protein levels of untreated or NF-κB inhibitor (10µM) treated Mel Wei, Mel Ho and A375 cells, β-Actin served as loading control.

By performing NF-κB inhibitor experiments the following human melanoma cell lines were analyzed, if a dependence of angiogenin expression on NF-κB exists: UACC-257, SK-MEL-5, RPMI-7951, MEWO, M14, MALME-3, MDA-MB-435, SK-MEL-3 and UACC-62. The cell lines were then sorted in two groups thereby one group comprising cell lines where angiogenin protein responding to NF-κB inhibition are shown in figure 41 and the other group comprising cell lines which did not respond to NF-κB inhibition are shown in figure 42.

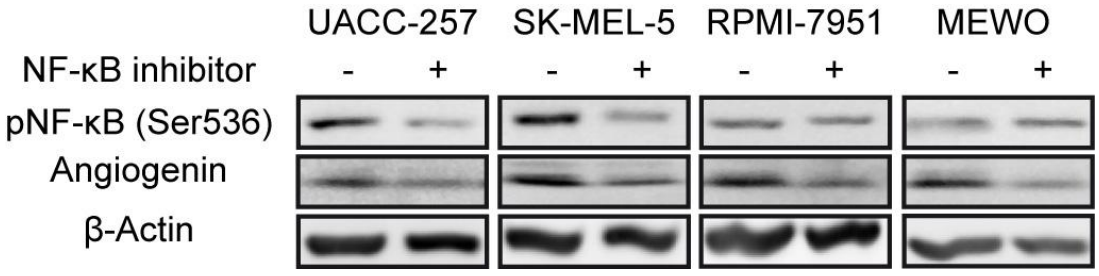


Figure 41: NF-κB-P-Ser536 and angiogenin protein levels of human melanoma cell lines responding to NF-κB inhibition.

Western blot of NF-κB-P-Ser536 and angiogenin protein levels of untreated or NF-κB inhibitor (10μM) treated human melanoma cell lines UACC-257, SK-MEL-5, RPMI-7951 and MEWO, β-Actin served as loading control.

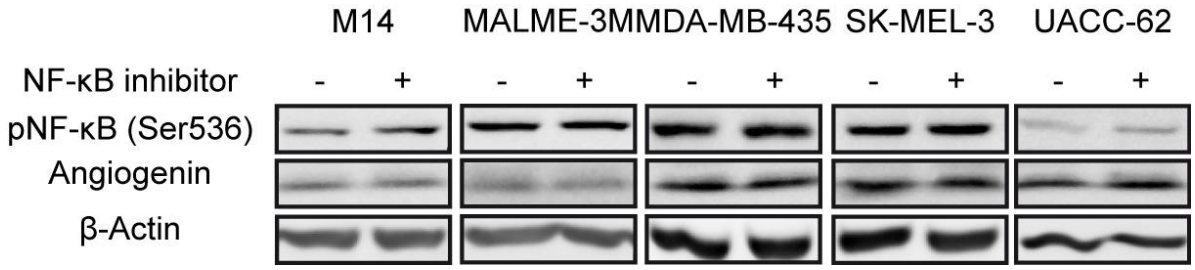


Figure 42: NF-κB-P-Ser536 and angiogenin protein levels of human melanoma cell lines not responding to NF-κB inhibition.
Western blot of NF-κB-P-Ser536 and angiogenin protein levels of untreated or NF-κB inhibitor (10μM) treated human melanoma cell lines M14, MALME-3, MDA-MB-435, SK-MEL-3 and UACC-62, β-Actin served as loading control.

It was previously described that angiogenin is upregulated by hypoxia in some melanoma cell lines [205]. To test if hypoxia, NF-κB, or both are relevant for angiogenin production in the melanoma cell lines used here, Mel Im, Mel Wei and A375 were kept for 24h under normoxic (20% O_2) or hypoxic (5% O_2) conditions and in absence or presence of NF-κB inhibitor. Subsequently, the protein levels of angiogenin were analyzed. Surprisingly, hypoxia did not lead to an increase, but rather a decrease of angiogenin levels, whereas NF-κB was required for angiogenin expression in all cases except in hypoxia-treated A375 cells (figure 43).

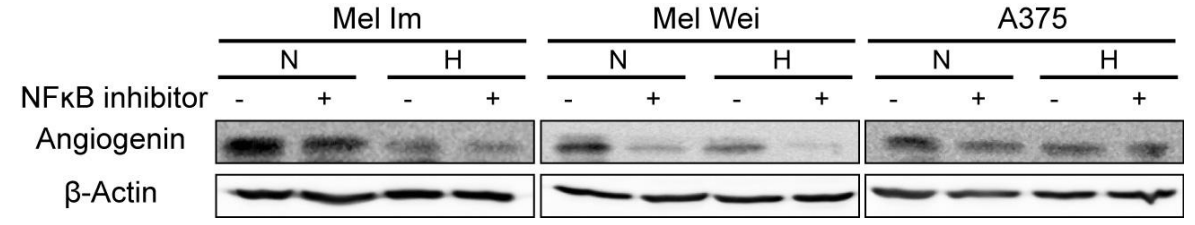


Figure 43: Angiogenin protein levels of Mel Im, Mel Wei and A375 cells in presence or absence of NF-κB inhibitor and under normoxia and hypoxia.
Normoxic condition = N and hypoxic condition = H. For hypoxia, cells were kept in an atmosphere with 5% O2, β-Actin served as loading control. Amount of NF-κB inhibitor: 10μM.

The same experiment was then performed in Xmrk expressing HEK293 cells (figure 44). Here, hypoxia had no effect on angiogenin expression at all, whereas NF-κB inhibition reduced angiogenin levels.

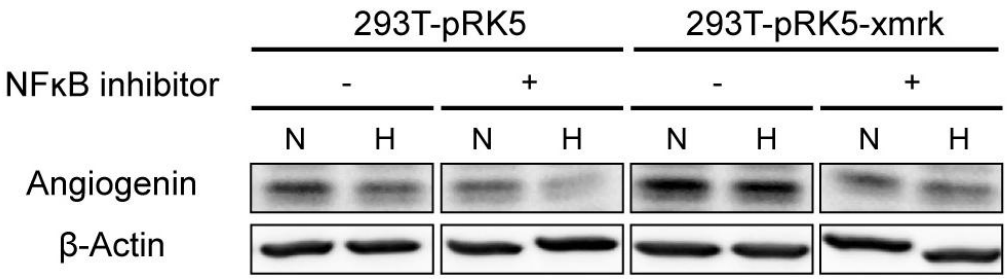


Figure 44: Protein levels of angiogenin in 293T-pRK5 or 293T-pRK5-xmrk cells in presence or absence of NF-κB inhibitor and under normoxia and hypoxia.
Normoxic condition = N and hypoxic condition = H. For hypoxia, cells were kept in an atmosphere with 5% O_2, β-Actin served as loading control. Amount of NF-κB inhibitor: 10μM.

In summary, all results demonstrated that in an experimental melanoma model angiogenesis can efficiently occur even in absence of hypoxia. It is instead regulated by ROS-driven NF-κB activation. Similar processes may occur in human melanoma cells, where strong angiogenesis inducers like angiogenin are upregulated by high intrinsic NF-κB activity.

4 DISCUSSION

4.1 Generation of a zebrafish Xmrk-melanoma model

Among other animal model systems, fishes are well suited for investigating tumor development, vascular development and angiogenesis due to their short reproduction cycle, their high transparency during early development and the ability to perform drug testing by simply applying a drug to the water (if soluble) and the ability to observe different developmental or tumorigenic processes in their natural tissue environment with high- resolution bioimaging techniques.

The present study revealed that injection of a Gal4 UAS-*xmrk* construct in zebrafish embryos lead to severe phenotypical effects. The fact that GFP-fluorescence is present in pigment cells or precursors of pigment cells proves that the pigment cell specific promotor *mitfa*, which is used to drive GFP expression in the injected plasmid, is functional. Further it was observed that injected embryos develop neoplasms or outgrowth of cells at different body sites. This suggests that the construct promotor *mitfa* is capable to drive *xmrk* expression too. Unfortunately none of the injected embryos which were visually sorted upon positive GFP-fluorescence survived the following weeks. This together with the observed severe phenotypic effects leads to the suggestion that enhanced *xmrk* expression in consequence of a 14-mer of UAS Gal4-binding site and subsequent protein production seems to be an invincible oncogenic burden for the embryos. It has been demonstrated that in a system with a lower number of UAS Gal4-binding sites (5-mer) it was possible to established a transgenic zebrafish line specifically expressing oncogenic human *HRAS* in the melanocytic lineage [50]. It is worthwhile noting that the oncogene *HRAS* is also less potent that *xmrk*. HRAS is not able to directly stimulate pathways in a way Xmrk is able to do. Xmrk can stimulate ROS production, migration, anti-apoptosis effects and even proliferation independent of RAS activation [37], [38], [39], [45], [46].

In conclusion, it is suggested that the high expression status of *xmrk* in this particular

case has a negative effect on early embryonic development and thus has led to the death of these embryos.

4.2 Xmrk mediated angiogenesis in an animal melanoma model system

With reference to the previous results, here it was demonstrated for the first time that melanoma angiogenesis in a transgenic *in vivo* melanoma model occurs at very early stages of tumor development.

In the present study it has been shown that *xmrk*-transgenic medaka developed a mesh of capillaries in the intra-fin ray areas of their fins. Interestingly, transformed cells which have not yet developed to three-dimensional tumors such as melanomas or xantoerythrophoromas can trigger angiogenesis. It is known that tumors, when reaching a certain volume which is above the oxygen diffusion limit of a certain tissue, are able to secrete hypoxia-induced pro angiogenesis factors such as VEGF or IL-8 [206]. This process is often mediated by hypoxia-inducible factor 1 and 2 (HIF-1/-2) [89]. According to the present observations in the medaka model, we can exclude that hypoxia is the main driving force of this angiogenetic phenomenon due to the following reasons: first, it was observed that even a few *xmrk*-transgenic cells can provoke an angiogenic sprouting towards these transformed cells; second: cross section of an adult medaka fin revealed that the lateral thickness of the caudal fin does not go beyond the oxygen tissue diffusion limit of approximately 1 mm [191], [192], third: the hypoxia indicator pimonidazole revealed that the respective fin tissue is not hypoxic [207].

Several experiments have brought to light that under different circumstances, melanoma cells similar to other tumor cells can express a broad spectrum of angiogenic factors like VEGF, bFGF, PlGF, IL-6, IL-8 [206]. Among these factors, VEGF, bFGF, and IL-8 have a special standing as they are strongly correlated with poor clinical outcome and are independent predictive factors for overall survival in

melanoma patients [208]. Surprisingly, prominent factors like VEGF or bFGF have not been detected in the previously described angiogenesis arrays of different cell systems in the current study. However, VEGF-signaling is still required for angiogenesis, as e.g. shown in the context of wound healing in fin amputation experiments in presence and absence of VEGFR2-inhibitor treatment.

Furthermore it his known that integrin signaling as well as certain metalloproteinases (MMPs) can contribute to melanoma tumor progression and angiogenesis [206]. It has been shown that several $a_v\beta_3$ integrin ligands such as osteopontin are able to modulate VEGF- as well as bFGF- induced tumor angiogenesis in animal models [209]. It has been demonstrated that MMPs are expressed by several melanoma and tumor stromal cells [210], [211]. MMPs are actively involved in matrix degeneration and can facilitate angiogenesis, metastasis and tumor growth [212], [213]. Interestingly, both classes of components are also regulated by Xmrk signaling [41], [43]. Several cell line or animal model studies showed that the balance between MMPs and their inhibitors (TIMPs) finally determines melanoma progression [206]. However, the role of TIMPs in melanoma tumor development is currently under controversial discussion. Overexpression experiments of TIMP-1, -2 and -3 on one hand resulted in a reduction of melanoma tumor cell invasion, migration, tumor growth, metastasis and neovascularization [214]. On the other hand other studies demonstrate a significantly enhanced tumor cell proliferation in human melanoma cells expressing TIMP-1 [215]. Furthermore transgenic mice overexpressing TIMP-1 in the peripheral blood showed a significant angiogenic response induced by B16 melanoma cells after intradermal injection [194]. In the present study it was observed that TIMPs were induced or upgregulated by Xmrk, which acts strongly angiogenic. Interestingly, it was also shown by our collaborators that a *TIMP1* knockdown in human melanoma cells reduced pro-angiogenic HUVEC sprouting [207]. These data indicate that TIMPs can clearly act pro-angiogenic in melanoma. However, whether this is MMP-dependent or –independent still has to be examined.
Further it was observed that IL-6 was induced upon Xmrk stimulation in HERmrk cells, but this was not observed for the 293T-pRK5-*xmrk* cell line. However, it has been shown that IL-6 can induce angiogenesis in a rat cornea micropocket [216].

And it had been demonstrated that IL-6 is also a target of NF-κB [196]. Furthermore it has been shown that in melanoma cells overexpressing ILK (Integrin-linked kinase) altered activity and subcellular localization of NF-κB subunit p65 occurred and this promotes enhanced binding of p65 to the IL-6 promotor [217]. It is suggested that in case of the HERmrk cell line IL-6 induction is mediated by Xmrk-induced NF-κB.

4.3 Xmrk mediated NF-κB expression

The data described in this thesis revealed that Xmrk mediates NF-κB actvation, which itself is involved in melanoma angiogenesis in medaka. There are several theories on how Xmrk can influence NF-κB signaling. It has been demonstrated that Xmrk can activate RAS/RAF signaling which in turn can indirectly activate NF-κB through constitutive action of ERK and the up-regulation of inflammatory cytokines [218].

Additionally, several recent studies demonstrate that MAP-kinases such as NF-κB inducing kinase (NIK) and MAP kinase kinase 1 (MEKK1) can participate in the activation of NF-κB in the cytoplasm as well as in the modulation of its transcriptional potential in the nucleus. It is suggested that NIK preferentially phosphorylates and activates IKKα, while MEKK1 preferentially phosphorylates and activates IKKβ [219].

It has also been shown that Xmrk activates PI3K signaling with a resulting AKT activation [36], [37]. It was described by Li and Stark 2002 [181], that AKT can lead to IKKα phosphorylation which leads than to phosphorylation of the NF-κB subunit p65. Furthermore, it was also shown that AKT alone is able to phosphorylate p65 which increases the binding of NF-κB complex to DNA [220].

As it was previously shown by Leikam et al. [46] high levels of Xmrk can lead to the generation of reactive oxygen species (ROS). It is suggested that Xmrk can mediate ROS induction by activation of NADPH oxidases which is similar to its human orthologue EGFR [221]. ROS are known as potent activators of NF-κB [197]. It was

demonstrated for human melanoma that ROS levels and NF-κB activity were linked together [203]. In the present study the influence of Xmrk-mediated ROS on angiogenesis were tested by applying a ROS scavenger (Tiron) *in vivo*. The *in vivo* data revealed that inhibiting ROS had indeed an influence on the angiogenic phenomenon as it leads to a significantly decreased number of sprouts. Furthermore, it was also shown by *in vitro* experiments that ROS scavengers like vitamin E and Tiron can reduce NF-κB protein levels in an Xmrk-mediated ROS caused system [207]. This together suggests that ROS have an influence on NF-κB signaling and angiogenesis in a *xmrk*-based cell system or *xmrk*-transgenic animal model.

In conclusion, there are several possible ways in which NF-κB might be activated by Xmrk, of which we consider ROS production to be the dominant one. However, it is likely that melanoma cells or Xmrk-transformed cells use different combinations of the mentioned pathways or specific pathways at different developmental stages to activate NF-κB.

4.4 NF-κB, angiogenin and human melanoma

It had been demonstrated that NF-κB is constitutively activated in several cancer types [146] where it modulates metastasis, chemoresistance and apoptosis prevention [222]. Most melanomas and melanoma derived cell lines show constitutive NF-κB signaling [173], [223].

In the present study, angiogenin has been found to be upregulated by using angiogenesis arrays and immunohistochemistry assays. Several reports have shown that angiogenin is a tumor-associated angiogenic factor [224–226]. It is a basic protein of about 14.1 kDa and belongs to the superfamily of pancreatic ribonucleases [227]. Angiogenin is able to bind specifically to the cellsurface of endothelial cells via endothelial cell surface smooth muscle-type α-actin (or α-actin-like protein) or an endothelial cell-surface 170 kDa polypeptide [227–231]. Angiogenin activity mediated by 170 kDa receptor is still not clearly understood and is under current investigation.

Interestingly the 170 kDa protein is not similar to receptors of known growth factors and cytokines which are able to induce cell proliferation and differentiation [232]. It has been demonstrated that angiogenin is able to increase levels of DNA synthesis as well as proliferation of endothelial cells but mainly in sparse cell cultures. Further after binding angiogenin is internalized by receptor-mediated endocytosis and is then localized in the nucleus of proliferating (subconfluent) endothelial cells [229]. It had been shown that enhancement of ribosomal RNA transcription is a nuclear function of angiogenin [233] and this serves as a crossroad in the process of angiogenesis induced by other angiogenic factors such as FGF and VEGF [234], [235].

Cell surface actin is assumed to have an impact on the degeneration of basement-membrane and extracellular matrix. Binding of angiogenin to cell surface actin lead to formation of angiogenin-actin-complexes which can dissociate from the cell surface. These complexes then can accelerate tissue-type plasminogen activator (tPA)-catalyzed generation of plasmin from plasminogen [236]. This in turn allows endothelial or other cells to penetrate and migrate into perivascular tissue [237]. In summary angiogenin contributes to angiogenesis by stimulating endothelial cell migration and invasion and is also able to promote cell proliferation and differentiation. It also mediates cell adhesion and activates cell-associated proteases [238–240].

Like the TIMPs, angiogenin is also regulated by NF-κB [200]. This is in line with the fact that the human angiogenin promotor exhibits NF-κB binding sites. Further it has been demonstrated that angiogenin could be induced by hypoxia [205]. As already mentioned some melanomas or melanoma-derived cell lines exhibit an intrinsic constitutive NF-κB activity. Due to this fact, it was tested in a couple of melanoma cell lines whether angiogenin induction is NF-κB and/or hypoxia dependent. I could demonstrate that angiogenin was induced by NF-κB activity, but not by hypoxia.

In summary, angiogenin induced by NF-κB was identified as an important and potent angiogenic factor in human melanoma cells and in melanoma models such as medaka. Importantly, I could reveal that highly efficient tumor angiogenesis can be

induced in a hypoxia-independent, but NF-κB-dependent manner by single cells already. Thus, the NF-κB pathway contributes to the early metastasic events which are observed for small melanoma lesions and which are a hallmark for melanoma progression [165], [85], [241].

4.5 Perspectives

The present medaka melanoma angiogenesis model is well suited for detection of angiogenesis at high resolution and is perfectly qualified as an in vivo model for the testing of new anti-angiogenesis inhibitors or new combinations of already known inhibiting components. This model system has the advantage of testing the inhibitor of interest by simple administration into the living media (freshwater) of the fishes instead of giving every animal an injection dose of the inhibitor. Furthermore it is able to observe putative angiogenesis effects without killing the animal at the end of the experiment. This benefits observation experiments over several days or weeks.

In this present study it has been shown that Xmrk is able to cause angiogenesis *in vivo*. It has been also demonstrated that Xmrk is able to lead to *in vitro* angiogenesis in a HUVEC system [207]. It was shown for the first time that Xmrk can activate NF-κB. As mentioned above there are a brought spectra of factor and pathways by which Xmrk can lead to NF-κB activation. Further it has been demonstrated that due to Xmrk-mediated activation of NF-κB secretion of pro-angiogenic factors like angiogenin, il-6 or TIMPs were induced. In conclusion, this study shows that targeting NF-κB pathway with its angiogenesis-dependent and –independent effects of tumor development might be a promising anti-tumor strategy and this is not limited to Xmrk-mediated tumors it might be also transferable to human melanoma as partially shown with NF-κB-inhibitor experiments in the human melanoma cell lines.

5 APPENDIX

5.1 List of literature

[1] A. Slominski, D. J. Tobin, S. Shibahara, and J. Wortsman, “Melanin pigmentation in mammalian skin and its hormonal regulation.,” *Physiological reviews*, vol. 84, no. 4, pp. 1155-228, Oct. 2004.

[2] B. K. Armstrong and A. Kricker, “The epidemiology of UV induced skin cancer.,” *Journal of photochemistry and photobiology. B, Biology*, vol. 63, no. 1–3, pp. 8-18, Oct. 2001.

[3] C. Garbe and A. Blum, “Epidemiology of cutaneous melanoma in Germany and worldwide.,” *Skin pharmacology and applied skin physiology*, vol. 14, no. 5, pp. 280-90.

[4] deutsche Dermatologische Gesellschaft, “Ergebnisse des Zentralregisters Malignes Melanom.” .

[5] N. K. Haass, K. S. M. Smalley, and M. Herlyn, “The role of altered cell-cell communication in melanoma progression.,” *Journal of molecular histology*, vol. 35, no. 3, pp. 309-18, Mar. 2004.

[6] A. J. Miller and M. C. Mihm, “Melanoma.,” *The New England journal of medicine*, vol. 355, no. 1, pp. 51-65, Jul. 2006.

[7] V. Gray-schopfer, C. Wellbrock, and R. Marais, “Melanoma biology and new targeted therapy,” *Nature*, vol. 445, no. February, 2007.

[8] B. A. Gilchrest, M. S. Eller, A. C. Geller, and M. Yaar, “The pathogenesis of melanoma induced by ultraviolet radiation.,” *The New England journal of medicine*, vol. 340, no. 17, pp. 1341-8, Apr. 1999.

[9] C. J. Hussussian et al., “Germline p16 mutations in familial melanoma.,” *Nature genetics*, vol. 8, no. 1, pp. 15-21, Sep. 1994.

[10] A. Kamb et al., “Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus.,” *Nature genetics*, vol. 8, no. 1, pp. 23-6, Sep. 1994.

[11] M. Serrano, G. J. Hannon, and D. Beach, “A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4.,” *Nature*, vol. 366, no. 6456, pp. 704-7, Dec. 1993.

[12] T. Kamijo, J. D. Weber, G. Zambetti, F. Zindy, M. F. Roussel, and C. J. Sherr, “Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 14, pp. 8292-7, Jul. 1998.

[13] J. Pomerantz et al., “The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2’s inhibition of p53.,” *Cell*, vol. 92, no. 6, pp. 713-23, Mar. 1998.

[14] F. J. Stott et al., “The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2.,” *The EMBO journal*, vol. 17, no. 17, pp. 5001-14, Sep. 1998.

[15] Y. Zhang, Y. Xiong, and W. G. Yarbrough, “ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways.,” *Cell*, vol. 92, no. 6, pp. 725-34, Mar. 1998.

[16] B. Vanhaesebroeck et al., “Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages.,” *Nature cell biology*, vol. 1, no. 1, pp. 69-71, May 1999.

[17] S. Cooray, “The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival.,” *The Journal of general virology*, vol. 85, no. Pt 5, pp. 1065-76, May 2004.

[18] D. Koul, Y. Yao, J. L. Abbruzzese, W. K. Yung, and S. A. Reddy, “Tumor suppressor MMAC/PTEN inhibits cytokine-induced NFkappaB activation

without interfering with the IkappaB degradation pathway.," *The Journal of biological chemistry*, vol. 276, no. 14, pp. 11402-8, Apr. 2001.

[19] H. Wu, V. Goel, and F. G. Haluska, "PTEN signaling pathways in melanoma.," *Oncogene*, vol. 22, no. 20, pp. 3113-22, May 2003.

[20] H. Tsao, V. Goel, H. Wu, G. Yang, and F. G. Haluska, "Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma.," *The Journal of investigative dermatology*, vol. 122, no. 2, pp. 337-41, Feb. 2004.

[21] M. Böhm et al., "Identification of p90RSK as the probable CREB-Ser133 kinase in human melanocytes.," *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research*, vol. 6, no. 3, pp. 291-302, Mar. 1995.

[22] C. Wellbrock, C. Weisser, E. Geissinger, J. Troppmair, and M. Schartl, "Activation of p59(Fyn) leads to melanocyte dedifferentiation by influencing MKP-1-regulated mitogen-activated protein kinase signaling.," *The Journal of biological chemistry*, vol. 277, no. 8, pp. 6443-54, Feb. 2002.

[23] C. Wellbrock and R. Marais, "Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation.," *The Journal of cell biology*, vol. 170, no. 5, pp. 703-8, Aug. 2005.

[24] K. V. Bhatt, L. S. Spofford, G. Aram, M. McMullen, K. Pumiglia, and A. E. Aplin, "Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling.," *Oncogene*, vol. 24, no. 21, pp. 3459-71, May 2005.

[25] J. T. Huntington et al., "Overexpression of collagenase 1 (MMP-1) is mediated by the ERK pathway in invasive melanoma cells: role of BRAF mutation and fibroblast growth factor signaling.," *The Journal of biological chemistry*, vol. 279, no. 32, pp. 33168-76, Aug. 2004.

[26] C. Cohen et al., “Mitogen-actived protein kinase activation is an early event in melanoma progression.,” *Clinical cancer research : an official journal of the American Association for Cancer Research*, vol. 8, no. 12, pp. 3728-33, Dec. 2002.

[27] V. Gray-Schopfer, C. Wellbrock, and R. Marais, “Melanoma biology and new targeted therapy.,” *Nature*, vol. 445, no. 7130, pp. 851-7, 2007.

[28] H. Davies et al., “Mutations of the BRAF gene in human cancer.,” *Nature*, vol. 417, no. 6892, pp. 949-54, Jun. 2002.

[29] A. Sharma, N. R. Trivedi, M. A. Zimmerman, D. A. Tuveson, C. D. Smith, and G. P. Robertson, “Mutant V599EB-Raf regulates growth and vascular development of malignant melanoma tumors.,” *Cancer research*, vol. 65, no. 6, pp. 2412-21, Mar. 2005.

[30] M. Gordon, “The Genetics of a Viviparous Top-Minnow Platypoecilus; the Inheritance of Two Kinds of Melanophores.,” *Genetics*, vol. 12, no. 3, pp. 253-83, May 1927.

[31] G. Häussler, “Über Melanombildungen bei Bastarden von Xiphophorus maculatus var. rubra,” *Klin. Wochenschr.*, no. 7, pp. 1561-1562, 1928.

[32] C. Kosswig, “Über Kreuzungen zwischen den Teleostiern Xiphophorus helleri und Platypoecilus maculatus,” *Z. Indukt. Abstammungs-Vererbungsl.*, no. 47, pp. 150-158, 1928.

[33] F. Anders, “Contributions of the Gordon-Kosswig melanoma system to the present concept of neoplasia.,” *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society*, vol. 4, no. 1, pp. 7-29, Feb. 1991.

[34] J. Wittbrodt et al., “Novel putative receptor tyrosine kinase encoded by the melanoma-inducing Tu locus in Xiphophorus.,” *Nature*, vol. 341, no. 6241, pp. 415-21, Oct. 1989.

[35] M. Schartl and R. U. Peter, “Progressive growth of fish tumors after transplantation into thymus-aplastic (nu/nu) mice.,” *Cancer research*, vol. 48, no. 3, pp. 741-4, Feb. 1988.

[36] S. Meierjohann and M. Schartl, “From Mendelian to molecular genetics: the Xiphophorus melanoma model.,” *Trends in genetics : TIG*, vol. 22, no. 12, pp. 654-61, 2006.

[37] M. Baudler, M. Schartl, and J. Altschmied, “Specific activation of a STAT family member in Xiphophorus melanoma cells.,” *Experimental cell research*, vol. 249, no. 2, pp. 212-20, Jun. 1999.

[38] J. C. Morcinek, C. Weisser, E. Geissinger, M. Schartl, and C. Wellbrock, “Activation of STAT5 triggers proliferation and contributes to anti-apoptotic signalling mediated by the oncogenic Xmrk kinase.,” *Oncogene*, vol. 21, no. 11, pp. 1668-78, Mar. 2002.

[39] C. Wellbrock and M. Schartl, “Activation of phosphatidylinositol 3-kinase by a complex of p59fyn and the receptor tyrosine kinase Xmrk is involved in malignant transformation of pigment cells.,” *European journal of biochemistry / FEBS*, vol. 267, no. 12, pp. 3513-22, Jun. 2000.

[40] C. Wellbrock, A. Gómez, and M. Schartl, “Melanoma development and pigment cell transformation in xiphophorus.,” *Microscopy research and technique*, vol. 58, no. 6, pp. 456-63, 2002.

[41] S. Meierjohann et al., “MMP13 mediates cell cycle progression in melanocytes and melanoma cells: in vitro studies of migration and proliferation.,” *Molecular cancer*, vol. 9, p. 201, Jan. 2010.

[42] J. Delfgaauw, J. Duschl, C. Wellbrock, C. Froschauer, M. Schartl, and J. Altschmied, “MITF-M plays an essential role in transcriptional activation and signal transduction in Xiphophorus melanoma.,” *Gene*, vol. 320, pp. 117-26, Nov. 2003.

[43] E. Geissinger, C. Weisser, P. Fischer, M. Schartl, and C. Wellbrock, “Autocrine stimulation by osteopontin contributes to antiapoptotic signalling of melanocytes in dermal collagen.,” *Cancer research*, vol. 62, no. 16, pp. 4820-8, Aug. 2002.

[44] C. Wellbrock and M. Schartl, “Multiple binding sites in the growth factor receptor Xmrk mediate binding to p59fyn, GRB2 and Shc.,” *European journal of biochemistry / FEBS*, vol. 260, no. 1, pp. 275-83, Feb. 1999.

[45] S. Meierjohann, E. Wende, A. Kraiss, C. Wellbrock, and M. Schartl, “The oncogenic epidermal growth factor receptor variant Xiphophorus melanoma receptor kinase induces motility in melanocytes by modulation of focal adhesions.,” *Cancer research*, vol. 66, no. 6, pp. 3145-52, Mar. 2006.

[46] C. Leikam, a Hufnagel, M. Schartl, and S. Meierjohann, “Oncogene activation in melanocytes links reactive oxygen to multinucleated phenotype and senescence.,” *Oncogene*, vol. 27, no. 56, pp. 7070-82, 2008.

[47] E. E. Patton et al., “BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma,” *Current Biology*, vol. 15, no. 3, pp. 249–254, 2005.

[48] M. Dovey, R. M. White, and L. I. Zon, “Oncogenic NRAS cooperates with p53 loss to generate melanoma in zebrafish.,” *Zebrafish*, vol. 6, no. 4, pp. 397-404, Dec. 2009.

[49] C. Michailidou, M. Jones, P. Walker, J. Kamarashev, A. Kelly, and A. F. L. Hurlstone, “Dissecting the roles of Raf- and PI3K-signalling pathways in melanoma formation and progression in a zebrafish model.,” *Disease models & mechanisms*, vol. 2, no. 7–8, pp. 399-411.

[50] V. Anelli, C. Santoriello, M. Distel, R. W. Köster, F. D. Ciccarelli, and M. Mione, “Global repression of cancer gene expression in a zebrafish model of melanoma is linked to epigenetic regulation.,” *Zebrafish*, vol. 6, no. 4, pp. 417-24, Dec. 2009.

[51] C. Santoriello et al., “Expression of H-RASV12 in a zebrafish model of Costello syndrome causes cellular senescence in adult proliferating cells.,” *Disease models & mechanisms*, vol. 2, no. 1–2, pp. 56-67.

[52] M. Schartl, B. Wilde, J. A. Laisney, Y. Taniguchi, S. Takeda, and S. Meierjohann, “A Mutated EGFR Is Sufficient to Induce Malignant Melanoma with Genetic Background-Dependent Histopathologies.,” *The Journal of investigative dermatology*, pp. 1-10, 2009.

[53] C. Winkler, J. Wittbrodt, R. Lammers, A. Ullrich, and M. Schartl, “Ligand-dependent tumor induction in medakafish embryos by a Xmrk receptor tyrosine kinase transgene.,” *Oncogene*, vol. 9, no. 6, pp. 1517-25, Jun. 1994.

[54] D. Winnemoeller, C. Wellbrock, and M. Schartl, “Activating mutations in the extracellular domain of the melanoma inducing receptor Xmrk are tumorigenic in vivo.,” *International journal of cancer. Journal international du cancer*, vol. 117, no. 5, pp. 723-9, Dec. 2005.

[55] M. Schartl, B. Wilde, J. A. G. C. Laisney, Y. Taniguchi, and S. Takeda, “A Mutated EGFR Is Sufficient to Induce Malignant Melanoma with Genetic Background-Dependent Histopathologies,” *Journal of Investigative Dermatology*, pp. 1-10, 2009.

[56] N. D. Lawson and B. M. Weinstein, “ARTERIES AND VEINS : MAKING A DIFFERENCE WITH ZEBRAFISH,” *Genetics*, vol. 3, no. September, 2002.

[57] W. J. Hamilton, J. D. Boyd, and H. W. Mossmann, “Human Embryology,” *Wiliam & Wilkins, Baltimore*, 1962.

[58] T. Veikkola and K. Alitalo, “VEGFs, receptors and angiogenesis.,” *Seminars in cancer biology*, vol. 9, no. 3, pp. 211-20, Jun. 1999.

[59] N. Ferrara, “Vascular endothelial growth factor: molecular and biological aspects.,” *Current topics in microbiology and immunology*, vol. 237, pp. 1-30, Jan. 1999.

[60] M. Presta, D. Moscatelli, J. Joseph-Silverstein, and D. B. Rifkin, “Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration.,” *Molecular and cellular biology*, vol. 6, no. 11, pp. 4060-6, Nov. 1986.

[61] F. Bussolino et al., “Role of soluble mediators in angiogenesis.,” *European journal of cancer (Oxford, England : 1990)*, vol. 32A, no. 14, pp. 2401-12, Dec. 1996.

[62] J. R. Jackson, M. P. Seed, C. H. Kircher, D. A. Willoughby, and J. D. Winkler, “The codependence of angiogenesis and chronic inflammation.,” *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, vol. 11, no. 6, pp. 457-65, May 1997.

[63] M. S. Pepper, “Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity.,” *Cytokine & growth factor reviews*, vol. 8, no. 1, pp. 21-43, Mar. 1997.

[64] Y. Sato et al., “Indispensable role of tissue-type plasminogen activator in growth factor-dependent tube formation of human microvascular endothelial cells in vitro.,” *Experimental cell research*, vol. 204, no. 2, pp. 223-9, Feb. 1993.

[65] R. S. Bar, M. Boes, B. L. Dake, B. A. Booth, S. A. Henley, and A. Sandra, “Insulin, insulin-like growth factors, and vascular endothelium.,” *The American journal of medicine*, vol. 85, no. 5A, pp. 59-70, Nov. 1988.

[66] M. P. Keane and R. M. Strieter, “The role of CXC chemokines in the regulation of angiogenesis.,” *Chemical immunology*, vol. 72, pp. 86-101, Jan. 1999.

[67] P. Dentelli et al., “Human IL-3 stimulates endothelial cell motility and promotes in vivo new vessel formation.,” *Journal of immunology (Baltimore, Md. : 1950)*, vol. 163, no. 4, pp. 2151-9, Aug. 1999.

[68] T. O. Daniel, H. Liu, J. D. Morrow, B. C. Crews, and L. J. Marnett, “Thromboxane A2 is a mediator of cyclooxygenase-2-dependent endothelial

migration and angiogenesis.," *Cancer research*, vol. 59, no. 18, pp. 4574-7, Sep. 1999.

[69] J. Badet, "Angiogenin, a potent mediator of angiogenesis. Biological, biochemical and structural properties.," *Pathologie-biologie*, vol. 47, no. 4, pp. 345-51, Apr. 1999.

[70] H. W. Schnaper, K. A. McGowan, S. Kim-Schulze, and M. C. Cid, "Oestrogen and endothelial cell angiogenic activity.," *Clinical and experimental pharmacology & physiology*, vol. 23, no. 3, pp. 247-50, Mar. 1996.

[71] P. Rooney, S. Kumar, J. Ponting, and M. Wang, "The role of hyaluronan in tumour neovascularization (review).," *International journal of cancer. Journal international du cancer*, vol. 60, no. 5, pp. 632-6, Mar. 1995.

[72] M. Slevin, J. Krupinski, S. Kumar, and J. Gaffney, "Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation.," *Laboratory investigation; a journal of technical methods and pathology*, vol. 78, no. 8, pp. 987-1003, Aug. 1998.

[73] M. Rusnati et al., "Interaction of fibroblast growth factor-2 (FGF-2) with free gangliosides: biochemical characterization and biological consequences in endothelial cell cultures.," *Molecular biology of the cell*, vol. 10, no. 2, pp. 313-27, Feb. 1999.

[74] D. Ribatti et al., "Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo.," *Blood*, vol. 93, no. 8, pp. 2627-36, Apr. 1999.

[75] A. E. Koch, M. M. Halloran, C. J. Haskell, M. R. Shah, and P. J. Polverini, "Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1.," *Nature*, vol. 376, no. 6540, pp. 517-9, Aug. 1995.

[76] M. Nguyen, N. A. Strubel, and J. Bischoff, “A role for sialyl Lewis-X/A glycoconjugates in capillary morphogenesis.,” *Nature*, vol. 365, no. 6443, pp. 267-9, Sep. 1993.

[77] A. J. Hayes, W. Q. Huang, J. Mallah, D. Yang, M. E. Lippman, and L. Y. Li, “Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells.,” *Microvascular research*, vol. 58, no. 3, pp. 224-37, Nov. 1999.

[78] T. I. Koblizek, C. Weiss, G. D. Yancopoulos, U. Deutsch, and W. Risau, “Angiopoietin-1 induces sprouting angiogenesis in vitro.,” *Current biology : CB*, vol. 8, no. 9, pp. 529-32, Apr. 1998.

[79] J. Folkman, “Angiogenesis in cancer, vascular, rheumatoid and other disease.,” *Nature medicine*, vol. 1, no. 1, pp. 27-31, Jan. 1995.

[80] S. Liekens, E. De Clercq, and J. Neyts, “Angiogenesis: regulators and clinical applications.,” *Biochemical pharmacology*, vol. 61, no. 3, pp. 253-70, Feb. 2001.

[81] N. Ferrara et al., “Vascular endothelial growth factor is essential for corpus luteum angiogenesis.,” *Nature medicine*, vol. 4, no. 3, pp. 336-40, Mar. 1998.

[82] A. E. Koch, “Review: angiogenesis: implications for rheumatoid arthritis.,” *Arthritis and rheumatism*, vol. 41, no. 6, pp. 951-62, Jun. 1998.

[83] N. Ferrara and K. Alitalo, “Clinical applications of angiogenic growth factors and their inhibitors.,” *Nature medicine*, vol. 5, no. 12, pp. 1359-64, Dec. 1999.

[84] J. Powell, “Update on hemangiomas and vascular malformations.,” *Current opinion in pediatrics*, vol. 11, no. 5, pp. 457-63, Oct. 1999.

[85] D. Hanahan, R. A. Weinberg, and S. Francisco, “The Hallmarks of Cancer Review University of California at San Francisco,” *Hormone Research*, vol. 100, pp. 57-70, 2000.

[86] G. Gasparini, “The rationale and future potential of angiogenesis inhibitors in neoplasia.,” *Drugs*, vol. 58, no. 1, pp. 17-38, Jul. 1999.

[87] J. Folkman, “Incipient angiogenesis.,” *Journal of the National Cancer Institute*, vol. 92, no. 2, pp. 94-5, Jan. 2000.

[88] V. Baeriswyl and G. Christofori, “The angiogenic switch in carcinogenesis.,” *Seminars in cancer biology*, vol. 19, no. 5, pp. 329-37, Oct. 2009.

[89] P. Carmeliet et al., “Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis.,” *Nature*, vol. 394, no. 6692, pp. 485-90, Jul. 1998.

[90] B. H. Jiang, F. Agani, A. Passaniti, and G. L. Semenza, “V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression.,” *Cancer research*, vol. 57, no. 23, pp. 5328-35, Dec. 1997.

[91] P. H. Burri and V. Djonov, “Intussusceptive angiogenesis--the alternative to capillary sprouting.,” *Molecular aspects of medicine*, vol. 23, no. 6S, pp. S1-27, Dec. 2002.

[92] P. H. Burri, R. Hlushchuk, and V. Djonov, “Intussusceptive angiogenesis: its emergence, its characteristics, and its significance.,” *Developmental dynamics : an official publication of the American Association of Anatomists*, vol. 231, no. 3, pp. 474-88, Nov. 2004.

[93] F. Hillen and A. W. Griffioen, “Tumour vascularization : sprouting angiogenesis and beyond,” *Cancer*, no. August, pp. 489-502, 2007.

[94] N. Ferrara, H.-P. Gerber, and J. LeCouter, “The biology of VEGF and its receptors.,” *Nature medicine*, vol. 9, no. 6, pp. 669-76, Jun. 2003.

[95] R. K. Jain, “Molecular regulation of vessel maturation.,” *Nature medicine*, vol. 9, no. 6, pp. 685-93, Jun. 2003.

[96] T. Asahara et al., “Isolation of putative progenitor endothelial cells for angiogenesis.,” *Science (New York, N.Y.)*, vol. 275, no. 5302, pp. 964-7, Feb. 1997.

[97] C. Kalka et al., “Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 7, pp. 3422-7, Mar. 2000.

[98] Y. Lin, D. J. Weisdorf, A. Solovey, and R. P. Hebbel, “Origins of circulating endothelial cells and endothelial outgrowth from blood.,” *The Journal of clinical investigation*, vol. 105, no. 1, pp. 71-7, Jan. 2000.

[99] T. Asahara et al., “VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells.,” *The EMBO journal*, vol. 18, no. 14, pp. 3964-72, Jul. 1999.

[100] K. Hattori et al., “Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment.,” *Nature medicine*, vol. 8, no. 8, pp. 841-9, Aug. 2002.

[101] D. Lyden et al., “Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth.,” *Nature medicine*, vol. 7, no. 11, pp. 1194-201, Nov. 2001.

[102] M. R. Machein, S. Renninger, E. de Lima-Hahn, and K. H. Plate, “Minor contribution of bone marrow-derived endothelial progenitors to the vascularization of murine gliomas.,” *Brain pathology (Zurich, Switzerland)*, vol. 13, no. 4, pp. 582-97, Oct. 2003.

[103] B. Larrivée et al., “Minimal contribution of marrow-derived endothelial precursors to tumor vasculature.,” *Journal of immunology (Baltimore, Md.: 1950)*, vol. 175, no. 5, pp. 2890-9, Sep. 2005.

[104] P. Wesseling, J. A. van der Laak, H. de Leeuw, D. J. Ruiter, and P. C. Burger, “Quantitative immunohistological analysis of the microvasculature in untreated

human glioblastoma multiforme. Computer-assisted image analysis of whole-tumor sections.," *Journal of neurosurgery*, vol. 81, no. 6, pp. 902-9, Dec. 1994.

[105] F. Pezzella et al., "Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis.," *The American journal of pathology*, vol. 151, no. 5, pp. 1417-23, Nov. 1997.

[106] J. Holash et al., "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF.," *Science (New York, N.Y.)*, vol. 284, no. 5422, pp. 1994-8, Jun. 1999.

[107] L. Zhang et al., "Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer.," *Cancer research*, vol. 63, no. 12, pp. 3403-12, Jun. 2003.

[108] B. Döme, S. Paku, B. Somlai, and J. Tímár, "Vascularization of cutaneous melanoma involves vessel co-option and has clinical significance.," *The Journal of pathology*, vol. 197, no. 3, pp. 355-62, Jul. 2002.

[109] E. S. Kim et al., "Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11399-404, Aug. 2002.

[110] R. Folberg et al., "The prognostic value of tumor blood vessel morphology in primary uveal melanoma.," *Ophthalmology*, vol. 100, no. 9, pp. 1389-98, Sep. 1993.

[111] M. J. C. Hendrix, E. A. Seftor, A. R. Hess, and R. E. B. Seftor, "Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma.," *Nature reviews. Cancer*, vol. 3, no. 6, pp. 411-21, Jun. 2003.

[112] E. K. Rofstad, H. Rasmussen, K. Galappathi, B. Mathiesen, K. Nilsen, and B. A. Graff, "Hypoxia promotes lymph node metastasis in human melanoma

xenografts by up-regulating the urokinase-type plasminogen activator receptor.," *Cancer research*, vol. 62, no. 6, pp. 1847-53, Mar. 2002.

[113] B. Bedogni, S. M. Welford, D. S. Cassarino, B. J. Nickoloff, A. J. Giaccia, and M. B. Powell, "The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation.," *Cancer cell*, vol. 8, no. 6, pp. 443-54, Dec. 2005.

[114] S. M. Rybak et al., "'Vasocrine' formation of tumor cell-lined vascular spaces: implications for rational design of antiangiogenic therapies.," *Cancer research*, vol. 63, no. 11, pp. 2812-9, Jun. 2003.

[115] D. W. J. van der Schaft et al., "Tumor cell plasticity in Ewing sarcoma, an alternative circulatory system stimulated by hypoxia.," *Cancer research*, vol. 65, no. 24, pp. 11520-8, Dec. 2005.

[116] M. S. Pepper and M. Skobe, "Lymphatic endothelium: morphological, molecular and functional properties.," *The Journal of cell biology*, vol. 163, no. 2, pp. 209-13, Oct. 2003.

[117] A. Kaipainen et al., "Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 8, pp. 3566-70, Apr. 1995.

[118] V. Joukov et al., "A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities.," *The Journal of biological chemistry*, vol. 273, no. 12, pp. 6599-602, Mar. 1998.

[119] S. A. Stacker et al., "VEGF-D promotes the metastatic spread of tumor cells via the lymphatics.," *Nature medicine*, vol. 7, no. 2, pp. 186-91, Feb. 2001.

[120] L. K. Chang et al., "Dose-dependent response of FGF-2 for lymphangiogenesis.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 32, pp. 11658-63, Aug. 2004.

[121] T. J. Lawley and Y. Kubota, “Induction of morphologic differentiation of endothelial cells in culture.,” *The Journal of investigative dermatology*, vol. 93, no. 2 Suppl, p. 59S-61S, Aug. 1989.

[122] S. Kanzawa, H. Endo, and N. Shioya, “Improved in vitro angiogenesis model by collagen density reduction and the use of type III collagen.,” *Annals of plastic surgery*, vol. 30, no. 3, pp. 244-51, Mar. 1993.

[123] E. A. Kruger et al., “Endostatin inhibits microvessel formation in the ex vivo rat aortic ring angiogenesis assay.,” *Biochemical and biophysical research communications*, vol. 268, no. 1, pp. 183-91, Feb. 2000.

[124] E. A. Kruger, P. H. Duray, D. K. Price, J. M. Pluda, and W. D. Figg, “Approaches to preclinical screening of antiangiogenic agents.,” *Seminars in oncology*, vol. 28, no. 6, pp. 570-6, Dec. 2001.

[125] V. R. Muthukkaruppan, B. L. Shinneers, R. Lewis, S. J. Park, B. J. Baechler, and R. Auerbach, “The chick embryo aortic arch assay: a new, rapid, quantifiable in vitro method for testing the efficacy of angiogenic and anti-angiogenic factors in a three-dimensional, serum-free organ culture system.,” *Proc. Am. Assoc. Cancer Res.*, no. 41, p. 65, 2000.

[126] J. Folkman, “Angiogenesis: initiation and control.,” *Annals of the New York Academy of Sciences*, vol. 401, pp. 212-27, Jan. 1982.

[127] C. a Staton, M. W. R. Reed, and N. J. Brown, “A critical analysis of current in vitro and in vivo angiogenesis assays.,” *International journal of experimental pathology*, vol. 90, no. 3, pp. 195-221, Jun. 2009.

[128] H. B. Vogel and R. G. Berry, “Chorioallantoic membrane heterotransplantation of human brain tumors.,” *International journal of cancer. Journal international du cancer*, vol. 15, no. 3, pp. 401-8, Mar. 1975.

[129] D. H. Ausprunk and J. Folkman, “Vascular injury in transplanted tissues. Fine structural changes in tumor, adult, and embryonic blood vessels.,” *Virchows Archiv. B: Cell pathology*, no. 1, pp. 31-44, Jul. 1976.

[130] M. A. Gimbrone, R. S. Cotran, S. B. Leapman, and J. Folkman, “Tumor growth and neovascularization: an experimental model using the rabbit cornea.,” *Journal of the National Cancer Institute*, vol. 52, no. 2, pp. 413-27, Feb. 1974.

[131] V. Muthukkaruppan and R. Auerbach, “Angiogenesis in the mouse cornea.,” *Science (New York, N.Y.)*, vol. 205, no. 4413, pp. 1416-8, Sep. 1979.

[132] T. Oikawa et al., “Effects of cytogenin, a novel microbial product, on embryonic and tumor cell-induced angiogenic responses in vivo.,” *Anticancer research*, vol. 17, no. 3C, pp. 1881-6.

[133] H. D. Papenfuss, J. F. Gross, M. Intaglietta, and F. A. Treese, “A transparent access chamber for the rat dorsal skin fold.,” *Microvascular research*, vol. 18, no. 3, pp. 311-8, Nov. 1979.

[134] B. Endrich, K. Asaishi, A. Götz, and K. Messmer, “Technical report--a new chamber technique for microvascular studies in unanesthetized hamsters.,” *Research in experimental medicine. Zeitschrift für die gesamte experimentelle Medizin einschliesslich experimenteller Chirurgie*, vol. 177, no. 2, pp. 125-34, Jan. 1980.

[135] H. A. Lehr, M. Leunig, M. D. Menger, D. Nolte, and K. Messmer, “Dorsal skinfold chamber technique for intravital microscopy in nude mice.,” *The American journal of pathology*, vol. 143, no. 4, pp. 1055-62, Oct. 1993.

[136] K. Stoletov, V. Montel, R. D. Lester, S. L. Gonias, and R. Klemke, “High-resolution imaging of the dynamic tumor cell–vascular interface in transparent zebrafish,” *Proceedings of the National Academy of Sciences*, vol. 104, no. 44, p. 17406, 2007.

[137] S. Nicoli, D. Ribatti, F. Cotelli, and M. Presta, “Mammalian Tumor Xenografts Induce Neovascularization in Zebrafish Embryos,” *Cancer*, no. 7, pp. 2927-2931, 2007.

[138] M. Haldi, C. Ton, W. L. Seng, and P. McGrath, “Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses

and stimulate angiogenesis in zebrafish.," *Angiogenesis*, vol. 9, no. 3, pp. 139-51, Jan. 2006.

[139] M. C. Mihm, W. H. Clark, and R. J. Reed, "The clinical diagnosis of malignant melanoma.," *Seminars in oncology*, vol. 2, no. 2, pp. 105-18, Jun. 1975.

[140] R. L. Barnhill, K. Fandrey, M. A. Levy, M. C. Mihm, and B. Hyman, "Angiogenesis and tumor progression of melanoma. Quantification of vascularity in melanocytic nevi and cutaneous malignant melanoma.," *Laboratory investigation; a journal of technical methods and pathology*, vol. 67, no. 3, pp. 331-7, Sep. 1992.

[141] H. Erhard, F. J. Rietveld, M. C. van Altena, E. B. Bröcker, D. J. Ruiter, and R. M. de Waal, "Transition of horizontal to vertical growth phase melanoma is accompanied by induction of vascular endothelial growth factor expression and angiogenesis.," *Melanoma research*, vol. 7 Suppl 2, pp. S19-26, Aug. 1997.

[142] C. H. Graham, J. Rivers, R. S. Kerbel, K. S. Stankiewicz, and W. L. White, "Extent of vascularization as a prognostic indicator in thin (< 0.76 mm) malignant melanomas.," *The American journal of pathology*, vol. 145, no. 3, pp. 510-4, Sep. 1994.

[143] A. Srivastava, P. Laidler, R. P. Davies, K. Horgan, and L. E. Hughes, "The prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma. A quantitative histologic study.," *The American journal of pathology*, vol. 133, no. 2, pp. 419-23, Nov. 1988.

[144] O. Straume, H. B. Salvesen, and L. A. Akslen, "Angiogenesis is prognostically important in vertical growth phase melanomas.," *International journal of oncology*, vol. 15, no. 3, pp. 595-9, Sep. 1999.

[145] T. Vlaykova et al., "Prognostic value of tumour vascularity in metastatic melanoma and association of blood vessel density with vascular endothelial growth factor expression.," *Melanoma research*, vol. 9, no. 1, pp. 59-68, Feb. 1999.

[146] M. M. Chaturvedi, B. Sung, V. R. Yadav, R. Kannappan, and B. B. Aggarwal, “NF-κB addiction and its role in cancer: ‘one size does not fit all’.,” *Oncogene*, vol. 30, no. 14, pp. 1615-30, Apr. 2011.

[147] S. Prasad, J. Ravindran, and B. B. Aggarwal, “NF-kappaB and cancer: how intimate is this relationship.,” *Molecular and cellular biochemistry*, vol. 336, no. 1–2, pp. 25-37, Mar. 2010.

[148] T. D. Gilmore, “RELevant gene amplification in B-cell lymphomas?,” *Blood*, vol. 103, no. 8, pp. 3243-3245, Apr. 2004.

[149] E. N. Hatada et al., “The ankyrin repeat domains of the,” vol. 89, no. March, pp. 2489-2493, 1992.

[150] Z. W. Li et al., “The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis.,” *The Journal of experimental medicine*, vol. 189, no. 11, pp. 1839-45, Jun. 1999.

[151] V. Dixit and T. W. Mak, “NF-kappaB signaling. Many roads lead to madrid.,” *Cell*, vol. 111, no. 5, pp. 615-9, Nov. 2002.

[152] M. Karin and Y. Ben-Neriah, “Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity.,” *Annual review of immunology*, vol. 18, pp. 621-63, Jan. 2000.

[153] S. Ghosh and M. Karin, “Missing pieces in the NF-kappaB puzzle.,” *Cell*, vol. 109 Suppl, pp. S81-96, Apr. 2002.

[154] H. J. Coope et al., “CD40 regulates the processing of NF-kB2 p100 to p52,” vol. 21, no. 20, pp. 5375-5385, 2002.

[155] E. Claudio, K. Brown, S. Park, H. Wang, and U. Siebenlist, “BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells.,” *Nature immunology*, vol. 3, no. 10, pp. 958-65, Oct. 2002.

[156] G. Xiao, E. W. Harhaj, and S. C. Sun, “NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100.,” *Molecular cell*, vol. 7, no. 2, pp. 401-9, Feb. 2001.

[157] U. Senftleben et al., “Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway.,” *Science (New York, N.Y.)*, vol. 293, no. 5534, pp. 1495-9, Aug. 2001.

[158] M. Naumann and C. Scheidereit, “Activation of NF-kappa B in vivo is regulated by multiple phosphorylations.,” *The EMBO journal*, vol. 13, no. 19, pp. 4597-607, Oct. 1994.

[159] H. Sakurai, H. Chiba, H. Miyoshi, T. Sugita, and W. Toriumi, “IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain.,” *The Journal of biological chemistry*, vol. 274, no. 43, pp. 30353-6, Oct. 1999.

[160] S. E. McNulty, N. B. Tohidian, and F. L. Meyskens, “RelA, p50 and inhibitor of kappa B alpha are elevated in human metastatic melanoma cells and respond aberrantly to ultraviolet light B.,” *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society*, vol. 14, no. 6, pp. 456-65, Dec. 2001.

[161] P. Dhawan and A. Richmond, “A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells.,” *The Journal of biological chemistry*, vol. 277, no. 10, pp. 7920-8, Mar. 2002.

[162] S. E. McNulty, R. del Rosario, D. Cen, F. L. Meyskens, and S. Yang, “Comparative expression of NFkappaB proteins in melanocytes of normal skin vs. benign intradermal naevus and human metastatic melanoma biopsies.,” *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society*, vol. 17, no. 2, pp. 173-80, Apr. 2004.

[163] J. Yang and A. Richmond, “Constitutive IkappaB kinase activity correlates with nuclear factor-kappaB activation in human melanoma cells.,” *Cancer research*, vol. 61, no. 12, pp. 4901-9, Jun. 2001.

[164] D. G. Uffort, E. A. Grimm, and J. A. Ellerhorst, “NF-kappaB mediates mitogen-activated protein kinase pathway-dependent iNOS expression in human melanoma.,” *The Journal of investigative dermatology*, vol. 129, no. 1, pp. 148-54, Jan. 2009.

[165] Y. Ueda and A. Richmond, “NF-kappaB activation in melanoma.,” *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society*, vol. 19, no. 2, pp. 112-24, Apr. 2006.

[166] N. Hayward, “New developments in melanoma genetics.,” *Current oncology reports*, vol. 2, no. 4, pp. 300-6, Jul. 2000.

[167] J. W. Rocco and D. Sidransky, “p16(MTS-1/CDKN2/INK4a) in cancer progression.,” *Experimental cell research*, vol. 264, no. 1, pp. 42-55, Mar. 2001.

[168] J. Bartkova et al., “The p16-cyclin D/Cdk4-pRb pathway as a functional unit frequently altered in melanoma pathogenesis.,” *Cancer research*, vol. 56, no. 23, pp. 5475-83, Dec. 1996.

[169] E. R. Sauter et al., “Cyclin D1 is a candidate oncogene in cutaneous melanoma.,” *Cancer research*, vol. 62, no. 11, pp. 3200-6, Jun. 2002.

[170] J. Utikal, M. Udart, U. Leiter, R. U. Peter, and G. Krähn, “Additional Cyclin D(1) gene copies associated with chromosome 11 aberrations in cutaneous malignant melanoma.,” *International journal of oncology*, vol. 26, no. 3, pp. 597-605, Mar. 2005.

[171] T. M. Becker et al., “Impaired inhibition of NF-kappaB activity by melanoma-associated p16INK4a mutations.,” *Biochemical and biophysical research communications*, vol. 332, no. 3, pp. 873-9, Jul. 2005.

[172] B. Wolff and M. Naumann, “INK4 cell cycle inhibitors direct transcriptional inactivation of NF-kappaB.,” *Oncogene*, vol. 18, no. 16, pp. 2663-6, Apr. 1999.

[173] Y. Ueda and A. Richmond, “NF-jB activation in melanoma,” *Cell*, 2006.

[174] A. P. Albino et al., “Mutation and expression of the p53 gene in human malignant melanoma.,” *Melanoma research*, vol. 4, no. 1, pp. 35-45, Feb. 1994.

[175] T. Papp et al., “Mutational analysis of N-ras, p53, CDKN2A (p16(INK4a)), p14(ARF), CDK4, and MC1R genes in human dysplastic melanocytic naevi.,” *Journal of medical genetics*, vol. 40, no. 2, p. E14, Feb. 2003.

[176] P. Ghiorzo et al., “INK4/ARF germline alterations in pancreatic cancer patients.,” *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, vol. 15, no. 1, pp. 70-8, Jan. 2004.

[177] H. Rizos, A. P. Darmanian, E. A. Holland, G. J. Mann, and R. F. Kefford, “Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF.,” *The Journal of biological chemistry*, vol. 276, no. 44, pp. 41424-34, Nov. 2001.

[178] L. Gu, N. Zhu, H. W. Findley, W. G. Woods, and M. Zhou, “Identification and characterization of the IKKalpha promoter: positive and negative regulation by ETS-1 and p53, respectively.,” *The Journal of biological chemistry*, vol. 279, no. 50, pp. 52141-9, Dec. 2004.

[179] J. T. Celebi, I. Shendrik, D. N. Silvers, and M. Peacocke, “Identification of PTEN mutations in metastatic melanoma specimens.,” *Journal of medical genetics*, vol. 37, no. 9, pp. 653-7, Sep. 2000.

[180] H. Tsao, M. C. Mihm, and C. Sheehan, “PTEN expression in normal skin, acquired melanocytic nevi, and cutaneous melanoma.,” *Journal of the American Academy of Dermatology*, vol. 49, no. 5, pp. 865-72, Nov. 2003.

[181] X. Li and G. R. Stark, “NFkappaB-dependent signaling pathways.,” *Experimental hematology*, vol. 30, no. 4, pp. 285-96, Apr. 2002.

[182] N. Sizemore, N. Lerner, N. Dombrowski, H. Sakurai, and G. R. Stark, “Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B.,” *The Journal of biological chemistry*, vol. 277, no. 6, pp. 3863-9, Feb. 2002.

[183] J. L. Norris and A. S. Baldwin, “Oncogenic Ras enhances NF-kappaB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways.,” *The Journal of biological chemistry*, vol. 274, no. 20, pp. 13841-6, May 1999.

[184] C. Castelli et al., “Expression of interleukin 1 alpha, interleukin 6, and tumor necrosis factor alpha genes in human melanoma clones is associated with that of mutated N-RAS oncogene.,” *Cancer research*, vol. 54, no. 17, pp. 4785-90, Sep. 1994.

[185] C. Gaggioli et al., “HGF induces fibronectin matrix synthesis in melanoma cells through MAP kinase-dependent signaling pathway and induction of Egr-1.,” *Oncogene*, vol. 24, no. 8, pp. 1423-33, Feb. 2005.

[186] J. Wittbrodt, R. Lammers, B. Malitschek, a Ullrich, and M. Schartl, “The Xmrk receptor tyrosine kinase is activated in Xiphophorus malignant melanoma.,” *The EMBO journal*, vol. 11, no. 11, pp. 4239-46, Nov. 1992.

[187] J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, 2001.

[188] I. A. N. K. Quigley, “Pigment Pattern Formation in Zebrafish : A Model for Developmental Genetics and the Evolution of Form,” vol. 455, no. February, pp. 442- 455, 2002.

[189] N. D. Lawson and B. M. Weinstein, “In vivo imaging of embryonic vascular development using transgenic zebrafish,” 2002.

[190] R. Cao, L. D. E. Jensen, I. Söll, G. Hauptmann, and Y. Cao, “Hypoxia-induced retinal angiogenesis in zebrafish as a model to study retinopathy.,” *PloS one*, vol. 3, no. 7, p. e2748, Jan. 2008.

[191] C. K. Griffith et al., “Diffusion limits of an in vitro thick prevascularized tissue.,” *Tissue engineering*, vol. 11, no. 1–2, pp. 257-66.

[192] R. S. Kerbel, “Tumor angiogenesis: past, present and the near future.,” *Carcinogenesis*, vol. 21, no. 3, pp. 505-15, Mar. 2000.

[193] J. Wittbrodt, R. Lammers, B. Malitschek, A. Ullrich, and M. Schartl, “The Xmrk receptor tyrosine kinase is activated in Xiphophorus malignant melanoma.,” *The EMBO journal*, vol. 11, no. 11, pp. 4239-46, Nov. 1992.

[194] M. S. de Lorenzo et al., “Altered tumor angiogenesis and metastasis of B16 melanoma in transgenic mice overexpressing tissue inhibitor of metalloproteinases-1.,” *In vivo (Athens, Greece)*, vol. 17, no. 1, pp. 45-50.

[195] F. Guadagni, P. Ferroni, R. Palmirotta, I. Portarena, V. Formica, and M. Roselli, “Review. TNF/VEGF cross-talk in chronic inflammation-related cancer initiation and progression: an early target in anticancer therapeutic strategy.,” *In vivo (Athens, Greece)*, vol. 21, no. 2, pp. 147-61.

[196] T. A. Libermann and D. Baltimore, “Activation of interleukin-6 gene expression through the NF-kappa B transcription factor.,” *Molecular and cellular biology*, vol. 10, no. 5, pp. 2327-34, May 1990.

[197] K. M. Wilczynska et al., “A novel mechanism of tissue inhibitor of metalloproteinases-1 activation by interleukin-1 in primary human astrocytes.,” *The Journal of biological chemistry*, vol. 281, no. 46, pp. 34955-64, Nov. 2006.

[198] J. M. O’Shea and N. D. Perkins, “Regulation of the RelA (p65) transactivation domain.,” *Biochemical Society transactions*, vol. 36, no. Pt 4, pp. 603-8, Aug. 2008.

[199] F. H. Sarkar, Y. Li, Z. Wang, and D. Kong, “NF-kappaB signaling pathway and its therapeutic implications in human diseases.,” *International reviews of immunology*, vol. 27, no. 5, pp. 293-319, Jan. 2008.

[200] D. a Chan, T. L. a Kawahara, P. D. Sutphin, H. Y. Chang, J.-T. Chi, and A. J. Giaccia, “Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment.,” *Cancer cell*, vol. 15, no. 6, pp. 527-38, Jun. 2009.

[201] S. Huang, A. Deguzman, C. D. Bucana, and I. J. Fidler, “Nuclear factor-kappaB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice,” *Clin. Cancer Res.*, vol. 6, pp. 2573-2581, 2000.

[202] S. Kuphal, I. Poser, C. Jobin, C. Hellerbrand, and A. K. Bosserhoff, “Loss of E-cadherin leads to upregulation of NFjB activity in malignant melanoma,” *Oncogene*, pp. 8509-8519, 2004.

[203] S. Kuphal, A. Winklmeier, C. Warnecke, and A.-K. Bosserhoff, “Constitutive HIF-1 activity in malignant melanoma.,” *European journal of cancer (Oxford, England : 1990)*, vol. 46, no. 6, pp. 1159-69, Apr. 2010.

[204] M. Zheng, S. Ekmekcioglu, E. T. Walch, C.-hui Tang, and E. A. Grimm, “Inhibition of nuclear factor-jB and nitric oxide by curcumin induces G 2 / M cell cycle arrest and apoptosis in human melanoma cells,” *Melanoma Research*, pp. 165-171.

[205] A. Hartmann et al., “Hypoxia-induced up-regulation of angiogenin in human malignant melanoma.,” *Cancer research*, vol. 59, no. 7, pp. 1578-83, Apr. 1999.

[206] G. H. Mahabeleshwar and T. V. Byzova, “Angiogenesis in melanoma.,” *Seminars in oncology*, vol. 34, no. 6, pp. 555-65, Dec. 2007.

[207] M. K. Schaafhausen et al., “Tumor angiogenesis is caused by single melanoma cells in a manner dependent on reactive oxygen species and NF-κB.,” *Journal of cell science*, vol. 126, no. Pt 17, pp. 3862-72, Sep. 2013.

[208] S. Ugurel, G. Rappl, W. Tilgen, and U. Reinhold, “Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival.,” *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, vol. 19, no. 2, pp. 577-83, Jan. 2001.

[209] D. G. Stupack and D. A. Cheresh, “Integrins and angiogenesis.,” *Current topics in developmental biology*, vol. 64, pp. 207-38, Jan. 2004.

[210] E. Kerkelä and U. Saarialho-Kere, “Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer.,” *Experimental dermatology*, vol. 12, no. 2, pp. 109-25, Apr. 2003.

[211] U. B. Hofmann, J. R. Westphal, G. N. Van Muijen, and D. J. Ruiter, “Matrix metalloproteinases in human melanoma.,” *The Journal of investigative dermatology*, vol. 115, no. 3, pp. 337-44, Sep. 2000.

[212] H. Nagase, R. Visse, and G. Murphy, “Structure and function of matrix metalloproteinases and TIMPs.,” *Cardiovascular research*, vol. 69, no. 3, pp. 562-73, Feb. 2006.

[213] C. M. Overall and C. López-Otín, “Strategies for MMP inhibition in cancer: innovations for the post-trial era.,” *Nature reviews. Cancer*, vol. 2, no. 9, pp. 657-72, Sep. 2002.

[214] B. Anand-Apte et al., “A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth.,” *Biochemistry and cell biology = Biochimie et biologie cellulaire*, vol. 74, no. 6, pp. 853-62, Jan. 1996.

[215] M. Bloomston, A. Shafii, E. Zervos, and A. S. Rosemurgy, “TIMP-1 antisense gene transfection attenuates the invasive potential of pancreatic cancer cells in vitro and inhibits tumor growth in vivo.,” *American journal of surgery*, vol. 189, no. 6, pp. 675-9, Jun. 2005.

[216] Q. Ebrahem, A. Minamoto, G. Hoppe, B. Anand-Apte, and J. E. Sears, “Triamcinolone acetonide inhibits IL-6- and VEGF-induced angiogenesis downstream of the IL-6 and VEGF receptors.,” *Investigative ophthalmology & visual science*, vol. 47, no. 11, pp. 4935-41, Nov. 2006.

[217] a a Wani, S. M. Jafarnejad, J. Zhou, and G. Li, “Integrin-linked kinase regulates melanoma angiogenesis by activating NF-κB/interleukin-6 signaling pathway.,” *Oncogene*, no. July 2010, pp. 1-11, Jan. 2011.

[218] J. L. Norris and A. S. Baldwin, “Oncogenic Ras enhances NF-kappaB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways.,” *The Journal of biological chemistry*, vol. 274, no. 20, pp. 13841-6, May 1999.

[219] K. I. Amiri and A. Richmond, “Role of nuclear factor-kappa B in melanoma.,” *Cancer metastasis reviews*, vol. 24, no. 2, pp. 301-13, Jun. 2005.

[220] D. Koul, Y. Yao, J. L. Abbruzzese, W. K. Yung, and S. A. Reddy, “Tumor suppressor MMAC/PTEN inhibits cytokine-induced NFkappaB activation without interfering with the IkappaB degradation pathway.,” *The Journal of biological chemistry*, vol. 276, no. 14, pp. 11402-8, Apr. 2001.

[221] L. I. Flinder, O. A. Timofeeva, C. M. Rosseland, L. Wierød, H. S. Huitfeldt, and E. Skarpen, “EGF-induced ERK-activation downstream of FAK requires rac1-NADPH oxidase.,” *Journal of cellular physiology*, vol. 226, no. 9, pp. 2267-78, Sep. 2011.

[222] A. C. Bharti, N. Donato, S. Singh, and B. B. Aggarwal, “Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis.,” *Blood*, vol. 101, no. 3, pp. 1053-62, Feb. 2003.

[223] K. Gao, D. L. Dai, M. Martinka, and G. Li, “Prognostic significance of nuclear factor-kappaB p105/p50 in human melanoma and its role in cell migration.,” *Cancer research*, vol. 66, no. 17, pp. 8382-8, Sep. 2006.

[224] S. Jimi et al., “Modulation by bovine angiogenin of tubular morphogenesis and expression of plasminogen activator in bovine endothelial cells.,” *Biochemical and biophysical research communications*, vol. 211, no. 2, pp. 476-83, Jun. 1995.

[225] J. W. Fett, J. L. Bethune, and B. L. Vallee, “Induction of angiogenesis by mixtures of two angiogenic proteins, angiogenin and acidic fibroblast growth factor, in the chick chorioallantoic membrane.,” *Biochemical and biophysical research communications*, vol. 146, no. 3, pp. 1122-31, Aug. 1987.

[226] J. W. Fett et al., “Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells.,” *Biochemistry*, vol. 24, no. 20, pp. 5480-6, Sep. 1985.

[227] D. J. Strydom, “The angiogenins.,” *Cellular and molecular life sciences : CMLS*, vol. 54, no. 8, pp. 811-24, Aug. 1998.

[228] G. F. Hu, S. I. Chang, J. F. Riordan, and B. L. Vallee, “An angiogenin-binding protein from endothelial cells.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 6, pp. 2227-31, Mar. 1991.

[229] G. F. Hu, J. F. Riordan, and B. L. Vallee, “A putative angiogenin receptor in angiogenin-responsive human endothelial cells.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 6, pp. 2204-9, Mar. 1997.

[230] G. F. Hu, D. J. Strydom, J. W. Fett, J. F. Riordan, and B. L. Vallee, “Actin is a binding protein for angiogenin.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 4, pp. 1217-21, Feb. 1993.

[231] J. Moroianu, J. W. Fett, J. F. Riordan, and B. L. Vallee, “Actin is a surface component of calf pulmonary artery endothelial cells in culture.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 9, pp. 3815-9, May 1993.

[232] a Wiedłocha, “Following angiogenin during angiogenesis: a journey from the cell surface to the nucleolus.,” *Archivum immunologiae et therapiae experimentalis*, vol. 47, no. 5, pp. 299-305, Jan. 1999.

[233] Z.-ping Xu, T. Tsuji, J. F. Riordan, and G.-fu Hu, “The nuclear function of angiogenin in endothelial cells is related to rRNA production.,” *Biochemical and biophysical research communications*, vol. 294, no. 2, pp. 287-92, Jun. 2002.

[234] X. Gao and Z. Xu, “Mechanisms of action of angiogenin Functions and Mechanisms of ANG in,” *Acta Biochimica et Biophysica Hungarica*, pp. 619-624, 2008.

[235] K. Kishimoto, S. Liu, T. Tsuji, K. A. Olson, and G.-F. Hu, “Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis.,” *Oncogene*, vol. 24, no. 3, pp. 445-56, Jan. 2005.

[236] G. Hu, J. F. Riordan, and B. L. Vallee, “Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 25, pp. 12096-100, Dec. 1994.

[237] F. Soncin, “Angiogenin supports endothelial and fibroblast cell adhesion.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 6, pp. 2232-6, Mar. 1992.

[238] G. Hu, J. F. Riordan, and B. L. Vallee, “Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 25, pp. 12096-100, Dec. 1994.

[239] C. H. Heldin, “Dimerization of cell surface receptors in signal transduction.,” *Cell*, vol. 80, no. 2, pp. 213-23, Jan. 1995.

[240] G. F. Hu, “Neomycin inhibits angiogenin-induced angiogenesis.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 17, pp. 9791-5, Aug. 1998.

[241] D. Hanahan and R. A. Weinberg, “Hallmarks of Cancer: The Next Generation,” *Cell*, vol. 144, no. 5, pp. 646-674, Mar. 2011.

5.2 List of figures

5.3 List of tables

5.4 List of abbreviations

A549	Alveolar basal epithelial cells
ALM	Acral lentiginous melanoma
ANG	Angiogenin
ANG-1	Angiopoietin 1
ARF (p14)	Alternate reading frame (ARF) product of the CDKN2A locus
BAFFR	B-cell activating factor receptor
BCL-3	B-cell lymphoma 3-encoded protein
BCL-X	B-cell chronic lymphocytic leukemia X
BF	Bright field
BRAF	V-Raf murine sarcoma viral oncogene homolog B1
BSA	Bovine serum albumin
$Ca(NO_3)_2$	Calcium nitrate
CAM	Chick chorioallantoic membrane assay
CD40R	Member of the TNF-receptor superfamily
CDK	Cyclin dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
COX-2	Cyclooxygenase-2
c-REL	V-rel reticuloendotheliosis viral oncogene homolog
ddH_2O	double dest. Water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1b promoter	Synthetic basal promoter derived from the carp β-actin gene
EGF	Epidermal growth factor
EGFP	Enhanced GFP
EGFR	Epidermal growth factor receptor
ENA-78	Epithelial neutrophil-activating protein 78
EPC's	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinases (MAPK)
E-selectin	Cell adhesion molecule expressed only on endothelial cells
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
fli promoter	Hemiangioblast specific promotor
Fyn	FAK-binding protein
Gal 4 PV16	Fusion of the yeast Gal4-DNA-binding domain with the herpes simplex virus transcriptional activation domain VP16
GCR	G-protein coupled receptor
G-CSF	Granulocyte-Colony Stimulating Factor
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
Grb2	Growth factor binding protein-2

GRO	Chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2 and CXCL3
GTP	Guanosine-5'-triphosphate
hEGF	Human epidermal growth factor
hEGFR	Human epidermal growth factor receptor
HEK293	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERmrk (Hm)	Extracellular part of human EGFR (HER) is fused to the intracellular part of Xmrk (mrk)
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor 1 alpha
hpi	Hours post injection
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
I-309	Human cytokine I-309 is a small glycoprotein secreted by activated T lymphocytes and structurally related to a number of inflammatory cytokines
IFNα	Interferon α
IFNγ	Interferon γ
IGF-I	Insulin-like growth factor 1
IGF-II	Insulin-like growth factor 2
IKK (IκB)	Inhibitor of nuclear factor kappa-B kinase
IKKα (IκBα)	Inhibitor of nuclear factor kappa-B kinase subunit α
IKKβ (IκBβ)	Inhibitor of nuclear factor kappa-B kinase subunit β
IKKγ (IκBγ)	Inhibitor of nuclear factor kappa-B kinase subunit gamma (NEMO)
IKKε (IκBε)	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-12 p70	Biologically active 70 kDa (p70) form of Interleukin 12
IL-13	Interleukin 13
IL-1a	Interleukin 1α
IL-1α/β	Interleukin 1α/β
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
INK4A (p16)	Inhibitor of cyclin–dependent kinase
I-TAC	Interferon-inducible T-cell alpha chemoattractant
KCl	Potassium chloride
LMM	Lentigo maligna melanoma
LTβR	Lymphohotoxin-β receptor
MAPK	Mitogene activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MCP-3	Monocyte chemotactic protein-3
MCP-4	Monocyte chemoattractant protein-4

MDM-2	Mouse double minute 2 homolog (MDM2) also known as E3 ubiquitin-protein ligase Mdm2
MEK	Mitogen-activated protein kinase kinase
Melan-a Hm	Mouse melanocytes transfected with HERmrk (Hm)
MgCl	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MIG	Monokine induced by gamma interferon
MITF	Microphthalmia transcription factor
MKP-1	MAP kinase phosphatase 1
MMP	Matrix metalloproteinase
MMP-1MM	Matrix metalloproteinase-1
MMP-9	Matrix metalloproteinase-9
mRNA	Messenger RNA
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NEMO	NF-κB essential modifier (IKKγ)
NF-κB	Nuclear factor kappa B
NIK	NF-κB-inducing kinase
NM	Nodular melanoma
O_2	Oxygen
OPN	Osteopontin
p100	Nuclear factor NF-kappa-B p100 subunit of NF-κB1
p105	Nuclear factor NF-kappa-B p105 subunit of NF-κB2
p50	Nuclear factor NF-kappa-B p50 subunit of NF-κB1
p52	Nuclear factor NF-kappa-B p52 subunit of NF-κB2
p53	p53 tumor suppressor protein
pA	SV40 polyadenylation site
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF-BB	Platelet-derived growth factor composed of two B (-BB) chains
PDK-1	Phosphoinositide-dependent-kinase-1
PECAM-1	Platelet endothelial cell adhesion molecule
pepmrk	Polyclonal anti-mrk recognizing the C- terminal part of Xmrk
PF-4	Platelet factor 4
PI3-K	phosphatidylinositol 3-kinase
PIGF	Placental growth factor
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
PKB/AKT	Protein kinase B
PTEN	Phosphatase and tensin homologue
RAF	Rapidly Accelerated Fibrosarcoma proteins (serine/threonine-specific protein kinases that are related to retroviral oncogenes)
RANTES	Regulated on activation normal T cell expressed and secreted
RAS	Rat sarcoma proteins (small GTPases)

RB	Retinoblastoma protein
REL family proteins	Reticuloendotheliosis (REL) family proteins
RELA (p65)	V-rel reticuloendotheliosis viral oncogene homolog A
RELB	V-rel reticuloendotheliosis viral oncogene homolog B
RGP	Radial growth phase
RHD	REL homology domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SCF	Stem-cell factor
SDS	Sodium dodecyl sulfate
SOS	Son of sevenless
SSM	Spreading malignant melanoma
STAT5	Signal transducer and activator of transcription 5
TAD	Transactivation domain
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TIE-2	Angiopoietin-1 receptor (TEK)
TIMP-1	TIMP metallopeptidase inhibitor 1
TIMP-2	TIMP metallopeptidase inhibitor 2
TNFα	Tumor necrosis factor α
TRAF3	Tumor necrosis factor receptor-associated factor 3
UAS	Upstream activating sequence
uPAR	Urokinase receptor
UV	Ultraviolet
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VEGF-D	Vascular endothelial growth factor D, needed for the development of Lymphatic vasculature surrounding lung bronchioles
VEGFR-3 (Flt-4)	Fms-related tyrosine kinase 4
VGP	Vertical growth phase
XE tumors	Xantoerythrophoromas
XMRK	Xiphophorus melanoma receptor kinase
ZM322881	VEGFR2-inhibitor

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ERKLÄRUNG

Gemäß §4, Abs. 3, Ziff. 3, 5 und 8

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Würzburg, März 2014

Maximilian K. Schaafhausen