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Animal Models of Angiogenesis and Lymphangiogenesis

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1. Introduction

Blood and lymphatic vessels are present in all tissues, and play important roles for their function, homeostasis and maintenance. Angiogenesis, the growth of new blood vessels, is therefore highly important during development, but is largely not observed in the adult, except for during the female reproduction cycle and during wound healing. In pathological situations, however, angiogenesis may be turned on, and in this case contribute to the onset and progression of most severe human pathologies characterized by high mortality, including cancer, diabetes, obesity and retinopathies (Carmeliet, 2003) or is insufficiently activated such as in the case of myocardial infarction and stroke (Y Cao et al, 2005). Thus, angiogenesis is one of the largest and fastest evolving areas of research today. Angiogenesis is a highly complicated process, involving many different cell types, and it is therefore highly recommended that researchers use *in vivo* animal models for their studies. Accordingly, today there are many *in vivo* models available.

The aim of this chapter is to give insights into the most commonly used *in vivo* angiogenesis models in both mice and zebrafish. We will provide detailed descriptions and discussions of the adipose tissue-, tumor-, ischemic hind limb- wound healing- and corneal micropocket angiogenesis models in mice and developmental-, tumor-, hypoxia-induced retinal- and regenerating tail fin angiogenesis models in zebrafish. We will provide a base for comparison between the different assays to quickly identify which model is best suited for a particular research focus.

1.1 Basic mechanisms of angiogenesis

Angiogenesis is a multistep process which is tightly regulated by an intimate balance between pro- and anti-angiogenic factors. Angiogenesis is stimulated by angiogenic factors the most commonly studied being members of the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF) or platelet derived growth factor (PDGF) families in the tissue. These factors either act locally, or are

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released into the blood stream and activate angiogenesis through binding to their corresponding receptors on pre-existing endothelial cells and/or perivascular cells (Y Cao, 2008). Upon ligand binding to the receptors, a cascade of intracellular signal transduction is triggered resulting in activation of the endothelial cell and initiation of the cellular mechanisms required for angiogenesis including basal membrane degradation, endothelial cell (EC) proliferation and migration, formation of tube like structures, and finally vascular maturation by coverage with smooth muscle cells or pericytes to ensure stability (Davis & Senger, 2005; Jensen et al, 2007).

The dynamics of angiogenesis as well as the morphology of the resulting vasculature differs depending on which factor induces the process (R Cao, 2004). In the case of the most studied angiogenic factor, VEGF, a mature vessel may be exposed to a gradient of VEGF induced by hypoxia in a nearby tissue such as a tumor. VEGF activates VEGF receptor 2 (VEGFR2) on endothelial cells leading to production of matrix-metalloproteases (MMPs) which disrupts EC-pericyte contacts, degrade the basal membrane and induce a tip-cell behavior in one cell, which is allowed to form cell processes important for cell migration such as filopodia and lammelopodia. The tip cell will via lateral inhibition signal to surrounding cells that they should not adopt this phenotype thus leading to an ordered sprouting of just one or a few neovessels from the original mother-vessel. The tip cell will start migrating up the angiogenic factor gradient, but remain in contact with the underlying and proliferating endothelial cells (stalk cells) and thus retain the connection to the original vessel. At a certain point, the tip cell will meet other tip cells, anastomose – i.e. fuse – and thus form a circulation loop. The endothelial chords will lumenize, allowing blood to flow through the vessel and the vessels will mature by recruiting perivascular mural cells which are tightly associated with the endothelial cells thus providing stability and trophic factors for the endothelial cells, leading to their maturation and re-entry into quiescence (Risau 1997).

1.2 Angiogenesis in physiology and pathology

During development the first vessels – the aorta and the cardinal vein – are formed by vasculogenesis - i.e. the *de novo* formation of blood vessels by aggregation and vascular morphogenesis of single endothelial progenitor cells. Following this process, the majority of the blood vessels in the body are formed by angiogenic expansion of this initial, primitive circulation loop (Risau 1997). Thus angiogenesis is very important during development. In adults however, most tissues and therefore also the vasculature have stopped growing and instead adopted a quiescent state. In some cases such as during the female reproductive cycle and during wound healing, angiogenesis is needed to regenerate the tissue, but otherwise the erroneous or insufficient induction of angiogenesis in adults is usually associated with pathology. For example, as a tumor is a growing tissue and therefore need blood vessels for supplying energy, angiogenesis is induced after the tumor has reached a certain size (approximately 1 cubic centimetre). Similarly in growing fat tissue new blood vessels are needed when the distance from the existing blood vessels are too large for efficient oxygenation. By blocking such tumor- or adipose-angiogenesis, it is possible to inhibit the growth of these tissues and therefore anti-angiogenic drugs have potential as treatment for cancer and obesity. In patients with retinopathy retinal hypoxia is a consequence of blocked or disrupted blood vessels and

also here lead to induction of retinal angiogenesis. The newly formed blood vessels are however often immature and prone to rupture which leads to micro-hemorrhages and thereby drives the pathological progression to advanced states of the disease (Carmeliet, 2003; Folkman, 1995). On the other hand, in other hypoxic tissues such as the hypoxic heart tissue downstream of a blocked coronary artery, it is important to speed up the angiogenic revascularization of the tissue in order to secure sufficient oxygen for the highly metabolically active myocardium such that it may sustain its function (Y Cao, 2010).
These aspects of physiological and pathological angiogenesis are accurately modeled by the angiogenesis models we will discuss in this chapter.

1.3 Mouse models of angiogenesis

Mouse models are the primary *in vivo* tools used in biomedical research. Most research institutions have their own mouse or rodent facilities, and today several strains have been genetically purified through serial in-breeding, which allow sophisticated investigations such as transplantation and grafting experiments to be done in a wildtype animal with a fully functional immune system. Furthermore, genetically manipulated mice have been generated for most of the genes important for angiogenesis and vascular biology. Such mice include conditional or global knock-outs or mice over-expressing angiogenic factors in defined tissues. As the murine and human vasculatures are highly similar, these tools have been very valuable in defining the role of angiogenesis and distinct angiogenic factors in the onset and progression of various human diseases.
Here we present five mouse assays of pathological angiogenesis that have shown tremendous power in the study of mechanisms behind human diseases. These are the adipose-angiogenesis models used to study obesity and diabetes, the xenograft models used to study cancer biology, the ischemia models used to study myocardial infarction and stroke, the wound healing models and the cornea micropocket models used to study basic mechanisms of angiogenesis and lymphangiogenesis.

2. Angiogenesis in adipose tissue

2.1 Introduction

According to a report of the World Health Organization in 2009, there are currently more than one billion people overweight and more than 300 million individuals considered clinically obese. The escalating number of obese individuals is no longer a problem faced only in high income countries. This adverse trend has also been adopted by low and middle income countries. Body Mass Index (BMI) is a commonly used method to categorize overweight (BMI ≥ 25 $kg*m^{-2}$) and obese (BMI > 30 $kg*m^{-2}$) individuals (Table 1) (Kopelman, 2000). However, rather than just being a cosmetic problem or being associated with social issues, obesity is a serious pre-disease that frequently leads to the development of severe complications and metabolic disorders that often have a fatal outcome. Amongst these diseases are dyslipidemias, fatty liver, sleep apnea, cardiovascular complications, stroke, type 2 diabetes as well as certain types of cancer such as prostate, breast and colon cancer (Y Cao, 2010; Y Cao, 2007). In the affected individuals, development of metabolic disorders is frequently related to endothelial dysfunction.

BMI ($kg*m^{-2}$)	WHO classification
< 18.5	underweight
18.5 - 24.9	-
25 - 29.9	grade 1 overweight
30 - 39.9	grade 2 overweight (obese)
> 40	grade 3 overweight (morbidly obese)

Table 1. Association of Body Mass Index (BMI) with overweight and obesity. A BMI value greater than 30 indicates obesity. Adapted from Kopelman, 2000.

The adipose tissue constantly experiences expansion and regression during growth and repair throughout adulthood. Interestingly, it is known that adipose tissue growth relies on angiogenesis. Already in embryos, the formation of the primitive fat organ is preceded by angiogenesis. Moreover, anti-angiogenic therapy can prevent the expansion and even induce regression of adipose tissue (Brakenhielm et al, 2004).

The adipose tissue vasculature plays an important role in supplying oxygen and nutrients, as well as plasma containing cytokines and growth factors. Furthermore, the vasculature provides the adipose tissue with bone marrow-derived stem cells, able to differentiate into pre-adipocytes and adipocytes, endothelial cells as well as pericytes. The blood vessels also facilitate infiltration of inflammatory cells, such as monocytes and neutrophiles, and play a role in the removal of metabolic waste products. Due to the close interaction of blood vessels and adipocytes, studying angiogenesis in the adipose tissue is a promising strategy to identify potential novel targets for anti-obesity/anti-diabetic therapy and might open new avenues in the prevention and treatment of metabolic disorders in the future.

There are two types of adipose tissue in the human adult, the white adipose tissue (WAT) and the brown adipose tissue (BAT). WAT is frequently regarded as the 'bad' fat which stores excess energy in the form of triglycerides. Indeed, this notorious WAT usually amasses in undesirable parts of the body such as in the intra-abdominal area resulting in a so called 'apple-shaped' body type or the thighs or hips leading to the so called 'pear-shaped' body type. WAT is essential to serve as heat insulation, mechanical cushion and to provide energy for the body. WATs are complex tissues consisting of different cell types such as endothelial cells, pericytes, macrophages and mesenchymal cells having close interaction with one another and collaboratively regulating processes in the adipose tissue. Being an endocrine organ, the WAT produces a myriad of cytokines and angiogenic factors including vascular endothelial growth factor (VEGF), leptin, adiponectin, resistin, interleukin-6 (IL-6), IL-8, hepatocyte growth factor (HGF), angiopoietin (Ang)-1 and -2 FGF-2, estrogen, TGF-α and -β as well as MMP-2 and -9 (Brakenhielm & Y Cao, 2008). These factors interact to regulate the survival, proliferation and differentiation of pre-adipocytes to adipocytes.

Remarkably, adipose tissue is one of the most highly vascularized tissues in the human body (Fig. 1 and 5). In BAT, blood vessel density is several folds higher than in WAT. This reflects the higher metabolic activity of BAT. Adipocytes in WAT are characterized by a large diameter, a spherical morphology and a large unilocular lipid droplet which is surrounded by a thin layer of cytoplasm whereas adipocytes originating from BAT are smaller, contain multilocular lipid droplets and higher cytoplasm content. Furthermore, brown adipocytes express uncoupling protein 1 (UCP1) which has an important role in energy metabolism.

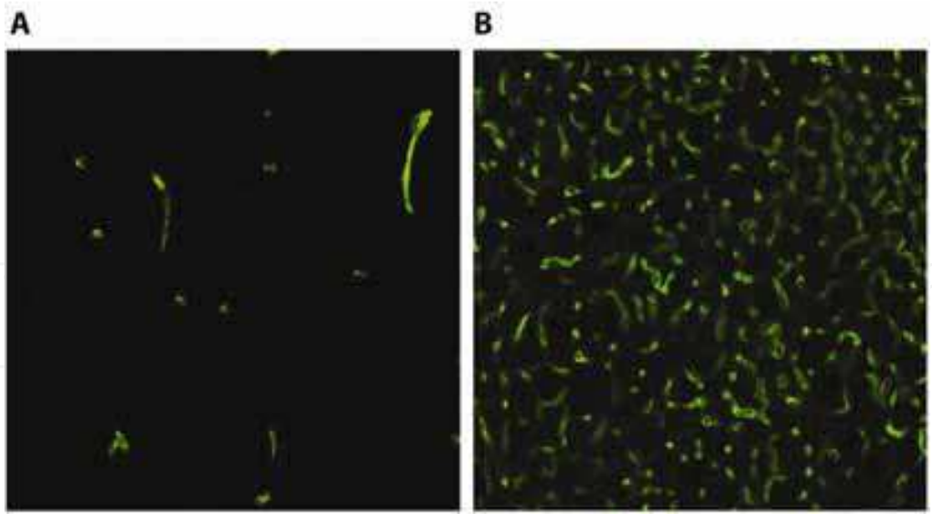


Fig. 1. Visualization of blood vessels in adipose tissue for quantitative analysis.
Fluorescence-based visualization of CD31+ endothelial cells (green) in white (A) and brown (B) adipose tissue.

Many decades ago, BAT was discovered as a thermogenic (heat generating) tissue, active in new born human babies, but it was thought to disappear or rather to be inactivated in adults (Cannon & Nedergaard, 2004). BAT is densely packed with mitochondria which explains the high metabolic status compared to white adipose tissue. In rodents, BAT is primarily located in the interscapular region, on the dorsal side between the front limbs, with minor amounts being found in the thymus, thorax and abdomen. The highly metabolically active, thermogenic BAT requires a high density of blood vessels to supply oxygen and substrates to the mitochondria and for waste removal.
In recent years, there has been accumulating evidence demonstrating the presence of active BAT in adult humans (Cypess et al, 2009; van Marken Lichtenbelt et al, 2009; Virtanen et al, 2009). Several clinical observations have shown the presence of BAT in patients, with tumors such as pheochromocytoma, following exposure to high levels of catecholamines or exposure to cold. Most research utilizes the uptake of 18F-fluoro-2-deoxygucose as a tracer in positron emission tomography (PET) and computer tomography (CT) to detect active BAT depots in adult humans. PET-CT reveals that the distribution of the BAT depot is located in the fascial plane in the ventral neck and thorax bilaterally instead of the interscapular region as seen in rodents and children. The human adult possesses approximately 10 g of BAT. If all the brown fat in the adult body was fully activated, it would be able to burn around 4.1 kg of white fat in a year. Histological studies of human BAT depots show high capillary density. Here, we address the possibilities of driving the activity of BAT, of conversion of WAT to brown-like adipose tissue and of using angiogenesis modulators to treat obesity.

2.2 Models/methods to study adipose tissue angiogenesis

To study adipose tissue angiogenesis, several mouse models including models in genetically manipulated mice, are currently available (Xue Y et al, 2010, Nat. Protoc). These models usually provide highly reproducible and robust results as the mice are inbred and therefore share a highly similar genetic background. However, in humans, the cause of developing

obesity is most frequently not genetic but rather due to overeating and a lifestyle based on high caloric intake and little physical exercise. Therefore, high-fat diet fed mouse models provide a powerful tool to study non-genetically related obesity.

2.3 Genetic models

2.3.1 Ob/ob mice

In 1950, obese mice carrying the mutation obese (ob) were described for the first time (Ingalls et al, 1950). The ob mutation was later shown to be located in the gene coding for a hormone known as leptin. Leptin is important in the regulation of appetite and food intake. Leptin signaling is mediated via binding to the leptin receptor (Ob-R) and subsequent signaling to the hypothalamus. Via this pathway, food uptake, energy expenditure as well as fat and glucose metabolism are regulated (Friedman & Halaas, 1998) (Fig. 2).

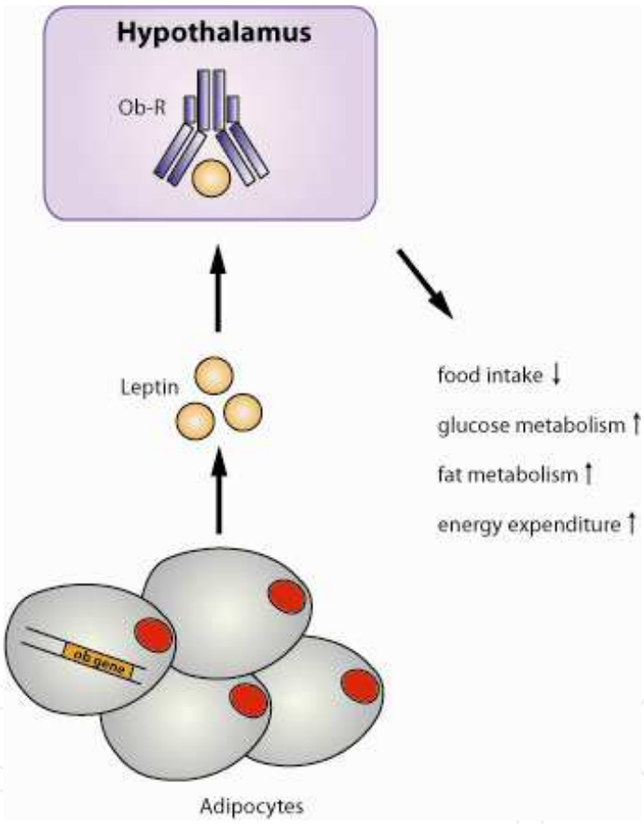


Fig. 2. Regulation of appetite and metabolism by Leptin. Leptin is the product of the *ob* gene and is expressed primarily in adipocytes. In the hypothalamus, Leptin binds to its receptor (Ob-R). It decreases food intake and increases energy expenditure, glucose as well as fat metabolism. Thereby, Leptin contributes to a satiety effect and a lean phenotype.

Due to a lack of leptin, these mice exhibit uncontrolled food intake. Constant overeating therefore results in a gain of body weight. Consequently, ob/ob mice can reach a weight that is three times higher compared to wild type littermates, and their body fat content can be elevated up to fivefold. Apart from this obvious phenotype, ob/ob mice also show decreased physical activity and energy expenditure, infertility and immune deficiencies.

Heterozygotes on the other hand do not display any phenotype as the mutation is recessive. The leptin deficient mouse can be used as an excellent model to study the role of angiogenesis in adipose tissue expansion. Obesity in these mice can be prevented by treatment with anti-angiogenic drugs (Brakenhielm et al, 2004) (this will be discussed more in depth in the Treatment section). Since these mice are comparable to morbidly obese humans regarding the obesity phenotype, using this model might be helpful to identify potential novel targets to treat obesity and obesity-related metabolic disorders in the future.

2.3.2 Db/db mice

The autosomal recessive mutation diabetes (db) was first described in 1966 in the mouse strain C57BL/KsJ (Hummel et al, 1966). These mice are deficient for the leptin receptor. Animals which are homozygous for this mutation, exhibit a phenotype that resembles human diabetes mellitus. This mutant strain is also characterized by an obese phenotype. Furthermore, homozygous mutants are infertile and hyperglycemic while heterozygotes are phenotypically indistinguishable from wild type littermates. These mice are excellent models for studying mechanisms of obesity-related diabetes and insulin insensitivity, and the role of angiogenesis in this regard.

2.4 Other mouse models of obesity

By injecting 3T3 preadipocyte cells subcutaneously into nude mice, researchers are allowed to study the close spatial and temporal correlation between neovascularization and adipose tissue development (Neels et al, 2004). 3T3 preadipocytes will differentiate into adipocytes, and start forming mature adipose tissue *in vivo*. The developing fat pad can be removed at different time points ranging from 1 to 21 days and stained for endothelial cell markers by immunohistochemistry (Fig. 3). Furthermore, the gene expression profile can be analyzed focusing on angiogenesis- as well as adipogenesis-specific genes. Due to the controlled onset and development of the fat pad in adult mice, this model also allowed for studying the origin of the cells that contribute to the formation of new blood vessels during vascularization of the growing adipose tissue.

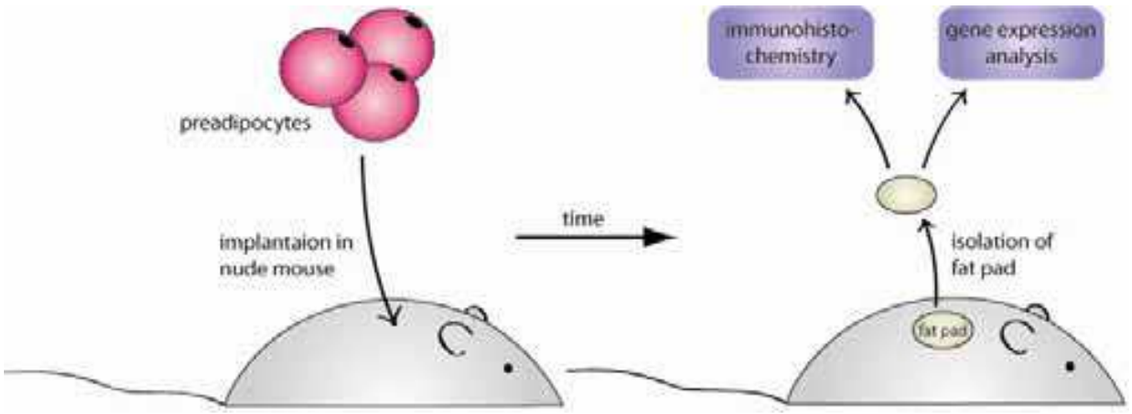


Fig. 3. A mouse model to study adipose tissue angiogenesis. In this model, 3T3 pre-adipocytes are injected into nude mice. Blood vessel formation and gene expression can be studied in the developing fat pad.

2.5 High-fat diet models

One of the first methods to obtain diet-induced obesity in rodent models was based on the so-called cafeteria diet. The cafeteria diet was first described in 1976 as a method to produce obesity in rats (Sclafani et al, 1976). Besides a nutritionally balanced diet, the rats were offered a variety of palatable food items such as cookies and chocolate, candy, salami or cheese and other foods containing high amounts of salt, sugar and fat. Interestingly, the animals ignored the nutritionally adequate chow in favor of the cafeteria food. Although this diet mimics the modern Western diet as consumed by millions of people, a major drawback of this diet is that the animals are allowed to choose independently from the available food. This self-selection may result in substantial differences in the choice of food and thereby nutrients and calories as well as the composition of protein, fat and carbohydrates consumed. Therefore, the cafeteria diet does not guarantee robust and reproducible results.

A more controlled setting to study diet-induced obesity in rodents can be achieved by providing diets in a pellet-form with a high content of fat. The typical standard chow for a laboratory mouse consists of 11.4 % of calories derived from fat, 62.8 % from carbohydrates and 25.8 % from protein, resulting in a nutritional value of 12.6 kJ/g food. A typical high-fat diet may be composed of 58 % fat, 25.6 % carbohydrate and 16.4 % protein. The high content of fat leads to a nutritional value of 23.4 kJ/g food (Winzell AM and Ahren B, 2004). Animals fed with this extreme diet elicit rapid weight gain and are prone to developing obesity.

2.6 Cold induced angiogenesis in adipose tissue

Some types of WAT in mice can acquire a BAT-like phenotype after exposure to cold temperature (4°C). Cold exposure leads to activation of the sympathetic nervous system which increases the capacity of non-shivering thermogenesis (NST) leading to increased heat production that is independent from non-productive muscle activity (shivering) (Xue et al, 2009). Acclimation of rodents to cold should be performed gradually. Mice should be adapted at 18°C for at least one week before transferring them to 4°C. The duration of adaptation is dependent on strains; genetically manipulated strains such as thermogenically incompetent UCP-1 knock-out mice require longer adaptation. Exposure of rodents to cold results in the transition of inguinal WAT to a BAT-like phenotype. Surprisingly, short term exposure (1 week) to cold is sufficient to regulate many genes involved in adipose tissue functions. For example, cold exposure results in up-regulation of BAT-related markers such as (UCP-1) and PGC-1α in the inguinal WAT. The density of blood vessels is highly correlated with the metabolic demand in the different adipose depots. During the transition from WAT to BAT-like, blood vessel density increases dramatically already after one week of cold exposure. After five weeks the WAT exhibits an even higher increase in blood vessel density. Blood vessels are constantly remodeling depending on the metabolic status of the adipose tissues. The transition of WAT to a BAT-like phenotype upon cold exposure is accompanied by the increase in pro-angiogenic factors such as VEGF. This example further demonstrates the importance of tight regulation of blood vessels and angiogenesis in adipose tissue remodeling and function.

2.7 Treatments

The major focus of biomedical research should be on interfering with physiological or pathological processes through treatment. Thus, while the models mentioned in this chapter

are valuable in studying the role of the vasculature in initiation and progression of diseases, they are also suitable for evaluation of potentially clinical benefits of novel or previously uncharacterized drugs.

2.7.1 Anti-angiogenic blockade of adipose tissue angiogenesis

Following the example of cold-induced transition from WAT to BAT-like depots described above, this change occurs in parallel with increased expression of VEGF receptors 1 and 2 coupled to increased density of blood vessels in the adipose tissue. Since VEGF-A activates signaling through binding to VEGFR1 and VEGFR2, treatment with neutralizing antibodies against VEGFR1 (MF1) or VEGFR2 (DC101) would provide mechanistic insights into the roles of these receptors. Inhibition with VEGFR2 abolishes the cold-induced vascularization, demonstrating that the VEGFR2 signaling pathway is involved in the regulation of the angiogenic switch in cold. On the other hand, inhibition with VEGFR1 (MF1) resulted in further increased angiogenesis in both WAT and BAT. This suggests that VEGFR1 could be involved in the negative regulation of angiogenesis in adipose tissues (Xue, 2009).

2.7.2 Angiogenesis inhibitors to counteract obesity

As mentioned, the expansion and regression of WAT is highly dependent on angiogenesis. Indeed, treatment with angiogenesis inhibitors, angiostatin, endostatin or thalidomide, results in the reduction of body weight in obese mice (Arbiser et al, 1999). Treatment of leptin deficient obese mice and high fat diet-fed wt C57Bl mice with TNP-470, a selective angiogenic inhibitor, has resulted in reduction of body weight and adipose tissue depot masses. The vascular density in the adipose tissue in TNP-470 treated animals was significantly lower indicating that TNP-470 exerts a direct anti-angiogenic effect on adipose tissues (Brakenhielm, 2004).

Leptin is considered to be a stimulator of angiogenesis. The secretion of leptin is proportionate to the size of adipocytes and regulated by the level of oxygen in adipose tissue. In hypoxic situations such as during hyperplasia or hypertrophy of adipocytes, VEGF protein as well as leptin levels are up-regulated thereby stimulating angiogenesis to provide adequate delivery of oxygen and nutrients to the adipocytes. Co-implantation of leptin with VEGF and FGF-2 in the avascular mouse cornea revealed a remarkable synergistic angiogenic stimulation. However, the treatment of obese individuals with leptin remains controversial. Despite that leptin stimulates angiogenesis, administration of leptin to individuals with a homozygous mutation in leptin genes confer beneficial outcome in terms of body weight reduction (Frederich et al, 1995).

2.8 Methods and assays to study blood vessels in tissues

The models mentioned in this chapter are further strengthened by (usually post mortem) analysis of the tissue in which the blood vessels are growing. In pathological situations the blood vessels are usually of poor quality and functionality, which greatly contributes to progression of the disease. In this section we will discuss several histological and functional tests that can be done to gain additional insight into the structure and quality of the blood vessels.

2.8.1 Hematoxylin/Eosin staining

For Hematoxylin/Eosin (HE) staining of tissues, it is recommended that sections with paraffin-embedded tissue of a thickness of 3 – 5 μm for interscapular BAT (iBAT) and

inguinal WAT (iWAT), respectively, are used. For non-adipose tissue types such as tumor, cornea, muscle or dermal tissues, a thickness 5 μm is recommended. Following de-paraffinization with xylene and rehydration using 99.7 %, 95 % and 70 % solutions of ethanol, the slides are stained for 3-5 min. with hematoxylin. This results in a clear blue/purple staining of the nuclei of the cells. Eosin is then used to stain the cytoplasmic contents of the cells pink/red. Depending on the different compartments within the cell, different shades of blue to pink can be observed (Fig. 4). With this method, the adipose tissue can be studied with regard to the size of adipocytes. Blood vessels however, cannot be visualized using this method.

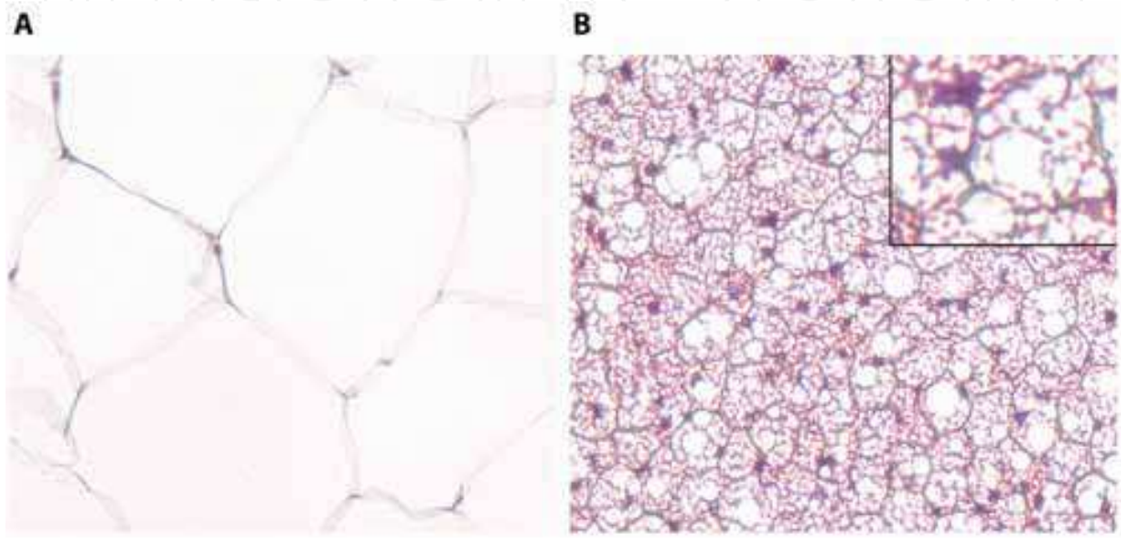


Fig. 4. Analysis of adipocytes by hematoxylin and eosin staining in white (A) and brown (B) adipose tissue. Nuclei are stained with hematoxylin (purple) and cytoplasmic components with eosin (pink).

2.8.2 Whole mount immunohistochemistry

The use of whole mount immunohistochemistry allows investigations of the vasculature in adipose and other tissues, especially in regard to its structure and functionality.

Different primary antibodies can be used to visualize endothelial cells. These antibodies target for example CD31, CD34 or isolectin all of which are expressed on endothelial cells. This provides a general overview of the vasculature in the tissue and its structure, i.e. whether the vessels are organized or disorganized, if their diameter is normal or if they are dilated and also gives information on the presence of microvessels and capillaries that might have been newly formed (Fig. 5). The tissue vascularity can be assessed by calculating the area of stained vessel signals per field. However, it should be noted that an increase of adipocyte size might imply that the number of vessels decreased even if that is not the case. This is due to the fact that with increasing adipocyte size the area per field that can be covered by vessels decreases. However, the ratio of vessels per adipocyte might not have been changed. In order to take this issue into account, it is recommended to calculate vessel number per adipocyte.

To study pericyte coverage, using an anti-NG2 antibody can provide insights into vessel maturation. Early premature vessels show a low pericyte coverage index and are therefore

prone to leakiness and can be considered less functional. To gain even further knowledge regarding vessel functionality, perfusion and leakiness, tetramethyl-rhodamine dextran can be used. Perfusion is studied by injecting 2000 kDa dextran, leakiness can be investigated by injecting 70 kDa dextran via the tail vein. The tissue is then fixed in 4 % paraformaldehyde (PFA) and the vasculature is counter-stained with anti-CD31 antibody using a non-red color secondary antibody (Fig. 6).

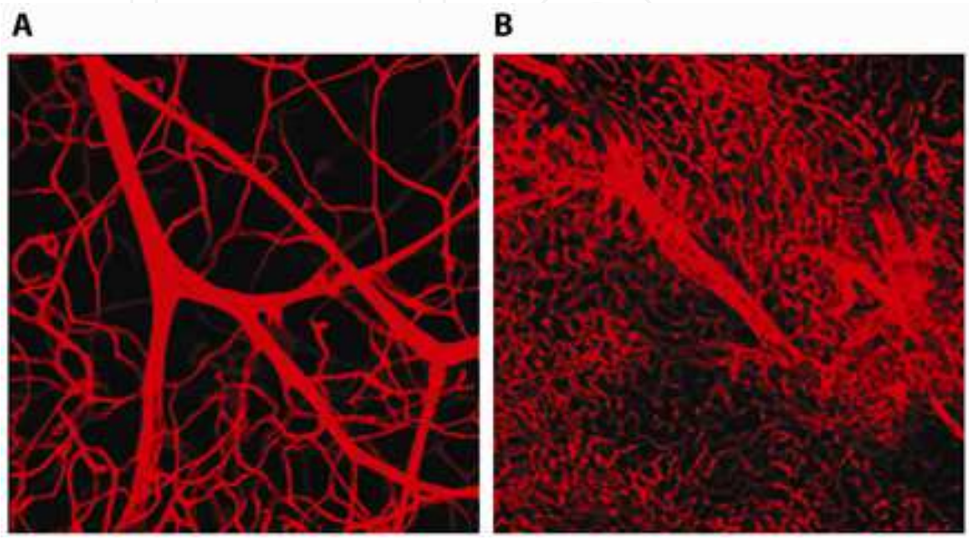


Fig. 5. Visualization of endothelial cells with the marker CD31 in whole mount immunohistochemistry. CD31+ cells (red color) are detected with a fluorochrome-linked secondary antibody in white (A) and brown (B) adipose tissue.

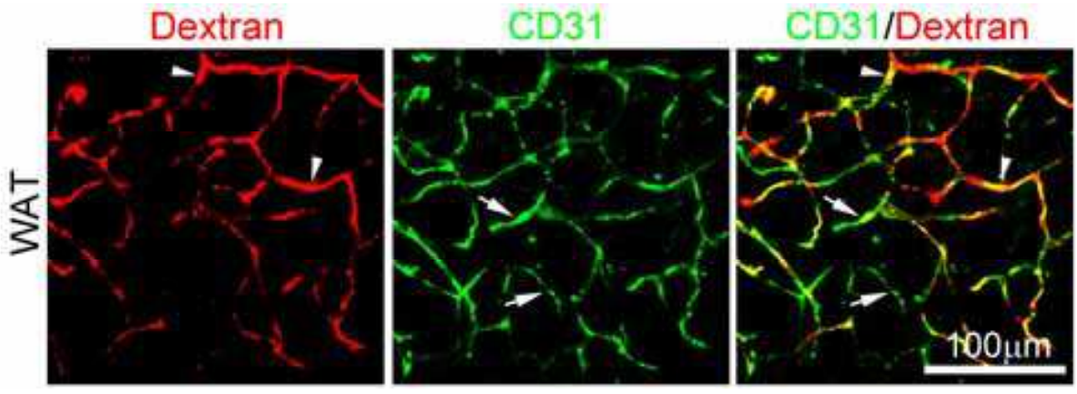


Fig. 6. Functionality test of the vasculature in adipose tissue. Dextran (red) was injected via the tail vein and perfused white adipose tissue (WAT) of a C57Bl mouse was further stained with anti-CD31 antibody (green). Perfused vessels are indicated with arrowheads and non-perfused vessels are indicated with arrows. Scale bar = 100 µm.

Whole mount staining also allows the investigation of lymphatic vessels in adipose and other tissues. Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), podoplanin or VEGFR-3 are common markers which can be targeted by specific antibodies to visualize lymphatic vessels (Fig. 13).

2.8.3 Immunohistochemistry on paraffin sections

While whole mount immunohistochemistry is a valuable tool to study vascular structure and functionality, different methods are advantageous to draw quantitative conclusions regarding the number of blood vessels per adipocyte. For this purpose, the use of thin sections of paraffin-embedded adipose tissue is recommended. In this approach, in contrast to whole mount immunohistochemistry where the thickness of the tissue varies as it is cut by hand, rather uniform tissue sections are prepared using a microtome. Immunohistochemistry on paraffin sections is based on the same principle as whole mount immunohistochemistry: the tissue is incubated with a primary antibody against a protein of interest. A fluorochrome or horse radish peroxidase (HRP)-labeled secondary antibody is then used to visualize the protein (Fig. 7 and 8).

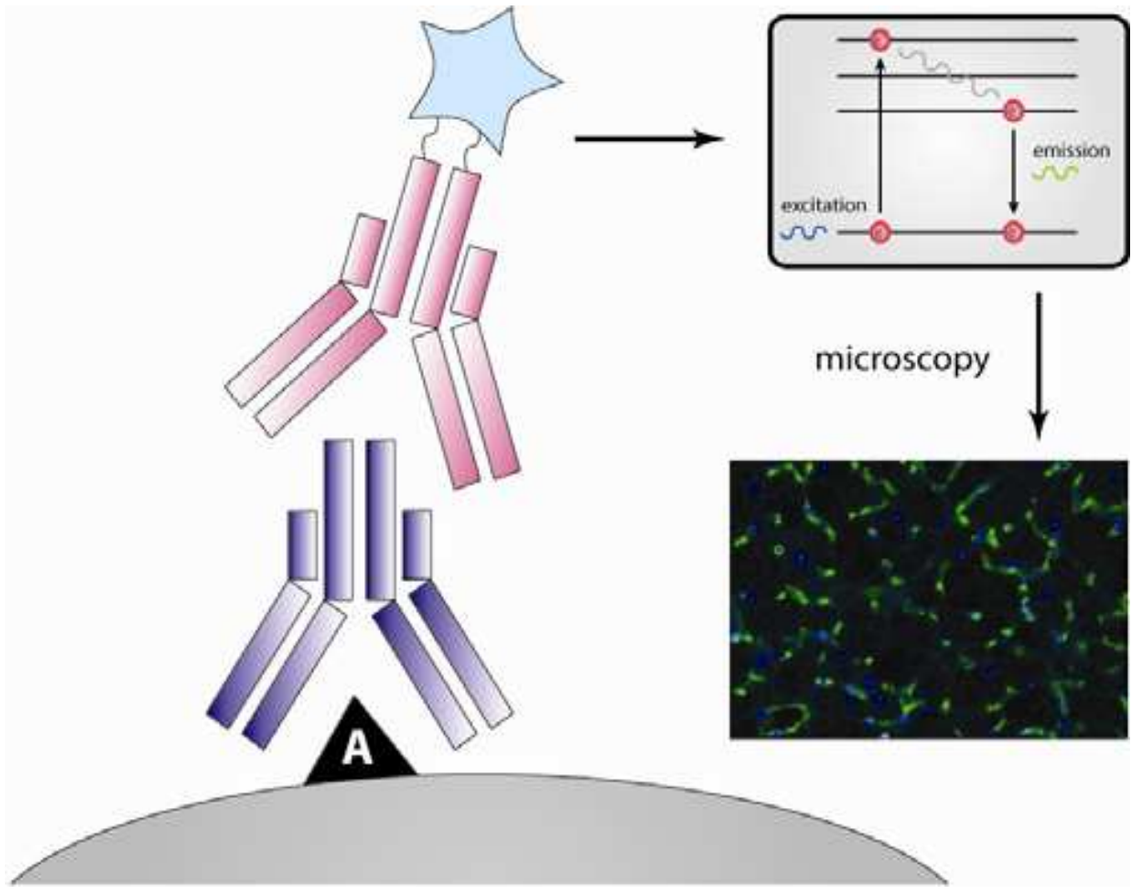


Fig. 7. Schematic representation of immunohistochemistry on paraffin sections using fluorescence-based detection of signals.

It is helpful to counterstain the tissue with DAPI, propidium iodide or Hoechst (for fluorescent stainings) or hematoxylin (for chromogenic HRP stainings) to visualize the nuclei of cells and thereby provide additional information on the structure of the tissue.

To study active angiogenesis, proliferating endothelial cells can be visualized by double staining with antibodies against an endothelial cell marker, such as CD31, and a proliferation marker, such as PCNA or Ki67. Another option is injection of 5-bromo-2'-

deoxyuridine (BrdU) into the tail vain of a mouse 1 min prior to sacrificing the animal. This synthetic thymidine analogue is incorporated into the DNA of proliferating cells. Using an anti-BrdU antibody thereby allows for detection of actively replicating cells.

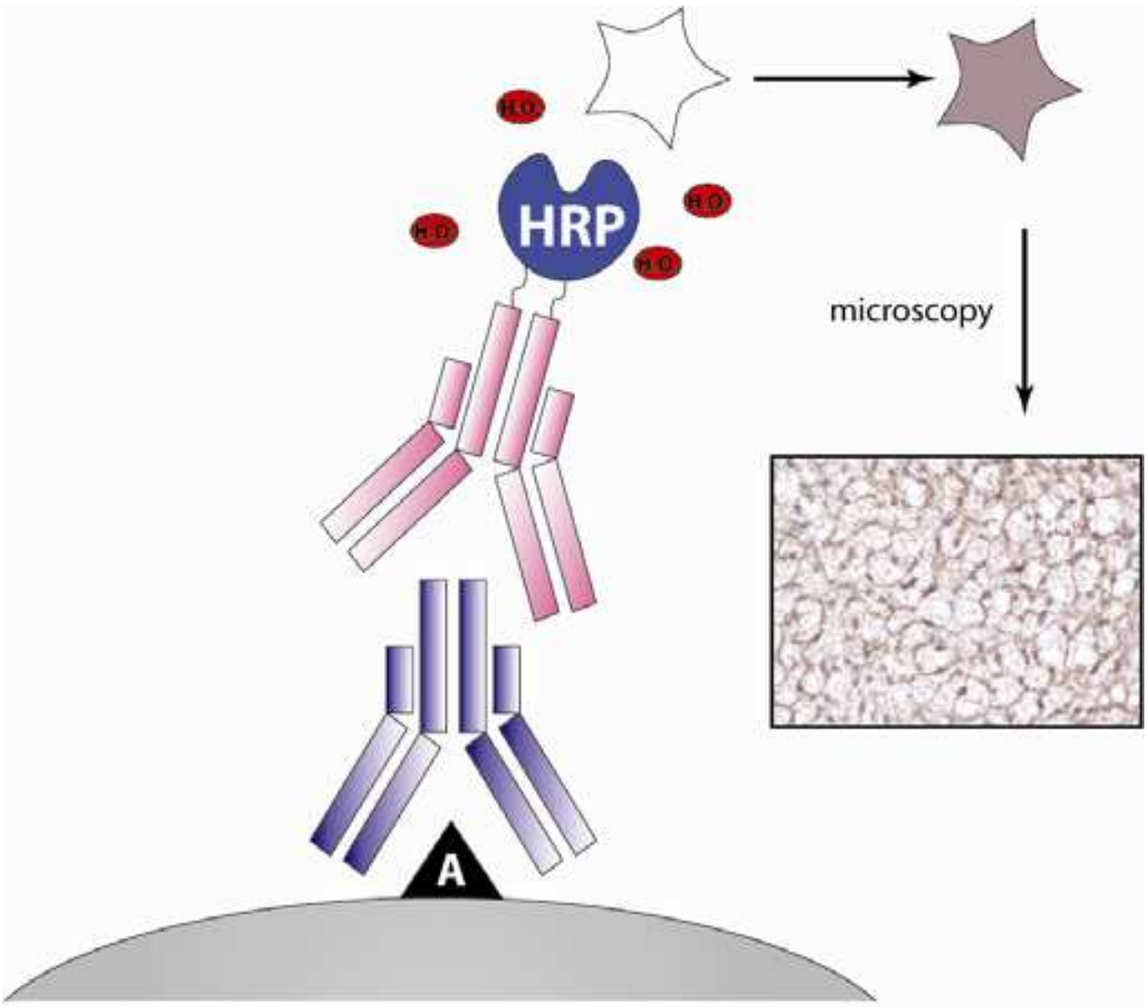


Fig. 8. Schematic representation of immunohistochemistry on paraffin sections using chromogenic detection of signals. The organic compound 3,3'-Diaminobenzidine is oxidized by HRP resulting in a brown color.

2.8.4 Immunohistochemisty on cryosections

Some antigens are masked by paraffin fixation, and in these cases staining has to be done on non-fixed, frozen tissues instead. Dissected adipose or other tissues should be embedded immediately in a plastic cryomold, snap frozen on dry ice and stored at -80°C until staining. Sectioning of cryo-embedded adipose tissue is slightly more challenging than other tissues due to its rather soft integrity. Hence, is it critical to lower the temperature of the cryotome to -30°C before sectioning cryo-embedded adipose tissue

samples. Thin sections of 15 μm should be adhered on Superfrost Plus microscope slides, subsequently fixed with cold acetone and stained with specific primary antibodies. However, it is important to note, when staining for lymphatic vessels in the adipose tissue that LYVE-1 is not as widespread on the lymphatic endothelium as podoplanin in this particular tissue (Fig. 9). Here, unlike other tissues, LYVE-1 staining mainly detects inflammatory cells.

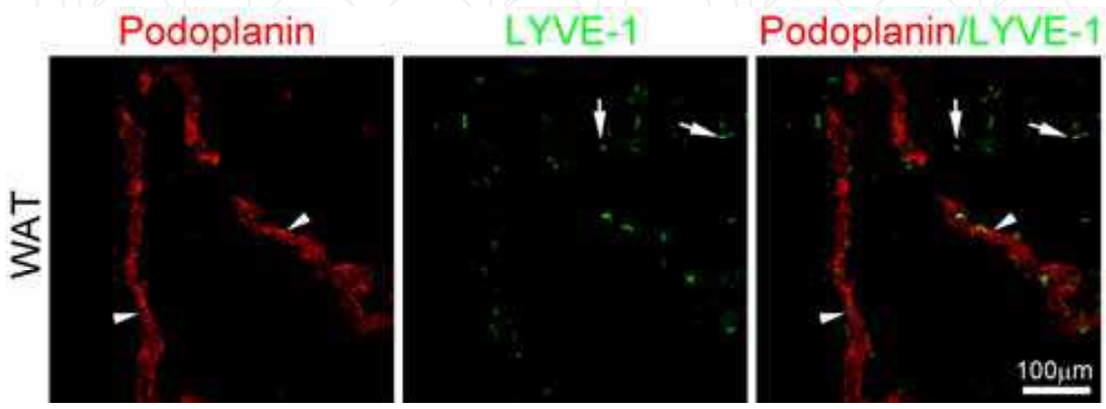


Fig. 9. Double cryosection immunostaining of the WAT of a mouse with podoplanin (red) and LYVE-1 (green). Podoplanin-positive lymphatic staining is indicated with arrowheads and LYVE-1-positive non- lymphatic staining is indicated with arrows.
Scale bar = 100 μm.

2.8.5 Hypoxia staining

Since hypoxia is one of the driving forces of angiogenesis, detection of the presence of hypoxia in tissues could also be performed. Hypoxyprobe-1 (pimonidazole hydrochloride) staining can be used to detect cell and tissue hypoxia. Pimonidazole hydrochloride has a molecular weight of 290.8 kD, ultraviolet absorbance at 324 nm and a plasma half-life of approximately 25 minutes in mice. The detection of hypoxia in tissues can be performed by intravenous or intraperitoneal injection or oral ingestion of pimonidazole hydrochloride at a dosage of 60 mg/kg, 15-90 minutes before sacrificing mice. Dissected tissues should be fixed in 4% PFA followed by paraffin embedding. Paraffin-embedded tissue sections of 3-5 μm is further stained using a peroxidase conjugated anti-pimonidazole antibodies and counterstained with nuclei staining.

3. Tumor models

3.1 Tumor models in general

Mouse tumor-angiogenesis models include xenograft/transplant models and spontaneous models in genetically engineered mice. Xenograft/transplant models are the most widely used, due to the homogeneous and fast onset and progression of the disease (Y Cao, 2005). In these models, human tumor cells are transplanted into athymic nude mice or severely compromised immunodeficient (SCID) mice (xenograft model), or murine tumor cells are transplanted into mice of the same genetic background (homologous transplantation model). Tumor cells can be injected under the skin (heterotypic transplantation, except skin

cancer) or into the same organ from which the tumors originate (orthotropic transplantation). It is important to remember when studying tumor angiogenesis that the tumor microvasculature might differ depending on the implantation sites. Orthotropic transplantation is considered to show a similar tumor vessel phenotype as the primary lesion, and thus more closely recapitulate the clinical situation. However, it is often easier and more accurate to follow the tumor growth in heterotypic models such as when the tumor grows under the skin, where it is easily visualized. Furthermore, depending on the tumor growth rate and size, the vessel structure in tumors can be different. Fast growing and big tumors (≥ 1.0 cm^3) usually have extensive necrosis in the center, which affects the vascular network in these areas. The appropriate tumor size for evaluation of tumor angiogenesis is considered to be 0.5-0.8 cm^3. Tumor cells can be modified to express high levels of angiogenic factors, such as VEGF and FGF, or reporter genes, such as green fluorescent protein (GFP), red fluorescent protein (RFP) or luciferase. These modifications allow us to study the function of specific angiogenic factors on tumor microenvironment in association with invasion and metastasis (R Cao et al, 2004). Recent technological developments have permitted us to use genetically manipulated animal models for tumor studies, in which the mice are over-expressing or deficient in angiogenesis related genes. The majority of these mice are however immunocompetent, requiring the use of murine tumor cells in such animals.

Another type of tumor-angiogenesis models are the spontaneous models. Such models are based on genetically engineered mice, where a tumor suppressor has been deleted, an oncogene is being over-expressed or both, often in a particular cell type such as the pancreatic beta-cell of the islets of Langerhans (Hanahan, 1985). These models provide a disease history that is more closely recapitulating the one in human patients, as tumors are generated from one single hyperplastic cell, and progress through steps which are highly similar to pathological progression of pre-malignant to malignant lesions in humans. In later stages of tumor development in these models, angiogenesis also becomes important and contributes to growth and metastasis of these primary lesions (Koh et al, 2010).

To study tumor angiogenesis using mouse models, control animals or tumor conditions must be incorporated in the study. If tumor cells are genetically altered, the proper control would be tumor cells which have an empty expression vector inserted in their genome instead of one coding for a particular angiogenic factor. For evaluation of treatment efficacy of anti-angiogenic drugs, the control group must be given the vehicle (solvent) in which the drug was prepared, because some vehicles can themselves affect the vascular structure in tumors. Age and genetic background of the mice must also be standardized for the different groups. Standard age for tumor experiments is 6-10 weeks. Sex is usually not important, unless the tumor cells under investigation are gender-specific such as in the case of breast or prostate cancer, but should nonetheless be the same in for example treatment and control groups.

3.2 Tumor models: Assessment of angiogenesis

Dr. Judah Folkman proposed in 1971 that all tumor growth is angiogenesis-dependent (Folkman, 1971). In general, because a limiting factor of tumor growth is the supply of sufficient levels of oxygen and nutrition, fast growing tumors are characterized by a more aggressive angiogenic phenotype which arises from the ability of the tumor cells to secrete angiogenic growth factors to support and change their microenvironments. For instance,

tumor cells transduced with the gene coding for VEGF grow much faster than the empty-vector transduced control cells, due to much higher intra-tumoral blood vessel density (Eriksson et al, 2002). In these tumors however, the vasculature consists of highly irregular and immature vessels and many vascular plexuses, which does not support efficient perfusion of blood compared to a less chaotic vasculature in VEGF non-transduced tumor tissue (Fig. 10). To assess angiogenesis in mouse models, there are thus three essential points which should be addressed.

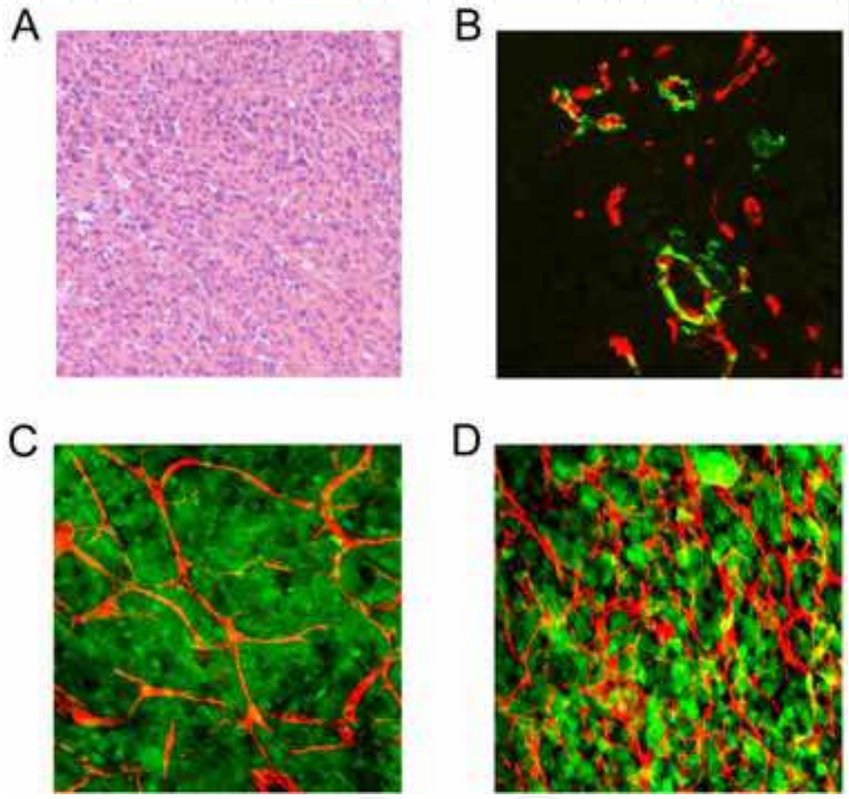


Fig. 10. Histological examinations of tumor tissue. (A) H&E staining of T241 fibrosarcoma. (B) Immunohistchemical staining of T241 fibrosarcoma visualized by fluorescence microscopy. Anti-endomucin labeling of endothelial cells is shown in red and anti-α-smooth muscle actin (SMA) labeling of pericytes/smooth muscle cells is shown in green. (C) Confocal imaging of T241 fibrosarcoma. GFP transfected tumors are shown in green, anti-CD31 labeling of endothelial cells is shown in red. (D) Confocal imaging of VEGF overexpresing T241 fibrosarcoma. GFP transfected tumors are shown in green, anti-CD31 labeling of endothelial cells is shown in red.

1. Tumor size (volume). Tumor growth depends on angiogenesis. Thus, tumor size is usually proportional to the degree of which tumor-angiogenesis is induced by the tumor. If the xenograft/transplant models are used and especially if tumors are transplanted dorsally under the skin, the size is easily measured and an accurate tumor growth curve can be generated by daily measurements of tumor width (W) and length (L) using calipers and calculating the volume (V) as $V=0.52*W^2*L$. If the tumor is not

visible because it is growing in a location inside the animal, special imaging systems, such as CT, magnetic resonance imaging (MRI) or bioluminesence imaging analysis (if tumors express luciferase) are needed to estimate the tumor size. The effect of anti-angiogenic drugs can be inferred from the reduction in tumor growth relative to vehicle treated controls.

2. Histology. Tumor tissues can be stained following the procedures listed in the section on adipose tissue angiogenesis. HE staining will give a general, structural view of the tumor, while immunohistochemical methods are used to visualize the tumor vasculature specifically. In tumors good antigens for visualizing the vasculature are for example von Willebrand Factor (vWF), CD31, CD34, endomucin, isolectin or VE-cadherin. These methods enable analysis of morphological changes and quantitative assessment of the tumor micro environment (Fig. 10). Immunohistochemical staining for multiple markers in the same sample may provide information on the localization of cells relative to each other, cell phenotypes, cell numbers, cell size, and cell conditions (i.e. apoptotic, proliferative).
3. Functional assay of tumor vessels. Tumor vasculatures are irregular and disorganized as well as leaky and poorly perfused. The stained vessels are not all functional; therefore evaluation of vessel functionality such as vessel permeability and perfusion, by using the dextran-injection method described in the adipose angiogenesis section, gives important qualitative information in all tumor models. Upon evaluation under the microscope, abluminal localization of dye indicates extravasation whereas functional vessels are characterized by retaining the dye within the vessel (Hedlund et al, 2009).

3.3 Lymphangiogenesis and lymphatic metastasis

It is well known that blood vessels can support tumor growth by providing oxygen and nutrients, and removing waste products, but the function of tumor lymphatic vessels remains poorly understood (Y Cao, 2008).

Lymphatic networks consist of lymphatic capillaries, collecting lymphatic vessels, and lymph nodes. Unlike blood vessels, lymphatic capillaries consist of one layer of lymphatic endothelial cells (LECs), discontinuous basement membrane and few vascular smooth muscle cells (VSMCs). Lymphatic vessels lack tight junctions between endothelial cells, but are instead equipped with one-way lymphatic valves which give these vessels the ability to collect fluids and macromolecules from the tissue and transport it back to the circulation. On the other hand, these features of the lymphatic endothelium also mean that malignant cells can easily enter into and disseminate via the lymphatic system, leading to lymphatic metastasis. Tumor lymphangiogenesis is therefore associated with cancer metastasis. In some common cancers, such as lung and breast cancer, lymphatic metastasis is the dominant route for tumor metastasis. Invasion of intra- or peri-tumoral lymphatics may result in dissemination of malignant cells to the lymphatic system, leading to lymphatic metastasis in regional lymph nodes (Y Cao Y, 2005; R Cao et al, 2004).

Similar to blood vessels, lymphatic vessels are quiescent in healthy individuals. The formation of lymphatic vessels in tumors is a multistep process that involves LEC proliferation, migration, tube formation and remodeling, which require up-regulation of lymphangiogenic stimulators and down-regulation of lymphangiogenic inhibitors (Y Cao, 2005). Lymphatic vessel growth may represent the imbalanced consequence between positive and negative regulators tipping toward positive regulation. Understanding the molecular mechanisms that control lymphangiogenesis is therefore an important step in

the development of therapeutic agents in the prevention and treatment of cancer metastasis.

Among the list of lymphangiogenic factors, members of the VEGF family are the best characterized. VEGF-A, which binds to VEGFR-2 and VEGFR-1, PlGF and VEGF-B which bind to VEGFR-1 and especially VEGF-C and VEGF-D which bind to VEGFR-3 are lymphangiogenic factors, the -C and -D isoforms being the most potent, which regulate both physiological and pathological lymphangiogenesis. VEGF-C/VEGF-D-VEGFR-3-mediated signals are also critical for the sprouting of the first lymphatic vessel from the developing veins in the embryo. This signaling pathway is essential for differentiation of endothelial progenitor cells into the lymphatic lineage (Kukk et al, 1996; Alitalo et al, 2005).

Primary tumors produce several lymphangiogenic factors, including VEGF-A, VEGF-C, VEGF-D, Insulin-like growth factor (IGF), hepatocyte growth factor (HGF) and PDGF-B. These factors induce angiogenesis and lymphangiogenesis both in the local environment and in the regional lymph nodes. Furthermore, they play an important role in establishing the pre-metastatic niche. The term pre-metastatic niche describes the adaptations of for example the lymph nodes which are needed to allow disseminating tumor cells, arriving at a later stage, to meet optimal conditions for growth in that particular site. In addition to signals produced by the tumor cells themselves, inflammatory cells such as macrophages are recruited to tumors by a wide range of tumor cell-derived cytokines and growth factors. At the tumor site, inflammatory cells play a critical role in mediating lymphangiogenesis most likely through the secretion of several lymphangiogenic cytokines.

To detect lymphatic vessels in or around tumors, immunohistochemical staining with lymphatic specific markers, such as VEGFR-3, LYVE-1 and podoplanin is recommended (see Fig. 13). Lymphangiogenesis can be evaluated using all of the models described in this chapter, by using antibodies against one or a combination of these factors either in whole mount staining or on thin sections of frozen or paraffin embedded tissue (Fig. 13).

4. Non-tumor, xenograft models

As tumor cells often produce a multitude of factors which may cooperate in inducing angiogenesis and lymphangiogenesis, these models are considered to be relatively "dirty" and unsuitable for studying the effects of just one or a few factors. In order to overcome this problem, a xenograft model for non-tumor angiogenesis has been developed. In this model, a matrigel plug is grafted onto the mouse which can be mixed with recombinant angiogenic factors prior to grafting.

4.1 Matrigel plug assay

Matrigel consist of purified basement membrane components (collagens, proteoglycans and laminin) and, while it is liquid at temperatures just above 0 degrees, it forms a gel when it is warmed to 37 °C. Thus, the material can be cooled and then injected in the mice, where it will form a three dimensional gel, in which host blood vessels can invade. Matrigel itself is a poor inducer of angiogenesis, but it can be mixed with angiogenic growth factors and/or cells prior to injection leading to a controllable induction of blood vessel growth into the plug. Plug vessels are usually evaluated 7-21 days after implantation by gross examination/photography as well as immunohistochemical staining as described above (Akhtar et al, 2002). If the plug contains functional vessels, the blood (red) vessels can be identified from the photograph. Alternatively, by using mice which express GFP in the

endothelium, immunohistochemical staining can be avoided. Also in this model, intravenous dye injection can be performed to evaluate vessel perfusion and leakiness.

5. Rat ischemic hind limb model

Most of the models mentioned above are very useful to study pathological angiogenesis, and therefore used to find novel anti-angiogenic treatment options including novel anti-angiogenic drugs, but other assays are needed to study diseases where therapy would consist of accelerated blood vessel growth, such as in the treatment of myocardial infarction (MI), stroke and wound healing/regeneration.

In MI an occluded coronary artery leads to a blockade of blood flow to a part of the cardiac muscle tissue, and thus leads to severe hypoxia (ischemia). The cardiac musculature is working constantly, has a very high metabolism and is therefore particularly sensitive to reduced oxygen (and sugar) levels. Therefore, unless new, so called collateral, arteries can be formed quickly, the affected cardiac tissue will perish, and usually so will the patient (Y Cao, 2010). Effective therapy would therefore be able to induce growth of highly functional arteries in the response to tissue hypoxia/ischemia.

An excellent model to study and manipulate the growth of blood vessels and in particular arteries in response to tissue hypoxia is the hind limb ischemia model, which can be performed in either mice or rats. In this model, all arteries supplying highly oxygenated blood to one of the back limbs of the animal are ligated in two steps, resulting in near-zero blood flow in the entire limb (Lundberg et al, 2002.) (Fig. 11). This leads to tissue

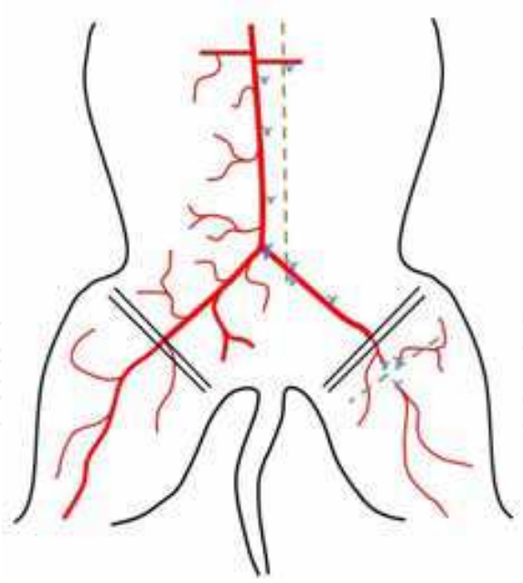


Fig. 11. Hind limb ischemia model. First, a midline incision is made (top dashed line) exposing the vessels. All branches originating from the aorta distal to the renal arteries and all branches from the left iliac artery are ligated by a small suture (blue knots). After a week a second inguinal incision is made (bottom dashed line) and the femoral artery and superficial gastric artery are ligated by a small suture (blue knots). Following this operation the left hind limb does not receive any blood and is considered ischemic.

ischemia and the induction of arteriogenesis from collateral arteries. Angiogenic factors under investigation can be injected into the limb musculature or antibodies against angiogenic or anti-angiogenic factors can be injected into circulation, thus modulating the arteriogenic response. As the hind limb is easily accessible, the circulation in the limb can be observed by Doppler-angiography and thus the same animal can be subjected to repeated investigations on how the blood flow improves over time. After euthanasia, the tissue can be excised and stained as mentioned for the adipose tissue, and the morphology of the blood vessels can therefore be studied post mortem (R Cao et al, 2003). This assay is the most commonly used assay to study therapeutic angiogenesis, and has been used in many seminal discoveries on how to therapeutically generate highly functional and stable arteries (Y Cao, 2010). However, the surgery needed to induce ischemia in the hind limb is very complicated and the assay therefore requires highly skilled and experienced surgeons. Also, there may be slight inter-individual differences in the residual blood flow in the limb after surgery – and therefore the degree of tissue hypoxia - within each experimental group.

6. Wound healing assay

The process of wound healing is divided into three stages; inflammation, new tissue formation and remodeling. Angiogenesis is critical for the formation of new tissue and vascular remodeling occurs in association with tissue remodeling at later stages. Therefore, the wound healing assay is a useful model to evaluate both angiogenesis and vascular maturation/remodelling. Wound healing models are usually performed on the skin as other accessible tissues such as the ears or the tail do not regenerate well. Usually, two circular, trans-dermal wounds are created on the back of anesthetized mice (Fig. 12) allowing one wound to serve as control while topical treatment can be administered on the other. Wound size, scar formation and re-epithelization of the wounds should be recorded daily by photography and by measuring the wound area with calipers, similar to how it is done in the tumor models. In this model, treatment given either systemically by oral administration or injection, or preferably topically on just one of the two wounds, can consist of pro- or anti-angiogenic compounds, and their effects on both the regenerative angiogenesis as well as on vessel morphology and function can be determined post mortem after the regenerated tissue has been excised, fixed and stained as mentioned for the adipose tissue. Also, transgenic or knock-out mice can be used, when available, to study the specific effects of particular genes (Xue et al, 2008).

The surgery required to create the wounds is very simple and the wounds are highly homogeneous in size and location for all animals used in the experiments. This assay is therefore very easy and robust, requires little practice and few animals as the experimental variation is relatively low. However, angiogenesis in this model occurs only in the skin, and in association with inflammation, blood clotting cascades and other highly complex biological processes, which encompass multiple cell types and a plethora of angiogenic and anti-angiogenic factors. Furthermore, regeneration of the skin is known to be quite different from regeneration of other tissues of higher clinical relevance such as the heart and nervous system, and can therefore not be expected to give much information on the role of angiogenesis in regeneration of other organs. Another drawback of this assay is that regeneration occurs by making new tissue rather than repairing/replacing damaged/dead tissue. This difference is thought to be important, as the latter is almost always the case in

clinical situations where for example ischemic insults result in large patches of dead tissue that is hard to replace.



Fig. 12. Wound healing model. Two circular holes of approximately 5 mm in diameter are punched with a tissue puncher through the dorsal skin of an anesthetized wildtype C57Bl6 mouse. No bandages or cover is needed, as the skin in this region has no major blood vessels, and the wound formation leads to very little if any bleeding. Wounds close within 2 weeks and would heal completely within a month.

7. Cornea models

The cornea is an avascular tissue consisting of two, thin, transparent layers in rodents. Thus it is possible to gently cut a tiny pocket between the two layers, and in this pocket insert a pellet containing factors which are to be investigated for their angiogenic or anti-angiogenic activities *in vivo*. All vessels detected in the cornea following a few days stimulation with the implanted factors can be considered newly formed vessels, and due to the transparent nature of the cornea and the strong red color of perfused blood vessels, the angiogenic response can be followed kinetically by simply taking photographs of the eye at different time points. This makes it possible to study the effects of angiogenic factors, either alone or in combination on different processes of angiogenesis such as initial angiogenic expansion, vascular remodeling, maturation and stability in the same animal over time.

Usually angiogenic or anti-angiogenic factors are prepared in slow release pellets consisting of hydron or alumni sucrose octo-sulfate, which are dried on a nylon mesh to ensure equal size and thereby amount of factor in each pellet which is then implanted in the corneal micropocket. Using this delivery system, factors are released constantly at low amounts for

weeks, thus giving rise to a continuous angiogenic stimulation or inhibition depending on the factor in question (R Cao et al, 2003). Alternatively, small pieces of tumor tissue can be implanted to study the angiogenic capacities of different tumors from different organs. Even primary human tumor samples can be investigated in this model by using immune-deficient nude or SCID mice (Jensen et al, 2009).

While blood vessels can be macroscopically studied by simply taking photographs of the cornea, more in-depth examinations on blood vessel structure and function including pericyte coverage, tip cell formation and vascular permeability require that the cornea is excised post mortem and subjected to immunohistochemical analysis such as those described for the fat tissue (Fig. 13).

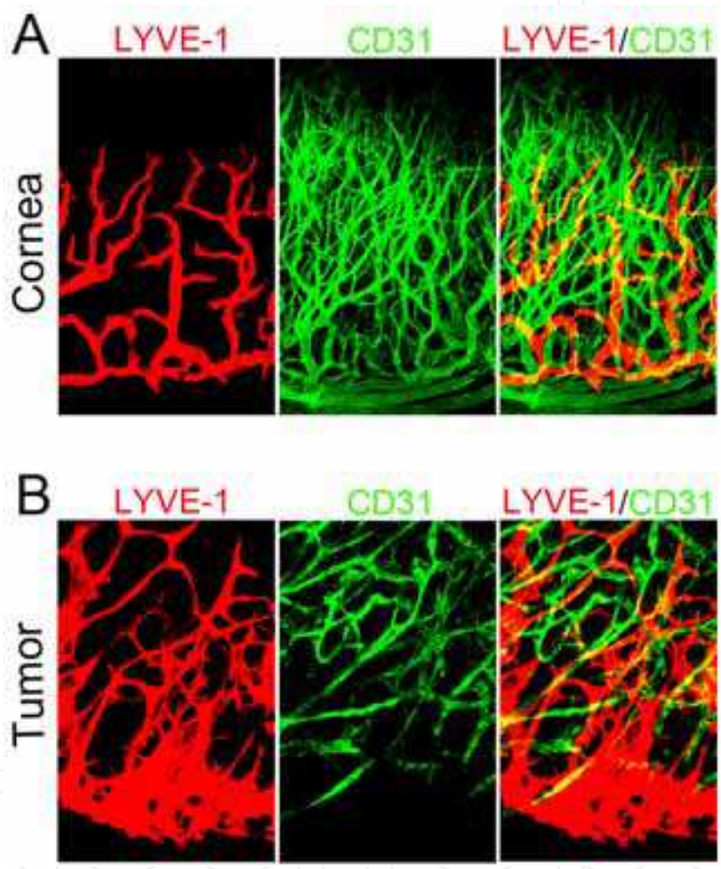


Fig. 13. Visualization of lymphangiogenesis and angiogenesis. A: Confocal analysis of mouse FGF2-induced corneal lymphatic vessels (red) stained with an anti-LYVE-1 antibody and blood vessels (green) stained with an anti-CD31 antibody. B: T241-VEGF-C tumor lymphatic vessels (red) stained with an anti-LYVE-1 antibody and blood vessels (green) stained with an anti-CD31 antibody.

Following staining with antibodies against markers specific for lymphatic endothelial cells, this assay is furthermore one of the strongest assays for investigating lymphangiogenesis. Furthermore, by treating the mice with drugs or neutralizing antibodies against particular receptors, this model can be used to parse out the specific contributions of one or a few receptors for a particular angiogenic factor in inducing angiogenesis and lymphangiogenesis.

This model is considered to be "clean" compared to the tumor models described above – i.e. the angiogenic or lymphangiogenic response can be induced by a defined factor or couple of factors. Considering all these benefits – this assay is probably the strongest *in vivo* assay to study the molecular biology of angiogenesis and lymphangiogenesis (Jensen et al, 2009).
However, the assay has been criticized for being poor at describing physiologic or pathological angiogenesis as the amount of factor used in the pellet usually give rise to supra-physiological concentrations in the cornea. However, the amount of factor used per pellet can be lowered accordingly, or instead of using purified recombinant growth factors, researchers may implant tumor tissue, or a small piece of suture, which lead to corneal inflammation, which then subsequently drives the angiogenic response although via a less defined and controllable pathway. The major limitation of the assay is the technical difficulty of implanting pellets into the mouse cornea. In the beginning, researchers circumvented this problem by using larger animals such as rats or rabbits, but in order to take advantage of the growing number of transgenic or knock-out mice available today it is a major benefit to use this animal if possible.

8. Zebrafish models of angiogenesis

Zebrafish have recently gained much attention as an angiogenesis model system. Zebrafish embryos develop outside of the uterus, which greatly facilitates imaging during development. Furthermore, pigmentation can be inhibited chemically, or by using non-pigmented strains, such that the embryos maintain transparency throughout development. Many eggs are produced each breeding cycle, zebrafish are relatively easy and cheap to maintain, compared to rodents, and their embryonic development is much faster. Additional benefits of zebrafish-based models include passive uptake of chemicals added to the water, which eliminate the invasive administration procedures which are commonly needed in rodents and their small size which means that all tissues can be oxygenated by passive uptake from the water during development, thus allowing researchers to study phenotypes of embryos with severely disrupted vasculature, which are embryonically lethal and therefore difficult to study in mice (Weinstein et al, 1995).
Because of these unique characteristics of the zebrafish embryo, several genes with essential functions in the vasculature have been discovered using this system. One approach which has yielded the identification and characterization of novel genes important for developmental angiogenesis and vascular maturation is to perform unbiased screens of mutant embryos generated by random mutagenesis induced either by radiation, chemicals or insertion of small genetic fragments (Gaiano et al., 1996; Haffter et al., 1996; Knapik et al., 1996). Such screens have in the past yielded information on human congenital disorders of which the genetic background was previously unknown (Alders et al, 2009), and have – combined with the ability of the embryos to passively take up chemicals added to the water – been used to screen for, and identify novel drugs which efficiently correct the pathological vascular phenotypes (Peterson et al, 2004). Such an approach is called chemical genetics, due to the screening of novel chemicals for therapeutic effects in genetically modified zebrafish models, and is today being frequently used as a discovery tool in the pharmaceutical industry.
Recently researchers have further expanded the benefit of zebrafish-based model systems by generating many transgenic zebrafish strains which express fluorescent markers in

particular cell types, organs or tissues, including endothelial cells of the vasculature (Jensen et al, 2009). By continuous observation of such transgenic embryos under the microscope, it is possible to follow the dynamics of growing vessels during zebrafish development in real time.

Such studies, which are difficult to perform in mice due to their *in utero* development, have yielded valuable insights into the process of vasculogenesis, which is the formation of the first embryonic vessels – the aorta, cardinal vein and thoracic duct – and on the origin of blood cells as well as the mechanism by which blood flow is initiated (Herbert et al, 2009; Lida et al, 2010; Yaniv et al, 2006).

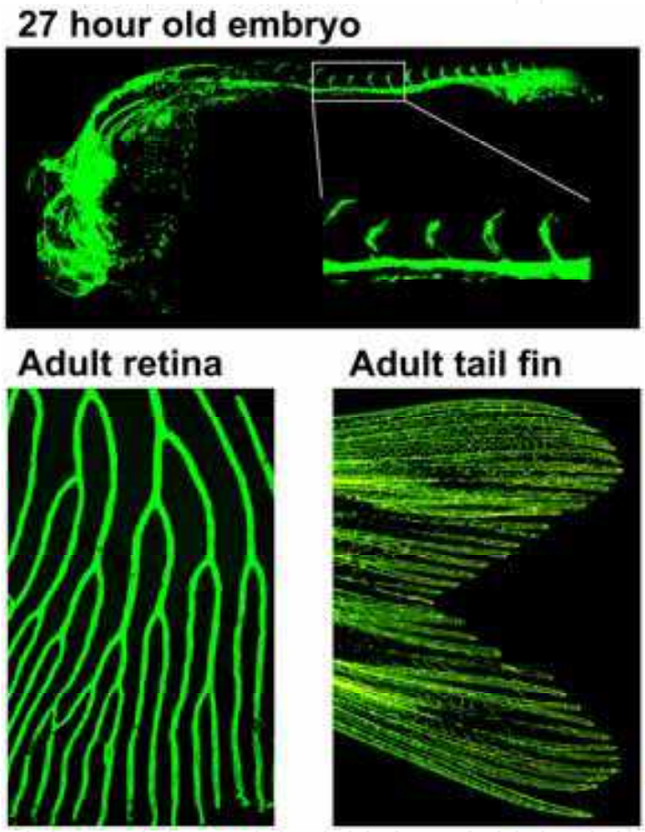


Fig. 14. Blood vessels in the *Fli1:EGFP* transgenic zebrafish line. In the *fli1:EGFP* transgenic zebrafish, the promoter drives EGFP expression specifically in the vasculature. The top image show EGFP positive blood vessels (green) in a 27 hours old embryo, on the bottom left is shown blood vessels in the adult retina and on the bottom right in the adult tail fin.

The zebrafish genome has been fully sequenced and annotated, which makes it easy to interfere with genes involved in vascular development by injecting specially designed synthetic RNA-like molecules called morpholinos. These morpholinos are designed to have a complementary sequence to a particular target mRNA to which they will anneal and thus specifically target that transcript for destruction. The addition of morpholic acid groups on the nucleotide backbone of morpholinos makes them stable inside the cell and

they are therefore not degraded along with their mRNA target (Nasevicius & Ekker, 2000). Morpholinos, as well as mature mRNA - if over-expression rather than inhibition of a particular gene is being investigated - can be injected in the yolk of newly fertilized 1-cell stage embryos, and will then be present in all cells during development. As the cell number increases, the concentration of morpholino per cell will gradually decrease, and most morpholinos will therefore only be effective for up to 4 days after fertilization. This period is – due to the fast development of the zebrafish embryo – usually more than sufficient for angiogenesis studies, and thus does usually not pose a restriction on the study.

Morpholino-mediated disruption of a particular gene product is called knock-down, due to its transient nature compared to permanent knock-outs, and is today a widely used method to study the involvement of particular genes in angiogenesis and vascular functions during zebrafish development.

By using a zebrafish strain with enhanced green fluorescent protein expressed in endothelial cells under the *fli1* promoter (Lawson & Weinstein, 2002) (Fig. 14), knock-down of VEGF leads to a concentration-dependent inhibition of angiogenesis which can be quantified already 24 hours after fertilization (Nasevicius et al, 2000). In mice, even heterozygous deletion of VEGF leads to early embryonic lethality (Carmeliet et al, 1996) and the developmental consequence of subtle concentration differences in this critical angiogenic factor can therefore only be studied in zebrafish. Thus, zebrafish have been used recently to show that VEGF is only important for arterial growth, whereas growth of veins are practically unaffected by reduced VEGF levels.

9. Embryonic zebrafish metastasis and tumor model

Zebrafish embryos in the first few weeks of development lack an adaptive immune system, which makes them unable to reject non-zebrafish grafts and they are therefore perfect recipients for implantation of mammalian tumor cells. Due to the transparent nature of zebrafish embryos, fluorescently labeled tumor cells may furthermore be traced in a completely non-invasive fashion, leading to continuous monitoring of the behavior of the tumor cells, including their dissemination from the primary tumor mass *in vivo* (Rouhi et al, 2010).

The induction of tumor angiogenesis is a hallmark of advanced tumors, and therefore also of established tumor cell lines. Thus, mammalian tumor cells, implanted into a space around the yolk sac called the perivitelline space of transgenic *fli1:EGFP* zebrafish embryos, is a powerful way of studying early events of tumor-induced angiogenesis and especially of tumor cell dissemination and metastasis via the vasculature (Lee et al, 2009; Rouhi et al, 2010). Depending on their invasive capabilities, implanted tumor cells may start disseminating from the primary site very shortly after implantation (Fig. 15). Angiogenic factors produced by the tumor cells can affect the host's existing and developing vasculature which furthermore is a good way of studying the function and morphology of vessels induced by the tumor-derived factors in question. Different kinds of manipulation e.g. knock-down or over-expression of a specific gene in the tumor cells can affect the dissemination and invasion pattern of a specific cell line implanted in the embryo. Genetic manipulation of tumor cells can be performed with help of different tools e.g. small interfering RNA (siRNA), small hairpin RNA (shRNA) or by retroviral integration of expression vectors containing angiogenic factors.

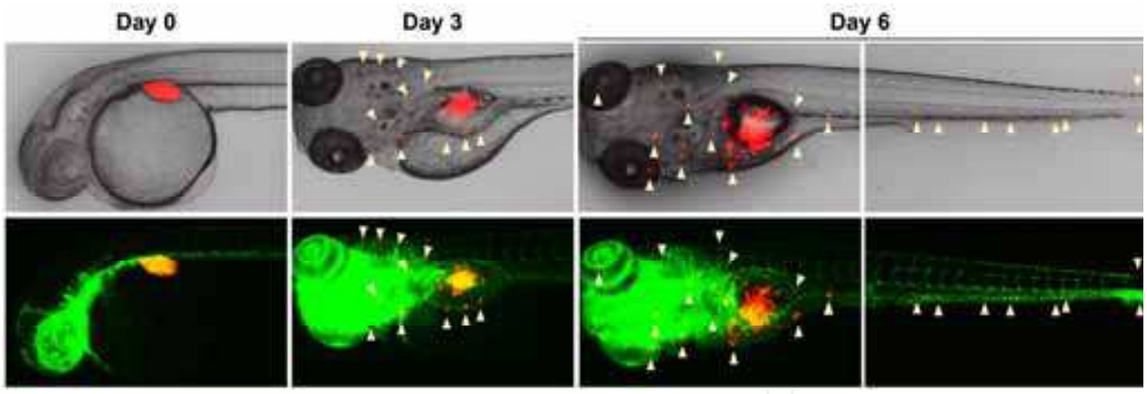


Fig. 15. Metastasis model in zebrafish embryos. DiI labeled tumor cells (red) are injected in the perivitelline space of approximately 2 day old zebrafish embryos (left panels). 3 days after injection many disseminated cells are observed (arrowheads) proximal to the tumor (middle panels). After 6 days tumor cells have spread throughout the embryo, via the vasculature (right panels). The top row shows brightfield images combined with the red-fluorescent signal from the tumor cells. The bottom row shows the EGFP-positive vasculature (green) in transgenic F*li1:EGFP* zebrafish embryos combined with the red-fluorescent signal from the tumor cells.

It is also possible to study the effects of host gene expression on tumor cell dissemination and metastasis by modifying expression of host genes by injecting either morpholinos or mature mRNA immediately after fertilization, and thus prior to tumor implantation, as described above (Lee et al, 2009). This model is thus a simple system to parse out the contributions of factors derived from the tumor relative to the host on tumor angiogenesis, dissemination and metastasis.

Since disseminated tumor cells in this model will be detected by fluorescent microscopy, they should either permanently express a fluorescent protein or they can – prior to injection – be labeled with fluorescent dyes such as DiI, which can be easily distinguished from the green fluorescent color emitted from the embryo vasculature. Tumor cells are preferably implanted in the non-vascularized area in the perivitelline cavity as it can otherwise be difficult to distinguish between existing vessels, those formed by normal developmental processes and those induced by the tumor mass.

Prior to implantation, embryos will be dechorionated, anesthetized and placed on a modified agarose gel. Transplantation of tumor cells is easily carried out using a micromanipulator which is connected to a microinjector. Approximately 100 cells will be implanted into each embryo.

After microinjection, based on the purpose of the study, embryos will be immediately transferred into appropriate embryo water such as E3 water or Danieu's buffer (Fig. 15). As mentioned above, drugs may be added to the water and their effects on tumor angiogenesis, dissemination and metastasis can thus easily be evaluated.

Each tumor bearing embryo may be placed in a separate well of a multi-well plate and examined individually to monitor tumor angiogenesis, dissemination and metastasis in as high temporal resolution as required in the experiment. This model furthermore allows generation of time-lapse video sequences of the tumor cells invading into and out from the vasculature at metastatic sites, and thus examine the tumor-endothelium communications involved in tumor dissemination and metastasis in detail.

As described in more detail below, zebrafish are – compared to mice – highly amenable to studies on the physiological and pathological effects of hypoxia. In order to investigate the effects of hypoxia on tumor angiogenesis, dissemination and metastasis, tumor cell-transplanted embryos can be placed in hypoxic water in a special aquarium (Lee et al, 2009). As tumor hypoxia in mammalian models is very difficult to control and monitor, this system allows studies of the effects of highly defined oxygen concentrations and periods of hypoxia exposure. In all conditions above fish embryos will be kept at 28.5°C, which is the standard temperature for rearing zebrafish embryos and larvae. Death rate is relatively high in hypoxia experiments and in order to have enough embryos at the end to make statistically correct conclusions, a relatively high number of embryos should be implanted with tumor cells and placed inside the hypoxia chamber.

10. Spontaneous tumor models in zebrafish

As it is the case for mice, there are also several genetically engineered spontaneous tumor models available in the zebrafish. While these models may be regarded to more closely model the clinical pathogenesis of cancer, the same limitations are relevant for the zebrafish models as those described for mice, most notably the very heterogeneous tumor onset and growth both for different tumors within each fish but also between different fish.

10.1 Peripheral nerve sheath tumors

Homozygous *tp53M214K* mutant zebrafish which carry a mis-sense mutation in exon 7 of the *P53* gene, giving rise to spontaneous formation of abdominal and periocular tumors at an age of 8-16.5 months. HE staining of ocular and abdominal tumors showed that these tumors are mainly composed of spindle cell and epitheloid cell neoplasms which recapitulate characteristics of human malignant peripheral nerve sheath tumor (MPNST).
Intraperitoneal implantation of extracted zMPNST cells into irradiated wild type adult zebrafish led to formation of typical zMPNST tumors after less than one month post transplantation (Berghmans et al, 2005).

10.2 Melanoma

By crossing the *tp53* mutant zebrafish with a transgenic line expressing a mutant, constitutively active form of BRAF (V600E, which is the same mutation frequently observed in human melanomas) under control of the melanocyte microphthalmia-associated transcription factor (*mitfa*) promoter, it was found that these fish develop melanomas spontaneously in preference to MPNSTs. In these fish formation of small nevi can be observed already at a young age, and these nevi continue to progress into large malignant, metastatic melanomas in multiple locations from approximately 4-6 months of age.
Malignant melanoma tumor cells from the tumor bearing fish can be transplanted into sub-lethally gamma irradiated wild type zebrafish, where they will grow and spread much like tumors in the mouse xenograft models described previously (Patton et al, 2005).

10.3 T-ALL

Aberrant expression of Myc in humans induces lymphoma and leukemia. Similarly in zebrafish a T cell acute lymphoblastic leukemia (T-ALL) zebrafish model was generated by microinjection of the mouse *c-myc* (*mMyc*) gene under control of the zebrafish *Rag2*

promoter (*zRag2*). Onset of tumors varied between 30 to 131 days post injection of the *Rag2-mMyc* expression plasmid. Fish developing T-ALL were characterized by inflated abdominal cavities and infiltration of malignant cells (transformed lymphoblasts) throughout the body, under the skin, into base of the pectoral fin, olfactory region, and the retrorbital soft tissue that led to splayed eyes.
These malignant lymphoblasts were transplantable into irradiated wild type adult zebrafish giving rise to small tumors appearing already one week post transplantation (Langenau et al, 2003)

10.4 Rhabdomyocarcoma

Rhabdomyosarcoma (RMS) is a very aggressive soft tissue sarcoma with high incidence seen among children compared to other types of cancer. The zebrafish RMS model was generated by injecting a Rag2-kRASG12D construct, expressing a constitutively active isoform of RAS under the Rag2 promoter, into the zebrafish embryo at one cell stage. Visible highly invasive tumors in liver, intestine, kidney, and testes appeared already 10 days post injection, in accordance with the early onset of these tumors in human patients, distinguishing this model as one of the fastest spontaneous, vertebrate tumor models available (Langenau et al, 2007).

11. Adult zebrafish models

While embryonic zebrafish models have yielded many valuable insights into human vascular or vessel-related pathologies, most diseases strike adult patients, and accordingly, their clinical symptoms are expected to be more closely recapitulated by adult disease models (Dahl Ejby Jensen et al, 2009). To this end there are today also a growing number of adult zebrafish models available which are highly valuable in the study of angiogenesis-related disorders including retinopathy, regeneration/wound healing and cancer.

11.1 Zebrafish retinal angiogenesis models

Diabetic retinopathy and age-related macular degeneration are severely debilitating disorders in which angiogenesis is a major driving force of the pathology. During progression of these diseases, retinal hypoxia induces pathological growth of immature and fragile blood vessels in the retina, which is associated with the progression to severe states of the diseases. Mice are not convenient to use for studies on retinal hypoxia, as the traditional methods of generating tissue hypoxia in mice (vessel occlusion/ligation) are not applicable in the retina, as there are no easily reachable arteries which can be ligated without causing excessive damage to the animal. Furthermore, mice cannot be exposed to severely hypoxic environments, as their respiration system is not adapted to withstand low atmospheric oxygen levels.
Zebrafish are much more robust to environmental hypoxia, and can withstand even very low oxygen levels in the water for a long time. In contrast to mammals, by incubating zebrafish in an aquarium, where the oxygen levels in the water can be tightly controlled by regulating the perfusion of nitrogen gas, researchers are given full control on the precise degree of hypoxia, the amount of time the tissue experiences hypoxia and the possibilities of distinguishing hypoxia-effects from other effects of restricted circulation such as acidosis and accumulation of waste products.
The zebrafish retinal vasculature is furthermore particularly amenable to studies on angiogenesis due to its remarkably simple structure (Fig. 16). As in mice and humans, the

retinal vasculature is supplied with blood from a major optic artery, which branches out at the center of the optic disc to form approximately 4-7 so-called grade I arteries which cover the inner surface of the retina. These arteries branch a few more times before anastomosing with protrusions from the circumferential vein. Thus, the zebrafish retina has a simple monolayer vasculature that is organized from the center to the periphery as arteries-capillaries-veins, which is in contrast to that in mice where the vasculature is multi-layered and arteries, capillaries and veins are co-localized throughout the retina (R Cao et al, 2008).

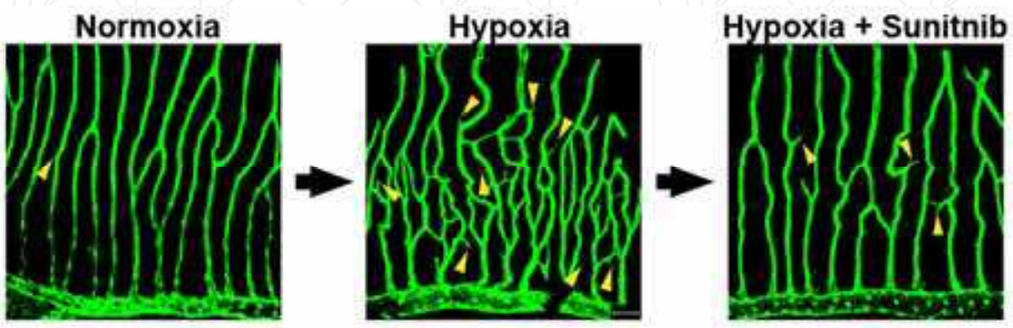


Fig. 16. Hypoxia-induced retinal angiogenesis model. Adult *Fli1:EGFP* transgenic zebrafish were incubated in 10% air-saturated water for 12 days, which induce marked angiogenic expansion of the capillary area of the retinal vasculature (middle figure) compared to fish in normoxic water (left figure). Hypoxia-induced retinal angiogenesis is dependent on VEGF signaling as the VEGF receptor inhibitor sunitnib is able to block the formation of new vessels under hypoxia in the zebrafish retina (right figure). Yellow arrowheads indicate angiogenic sprouts. Figure adapted from R Cao et al, 2008.

This retinal vasculature is highly sensitive to hypoxia. After only a few days of exposure to environmental hypoxia, a marked angiogenic expansion of the capillary area is clearly observed, which can be modulated either by the degree, or the amount of time, the fish is kept in hypoxia. An example of hypoxia-induced retinal angiogenesis in the adult zebrafish is given in Fig. 16.

In this model, adult zebrafish are put into a specifically designed aquarium where the water oxygen levels can be automatically controlled and monitored using an oxygen sensor coupled to a valve which opens for perfusion of nitrogen gas when the oxygen levels are above a set level defined by the researcher. Zebrafish can withstand very low oxygen levels better when being gradually adapted, so by slowly reducing the oxygen concentration in the hypoxia aquarium over the course of 1-2 days, the fish can be maintained in water with an oxygen concentration of only 8-10% of that in fully air-saturated water (Z Cao et al, 2010). After exposure to this level of hypoxia for a few days, retinal neovascularization is readily detected post mortem, under a fluorescent microscope. Also in this assay, it is convenient to add orally active drugs to the water during exposure to hypoxia, to study how such drugs may interfere with hypoxia-induced retinal neovascularization. Hypoxia-induced newly formed vessels can easily be distinguished due to the normally very simple and organized vasculature of the retina in adult zebrafish. Quantifying the number of sprouts, new branches and vascularization area are convenient ways of comparing the effects of the investigated drugs compared to non-treated controls (Z Cao et al, 2010). While capillary sprouting begins on day 3 after exposure to hypoxia, formation of a neovascular network becomes obvious on day 6 after exposure to hypoxia (R Cao et al, 2008).

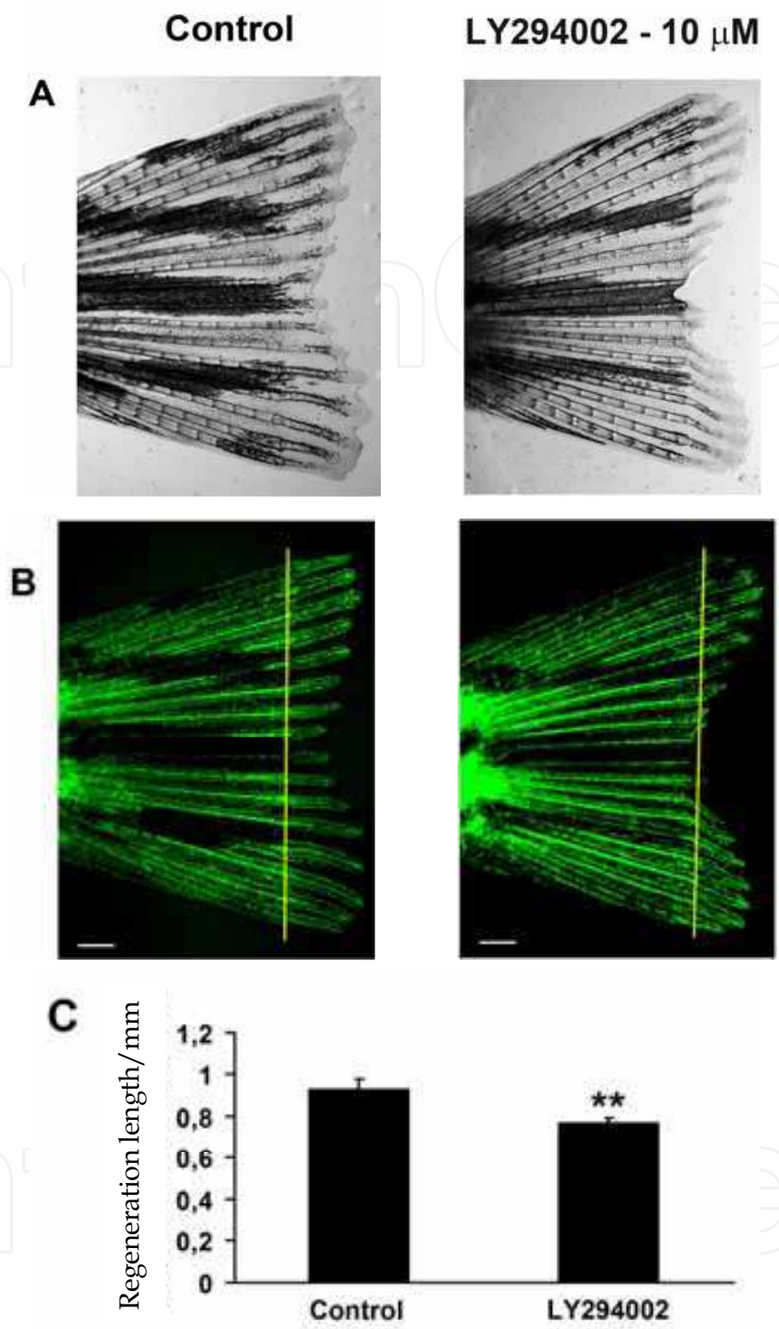


Fig. 17. Regenerating tail fin assay. The distal 1/3 of adult *Fli1:EGFP* transgenic zebrafish tail fins was amputated under anaestisia, and the fish were placed in either clean water (left panels) or water containing the phosphatidyl-inositol-3-kinase (PI3K) inhibitor LY294002 (right panels). After 6 days the fish were sacrificed, the tail fins were fixed in 4% PFA and the regeneration could be determined by bright-field microscopy (A). The vasculature in the regenerating tail fin tissue was observed by fluorescence microscopy (B). Quantification of the regeneration length is an easy and convenient way to demonstrate the effects of the tested drugs (C). Bars indicate 0.5 mm. Figure reproduced from Alvarez et al, 2009.

This experiment allows researchers to study the mechanisms of hypoxia-induced angiogenesis *in vivo*, to study neovascularization, vascular remodeling, and leakiness under pathological settings, to correlate vascular changes with disease development, to screen potential orally active therapeutic agents, and to assess the beneficial effects of known antiangiogenic agents for the treatment of retinopathy. However, there are also limitations. Unlike the clinical situation, this model does not induce local tissue hypoxia but rather rely on systemic exposure to hypoxia. Also, owing to the limitation of available antibodies against zebrafish proteins, staining of particular cell subsets in the retina may be difficult, reducing the amount of information that can be obtained from histological examinations compared to mouse models.

11.2 Angiogenesis in the regenerating zebrafish fin

Adult zebrafish have a remarkable regenerative capability. Many tissues which may not be regenerated in mammals are quickly regenerated in zebrafish. Among these are the heart, retina, maxillary barbell and fins (Poss et al, 2002; Vihtelic & Hyde, 2000; Alvarez et al, 2009). Importantly, as they regenerate, new blood and lymph vessels grow into the regenerating tissue – which enables studies on regenerative angiogenesis. One commonly used assay in the adult zebrafish, based on this principle is the regenerating tail fin. After amputation, the tail fin will re-grow and after approximately 1 month, the fin is back to its original size (Huang et al, 2003). This process of fin regeneration encompasses many of the same mechanisms as in human wound healing and regeneration, and is therefore a good model of regenerative angiogenesis. As the fin is largely transparent, and as morpholinos can be introduced by microinjection and electroporation, this assay is almost as versatile as the zebrafish developmental angiogenesis assays, the major difference being that it is performed in an adult animal. Also in the fins, the vasculature is remarkably simple (Fig. 17), and as the fin grows back various levels of vascular remodeling can be observed and therefore studied in detail (Huang et al, 2003). This regeneration model is probably the most commonly used, adult zebrafish angiogenesis model, and is considered to be complementary to the developmental angiogenesis models.

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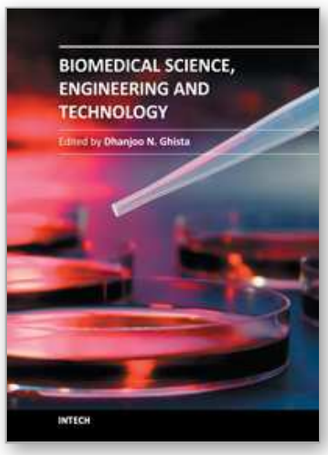
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