Cyclooxygenase-2 blockade can improve the efficacy of VEGF-targeting drugs

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1. Introduction

1.1. Breast cancer

1.1.1. Breast cancer statistics

Breast cancer is the second most common cancer in the world and the most frequent among women in both developed and developing countries[1]. Worldwide, 1.67 million new cases of breast cancer were diagnosed in 2012, corresponding to 25% of all cancers in women and 522.000 women died of this disease in the same year[1]. In Germany, the Robert Koch Institute registered 69.700 cases of breast cancer in 2011, which corresponds to 30.5% of all diagnosed cancer types in women (Seedat et al. 2015).

1.1.2. Development of breast cancer

The human female breast is composed of 15 to 20 sections named lobes, which consist of many smaller sections called lobules. The lobules contain the mammary glands, which are responsible for the milk production during pregnancy and after childbirth. The produced milk flows from the lobules trough the milk ducts to the nipple, in order to feed the newborn. The space between lobules and milk ducts is filled by fibrous tissue and fat, which determine form and size of the female breast[2] (Figure 1).

1 World Health Organization, International Agency for Research on Cancer
Globocan 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide 2012
Available at: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx
[Accessed on 14 January 2016]

2 National Cancer Institute
General Information About Breast Cancer
Available at: http://www.cancer.gov/types/breast/patient/breast-treatment-pdq
[Accessed on 14 January 2016]

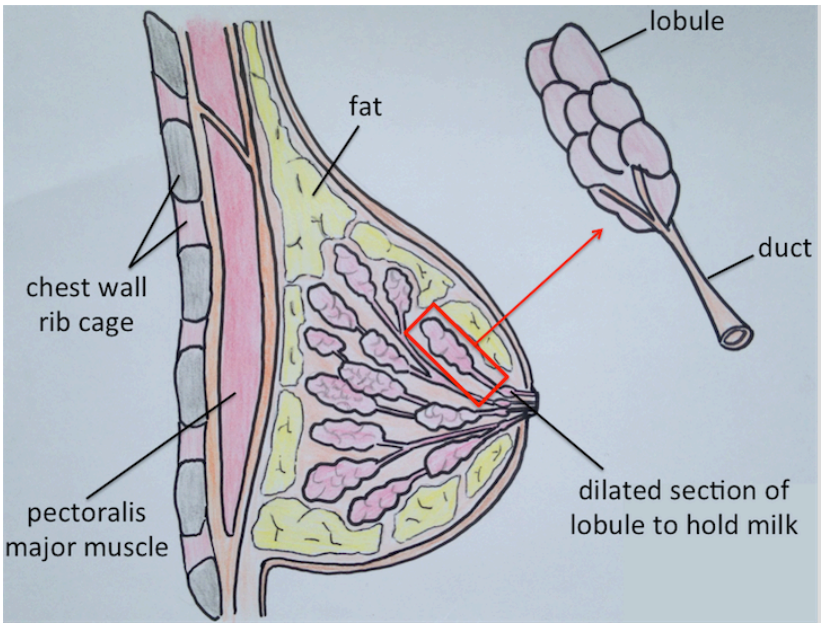

Figure 1: Structure of the human female breast. The human female breast consists of 15-20 lobes, which are embedded in fibrous tissue and fat. The mammary glands are located in the lobules that build up the lobes and ensure the production of milk, which ultimately flows to the nipple through the milk ducts (Picture adapted from images from www.cancer.gov).

Breast cancer is a clonal disease that develops from a single cell through uncontrolled proliferation and can affect any part of the breast. Abnormal proliferation results from sequential alterations of genes that regulate the normal cell cycle. In most cases, cancer cells develop from epithelial tissue that line the milk ducts or the lobules and are referred to as carcinomas [3]. The malignant progression of breast cancer includes several stages, which are classified according to differences in the structural organization of the epithelium. At the initial stage, increased cellular division results in hyperplasia, a premalignant lesion where cells have normal morphology but are increased in number. In the next stage, known as dysplasia, abnormally proliferating cells are morphologically heterogeneous, which causes gradual loss of the normal tissue arrangement. Carcinoma *in situ* (CIS), a more advanced stage, is characterized by changes in cell morphology and increased proliferation. At this stage, however, the cancer still does not spread beyond the original tissue due to the basement membrane that remains intact. In contrast, cancer cells from an invasive carcinoma break through the basement membrane and acquire the potential to spread to other parts of the body, a process known as metastasis[4].

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3 American Cancer Society, *Types of breast Cancer*, Available at: http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-breast-cancer-types, [Accessed on 14 January 2016]

4 National Cancer Institute, *What is Cancer*, Available at: http://www.cancer.gov/about-cancer/what-is-cancer, [Accessed on 14 January 2016]

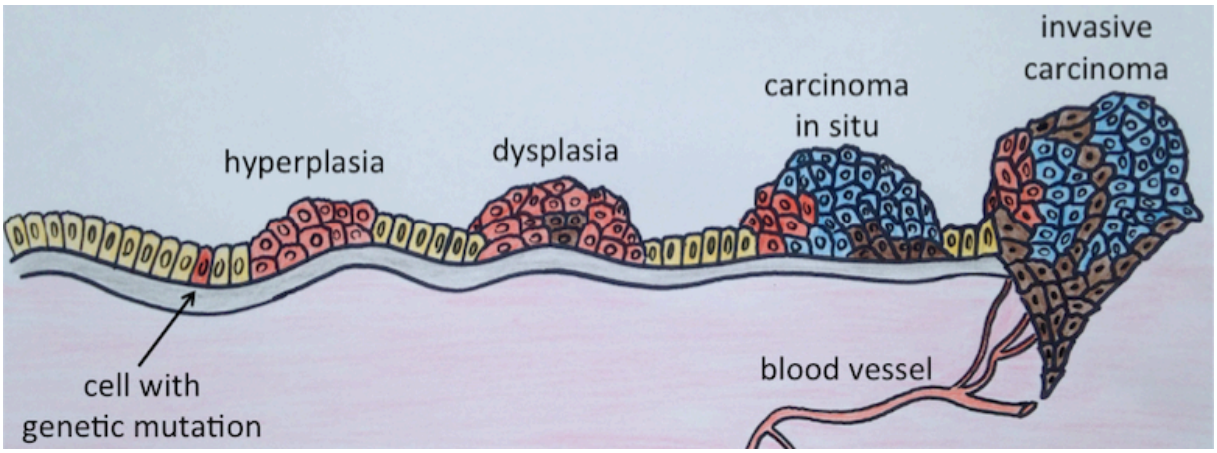

Figure 2: Steps of tumour development. Genetic alterations lead to the transformation of normal cells into tumour cells, which are characterized by an uncontrolled proliferation. Malignant progression of breast cancer is marked by a gradual loss of tissue arrangement as well as decreased cell differentiation. A precancerous condition known as hyperplasia is characterized by an increased number of cells. A gradual loss of normal tissue arrangement besides abnormal increased cell proliferation leads to dysplasia. A non-invasive tumour mass, referred to as carcinoma *in situ*, shows a higher grade of dedifferentiation compared to normal tissue as well as an intact basement membrane. Invasive carcinomas have the ability to invade the surrounding tissue and metastasize to other parts of the body. (Picture adapted from images from www.science.education.nih.gov)

1.1.3. Aetiology of breast cancer

The aetiology of breast cancer is often unknown. Diverse studies have identified many epidemiological risk factors increasing the chance of developing this disease. These include increased age, nulliparity, family history of breast cancer and the use of menopausal hormone therapy, among others (Lacey et al. 2009). The sequential genetic alterations that cause cancer can be grouped into sporadic somatic mutations, which can arise during a person's lifetime or germ-line mutations, which can be inherited from progenitor cells[4]. Two main groups of these genes are affected by these mutations:

- **Oncogenes**, which result from modifications of normal genes termed proto-oncogenes. Proto-oncogenes encode proteins that positively regulate the normal cell cycle, i.e. the series of events that occurs in the cell leading to its duplication. These proteins include transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators. As a consequence of activated oncogenes, cells can proliferate uncontrollably and become malignant (Croce 2008). Examples of proto-oncogenes include Myc, Ras and Her2/neu, among others.
- **Tumour-suppressor genes,** which encode proteins that have a repressive effect on the regulation of the cell cycle, repair the DNA damage and promote apoptosis. Hence, tumour-suppressor genes protect normal cells to transform into malignant

cells (Croce 2008). Some examples of proteins encoded by tumour-suppressor genes include the breast cancer 1 and/or breast cancer 2 genes (BRCA1 and/or BRCA2), which play an important role for DNA repair. The most common cause of hereditary breast cancer accounting for 30% of all cases consists of inherited mutations in these genes (Economopoulou et al. 2015). Other gene mutations implicated with hereditary breast cancer are less common. These mutations affect other tumour suppressor genes that encode proteins such as p53 and ATM (ataxia telangiectasia mutated), which mediate cell cycle arrest, initiation of apoptosis and activation of DNA repair (Economopoulou et al. 2015).

1.2. Angiogenesis

The normal physiological functionality of tissues and organs depends on the supply of oxygen and nutrients, which are transported through a network of blood vessels. Blood vessels can be classified according to their structure and function into arteries, veins and capillaries. Arteries and veins are composed of three layers. The innermost layer is the tunica intima and consists of a single layer of endothelial cells (ECs) that build the endothelium and is embedded in an extracellular matrix (ECM). The tunica media surrounds this monolayer of ECs and contains mural cells such as fibroblasts as well as smooth muscle cells (SMCs). The outermost layer is the tunica adventitia and is entirely composed of connective tissue (Michel et al. 2007). Capillaries are the smallest vessels and in contrast to arteries and veins consist of a monolayer of ECs surrounded by pericytes (PCs) (Carmeliet 2003). The formation of the vascular system is a physiological process occurring during the development of vertebrates through two major processes known as vasculogenesis and angiogenesis (Patel-Hett & DAmore 2011). Angiogenesis can also occur under pathological conditions and is an important feature during tumour progression, since tumours need to be sustained with oxygen and nutrients in order to keep up with their rapid growth (Carmeliet 2005). Therefore, tumour angiogenesis represent an attractive therapeutic target for the development of new therapies.

1.2.1. Physiological angiogenesis

Vasculogenesis occurs first during the early embryogenesis and represents the *de novo* formation of blood vessels, as endothelial precursor cells, also known as

angioblasts, differentiate into ECs (Schmidt et al. 2007). These vessels establish a primary vascular plexus that progressively expands by angiogenesis, a process consisting in the formation of new blood vessels from a pre-existing vasculature. During angiogenesis, new capillaries are formed by sprouting or by splitting from their vessel of origin and a highly organized vessel network emerges (Risau 1997; Carmeliet 2005). In this process, the basement membrane is degraded by enzymes synthesised by ECs such as matrix metalloproteases (MMPs), and interendothelial contacts are weakened, consequently ECs can detach and migrate into the connective tissue (Ribatti & Crivellato 2012; van Hinsbergh & Koolwijk 2008). ECs express a wide variety of receptors such as growth factor and cytokine receptors, which enable these cells to rapidly divide and form new blood vessels in response to diverse pro-angiogenic stimuli. Initially, the nascent vessels consist of naked ECs, which mature in further steps of angiogenesis. These maturation steps involve the recruitment of mural cells as well as the development of an extracellular matrix, which provide strength and stability to the new vessels (Carmeliet 2003)(Figure 3).

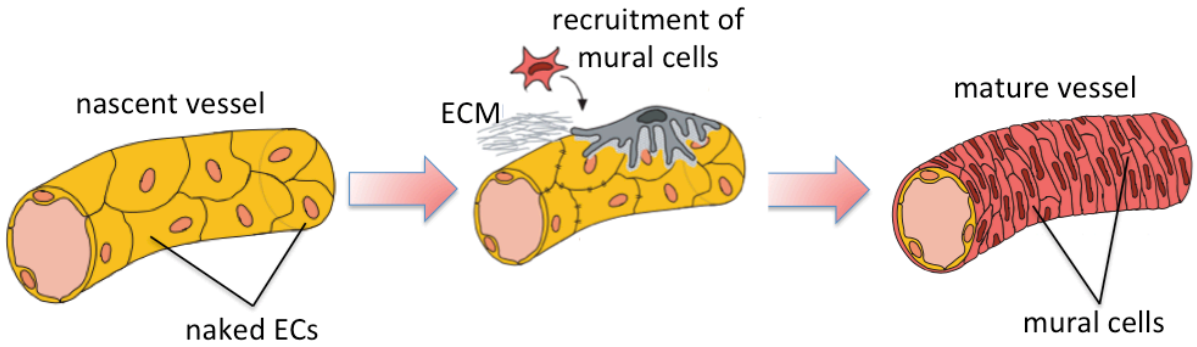

Figure 3: Maturation of nascent blood vessels. Nascent vessels consist initially of naked ECs that form endothelial tubes. Through secretion of angiogenic factors, mural cells that include PCs and SMCs are recruited to the nascent vessels in order to confer strength and stability to the new mature blood vessel (Graphic adapted from Carmeliet 2003).

In postnatal life, angiogenesis still contributes to organ growth but this process partially subsides in the adulthood, occurring only in the cycling ovary and in the placenta during pregnancy. Nevertheless, angiogenesis can be reactivated in response to a pathological stimulus, as for example wound healing and tissue repair (Carmeliet 2005). Although angiogenesis is an important physiological process during the growth and development of organs as well as wound healing, it also represents a pathological process implicated in many diseases including the growth of solid tumours.

1.2.2. Pathological angiogenesis

The first scientific observations about angiogenesis date back to 1796, when the Scottish surgeon and scientist John Hunter suggested that the extent of the vasculature directly depends on the metabolic activity of tissue (Hunter 1840). However, the American medical scientist Judah Folkman introduced for the first time the term angiogenesis only in 1971 when he hypothesized that tumour growth is angiogenesis-dependent (Folkman 1971).

In early stages of carcinogenesis, the tumour consists of a mass of aberrant cells *in situ*, which is in a dormant state and has a microscopic size that does not exceed 1 mm^3 (Folkman 2006). Since the diffusion limit of oxygen is approximately 100 μm, cells need to be located within this distance from a blood vessel to ensure their oxygen supply. Thus, a microscopic tumour is highly dependent on the surrounding vasculature of its microenvironment in order to be supplied with oxygen and other nutrients by diffusion (Torres Filho et al. 1994; Folkman 2006). Once the avascular tumour exceeds a certain critical size, tumour-induced angiogenesis is triggered to expand the tumour mass. The success of the tumour progression critically depends on the ability to change from the non-angiogenic to the angiogenic phenotype, a step known as the "angiogenic-switch". One of the major driving-forces for the activation of the angiogenic switch is hypoxia, a condition that is present in solid tumours due to deprivation of sufficient oxygen supply (Longatto Filho et al. 2010). Tumour vessels are structurally and functionally abnormal due to unbalanced overexpression of cytokines that promote angiogenesis (Nagy et al. 2009). This abnormal vasculature results in a hostile tumour microenvironment characterized by low pH and high interstitial fluid pressure, besides hypoxia (Carmeliet & Jain 2011). Hypoxia promotes tumour angiogenesis through the upregulation of several angiogenic factors. Among all factors involved in angiogenesis, the members of the vascular endothelial growth factor (VEGF) family stand out for their importance in this process. Carmeliet et al. showed the crucial role of VEGF in mice embryos harbouring a deletion of a single VEGF allele. As a consequence of this deletion, the embryos exhibited impaired blood vessel formation that resulted in lethality in utero, thus demonstrating the importance of VEGF for the blood vessel formation (Carmeliet et al. 1996) (Figure 4).

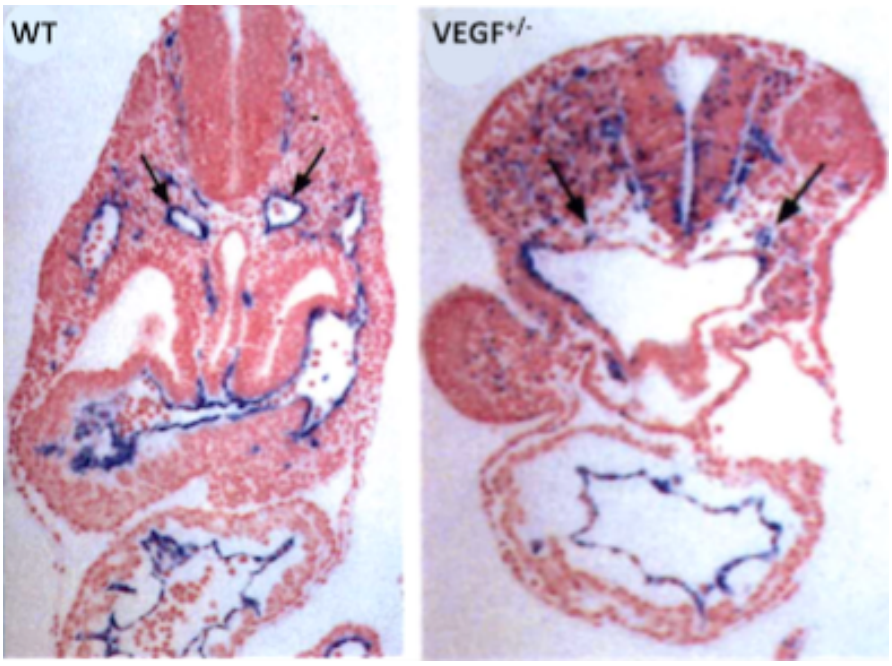

Figure 4: VEGF is a key molecule for angiogenesis. Experiments in mouse embryos with a single deletion of a VEGF allele showed an impairment in the formation of blood vessels (stained in blue), which resulted in lethality (right panel). The left panel shows a WT embryo (Graphic adapted from Carmeliet et al. 1996).

Based on Folkman's breakthrough findings in 1971, several studies in the decades that followed focused on the investigation of mechanisms regulating tumour angiogenesis. The crucial role of VEGF in tumour angiogenesis demonstrated in the years thereafter led to the development of anti-angiogenic therapies targeting VEGF or its receptors with the expectation of cutting off the tumour supply with oxygen and nutrients, thereby killing the tumour cells.

1.3. Role and targeting of the vascular endothelial growth factor family

1.3.1. The vascular endothelial growth factor family

VEGF proteins represent a family of secreted polypeptides that include VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). The biological functions of the VEGFs are mediated by interaction with three main VEGF receptors (VEGFRs) to which they bind with different affinity. The VEGF receptors are a family of receptor tyrosine kinases consisting of VEGFR-1 (Flt1), VEGFR-2 (KDR in humans and Flk1 in mouse) and VEGFR-3 (Flt4) (McMahon 2000). The most studied member of the VEGF family is VEGF-A, which is mostly referred to as VEGF. This ligand signals mainly through the VEGFR-2, which is the major VEGF receptor mediating angiogenesis (Kerbel 2008). Additionally, different isoforms of VEGF-A generated by differential splicing of

the human VEGF gene can bind to heparan sulphate proteoglycans and non-tyrosine kinase receptors such as neuropilins (NRPs) (Holmes & Zachary 2005). VEGFR-1 is able to bind VEGF-A with approximately 10-fold increase in affinity compared to VEGFR-2, although its signal transduction properties are very weak. In the case of VEGF-C and VEGF-D, these ligands bind both VEGFR-2 and VEGFR-3, whereas PlGF and VEGF-B bind only to VEGFR-1 (Kerbel 2008) (Figure 5).

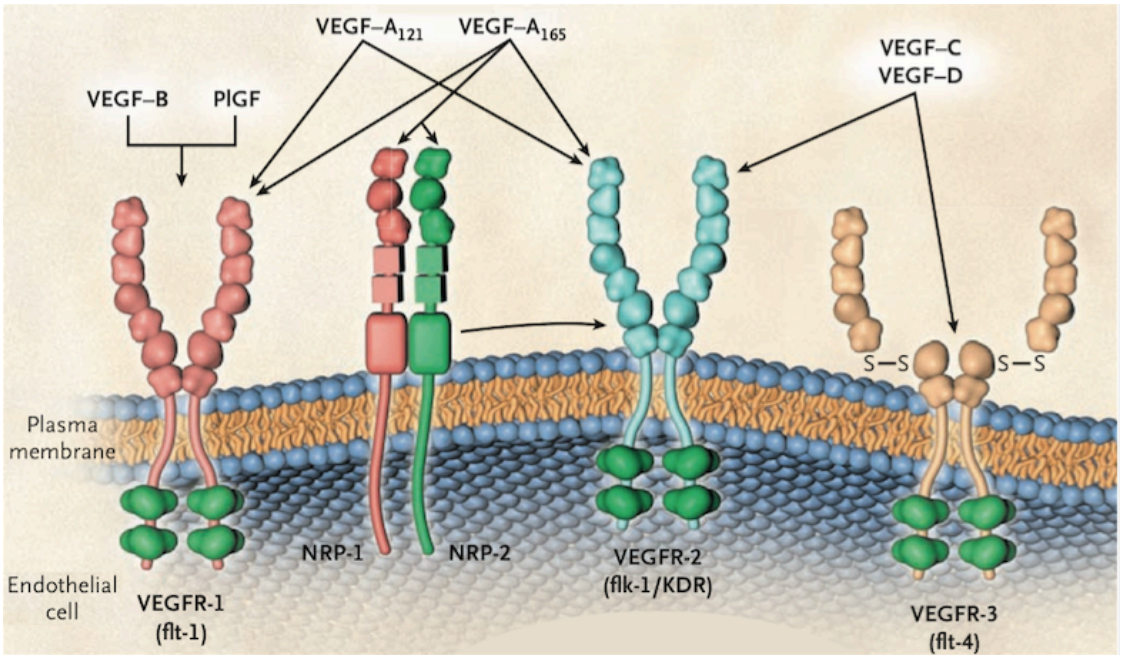

Figure 5: The VEGF family and their receptors. The VEGF family is comprised of five ligands, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF. These ligands bind with different affinity to VEGF receptors VEGFR-1, VEGFR-2 and VEGFR-3. Additionally, VEGFs signal through neuropilins NRP-1 and NRP-2, which are transmembrane glycoproteins with short cytoplasmic domains and function as VEGFR co-receptors in ECs (Graphic adapted from Kerbel 2008).

The signalling molecule VEGF is secreted by cancer cells and targets primarily VEGFR-2 expressing ECs to mediate tumour angiogenesis (Sakurai & Kudo 2011). Upon binding of VEGF to its receptor, VEGFR-2 transduce a series of signalling events in ECs promoting survival, proliferation, migration and formation of vascular tubes (Koch et al. 2011). Several VEGFR-2 tyrosine residues become phosphorylated, and subsequently function as docking sites for different downstream signalling mediators. Upon activation of these mediators, diverse signalling pathways become induced. Some of these signal transduction intermediates include Ras and phospholipase Cγ (PLCγ), which induce the MAPK/Erk signalling pathway, leading to cell proliferation (Meadows et al. 2001; Takahashi et al. 2001). Other mediators such as Gab1 activate signalling pathways including the PI3K/Akt pathway, which is known to regulate cell migration and survival (Datta et al. 1999; Laramee et al. 2007). The activation of these signalling pathways can

also occur independently of VEGF. For instance, the PI3K/Akt signalling pathway can also be activated via Gab1 through flow shear stress that stimulates the VEGFR-2 activation in the absence of VEGF (Jin et al. 2005). Similarly, other growth factors can stimulate the activation of different receptors that stimulate the activation of signalling pathways important for the angiogenic process as will be discussed in some detail below.

1.3.2. Therapies targeting the vascular endothelial growth factor pathway

Angiogenesis represents a vital process for tumour growth and development. Several studies *in vitro* and *in vivo* conducted in mice have highlighted the importance of VEGF and its receptor VEGFR-2 for angiogenesis and have been fundamental for the development of current anti-angiogenic drugs (Carmeliet et al. 1996). As such, the blockade of the murine equivalent of VEGFR-2 (the Flk-1 receptor) using the murine monoclonal antibody DC101 (the murine homolog of the human monoclonal antibody ramucirumab), showed an inhibition of the tumour growth in diverse mouse models as well as decreased formation of blood vessel within the tumours (K. Zhang & Waxman 2013; Cheong et al. 2011). Because of the importance of these receptors and their ligands for angiogenesis, several drugs targeting these growth factors and signalling pathways have been developed in order to inhibit the growth and proliferation of ECs, thus blocking the formation of a vasculature that supplies the tumour with oxygen and nutrients (Figure 6). To date, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved different anti-angiogenic agents including monoclonal antibodies and small-molecule tyrosine kinase inhibitors (TKIs).

Monoclonal antibodies are directed against specific pro-angiogenic factors and/or their receptors. Among these antibodies is bevacizumab, which has been most extensively studied in diverse clinical trials. Other monoclonal antibodies include ramucirumab and ranibizumab, which have recently been approved by the FDA for the treatment of cancer and other disorders as described in the following section.

- **Bevacizumab** (Avastin®, Genentech Inc.) is the first FDA approved angiogenesis inhibitor (Ferrara et al. 2004). It consists of a humanized monoclonal antibody directed against the VEGF-A ligand, thereby preventing it from binding to its receptor (VEGFR-2) and activating signalling pathways that mediate tumour angiogenesis (Buzdar 2011). This monoclonal antibody was shown to improve

the overall survival by about five months in patients with metastatic colorectal cancer when given as a combination treatment along with standard chemotherapy drugs (Hurwitz et al. 2004). As a result of the promising clinical activity against metastatic colorectal cancer, the FDA approved bevacizumab in 2004 as a first-line therapy for the treatment of this cancer type.

In 2008, the FDA approved bevacizumab as part of an accelerated approval program for the treatment of metastatic breast cancer. This accelerated approval was based on promising results that showed a prolonged progression-free survival in first-line treatments with bevacizumab and paclitaxel (Miller et al. 2007). However, this approval was revoked in 2010 after subsequent studies did not show any significant effects in terms of prolonged progression-free and overall survival in patients with breast cancer in both early and advanced stages of disease (Montero et al. 2011). Nevertheless, the EMA currently approves bevacizumab for the treatment of metastatic breast cancer in combination with other chemotherapy drugs including paclitaxel or capecitabine. Besides, bevacizumab has also been approved for the treatment of many other cancers in advanced stages such as non-small cell lung carcinoma in combination with chemoterapeutic drugs (Sandler et al. 2006) and in combination with interferon alpha for the treatment of patients with metastatic renal cell carcinoma (Escudier et al. 2007).

- **Ramucirumab** (Cyramza, Eli Lilly and Company) is a recombinant monoclonal antibody that binds to VEGR-2, thereby acting as receptor antagonist that blocks its activation. This monoclonal antibody was first approved in 2014 for use as single agent in the treatment of patients with advanced gastric cancer or gastroesophageal junction adenocarcinoma (Fuchs et al. 2014). Subsequently, the FDA expanded its use to treat patients with advanced gastric cancer in combination with the chemotherapy drug paclitaxel (Wilke et al. 2015). Besides, ramucirumab is also approved for the treatment of non-small cell lung carcinoma and metastatic colorectal carcinoma in combination with chemotherapy (Garon et al. 2014; Tabernero et al. 2015).

- **Ranibizumab** (Lucentis, Genentech Inc.) is a humanized anti-VEGF antibody that was approved in 2006 for the treatment of patients with macula degeneration. So far this antibody is not being used in the treatment of cancer (Heier et al. 2006).

Small-molecule TKIs block diverse pro-angiogenic growth factor receptors, including VEGF receptors such as VEGFR-2. The initial successes of bevacizumab were paralleled by the approval of many oral small-molecule TKIs. Some of the most clinically relevant TKIs include sunitinib and sorafenib, among others.

- **Sunitinib** (Sutent®, Pfizer) is a small molecule that inhibits multiple receptor tyrosine kinases including PDGF receptors (PDGFRα and PDGFRβ), VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3) and stem cell factor receptor (cKIT), among others (Aparicio-Gallego et al. 2011). The FDA approved sunitinib for the treatment of patients with pancreatic neuroendocrine tumours, renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumour (Goodman et al. 2007; Blumenthal et al. 2012). Several studies have evaluated the efficacy of sunitinib in addition to chemotherapy in patients with advanced or metastatic breast cancer. The results of these studies did not show any substantial efficacy of sunitinib, which has also been shown to cause sever side effects in some patients (Bergh et al. 2012; Crown et al. 2013).

- **Sorafenib** (Nexavar®, Bayer AG) is a TKI that targets receptors such as VEGFR-1, VEGFR-2 and VEGFR-3 and PDGFRβ, among other kinases. The FDA approved this inhibitor for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma (Kane et al. 2006; Llovet et al. 2008). Preclinical studies have demonstrated anti-proliferative, anti-angiogenic and pro-apoptotic effects of the sorafenib treatment in cancer models that include renal cell, hepatocellular, breast and colorectal carcinomas (Wilhelm et al. 2008). Some phase II clinical trials showed that sorafenib in combination with chemotherapy drugs such as gemcitabine or capecitabine improved the progression-free survival in patients with Her2/neu negative advanced breast cancer, thus leading to further phase III trials for these combinatory therapies (Baselga et al. 2012; Schwartzberg et al. 2013). In contrast, additional phase II trials showed no benefits of these combinations in advanced breast cancer. This is also the case when using sorafenib in combination with paclitaxel (Gradishar et al. 2013). Nevertheless, all of these trials consistently demonstrate adverse effects induced by sorafenib treatment, thereby requiring a reduction of the administered dose.

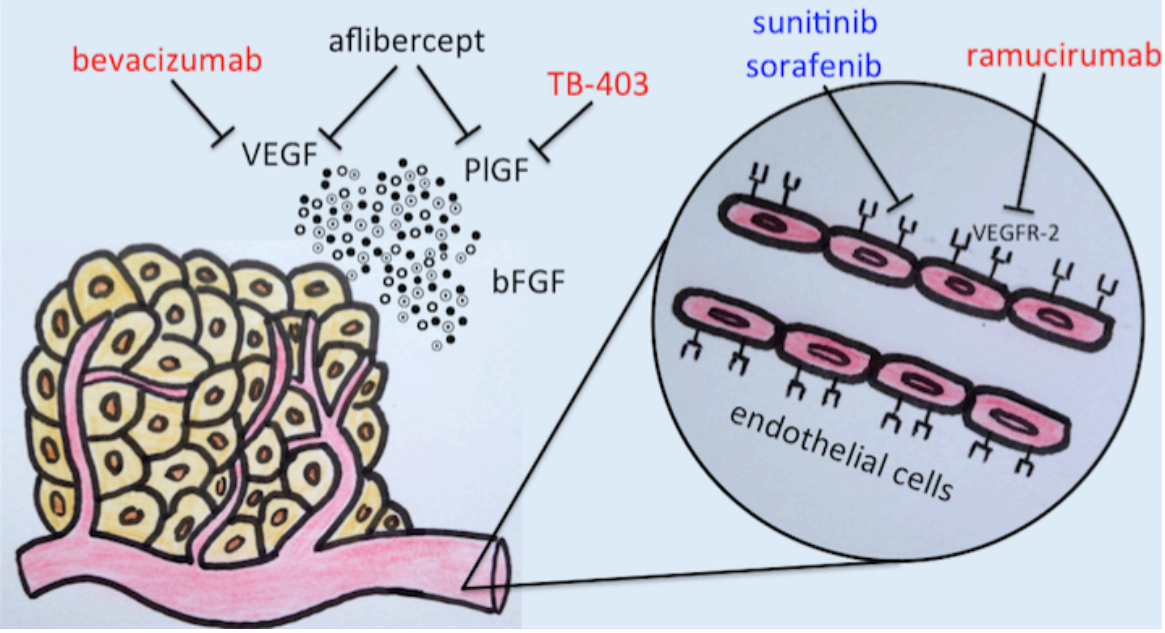

Figure 6: Anti-angiogenic therapies for the treatment of cancer. A common trait of solid tumours is their ability to secrete a plethora of growth factors, which upon binding to their correspondent receptors induce tumour angiogenesis. Different anti-angiogenic drugs consist of monoclonal antibodies (marked red) or small molecule TKI (marked blue). Monoclonal antibodies used in anti-angiogenic therapies include bevacizumab and ramucirumab, which target VEGF and VEGFR-2, respectively. Small-molecule TKIs include among others sunitinib and sorafenib. These inhibitors can block the activity of several receptors on ECs including VEGFR-2, thereby interfering with the angiogenic process. (© Cubas Cordova)

Despite the clinical benefits of VEGF(R) inhibitors reported in diverse clinical studies, their efficacy in the treatment of cancer remains limited in part due to innate and acquired tumour resistance mechanisms. So far, the clinical success of these therapeutics consist in prolonging the overall survival of cancer patients by only months rather than providing a curative effect (Loges et al. 2009). Among the factors responsible for these limited benefits are hypoxia and tumoral stromal cells, which are considered important mediators of tumour resistance.

1.4. Factors promoting tumour resistance against anti-angiogenic treatment

1.4.1. Hypoxia

Hypoxia initially arises due to oxygen diffusion limitations in avascular primary tumours. Later on, it results from the spatial disorganization and the unstable blood flow through an abnormal tumour vasculature, which is not able to rectify this oxygen deficit (Wilson & Hay 2011). Anti-angiogenic drugs can aggravate this condition, thereby causing elevated levels of tumour hypoxia as a result of reducing the tumour

microvessel density (vessel pruning) and the vessel blood flow (Franco et al. 2006). As a consequence, treatment-induced hypoxia contributes to a hostile environment that leads to an increased selection of hypoxia-tolerant and pro-angiogenic cancer stem cells (CSCs), radiation and chemotherapeutic resistance, invasiveness and metastasis (Loges et al. 2010; Carmeliet & Jain 2011; Wilson & Hay 2011). In addition, treatment-induced hypoxia fosters the recruitment of stromal cells such as cancer-associated fibroblast (CAFs), tumour-associated macrophages (TAMs) and mesenchymal-derived suppressor cells (MDSCs), and causes the secretion of pro-angiogenic factors that sustain tumour angiogenesis (Rapisarda & Melillo 2009; Riabov et al. 2014; Zheng et al. 2014). Therefore, hypoxia is considered an important driving-force of stroma-mediated resistance to anti-angiogenic therapies due to its ability to upregulate the production of angiogenic factors that aggravate the vessel disorganization and fuel angiogenesis (Carmeliet & Jain 2011). Hypoxia also regulates several genes that have an important role in the regulation of tumour angiogenesis. One of these genes is cyclooxygenase-2 (Cox-2), an enzyme that promotes angiogenesis and is upregulated under hypoxic conditions (Schmedtje et al. 1997; Rüegg et al. 2004; Lee et al. 2010). Hence, hypoxia-induced genes such as Cox-2 may be useful targets in order to overcome the onset of tumour resistance against anti-angiogenic treatments and will be discussed below.

1.4.2. Tumour stroma

Besides consisting of proliferating cancer cells, tumours are complex tissues composed of multiple host cell types that interact with one another and contribute actively to tumorigenesis. These normal cells form the tumour-associated stroma and include ECs, PCs, CAFs, and immune inflammatory cells (Hanahan & Weinberg 2011). Besides tumour cells, stromal cells contribute to resistance against anti-angiogenic therapy, as they produce a myriad of angiogenic factors that can substitute for one another, thus rescuing tumour vascularization. VEGF-targeting therapies induce the release of cytokines by healthy tissues, which induce a systemic 'pseudo-inflamed' state that may promote the recruitment of stromal cells into the tumour (Loges et al. 2010). Preclinical models have showed that VEFGR-2 blockade up regulates the expression of angiogenic factors such as placental growth factor (PlGF), VEGF, angiopoetin-1, fibroblast growth factor (FGF) family members, among others (Loges et al. 2010). For instance, FGFs and their receptors can mediate the activation of signalling pathways such as MAPK/Erk or PI3K/Akt, which are important for the regulation of cellular

proliferation, migration and survival, and can promote angiogenesis by stimulating proliferation of ECs (Turner & Grose 2010). Hence, the secretion of these factors may compensate the VEGF signalling blockade caused by anti-angiogenic drugs, thus restoring tumour angiogenesis. Due to their role mediating angiogenesis rescue and resistance against anti-angiogenic therapies, stromal cells have gained great interest as possible new targets for therapies:

- **Cancer-associated fibroblasts** are among the most common cell types in the microenvironment of solid tumours, being particularly abundant in carcinomas of breast, pancreas, prostate and colon (Allen & Louise Jones 2010). CAFs display an 'activated' phenotype in contrast to normal quiescent fibroblasts and are characterized by having a higher proliferative activity. A further hallmark of activated CAFs is the expression of markers such as α-smooth muscle actin (α-SMA) and fibroblast-activation protein (FAP) (Kalluri & Zeisberg 2006). The activation of CAFs occurs through the release of pro-fibrotic growth factors by tumour cells, which include TGFβ (Kalluri & Zeisberg 2006). When active, CAFs can contribute to tumour growth and spread, mediated through the release of growth factors, such as EGF, TGFβ and HGF, as well as multiple chemokines that have been shown to influence different aspects of tumour cell behaviour (Allen & Louise Jones 2010). In addition to tumour progression, growth and metastasis, activated CAFs are involved in the induction of angiogenesis through the expression of pro-angiogenic factors other than VEGF, such as FGF1, FGF2, PDGF-B and PDGF-C (Luo et al. 2015). Moreover, CAFs play a causative role of chemotherapy and target therapeutic resistance in breast cancer treatment and increased evidence has revealed a correlation between CAFs and a poor clinical outcome in breast cancer patients (Luo et al. 2015). For instance, collagen type I secreted by CAFs decrease uptake of chemotherapeutic drugs into tumours, while HGF contributes to resistance to EGFR inhibitors in triple negative breast cancer cells (Loeffler 2006; K. L. Mueller et al. 2012). Based on the important role of CAFs in tumour progression and angiogenesis, and due to their genetic stability compared with frequently mutant tumour cells, many studies of targeted therapy have focused on this stromal cell population (Cirri & Chiarugi 2011). In particular, anti-CAF therapy might exert anti-carcinogenic and anti-angiogenic effects and have the ability to increase tumour uptake of chemotherapeutic drugs (K. Zhang & Waxman 2013). A potential

therapeutic strategy includes inhibition of the FAP enzymatic activity, which is selectively expressed in activated CAFs. Accordingly, many new agents have undergone preclinical evaluation and clinical trials and provided encouraging results that may lead to the translation of this strategy into future clinical applications (K. Zhang & Waxman 2013).

- **Tumour-associated macrophages** are another important cell population within the tumour microenvironment that are actively recruited by chemokines, cytokines and growth factors derived from tumour and stromal cells such as CAFs (Gacche & Meshram 2014; Tripathi et al. 2014). Macrophages can be differentiated according to their phenotype into classically activated M1 macrophages and alternatively activated M2 macrophages. M1 macrophages are responsible for the microbicidal and tumoricidal response while M2 macrophages stimulate angiogenesis, invasion and immunosuppression (Tripathi et al. 2014; Loges et al. 2010). TAMs closely resemble the M2 phenotype and release diverse potent pro-angiogenic growth factors and cytokines such as VEGF, TNF-α, IL-8 and FGF2 (Lewis 2006). They accumulate preferentially in hypoxic and necrotic areas of the tumour and their numbers correlate with adverse clinical outcome and poor overall survival in various cancer types, including breast cancer (Murdoch 2004; DeNardo et al. 2011). TAMs have been reported to decrease the efficacy of anti-angiogenic drugs and promote the development of tumour resistance to these therapeutics (Bergers & Hanahan 2008). Hypoxic tumour cell-derived cytokines, such as oncostatin M and eotaxin, are critical regulators of macrophage recruitment and their polarization to M2 skewed macrophages and blocking of these cytokines decreased resistance in syngeneic mouse models (Tripathi et al. 2014). Furthermore, blockade of angiopoetin-2 (Ang2), a Tie2 ligand secreted by ECs, showed in mammary and pancreatic tumour models to inhibit upregulation of Tie2 by TAMs. As a consequence their association with blood vessels and their ability to restore angiogenesis in response to anti-angiogenic therapy was blocked, indicating that the Ang2/Tie2 pathway regulates the pro-angiogenic activity of TAMs (Mazzieri et al. 2011). Hence, TAMs represent also an interesting stromal target as they point towards new combination therapies whereby inhibition of recruitment and M2 polarization of macrophages may lead to an increased efficacy of anti-angiogenic treatments.

- **Myeloid-derived suppressor cells (MDSCs)** comprise a heterogeneous population of immature myeloid cells, which are observed in the peripheral blood and tumour microenvironment of cancer patients (Goedegebuure et al. 2011). The number of MDSCs correlates with a poor prognosis and tumours vasculogenesis (Talmadge & Gabrilovich 2013). Two main subsets of MDSCs have been identified in cancer patients: a monocytic (mMDSCs) and a granulocytic (gMDSCs) subset. The prevalence of each subset appears to be disease-related, as gMDSCs were detected in patients with renal, lung, pancreas, and breast cancer, whereas mMDSCs were detected in patients with glioblastoma, liver and ovarian cancer (Goedegebuure et al. 2011; Katoh & Watanabe 2015). However, both subsets of MDSCs actively suppress host immunity (Goedegebuure et al. 2011). In addition to immune suppression, MDSCs enhance angiogenesis through the secretion of pro-angiogenic factors such as VEGF and TGFβ, and promote tumour progression and metastasis by secreting matrix-degrading enzymes such as MMPs (Bierie & Moses 2010). *In vivo* studies have also demonstrated that the recruitment of gMDSCs is mediated by tumour-derived granulocyte colony stimulating factor (G-CSF) and represents a mechanism underlying resistance to anti-VEGF therapy (Shojaei et al. 2009). Besides, gMDSCs express Bv8 and mediate tumour angiogenesis via STAT3 signalling pathway (Shojaei et al. 2007; Xin et al. 2013). Therefore, blockade of pathways and mechanisms that mediate the recruitment and activation of these stromal cells might help to improve response to anti-angiogenic therapies.

1.5. Role of alternative pro-angiogenic factors in angiogenesis

1.5.1. Alternative pro-angiogenic factors in human tumours

Besides the VEGF ligands, other pro-angiogenic factors are expressed in many malignant tumours. Some of these pro-angiogenic factors include:

Fibroblast growth factors (FGFs)

The FGF family members consist of eighteen different ligands which are involved in a plethora of developmental processes as well as in angiogenesis and wound healing (Beenken & Mohammadi 2009). Among the FGF ligands, FGF2 is the most extensively studied peptide of this family. This ligand features a potent angiogenic activity and is

expressed in many malignant tumours including breast, pancreas and prostate cancer by both cancer and stromal cells (Korc & Friesel 2009; Cirri & Chiarugi 2011). Besides the pro-angiogenic traits of FGF2, it can act in a paracrine and autocrine manner on the tumour, thus promoting its growth. FGFs can elicit signal transduction through the activation of their tyrosine kinase receptors FGFR1-4. FGF2 binds FGFR1, which is the main FGF receptor expressed on ECs (Korc & Friesel 2009). Together with other pro-angiogenic factors, FGF2 is implicated in the emerging resistance to the inhibition of VEGFR-2, which results in the reactivation of tumour angiogenesis (Gacche & Meshram 2014).

Platelet-derived growth factors (PDGFs)

The PDGF family consists of four ligands referred to as PDGF-A, PDGF-B, PDGF-C and PDGF-D, which can form homodimers or a PDGF-AB heterodimer (Patel-Hett & DAmore 2011). Receptors for PDGFs include PDGFRα and PDGFRβ, both receptor tyrosine kinases. The PDGF ligands play a key role in angiogenesis as it could be observed in *in vivo* models. For instance, PDGF-B is expressed in proliferating ECs while its receptor PDGFRβ is expressed on the surface of PCs (Gacche & Meshram 2014). The activation of these receptors plays an important role in the recruitment of PCs to blood vessels, which is an essential step during angiogenesis, promoting maturation and stabilization of new blood vessels (Gacche & Meshram 2014). PDGF-B can also directly induce the proliferation and migration of ECs as well as tube formation in contrast to PDGF-A, which lacks these effects (Gacche & Meshram 2014).

Hepatocyte growth factor (HGF)

HGF is a mesenchyme-derived pleiotropic cytokine that strongly stimulates angiogenesis. It acts directly on proliferating ECs by binding to its receptor c-Met, a transmembrane tyrosine kinase encoded by the MET proto-oncogene (Ding et al. 2003). The expression of HGF and the c-Met receptor has been observed in many solid tumours including breast cancer (Parr et al. 2004). Interestingly, the HGF/c-Met pathway has been demonstrated in an experimental *in vivo* model to act as an alternative angiogenic pathway responsible for the onset of tumour resistance against anti-angiogenic treatments (Shojaei et al. 2010). Despite HGF being expressed and secreted by tumour cells, this cytokine is also expressed by components of the tumour stroma. For instance, ECs from multiple myeloma patients were shown to express both

HGF and c-Met, which act in an autocrine manner thereby sustaining angiogenesis (Ferrucci et al. 2014). In addition, CAFs also markedly express HGF, thus promoting tumour growth and progression as observed in specimens of squamous cell carcinoma (Xu et al. 2013).

Transforming growth factor beta (TGFβ)

The TGFβ family consists of three isoforms (TGFβ1-3) that bind two types of receptors referred to as TGFβRI and TGFβRII (Patel-Hett & DAmore 2011). TGFβ exerts pleiotropic effects in the progression of breast cancer, since it can inhibit angiogenesis at early stages of the disease but is able to stimulate this process in advanced disease stages by positively regulating EC proliferation and migration (Li et al. 2001). In addition, *in vitro* studies using breast cancer cells showed that blocking of TGFβ was sufficient to inhibit cell invasiveness, migration and angiogenesis (X. G. Wang et al. 2014). TGFβ is very frequently overexpressed in tumours including breast, oesophageal, lung and prostate cancer but its expression is also localized in adjacent stromal tissues (Levy & Hill 2006). Besides tumour cells, stromal cells such as MDSCs are known to be an abundant source of TGFβ (Bierie & Moses 2010).

Interleukin 6 (IL-6)

IL-6 is a multifunctional cytokine, which plays an important role in diverse tumoral processes such as modulation of growth and differentiation. Abnormal overexpression of IL-6 is associated with tumour progression through inhibition of apoptosis, stimulation of angiogenesis and increased drug resistance. Diverse clinical studies showed that increased IL-6 serum levels are associated with advanced tumour stages of various cancers including breast cancer (Guo et al. 2012). IL-6 is expressed at high levels by CAFs in breast cancer tumours (Erez et al. 2013). Therefore, blocking the IL-6 signalling pathway might be of therapeutic potential in order to overcome tumour resistance towards VEGF-anti-angiogenic therapies.

Taken together, these and other alternative pro-angiogenic factors are implicated in the induction of tumour angiogenesis and can be secreted by both malignant and non-malignant cells. Therefore, these factors might have a pivotal role in angiogenesis rescue, since they could compensate the inhibition of VEGF by anti-angiogenic therapies, thus being responsible for a low efficacy of these therapies. For this reason,

they have generated great interest as possible targets for new therapies that aim to inhibit angiogenesis in addition to blocking the VEGF signalling pathway.

1.5.2. Novel anti-angiogenic therapies

Despite the positive effects that angiogenesis VEGF inhibitors showed in the treatment of some cancers both in preclinical and clinical trials, the translation of these results into the clinical practice faces some major challenges that include:

- Adverse effects caused by anti-angiogenic agents due to endothelial dysfunction and vessel pruning in healthy tissues (Loges et al. 2010).
- Little or no efficacy in some types of cancer such as pancreatic cancer due to intrinsic tumour resistance (Loges et al. 2010).
- Short-lived efficacy since withdrawal of anti-angiogenic agents induce rapid regrowth of tumour vessels and restoration of tumour growth and progression (Loges et al. 2010).

In breast cancer therapies, diverse clinical trials have failed to consistently demonstrate clinical benefit out of the use of anti-angiogenic drugs that target the VEGF signalling pathway. For instance, treatments with bevacizumab in patients with colon and metastatic breast cancer were not able to not prolong the overall survival (Allegra et al. 2013; Miller et al. 2007). However, trials with other drugs such as ramucirumab showed improved overalls survival in patients with metastatic colorectal cancer and advanced gastric cancer (Tabernero et al. 2015; Wilke et al. 2015). The limitation of these anti-angiogenic drugs might be caused by pro-angiogenic factors different from VEGF that are secreted by tumour and stromal cells and due to treatment-induced intratumoral hypoxia, which mediates the upregulation of many cytokines able to foster tumour angiogenesis (Loges et al. 2010; Bergers & Hanahan 2008). The secretion of these alternative pro-angiogenic factors upon VEGF inhibition could be observed as a mechanism that the tumour uses to compensate the blocking of VEGF and thus sustain tumour angiogenesis via many other signalling pathways. Therefore, it is necessary to identify new therapeutic targets besides the VEGF axis, which might be useful to increase the efficacy of anti-angiogenic treatments.

The number of anti-angiogenic therapies targeting pro-angiogenic factors besides VEGF is still scarce at the present time. Some novel anti-angiogenic inhibitors include

Aflibercept (Zaltrap®, Sanofi and Regeneron Pharmaceuticals, Inc.), which is a human fusion protein that binds PlGF-1 and PlGF-2 in addition to VEGF-A and VEGF-B and acts as a trap for these growth factors. Aflibercept showed efficacy in the treatment of metastatic colorectal cancer in phase III clinical trials, thus leading to the FDA approval in 2012 for the treatment of this cancer type (Lockhart et al. 2012). However, a phase II trial using this fusion protein in patients with metastatic breast cancer who were previously treated with chemotherapy did not show significant clinical benefits and adverse effects expected for VEGF-treatments occurred (Sideras et al. 2012).

1.6. The cyclooxygenase-2

1.6.1. Role of cyclooxygenase-2 in tumour angiogenesis

Cyclooxygenase (Cox) is the rate-limiting enzyme responsible for the conversion of arachidonic acid to prostaglandins, which play a pivotal role in inflammatory processes and also in carcinogenesis and tumour growth (Hoellen et al. 2011). There are two isoforms of this enzyme that have been described:

- Cox-1 is constitutively expressed in several tissues and its enzymatic activity is linked to renal function, gastric mucosal maintenance stimulation of platelet aggregation and vasoconstriction (D. Wang & DuBois 2006).
- Cox-2 is an inducible isoform that is normally absent in most cells and tissues but is highly induced in response to proinflammatory cytokines, hormones and tumour promoters (D. Wang & DuBois 2006).

The induction of Cox-2 caused for instance by hypoxia quickly results in the biosynthesis of diverse structurally related prostaglandins, particularly of prostaglandin E_2 (PGE_2), which are able to induce angiogenesis, invasion or loss of apoptosis upon binding and activation of the prostaglandin receptors EP1-4 (Harris 2014) (Figure 7). Diverse studies have demonstrated overexpression of Cox-2 as well as high levels of PGE_2 in several human cancers including oesophagus, colon, ovary and breast (Eberhardt et al. 1994; Zimmermann et al. 1999; Ali-Fehmi et al. 2010). In breast cancer, the overexpression of Cox-2 is known to occur throughout the entire development and progression of the malignancy (Harris 2014). Furthermore, both Cox-2 overexpression and increased levels of PGE_2 have been defined in many studies as biomarkers that

correlate with a poor prognosis (Hoellen et al. 2011). *In vitro* experiments with Cox-2 expressing tumour cells showed that these cells expressed increased levels of angiogenic factors including VEGF, FGF2, PDGF and endothelin, and were able to stimulate both endothelial migration and tube formation (Tsujii et al. 1998). Besides, it has been reported that Cox-2-derived PGE_2 can directly stimulate endothelial cell migration and angiogenesis (Gately 2000). Therefore, the blockade of Cox-2 might be an attractive target to inhibit tumour progression.

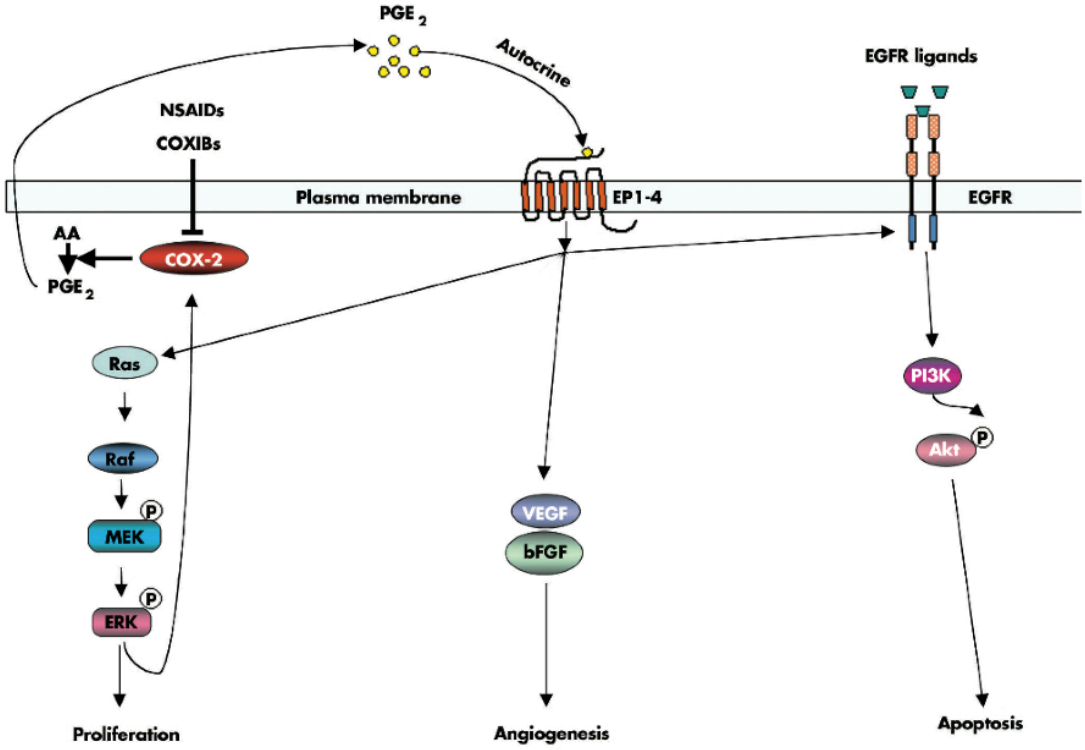

Figure 7: The PGE_2 signalling pathway. COX-2 catalyses the key step of the synthesis of PGE_2 from arachidonic acid. This prostaglandin can act either in a paracrine or autocrine manner through the binding to the EP receptors, mediating several responses through the activation of multiple signalling pathways including MAPK/Erk and PI3K/Akt. Activation of these pathways mediate among others cell proliferation, angiogenesis and inhibition of apoptosis (Graphic adapted from D. Wang & DuBois 2006).

1.6.2. Signal pathways activated by cycloocygenase-2

PGE_2 is the major proinflammatory bioactive lipid in many human solid tumours and is produced from arachidonic acid through the actions of Cox-2 and PGE synthases (D. Wang & DuBois 2006; O'Callaghan & Houston 2015). PGE_2 binds to its cognate prostaglandin receptors, thus triggering the activation of many signalling pathways that mediate diverse effects including tumour cell proliferation, angiogenesis, inhibition of apoptosis, stimulation of invasion/motility and suppression of the immune response (D. Wang & DuBois 2006) (Figure 7). Among these pathways, two of the most important include the PI3K/Akt and MAPK/Erk signalling pathways.

The PI3K/Akt signalling pathway

The PI3K/Akt signalling pathway plays an important role in multiple cellular processes such as cell proliferation and apoptosis. A study conducted in estrogen receptor negative and Her2/neu-positive breast tumours provided evidence that high expression of Cox-2 is associated with the activation of this signalling pathway and is a marker of poor outcome (Glynn et al. 2010). A different study showed also that phosphorylation of Akt strongly correlated with the expression of Cox-2 in breast tumours and *in vitro* experiments using a breast cancer cell line demonstrated that PGE_2 induces the phosphorylation of Akt (Prueitt et al. 2006). Furthermore, PGE_2 can transactivate the EGF receptor, resulting in stimulation of cell migration through increased PI3K/Akt signalling in colorectal cancer cells (Pai et al. 2002). Inhibition of Cox-2 by celecoxib was reported to induce apoptosis in human prostate cancer cells by blocking the Akt activation (Hsu et al. 2000). Similarly, a recent study showed that celecoxib suppresses the effects elicited by the activation of Akt through the upregulation of PTEN in hepatic cancer cells, which acts as an inhibitor of the PI3K/Akt signal pathway (Chu et al. 2014). Taken together, diverse studies indicate that Cox-2 stimulates cell proliferation and inhibits cell apoptosis in different cancer types through Akt activation.

The MAPK/Erk signalling pathway

The MAPK/Erk pathway is one of the major intracellular signalling pathways responsible for cell proliferation. This signalling pathway is activated in response to PGE_2, as it was observed in colorectal cancer cells (D. Wang et al. 2005). The effects of the MAPK/Erk signalling pathway were shown to be suppressed *in vitro* by blocking specifically Cox-2 in a colorectal cancer cell line using the inhibitor SC-236, thus impairing the cell proliferation (Sobolewski et al. 2010). Besides the effects on tumour cell proliferation, PGE_2-mediated activation of this signalling pathway increases the expression of VEGF and FGF2 in ECs, thereby fostering tumour angiogenesis (D. Wang & DuBois 2006).

1.6.3. Inhibitors of cyclooxygenase

Two main groups of Cox inhibitors consist of non-selective inhibitors referred collectively to as non-steroidal anti-inflammatory drugs (NSAIDs) and of Cox-2-specific inhibitors (COXIBs).

- **NSAIDs** inhibit the activity of both Cox-1 and Cox-2, thereby blocking the biosynthesis of prostaglandins. Acetylsalicylic acid (aspirin) and isobutylphenyl propanoic acid (ibuprofen) are some of the best-known drugs categorized in this group. Clinical and experimental evidence indicates that NSAIDs have protective effects against cancer and use of these drugs in animal models of mammary carcinogenesis reduced the growth and progression of breast tumours (Harris 2014).
- **COXIBs** specifically block the activity of Cox-2, thus supressing the tumour growth in animal models. For instance, celecoxib, a specific Cox-2 inhibitor has been reported to reduce the incidence, multiplicity and volume of carcinogen-induced breast cancer in rats (Harris et al. 2000). Furthermore, celecoxib impairs the expression of angiogenic proteins such as VEGF and FGF2 and is able to block the ability of highly invasive human breast cancer cells to form vascular channels, an alternate neoangeonesis pathway known as vascular mimicry, which is independent of neoangiogenesis (Basu et al. 2005). Similarly, other Cox-2-specific inhibitors such as SC-236 decreased angiogenesis and VEGF levels in a murine breast cancer model (Connolly et al. 2002).

Taken together, the role of Cox-2 in tumorigenesis and tumour progression has been demonstrated in several animal models. The overexpression of Cox-2 in different cancer types induces the biosynthesis of PGE_2, which upon binding to its EP receptors elicits the activation of multiple signalling pathways that control proliferation, angiogenesis and survival, among other processes. Since VEGF and additional angiogenic factors are down regulated upon Cox-2 inhibition, the use of NSAIDS or COXIBs in combination with anti-angiogenic therapies is an attractive idea to increase efficacy of treatment in experimental breast cancer models.

2. Aim of the thesis

The effects of anti-angiogenic treatments have been proved to be modest in several cancers in part because of the existence of inherent and acquired tumour resistance. Several studies have shown that angiogenesis inhibitors targeting the VEGF signalling pathway elicit hypoxia, which is considered a driving force for the development of resistance against these drugs. Hypoxia can promote tumour resistance through many mechanisms including the recruitment of host-related stromal cells, which can rescue tumour angiogenesis by secreting diverse pro-angiogenic factors. Besides, hypoxia is responsible for the regulation of several genes that might play an important role in resistance. Cox-2 is a gene commonly upregulated by hypoxia which is overexpressed in diverse cancer types and is involved in the recruitment of stromal cells into the tumour. Hence, Cox-2 might be an interesting target molecule in order to decrease the development of tumour resistance upon treatment with anti-angiogenic therapies.

The major aims of this thesis were:

- Investigate the therapeutic efficacy of anti-angiogenic drugs in combination with Cox-2 inhibitors in breast cancer tumour models.
- Elucidate the role of different cell populations in the tumour stroma that might mediate resistance to anti-angiogenic therapies.
- Study Cox-2-related mechanisms that might induce resistance to anti-angiogenic therapies.

3. Materials and Methods

3.1. Animal experiments

3.1.1. Mouse line

Six to eight weeks old female Balb/C mice were purchased from Charles River Laboratories International (Sulzfeld, Germany). Housing and breeding were carried out under a 12h light – 12h dark cycle and standard laboratory conditions, i.e. temperature of 22±1°C, 55% humidity, food and water *ad libitum* and 150-400 lx light intensity during the light phase.

3.1.2. Breast cancer models

Animal experiments were performed in collaboration with Dr. Isabel Ben Batalla using the murine breast cancer cell lines 4T1 (previously stably transfected with eGFP as described below) and 66cl4, both with a BALB/c background. Either $5x10^5$ 4T1 or $1x10^6$ 66cl4 murine breast cancer cells were orthotopically implanted into the second mammary gland of syngeneic BALB/c mice. Randomization of animals was carried out approximately 7-10 days after tumour cell implantation, when tumours reached a mean size of 80-150mm^3. Animal experiments were approved by the Behörde für Soziales, Familie, Gesundheit, Verbraucherschutz; Amt für Gesundheit und Verbraucherschutz, Hamburg, Germany according to the project number 98/10. All experiments were performed according to the institutional guidelines for the welfare of animals.

3.1.3. Drugs

Acetylsalicylic acid, also known as aspirin (ASA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in a 0.9% NaCl solution with dimethyl sulfoxide (DMSO). The final concentration of DMSO in cell culture medium was 1/1000 (v/v). The solution was freshly prepared. The specific Cox-2 inhibitor SC-236 was purchased from Cayman Chemical (Ann Arbor, MI, USA). SC-236 was dissolved in ethanol with phosphate buffered saline (PBS) pH 7.6 in a 1:4 solution of ethanol:PBS and stored at -20°C. Sunitinib malate was obtained from Pfizer (New York, NY, USA) and was suspended in a carboxymethylcellulose (CMC) solution (0.5% CMC, 1.8% NaCl, 0.4% Tween 80, and 0.9% benzyl alcohol in distilled water) for *in vivo* experiments. Drug aliquots were prepared once weekly and kept in the dark at 4°C. The murine anti-

VEGFR-2 antibody DC101 was obtained from ImClone Systems (Branchburg, NJ, USA) and was diluted in PBS. Aliquots were stored at -20°C. PGE_2 was purchased from Cayman Chemical, dissolved in DMSO and stored at -20°C. The Akt inhibitor MK-2206 was purchased from Merck Millipore (Darmstadt, Germany), dissolved in PBS with 20% ethanol and stored at -20°C.

3.1.4. Treatment of animals

All single and combinatory treatments were started directly after randomization of animals. Animals were treated with daily intraperitoneal (i.p.) injections of 12.5, 25 or 100mg/kg ASA. Similarly, single treatments with 1.5mg/kg of the specific Cox-2 inhibitor SC-236 and 10, 15 or 40mg/kg of the anti murine VEGFR-2 antibody DC101 were administered three times per week as i.p. injections. Treatments with the pan-VEGFR inhibitor sunitinib were administered by oral gavage once per day, at doses of 10, 20, 40 or 60mg/kg. All combinatory treatments were carried out as described in the corresponding graphs and were started at the same day.

3.1.5. Analysis of tumour growth and tissue

The tumour growth was monitored by calliper and its volume was calculated according to the formula V=(longer length^2 x shorter length)/2. Animals were sacrificed according to ethical regulations when the first tumour in the control group reached the maximum allowed size of 1500 mm^3. For analysis of tumour cell proliferation and hypoxia, animals were intraperitoneally injected with bromodeoxyuridine (BrdU; Sigma-Aldrich) and hypoxyprobe (pimonidazole HCl, Hypoxyprobe™-1 Omni Kit, Hypoxyprobe, Inc., Burlington, MA, USA) twelve and two hours prior to sacrification, respectively. After sacrification of mice, tumours were excised and weighed. The whole tumours were cut transversely in two equal pieces, one piece was embedded in paraffin for further immunohistochemistry (IHC) or immunofluorescence (IF) analyses and the other piece of fresh tumour tissue was frozen for protein and RNA extractions. For paraffin embedding, tumour tissues were put into tissue embedding cassettes and fixed in a 1% neutral-buffered formalin solution (Grimm med Logistik GmbH, Torgelow, Germany) over night at 4°C. The tumour pieces were subsequently dehydrated prior paraffin embedding to remove all traces of water. Dehydration was accomplished by passing the cassettes with the tumour tissues through a series of increasing ethanol concentrations, i.e. 70%, 80%, 96% and 100% ethanol. The cassettes containing the

tumour tissues were then placed into a pot of melted paraffin for embedding of tumour pieces into a paraffin block. Paraffin-embedded tumour tissues were stored at room temperature and frozen tumour tissues at -80°C.

3.2. Cell Culture

3.2.1. Cell lines

The murine breast cancer cell line 4T1 was kindly provided by Peter Carmeliet from the Vesalius Research Centre, K.U. Leuven, Belgium. The murine breast cancer cell line 66cl4 was obtained from Fred Miller from the Karmanos Cancer Institute and Wayne State University, Detroit, MI, USA. The human embryonic fibroblast cell line MRC-5 and the primary human cancer associated fibroblasts (CAFs), which were isolated from tumour tissue of lung cancer patients (n=2), were kindly provided by Iñigo-Martinez-Zubiaurre from the Arctic University of Norway, Tromsø, Norway.

3.2.2. Cell culture

The murine breast cancer cell line 4T1 was cultivated in RPMI-1640 medium (Life Technologies, Darmstadt, Germany). Primary human CAFs as well as the murine breast cancer cell line 66cl4 were both cultivated in DMEM medium (Life Technologies). Culture media were supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptavidin. The human embryonic fibroblast cell line MRC-5 was cultivated in MEM medium supplemented with 1% sodium pyruvate. Murine cell lines and primary human CAFs were grown in cell culture flasks or six well plates in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Confluent cell lines were washed with pre-warmed DPBS (Life Technologies) and incubated for two minutes with 0.05% trypsin-EDTA for cell detachment. Cells were subsequently resuspended in fresh culture medium and centrifuged for 5 minutes at 1.500-1.600 rpm. The supernatant was discarded and the pelleted cells were resuspended in fresh culture medium to be seeded at a dilution of 1:2 to 1:10. Determination of the cell number was done using a Neubauer haemocytometer. Dead cells were stained with trypan blue stain (Biochrom AG, Berlin, Germany) prior to cell counting.

3.2.3. Lentiviral transduction of 4T1 cells

Murine breast cancer 4T1 cells were stably transduced with lentiviral particles carrying a LeGO-G2 vector expressing enhanced *green fluorescent protein* (eGFP) and a resistance gene against puromycin. Lentiviral particles were kindly provided by Kristoffer Riecken, Clinic for Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. For lentiviral transduction, $5x10^4$ murine breast cancer 4T1 cells were seeded into 24-well dishes. On the day of transduction, culture medium was changed to fresh culture medium supplemented with 8µg/ml polybren and supernatant containing lentiviral particles was added to the cells. The 6-well dish was thereafter centrifuged at 1.000g and 24°C for one hour. Transduced 4T1 cells were incubated for one day before the virus supernatant was removed by replacing the culture medium. Analysis of the transduction efficiency was carried out by flow cytometry two days after transduction based on the fluorescence emitted by eGFP. Selection of 4T1-transduced cells was carried out by incubating the cells in culture medium containing 5µg/µl puromycin until a purity of at least 90% was achieved.

3.2.4. Treatment of cells

Primary CAFs and MRC-5 cells were starved in serum-free medium over night. For qRT-PCR and cell viability assays, $1x10^5$ or $9x10^3$ cells were seeded in 6 well plates or 96 well plates, respectively. Cells were then cultivated for 48 hours in fresh serum-free medium containing either 10ng/ml PGE_2, 5mM ASA, 15µM SC-236 and 7.5µM of the Akt inhibitor MK-2206 or any combination of these reagents as described in the corresponding graphs below. For migration assays, $1.1x10^4$ cells were seeded onto migration inserts and placed in 24 well plates, followed by treatments for 24 hours with 10ng/ml PGE_2, 5mM ASA, 15µM SC-236 and 7.5µM MK-2206 previously dissolved in serum-free medium alone or in combination. Likewise, treatments for western blot analyses were performed using $3x10^6$ cells incubated with the same concentrations of reagents mentioned before for 3 hours. For qRT-PCR and western blot analyses, cells were harvested and lysed immediately after treatments.

3.3. Fluorescence-activated cells sorting (FACS)

The following solutions were used for the homogenization of tumours and for FACS sorting:

- **Protease solution**

  0.2mg/ml Collagenase A (Roche Diagnostics GmbH, Mannheim, Germany)
  Collagenase A was dissolved in 50ml RPMI-1640 medium (Life Technologies) following incubation at 37°C to dissolve.

- **PBS/DNase solution**

  0.015mg/ml DNase (Roche Diagnostics GmbH)
  DNase was dissolved in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies)

- **FACS buffer**

  200ml 10x PBS
  40ml FBS (heat inactivated)
  36ml 5% NaN_3
  4.8ml 10% EDTA
  All components were diluted and dissolved in 2L of distilled water. The FACS buffer was stored at 4°C.

For sorting of tumour cells and tumour infiltrating cells, the resected tumours were first cut into small pieces and subsequently digested in 15ml of protease solution for 1 hour at 37°C on a rotating table. Thereafter, the supernatants were removed and kept on ice, while the tumour pellets were incubated for another 30 minutes at 37°C on a rotating table using 15ml of fresh protease solution. After incubation, 10ml PBS/DNase was added to the tumour pellets and the incubation was continued for further 30 minutes. Single cell suspensions were obtained by filtering the digested tumours through a 70µm pore cell strainer. The single cell suspensions were incubated with Fc-block (anti-CD16/32; BioLegend, San Diego, CA, USA) for 10 min on ice to block unspecific binding sites on the cell surface. Thereafter, cells were incubated for 30 min at 4°C with antibodies described in Table 1. After incubation cells were rinsed with 1ml FACS buffer, centrifuged at 1.200 rpm for 5 min and pellets were resuspended in FACS buffer. The specificity of all antibodies was determined by using appropriate corresponding isotype controls. Exclusion of dead cells was accomplished by co-staining with 7-AAD or DAPI (BioLegend Inc., San Diego, CA, USA). Multicolour-flow cytometry was used to sort tumour cells ($GFP^+/CD45^-$), tumour associated macrophages (TAMs; $F4/80^+/CD11b^+$), endothelial cells (ECs; $CD31^+/CD45^-/GFP^-$), cancer-associated fibroblasts (CAFs;

PDGFR$^+$/CD45$^-$/GFP$^-$), granulocytic myeloid-derived suppressor cells (gMDSCs; CD11b$^+$/Ly6G$^+$Ly6C$^{low/-}$) and monocytic MDSCs (mMDSCs; CD11b$^+$/Ly6C$^+$Ly6G$^-$). FACS sorting was performed with a FACS Aria IIIu using the FACS Diva software version 6.1.3. Sorted cells were recollected in RNA lysis buffer.

Antibody	Manufacturer	Catalog No.	Dilution	Conjugate
monoclonal rat anti-mouse CD140a (PDGFR-α)	BioLegend	135907	1:50	APC
monoclonal rat anti-mouse F4/80	eBioscience	12-4801	1:50	PE
monoclonal rat anti-mouse CD11b	eBioscience	25-0112	1:50	PE-Cy7
monoclonal rat anti mouse Ly-6G	BD Pharmingen	551461	1:50	PE
monoclonal rat anti-mouse Ly-6C	eBioscience	45-5932	1:50	PerCP-Cy5.5
monoclonal rat anti-mouse CD45	eBioscience	17-0451	1:50	APC
monoclonal rat anti-mouse CD31	eBioscience	25-0311	1:50	PE-Cy7
CD16/32 (Fc Block)	BioLegend	101319	1:50	none
rat IgG2a K isotype control (RTK2758)	BioLegend	400511	1:50	APC
rat IgG2a K isotype control (eBR2a)	eBioscience	12-4321	1:50	PE
rat IgG2b K isotype control (eB149/10H5)	eBioscience	25-4031	1:50	PE-Cy7
rat IgG2a K isotype control (R35-95)	BD Pharmingen	553930	1:50	PE
rat IgG2b K isotype control (A95-1)	BD Pharmingen	553991	1:50	APC
rat IgG2a K isotype control (eBR2a)	eBioscience	25-4321	1:50	PE-Cy7

Table 1: Primary and isotype antibodies used for flow activated cell sorting (FACSs).

3.4. ELISA assay

For preparation of tumour lysates for ELISA assays, the following solutions were prepared and used:

- **1M K_2HPO_4 solution**

  87.1g K_2HPO_4

  Dipotassium phosphate was dissolved in 500ml of distilled water and stored at room temperature.

- **1M KH_2PO_4 solution**

  68g KH_2PO_4

  Monopotassium phosphate was dissolved in 500ml of distilled water and stored at room temperature.

- **0.1M Potassium Phosphate Buffer, pH 7.4**

  80.2ml 1M K_2HPO_4

  19.8ml 1M KH_2PO_4

Buffers were mixed as indicated above and dissolved to a final volume of 1L with distilled water. The pH was set to 7.4 with HCl and the buffer stored at room temperature.

For ELISA analyses of PGE_2 in tumours, frozen tumour tissues were mechanically disaggregated with a homogeniser in 300µl of 0.1M potassium phosphate buffer containing protease inhibitors (cOmplete EDTA-free, Roche Diagnostics GmbH) and kept on ice. The homogenization of tumours was completed by sonication using an ultrasonic device (Hielscher Ultrasonic GmbH, Teltow, Germany). Tumour lysates were then centrifuged at maximum speed at 4°C for 10 min and supernatants were collected and stored at -20°C or -80°C for long-term storage. The protein concentration of tumour lysates was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany) according to the manufacturer's specifications. Reactions were set up in 96-well plates and absorbance was detected at 595nm in an ELISA reader (Infinite 200M; Tecan Group Ltda., Männedorf, Switzerland). Protein concentrations were calculated using BSA (New England BioLabs GmbH, Frankfurt am Main, Germany) as protein standard, which was diluted in in 0.1M potassium phosphate buffer to create a calibration curve ranging from 0 to 10 µg/µl.

The PGE_2 levels in tumour lysates were determined using the Prostaglandin E_2 Parameter™ ELISA kit from R&D Systems (Minneapolis, MN, USA). The competitive ELISA assays were performed using a total protein amount of 3µg. In detail, 150µl of PGE_2 standards and samples were pipetted into the wells of strips coated with a goat anti-mouse polyclonal antibody. Thereafter, 50µl of a primary antibody solution containing a mouse monoclonal antibody to PGE_2 was pipetted into the wells. The microplate was covered with a sealer and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker at 500rpm. After incubation, 50µl of PGE_2 conjugated to horseradish peroxidase (HRP) was added to the wells and the microplate was covered and incubated for 2 hours at room temperature on the shaker. The wells were then washed four times using the wash buffer provided with the kit. Afterwards, 200µl of substrate solution, containing tetramethylbenzidine (TMB) was pipetted into the wells. The microplate was incubated for 30 min at room temperature protected from light without shaking. The enzymatic reaction of HRP was stopped by adding 100µl of 2N sulphuric acid to the wells. The optical density was determined using an ELISA

reader set to 450nm with a correction wavelength set to 540nm. The PGE_2 levels in cell culture supernatants were determined using a competitive PGE_2 ELISA kit purchased from Enzo Life Science (Farmingdale, NY, USA). In detail, 100µl PGE_2 standards and supernatants samples were pipetted to the wells of strips coated with a goat anti-mouse antibody. Thereafter, 50µl of a solution of alkaline phosphatase (AP) conjugated PGE_2 and 50µl of a solution of a monoclonal antibody to PGE_2 were pipetted to each well. The microplate was sealed and incubated at room temperature for 2 hours on a horizontal orbital microplate shaker at 500rpm. After incubation, the wells were rinsed three times using the wash buffer provided with the kit and 200µl of a pNpp substrate solution (para-nitrophenyl phosphate) was pipetted in each well. The microplate was incubated for 45 min at room temperature protected from light without shaking. The enzymatic reaction of AP was stopped by pipetting 50µl of a solution of trisodium phosphate in water to each well. The optical density was measured with an ELISA reader at 405nm with a correction wave set to 570nm. The PGE_2 concentrations were calculated using a four parameter logistic (4-PL) curve.

3.5. Immunohistological analysis

For immunohistological analyses, the following solutions were prepared and used:

- **10x Phosphate-buffered saline (PBS)**

1.37M NaCl	160g NaCl
26.8 mM KCl	4g KCl
64.6 mM $Na_2HPO_4.2H_2O$	23g $Na_2HPO_4.2H_2O$
14.7mM KH_2PO_4	4g KH_2PO_4

All components were dissolved in 1.5L of distilled water. The pH was set to 7.4 with HCl and the final volume was adjusted to 2L. The solution was stored at room temperature and diluted 1:10 with distilled water to obtain a 1x working solution.

- **1M Tris buffer, pH 7.5**

121,4g Tris Base

Tris Base was dissolved in 800ml of distilled water. The pH was set to 7.5 with HCl and the final volume adjusted to 1L. The buffer was stored at room temperature.

- **5M Sodium chloride solution (NaCl)**

  290g NaCl

  Sodium chloride was dissolved in 1L of distilled water and stored at room temperature.

- **Blocking reagent**

1% (w/v) bovine serum albumin	5g BSA (Sigma-Aldrich)
0.3% (v/v) Triton-x	1.5ml Triton-x

  All components were dissolved in 500ml of 1x PBS. The blocking reagent was stored at 4°C.

- **10x Tris-buffered saline (TBS buffer)**

500mM Tris Base	60.6g Tris Base
1.5M NaCl	87.6g NaCl

  All components were dissolved in 800ml of distilled water. The pH was set to 7.5 with HCl and the final volume was adjusted to 1L. The solution was stored at room temperature and diluted 1:10 with distilled water to obtain a 1x working solution.

- **1x TNT buffer**

100mM Tris Base	100ml 1M Tris, pH 7.5
150mM NaCl	30ml 5M NaCl
0.05% (v/v) Triton-x	500µl Triton-x

  All components were dissolved in 800ml of distilled water. The pH was set to 7.5 with HCl and the final volume adjusted to 1L. The solution was stored at room temperature.

- **DAB**

  25mg DAB

  200ml 1x Tris

  65µl H_2O_2 30%

  The solution was freshly prepared for IHC stainings. DAB was dissolved in 1x Tris and the solution was filtered using Whatman filter paper. After filtration, hydrogen peroxide was added to the solution.

- **Harris Haematoxylin solution**

  100ml Harris Haematoxylin

  100ml dH_2O

  2ml acetic acid

  Harris haematoxylin was diluted in distilled water and filtered using Whatman filter paper. In a final step, acetic acid was added to the solution, which was stored at room temperature and kept for at least 2 months.

- **Mowiol® 4-88 mounting medium**

  2.4g Mowiol® 4-88

  6g glycerol

  6ml dH_2O

  12 ml 0.2M Tris buffer (pH 8.5)

  Mowiol® 4-88 and Glycerol were stirred to mix for 1 hour at room temperature. Distilled water was added and the mixture was further stirred over night at room temperature. The 0.2M Tris buffer was added to the mixture followed by incubation for 30 min at 50°C. After the Mowiol® 4-88 dissolved, the solution was clarified by centrifugation at 5.000g for 15 min at room temperature. Aliquots of mounting medium were prepared and stored at -20°C.

3.5.1. Immunohistochemistry (IHC)

Paraffin-embedded tissues were sectioned at 4µm using a sliding microtome Leica SM2000 R (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and mounted on superfrost slides (VWR International BVBA, Leuven, Belgium), followed by incubation at 60°C for 30 min. Sections were first deparaffinised in xylene for 20 min and subsequently rehydrated through a series of graded ethanol, i.e. 70%, 80%, 96% and 100% ethanol, two times respectively. Sections were then rinsed in distilled water and in TBS buffer. Antigen retrieval was performed in pre-heated citrate buffer (Dako, distributed through Agilent Technologies, Glostrup, Denmark) using a steamer for 20 min. After cooling to room temperature, sections were incubated in methanol with 0.3% hydrogen peroxide for 20 min at room temperature to inactivate endogenous peroxidases, followed by rinsing with TBS buffer. Incubation with 1% BSA and 0.3% triton in PBS was performed to block unspecific binding sites. Incubation with primary antibodies for CD31 or Cox-2 was performed overnight at 4°C (Table 2). For the hypoxia

staining, sections were incubated with anti-pimonidazole rabbit antisera (1:300, Hypoxiprobe™-1 Omni Kit, Hypoxyprobe, Inc.) for 40 minutes at room temperature. After incubation with the primary antibodies, sections were rinsed with TNT buffer and incubated with HRP conjugated secondary antibody for two hours. In a next step, tumour sections were rinsed again with TNT buffer followed by incubation with the DAB solution until signals of the tissues were visible. The reaction was stopped with distilled water following the counterstaining with haematoxylin for 10-20 sec. The tumour sections were permanently mounted with glass coverslips in mowiol.

Antibody	Manufacturer	Catalog No.	Dilution	Conjugate
polyclonal rabbit anti-mouse CD31	Abcam	ab28364	1:50	none
polyclonal rabbit anti-mouse Cox-2	Abcam	ab15191	1:500	none
anti-pimonidazole rabbit antisera	Hypoxyprobe, Inc.	Omni Kit	1:50	none
goat anti rabbit IgG	Abcam	ab97051	1:200	HRP

Table 2: Primary and secondary antibodies used for IHC staining

3.5.2. Immunofluorescence (IF)

Immunofluorescence staining was performed on paraffin-embedded tissues after deparaffinization, antigen retrieval and blocking of unspecific binding sites as described above. An additional incubation step with 1% BSA in TNT buffer was performed to permeabilize sections prior to the overnight incubation with the primary antibodies (Table 3). For the staining of (activated) CAFs, tumour sections were incubated with primary antibodies directed against vimentin and α-SMA conjugated with Cy3 at 4°C. Likewise, staining of proliferating CAFs was performed using primary antibodies against vimentin and BrdU. After incubation with primary antibodies, tumour sections were rinsed with TNT buffer and incubated with secondary antibodies conjugated with Alexa Fluor dyes (1:200, Dianova, Hamburg, Germany and Molecular Probes distributed through Life Technologies Darmstadt, Germany) for two hours at room temperature. Subsequently, sections were rinsed with TNT buffer, incubated for 2 min with DAPI (1:500) to visualize the nuclei and rinsed again with TNT buffer. The labelled sections were permanently mounted with glass coverslips in mowiol.

Antibody	Manufacturer	Catalog No.	Dilution	Conjugate
monoclonal rabbit anti-mouse Vimentin	Abcam	ab92547	1:500	none
monoclonal mouse anti-mouse α-SMA	Sigma-Aldrich	C6198	1:200	Cy3
polyclonal chicken anti-mouse Vimentin	Novus	NB300-223	1:1000	none
monoclonal rat anti BrdU	AbD Serotec	MCA2060	1:200	none
donkey anti rabbit IgG	Dianova	711-495-152	1:500	DyLight 649
goat anti chicken IgY	Molecular Probes	A-21437	1:200	Alexa Fluor 555
rabbit anti rat IgG	Antibodies Online	ABIN376000	1:200	biotin

Table 3: Primary and secondary antibodies for IF staining

3.5.3. Imaging and evaluation of IHC and IF sections

Imaging of tumour tissues was carried out using a Zeiss Axio Scope.A1 (Carl Zeiss Microscopy, Jena, Germany) for IHC sections or a Leica DM5000 B (Leica Microsystems, Wetzlar, Germany) for IF sections. Image analysis was performed using the imaging software AxioVision (Carl Zeiss Microscopy). Counting of immunostained cells in the tumour stroma was performed by choosing 10-12 sequences of 20x fields of the tumour section covering the entire slide. Microvessel and CAF densities were calculated as the product of the counted $CD31^+$ as well as α-SMA^+ and/or $Vimentin^+$ cells divided by the total area of analysed tumour stroma, respectively. $Hypoxia^+$ and $BrdU^+$ areas were determined semi-automatically by the imaging software based on the stained regions in the tumour stroma or stained nuclei, respectively.

3.6. Quantitative real time PCR (qRT PCR)

Total cellular mRNA was extracted from sorted or cultured cells using the Ambion PureLink® RNA Mini Kit (Life Technologies). Sorted or cultured cells were lysed in fresh prepared lysis buffer containing 1% of beta-mercaptoethanol. Manual homogenization of the samples was performed by passing the lysates five to ten times through a 1,10x30mm syringe needle (Braun, Melsungen, Germany). One volume of 70% ethanol was added to each volume of cell homogenate following thoroughly mixing with a vortex. The samples were transferred to spin cartridges delivered with the kit and centrifuged at 12.500 x g for 30 sec at room temperature. After discarding the flow-through, 350µl of Wash Buffer I was pipetted to the spin cartridges containing the bound RNA followed by centrifugation at 12.500 x g for 30 sec at room temperature. Subsequently, 80µl of PureLink™ DNase mixture were pipetted directly onto the surface of the spin cartridge membranes, which were subsequently incubated for 15 min at room temperature. Following the incubation, 350µl Wash Buffer I was pipetted to the

spin cartridges and centrifugation at 12.000 x g for 30 sec at room temperature was carried out. Flow-through was again discarded and spin cartridge membranes were rinsed two times with 500µl Wash buffer II. Spin cartridges were centrifuged at 12.000 x g for 1-2 min to dry the membranes. Finally, the spin cartridges were inserted in recovery tubes and 35µl of RNase free water was pipetted to the centre of the spin cartridge. After 1 min incubation at room temperature, the bound RNA from the membrane was eluted into the recovery tube by centrifugation for 2 min at maximal speed. The RNA concentration of the samples was determined using a Nanodrop2000 (Peqlab Biotechnology GmbH, Erlangen, Germany). Synthesis of cDNA was carried out using the First Strand cDNA Synthesis Kit from Thermo Fisher Scientific (distributed through Life Technologies) according to manufacturer's instructions. The transcription from RNA into cDNA was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using 1µg of isolated RNA. The relative levels of gene expression for each experimental sample were determined by quantitative real-time PCR (qRT-PCR). The mRNA levels of target genes were quantified using the SYBR Green qPCR Master Mix and ABI 7500 instrument (Applied Biosystems, distributed through Life Technologies). Expression of GAPDH served as an endogenous control. All primers were synthesized by Eurofins MWG (Ebersberg, Germany) and are shown in Table 4 and Table 5. The relative mRNA levels in each sample were calculated based on their threshold cycle (Ct) normalized by their respective Ct value of GAPDH using the ΔΔCt-method.

Name	Forward primer	Reverse primer
murine *Ptgs2* (Cox-2)	5'-GTCTTCCAGCCCATTGAACCT-3'	5'-GGAACACAGCT ACGAAAACCC- 3'
murine *Tgfβ1*	5'-AAATCAACGGGATCAGCCCC-3'	5'-CGCACACAGCAGTTCTTCTC-3'
murine *Fgf2*	5'-ACCCACACGTCAAACT ACAACT- 3'	5'-ACTGGAGT ATTTCCGTGACCG- 3'
murine *Il-6*	5'-AGAAAGACAAAGCCAGAGTCCT- 3'	5'-CTTGGTCCTT AGCCACTCCTT-3'
murine *Vegf-a*	5'-TGCGGATCAAACCTCACCAA-3'	5'- TGTTCTGTCTTTCTTTGGTCTGC-3'
murine *Hgf*	5'-GCCCT ATTTCCCGTTGTGAAG-3'	5'-CCGCAGTTGTTTTGTTTTGGC-3'
murine *Pdgf-d*	5'-CCCTCCAAGGAT AACGTCAAGA- 3'	5'- ACACCCCAGAGAAAGAGCTTGT- 3'
murine *Gapdh*	5'- TCAAGCTCATTTCCTGGTATGACA- 3'	5'-CTCTTGCTCAGTGTCCTTGCTG- 3'

Table 4: List of murine primers used for qRT-PCR

Name	Forward primer	Reverse primer
human *PTGS2* (Cox-2)	5'-GTGCATTGGAATCAAGCCTGG- 3'	5'-GGCAGAGTCCAAAGAAAGTGAAC- 3'
human *ACTA2* (α-SMA)	5'-CGTTACTACTGCTGAGCGTGA- 3'	5'-GATGGCTGGAACAGGGTCTC-3'
human *FAP*	5'-GGGATGGTCATTGCCTTGGT-3'	5'-CTCCAT AGGACCAGCCCCATA-3'
human *GAPDH*	5'-AGGGCTGCTTTTAACTCTGGT-3'	5'-CCCCACTTGATTTTGGAGGGA-3'

Table 5: List of human primers used for qRT-PCR

3.7. Cell viability assay

The cell viability assay was performed by Dr. Isabel Ben Batalla using a WST-1 assay (Roche Applied Science, Mannheim Germany) as described by the manufacturer. In addition, viability of cells was assessed using trypan blue exclusion. The number of viable cells was determined using a Neubauer chamber.

3.8. Migration assay

The migration assay was performed by Dr. Isabel Ben Batalla using BD BioCoat matrigel invasion chambers (BD Biosciences). To assess cellular migration, inserts without matrigel (8µm pore size) were utilized and 1.1 x 10^4 cells were seeded onto the inserts containing 400µl of serum-free medium. The insert was placed in a 24-well plate and 400µl of serum free medium was added to the bottom of each well. The cells were kept in culture for 24 hours in standard conditions with the different treatments. After this time, the cells that had not migrated were gently removed by washing with a cotton swab. The cells that remained in the inserts were fixed with 100% methanol for 2 minutes and stained with cristal violet. The stained cells were imaged using an Axio-Scope.A1 microscope (Carl Zeiss Microscopy) and counted to evaluate their migratory activity.

3.9. Western blot analysis

Western blot analyses were performed by Dr. Isabel Ben Batalla. For western blot analyses, following solutions were used:

- **RIPA buffer**

  1ml Nonidet P40 (1%)
  500mg Na-Doxycholat (0.5%)
  1ml SDS 10% (0.1%)
  10ml 10x PBS

All components were dissolved in 100ml of distilled water. Aliquots were prepared and stored at -20°C.

- **1.5M Tris, pH8.8**

  91.05g Tris Base

  Tris Base was dissolved in 250ml of distilled water and the pH was set to 8.8 with HCl. The final volume was adjusted to 500ml with distilled water.

- **1M Tris, pH6.8**

  30.35g Tris Base

  Tris Base was dissolved in 100ml of distilled water and the pH was set to 6.5 with HCl. The final volume was adjusted to 250ml with distilled water.

- **6% SDS-Polyacrylamide gel (resolving gel)**

H_2O	5.3 ml
30% acrylamide	2.0ml
1.5M Tris (pH 8.8)	2.5ml
10% SDS	0.1ml
10% $(NH_4)_2S_2O_8$	0.1ml
TEMED	0.008ml

- **5% SDS-Polyacrylamide gel (stacking gel)**

H_2O	2.1ml
30% acrylamide	0.5ml
1M Tris (pH6.8)	0.38ml
10% SDS	0.03ml
10% $(NH_4)_2S_2O_8$	0.03ml
TEMED	0.003ml

- **10x Running buffer**

1.92M glycine	144g glycine
250mM Tris Base	30g Tris Base
35mM SDS	10g SDS

All components were dissolved in 1L of distilled water and solution was stored at room temperature. The solution was diluted 1:10 with distilled water to make a 1x working solution.

- **10x Transfer buffer**

  1.92M Glycine 144g Glycine

  250mM Tris Base 30g Tris Base

  All components were dissolved in 1L of distilled water and solution was stored at room temperature. A 1x working solution was prepared by mixing 100ml of 10x transfer buffer with 200ml of methanol and adjusting the final volume to 1L with distilled water.

- **Wash buffer**

  100ml 1x PBS

  300µL Tween-20

3.9.1. Cell lysis

Prior to lysis, primary cells and cell lines from *in vitro* experiments were washed twice with ice cold DPBS (Life Technologies) to remove fetal bovine serum (FBS). Cell pellets in 1.5ml tubes were resuspended in RIPA lysis buffer supplemented with protease and a cocktail of four phosphatase inhibitors (Merck Millipore, Darmstadt, Germany) and incubated for 20 min on ice. Cellular debris was pelleted by centrifugation at maximal speed and 4°C for 10min. The cleared cell lysate was transferred to a new 1.5 ml tube and stored at -20°C or -80°C for long-term storage. Primary human CAFs and human embryonic fibroblast MRC-5 cells were lysed in 200µl RIPA lysis buffer per $5x10^6$ cells. To determine protein concentrations, the Bio-Rad Protein Assay (Bio-Rad Laboratories) was used as described above. Protein concentrations were calculated using BSA (New England BioLabs GmbH) as a protein standard, which was diluted in RIPA lysis buffer to create a calibration curve ranging from 0 to 10 µg/µl.

3.9.2. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins in cell lysates depending on their molecular weight. For SDS-PAGE, gels consisting of a 6% resolving gel and a 5% stacking gel were prepared and put into a Mini-PROTEAN® Tetra System electrophoresis cell (Bio-Rad Systems) containing 1x running buffer. A total protein amount of 30µg cell lysate was diluted in loading buffer and denatured by heating at 95°C for 5 min. As a molecular weight marker, the

prestained SeeBlue Plus2 Marker (Invitrogen) was used. Samples were loaded into the gel and were run at 140V.

3.9.3. Western blotting

Proteins separated by SDS-PAGE were transferred to (polyvinylidene fluoride) PVDF membranes (Immobilion®-P, Millipore) in a wet-blot chamber. The protein transfer was done in a tank blot filled with cold transfer buffer. PVDF membranes were activated through incubation in methanol for 1 min, followed by rinsing with distilled water for 3-5 min. The activated PVDF membrane and the polyacrylamide gel were put between three layers of Whatman paper sheets, previously soaked in transfer buffer. This assembly was fixed in a grid, which was vertically inserted into the tank blot chamber. Proteins were blotted with a constant current of 300 mA for 1 hour at 4°C. After blotting, the membrane was incubated with blocking buffer consisting of 5% of skim milk powder dissolved in washing buffer. Subsequently, the primary antibodies were applied and the PVDF membranes were incubated at 4°C over night. Following the incubation with the primary antibodies and after rinsing three times for 5 minutes with washing buffer containing 0.3% Tween-20, the membranes were incubated with an appropriate HRP-conjugated secondary antibody (previously diluted in blocking buffer) for 1 hour at room temperature. Finally, the membranes were incubated with an Amersham ECL solution (GE Healthcare, Freiburg, Germany) and exposed on an autoradiographic film (Amersham Hyperfilm MP, 18 × 24 cm, GE Healthcare) in a darkroom, followed by photographic development. Dilutions and incubation conditions of used western blot antibodies are given in Table 6.

Antibody	Manufacturer	Catalog No.	Dilution	Conjugate
polyclonal rabbit anti mouse Akt	Cell Signaling	9272	1:1000	none
monoclonal rabbit anti mouse pAkt (Ser473)	Cell Signaling	4058	1:1000	none
monoclonal mouse anti p44/42 MAPK (tErk)	Cell Signaling	9107	1:2000	none
monoclonal rabbit anti p44/42 MAPK (pErk)	Cell Signaling	4370	1:2000	none
monoclonal mouse anti mouse β-Actin	Santa Cruz Biotechnology	sc-47778	1:10000	none
polyclonal swine anti-rabbit	Dako	P0217	1:1000	HRP
polyclonal rabbit anti-mouse	Dako	P0260	1:1000	HRP

Table 6: List of primary and secondary antibodies for western blotting

3.10. Data analysis

Data represent mean ± SEM (standard error of the means) of representative experiments, unless otherwise stated. Statistical significance was calculated by Student's t-test. The dependence of numerical dependent parameters of n>2 categorial variables was studied using the ANOVA setting where indicated. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

4. Results

4.1. Anti-angiogenic therapies enhance PGE_2 levels as a consequence of increased hypoxia and Cox-2 expression in 4T1 tumours

Anti-angiogenic treatments have been shown to decrease tumour progression in different tumour models (Brekken et al. 2000; Chinchar et al. 2014). In line with these data, treatments with the anti-angiogenic drugs DC101 and sunitinib at a dose level of 40mg/kg showed inhibitory effects on 4T1 tumour growth compared to a control group. For treatments with DC101, the tumour growth was significantly reduced by 44.6% when compared to 4T1 tumours from a control group (Figure 8A). Treatment with sunitinib induced a tumour growth reduction by 73.3% (Figure 8B). Hence, treatments with both anti-angiogenic drugs inhibited tumour growth in 4T1 tumour-bearing animals.

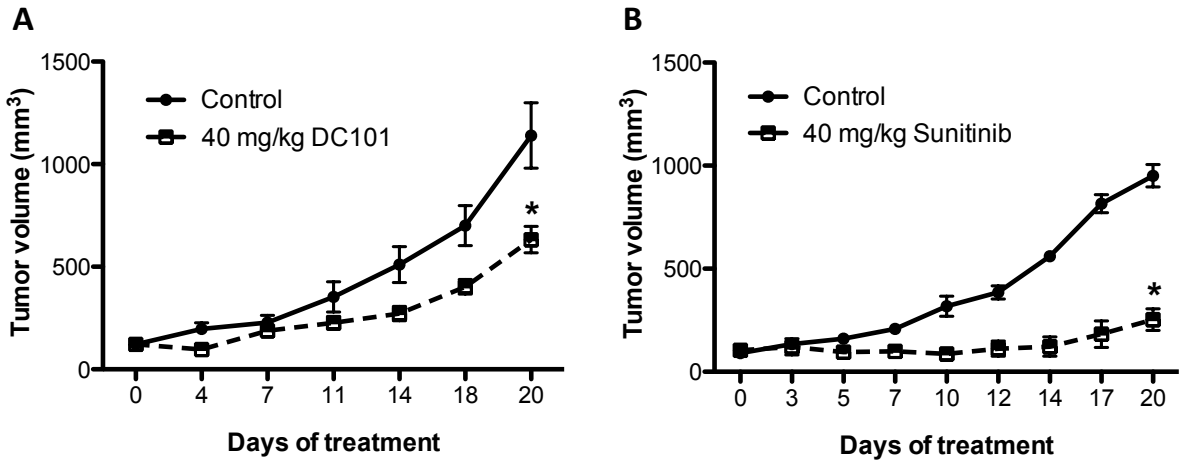

Figure 8: Anti-angiogenic treatments inhibit 4T1 tumour growth. Tumour growth was monitored in 4T1 tumour-bearing animals treated with DC101 or sunitinib at a dose level of 40mg/kg. **(A)** Treatments with DC101 showed a significant inhibition of tumour growth corresponding to a decrease by 44.6% compared to the control group; n=5; *P<0.05. **(B)** Treatments with sunitinib caused also a significant inhibition of tumour growth by 73.3%; n=5; *P<0.0001. Experiments performed in collaboration with Dr. Isabel Ben Batalla.

Several studies in breast cancer animal models have demonstrated that treatments with anti-angiogenic therapies induce intratumoral hypoxia (Franco et al. 2006). Hypoxia mediates the regulation of several genes that might be responsible for the development of tumour resistance against anti-angiogenic therapies, among which is Cox-2 (Lee et al. 2010; Schmedtje et al. 1997). Based on this evidence, the mRNA expression levels of Cox-2 were analysed via qRT-PCR in end-stage 4T1 tumours upon treatment with anti-angiogenic therapies. The Cox-2 mRNA expression levels in 4T1 tumours from animals treated with DC101 showed a 2.5-fold significant increase in comparison to the control

group (Figure 9A). Similarly, treatments with sunitinib induced a 2.3-fold Cox-2 mRNA expression in 4T1 tumours in relation to the control group (Figure 9B). Therefore, these results indicate that the treatments with both anti-angiogenic therapies induced the expression of Cox-2 at the mRNA level in end-stage 4T1 tumours.

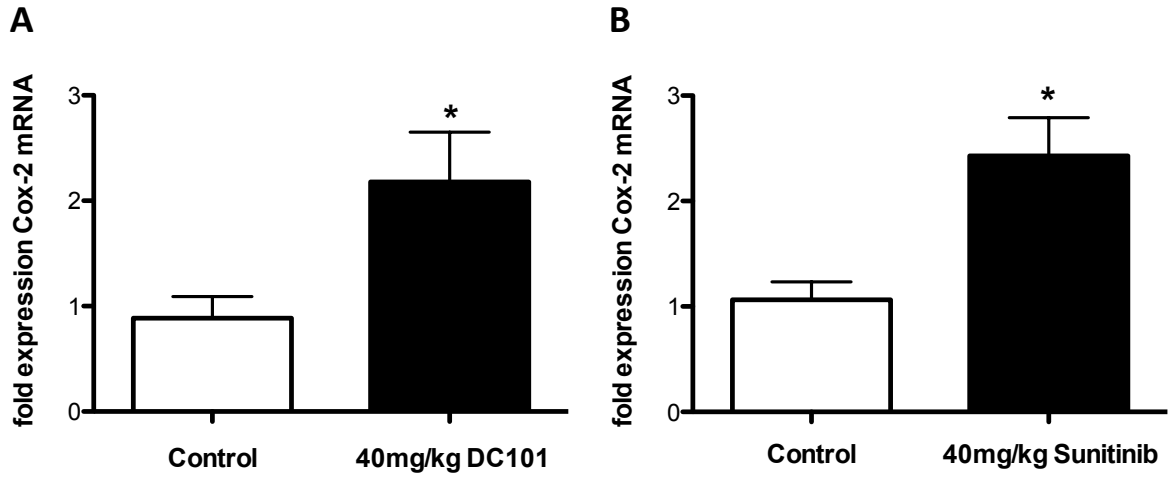

Figure 9: Anti-angiogenic treatments upregulate Cox-2 mRNA expression in 4T1 tumours. Cox-2 mRNA levels in 4T1 tumour tissues from animals treated with DC101 or sunitinib at a dose level of 40mg/kg were analysed via qRT-PCR. **(A)** 4T1 tumours treated with DC101 showed a 2.5-fold higher Cox-2 mRNA expression compared to 4T1 tumours from animals of the control group; n=5; *P<0.05. **(B)** In accordance, 4T1 tumours from animals treated with sunitinib showed 2.3-fold higher Cox-2 mRNA expression levels; n=5; *P=0.01.

The upregulation of Cox-2 mRNA expression induced by anti-angiogenic therapies may result in elevated levels of PGE_2, which is the main product synthesised by this enzyme (D. Wang & DuBois 2006). In order to elucidate whether the Cox-2 induction had functional consequences, the PGE_2 levels were measured in 4T1 tumour lysates using an ELISA assay. Indeed, the PGE_2 levels were increased in 4T1 tumours upon treatments with different doses of anti-angiogenic therapies. In the case of DC101, a 1.4-fold slight increase of the PGE_2 levels compared to control was observed at a low dose of 10mg/kg DC101, while these levels significantly increased 1.7-fold upon treatment with 40mg/kg DC101 (Figure 10A). Similarly, a slight increase of the PGE_2 levels was observed upon treatments with 40mg/kg sunitinib, which was 2-fold higher compared to the control group, whereas treatment with 60mg/kg sunitinib resulted in a 5.2-fold increase (Figure 10B). Thus, treatment with anti-angiogenic therapies led to increased levels of Cox-2 and of its product PGE_2 in 4T1 tumours.

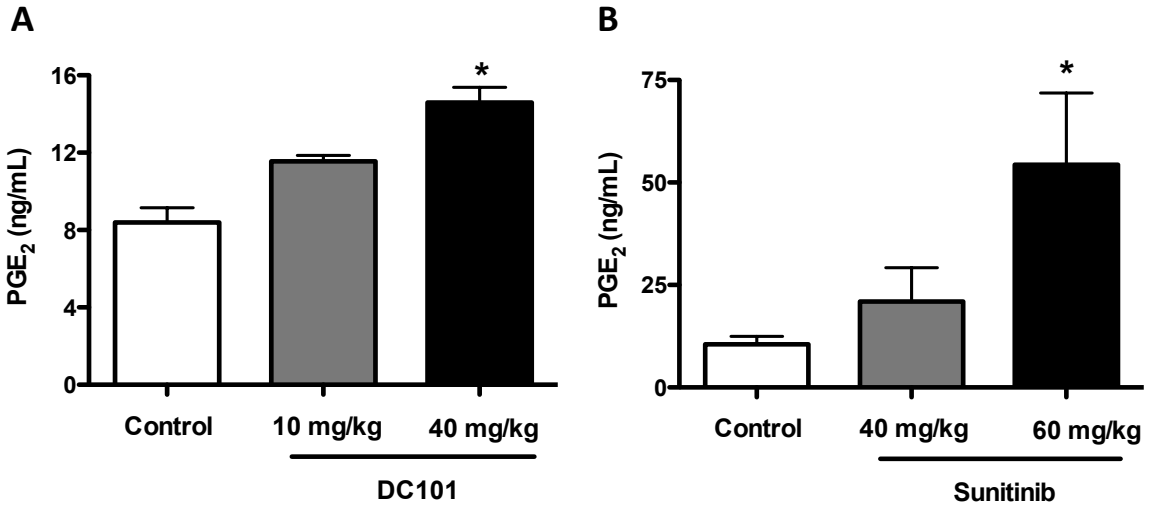

Figure 10: Anti-angiogenic treatments increase PGE_2 levels in 4T1 tumours. PGE_2 levels in 4T1 tumour lysates from animals treated with different doses of DC101 or sunitinib were analysed via ELISA assays. **(A)** 4T1 tumours showed slightly elevated PGE_2 levels upon treatment with 10mg/kg DC101 (1.4-fold), which were significantly increased upon treatment with 40mg/kg DC101 (1.7-fold); n=5; *P<0.005. **(B)** Similarly, 4T1 tumours treated with 40mg/kg sunitinib displayed slightly increased PGE_2 levels (2-fold), while 60mg/kg sunitinib significantly enhanced these levels (5.2-fold); n=5; *P=0.03.

Based on the evidence that anti-angiogenic therapies induce hypoxia, immunohistochemical analyses were performed in 4T1 tumours from animals treated with sunitinib in order to determine the levels of hypoxia and correlate them to the levels of PGE_2. Histomorphometric analyses showed a 2.6-fold increase of the hypoxia levels compared to those in 4T1 control tumours (Figure 11A). The levels of secreted PGE_2 and intratumoral hypoxia showed a correlation between these two parameters, indicating that treatment-induced hypoxia might lead to increased PGE_2 levels (Figure 11B).

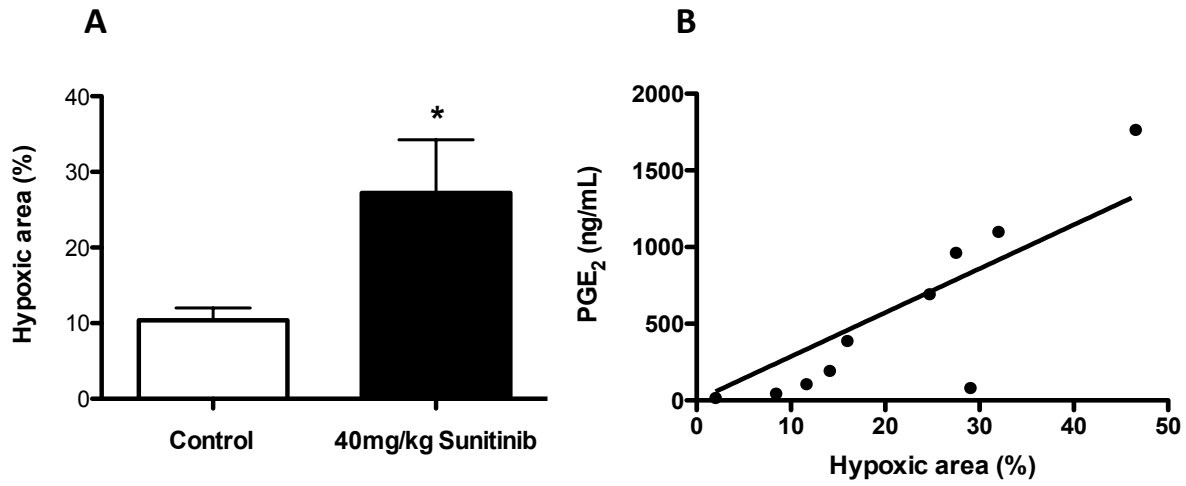

Figure 11: Sunitinib-induced hypoxia correlates with intratumoral PGE_2 levels. (A) Animals treated with 40mg/kg sunitinib showed a 2.6-fold increase of intratumoral hypoxic levels in comparison to hypoxia levels in control tumours, as determined by immunohistochemical analysis in 4T1 tumour sections; n=5; *P<0.05. **(B)** Intratumoral hypoxia levels correlated with the PGE_2 levels in 4T1 tumours from animals treated with sunitinib; n=10; r=0.81; *P=0.058.

Taken together, sunitinib and DC101 decreased tumour progression in 4T1 tumour-bearing animals. However, both angiogenesis inhibitors simultaneously increased the levels of intratumoral hypoxia and the expression of Cox-2 mRNA that ultimately resulted in elevated PGE_2 levels in 4T1 tumours.

4.2. Inhibition of Cox-2 improves the efficacy of anti-angiogenic drugs

Since anti-angiogenic therapies induce hypoxia and subsequently enhance Cox-2 mRNA expression in 4T1 tumours, Cox-2 is an interesting target due to its crucial role in the development of tumour resistance against anti-angiogenic drugs (Franco et al. 2006; Lee et al. 2010). Therefore, blocking of Cox-2 might be helpful for overcoming the development of resistance, thus enhancing the efficacy of anti-angiogenic therapies. In order to investigate the implication of Cox-2, the pan-Cox inhibitor acetyl salicylic acid (ASA) was used both in single treatments and in combination with different doses of anti-angiogenic drugs. Initially, 4T1 tumour-bearing animals were treated either with high doses of sunitinib (60mg/kg) or ASA (100mg/kg) as well as the combination of both drugs. Both single therapies exhibited a significant inhibitory effect on the tumour growth, which was reduced by 69.1% with sunitinib and 59.1% with ASA compared to the control group (Figure 12A). Strikingly, upon combination of sunitinib and ASA the inhibitory effect on the tumour growth was increased to 83% (Figure 12A). The PGE_2 levels were again determined in 4T1 tumours from treated animals and showed a 4.6-fold increase upon administration of sunitinib (Figure 12B), while these levels were decreased 14.1-fold in comparison to the 4T1 control tumours upon treatment with ASA (Figure 12B). Interestingly, combinatory treatments with sunitinib and ASA normalized PGE_2 levels to those observed in 4T1 control tumours (Figure 12B).

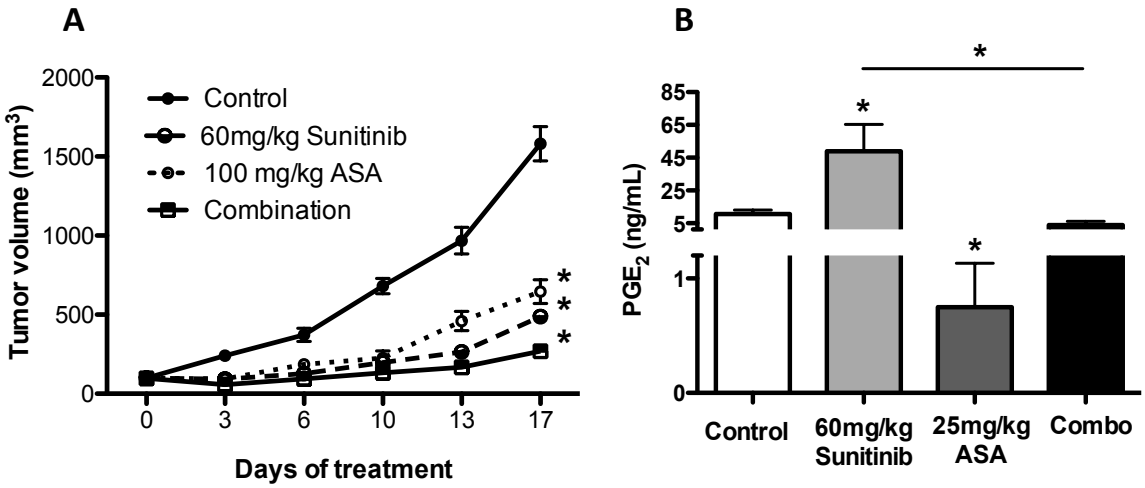

Figure 12: Combination of sunitinib and ASA has additive inhibitory effect on 4T1 tumour growth and normalizes intratumoral PGE_2 levels. (A) Single treatments with 60mg/kg sunitinib or 100mg/kg ASA elicited a significant decrease of the 4T1 tumour growth by 69.1% and 59.1%, respectively. The combination of both drugs showed a significant additive effect, leading to a decrease by 83%; n=7; *P<0.0001; experiment performed in collaboration with Dr. Isabel Ben Batalla. **(B)** Analyses of the PGE_2 levels in 4T1 tumours revealed a 4.6-fold increase of these levels in 4T1 tumours upon treatment with sunitinib, while ASA decreased the PGE_2 levels 14.1-fold. The combination of sunitinib and ASA normalized the PGE_2 levels to those measured in 4T1 tumours from the control group; n=7; *P<0.03.

Treatments performed with intermediate doses (15mg/kg DC101; 20mg/kg sunitinib) of anti-angiogenic therapies showed inhibitory effects on the tumour growth of 32.2% and 32.6% for sunitinib and DC101, respectively (Figure 13A). Single treatments with ASA were shown to be effective in the inhibition of tumour growth, which was decreased to 43.2%. Interestingly, the combination of ASA either with sunitinib or DC101 was sufficient to significantly reduce the tumour growth by 61.1% and 56.9%, respectively, thus showing an additive inhibitory effect (Figure 13A, B).

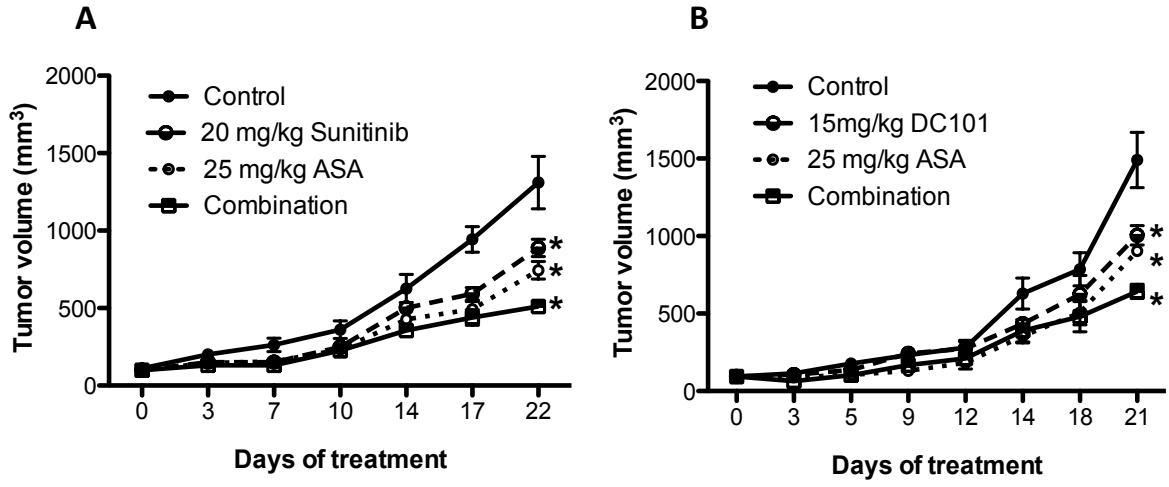

Figure 13: Combination of intermediate doses of anti-angiogenic therapies and ASA has additive inhibitory effect on 4T1 tumour growth. (A) Single treatments with 20mg/kg sunitinib and 25mg/kg ASA elicited a significant decrease of the 4T1 tumour growth by 32.2% and 43.2%, respectively. The combination of both inhibitors showed a significant additive effect, leading to a decrease by 61.1%; n=7; *P<0.0001. **(B)** Single treatments with 15mg/kg DC101 and 25mg/kg ASA elicited likewise a significant decrease of the 4T1 tumour growth by 32.6% and 39.3%, respectively. The combination of both drugs elicited a significant decrease by 56.9% compared to control tumours; n=7; *P<0.0001. Experiments performed in collaboration with Dr. Isabel Ben Batalla.

Further dose reductions of both sunitinib (10mg/kg) and ASA (12.5mg/kg) showed no significant inhibitory effects on the 4T1 tumour growth when administered as single treatments (Figure 14). However, the previously observed additive inhibitory effect of anti-angiogenic drugs in combination with ASA was still present and caused a reduction by 46.1% of the tumour volume (Figure 14).

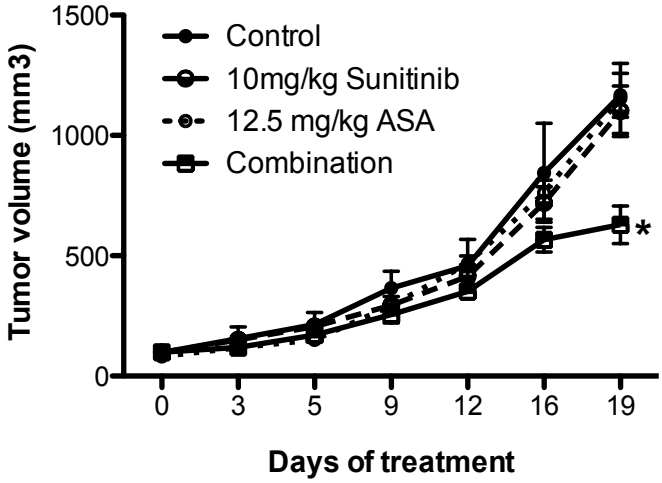

Figure 14: Combination of low doses of sunitinib and ASA has additive inhibitory effect on 4T1 tumour growth. Single treatments with 10mg/kg sunitinib and 12.5mg/kg ASA showed minor inhibitory effects on the 4T1 tumour growth compared to 4T1 control tumours. In contrast, the combination of both inhibitors exhibited a significant decrease by 46.1%; n=7; *P<0.0001. Experiment performed in collaboration with Dr. Isabel Ben Batalla.

In conclusion, both anti-angiogenic drugs and ASA exerted dose-dependent inhibitory effects on the tumour growth as single treatments. Interestingly, the combination of both anti-angiogenic therapies and ASA exerted profound additive inhibitory effects. The concomitant administration of ASA with anti-angiogenic drugs seemed to enhance therapeutic efficacy and could be useful in reducing the administered doses of anti-angiogenic drugs in order to reduce side effects while maintaining efficacy.

The observed normalization of the PGE_2 levels in 4T1 tumours treated with the combination of ASA and sunitinib might be the result of a decrease of Cox-2 protein levels. In order to investigate this, immunohistochemical analyses in 4T1 tumour tissues were performed and Cox-2 positive areas were quantified. Single treatments with low doses sunitinib did not influence the protein levels of intratumoral Cox-2, which remained similar to those in 4T1 control tumours (Figure 15A). Cox-2 protein levels were significantly decreased by 65% upon ASA treatment and the combination of ASA and sunitinib resulted in a decrease by 74.5% compared to the Cox-2 levels determined in 4T1 control tumours (Figure 15A). Hence, the normalization of PGE_2 levels observed during the combinatory therapy could be due to a reduction of the Cox-2 protein levels

in 4T1 tumours, which was induced by ASA (Figure 15B). The observed normalization of PGE_2 levels might in turn lead to an increased efficacy of anti-angiogenic drugs, since PGE_2 promotes tumour progression and angiogenesis.

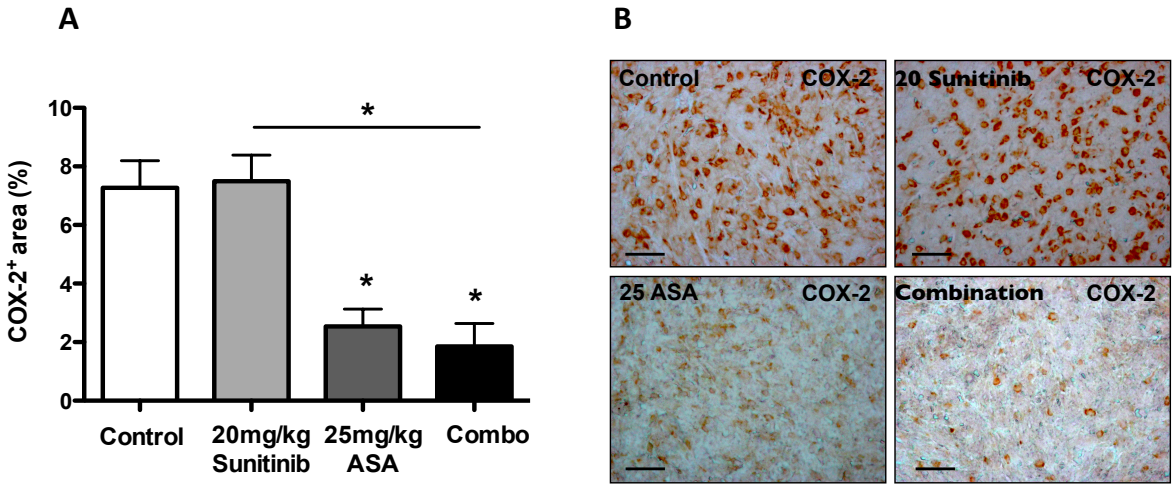

Figure 15: Treatments with ASA decrease Cox-2 protein levels. (A) Quantification of Cox-2 positive areas in 4T1 tumours by morphometric analyses showed no significant differences of intratumoral Cox-2 between 4T1 control tumours and tumours from animals treated with 20mg/kg sunitinib. Cox-2 was significantly decreased upon treatments with 25mg/kg ASA (65%) and the combination of both sunitinib and ASA (74.5%); n=7; *P<0.0005. **(B)** Representative images of 4T1 tumour tissues from control animals and animals treated with sunitinib, ASA or the combination of both inhibitors. Scale bar: 50µm.

Based on the findings that the efficacy of anti-angiogenic drugs was still improved at rather lower doses when administered in combination with ASA, animal experiments were performed using 20mg/kg sunitinib and 25mg/kg ASA. To corroborate that the observed additive inhibitory effects on tumour progression upon concomitant inhibition of Cox-2 with anti-angiogenic drugs does not only occur in the 4T1 breast cancer model but also apply to other tumour models, animals transplanted with the murine breast cancer cell line 66cl4 were treated with sunitinib and ASA. As observed in the 4T1 breast cancer model, the tumour growth in 66cl4 tumour-bearing animals was significantly impaired upon treatment with sunitinib or ASA alone by 45.1% and 33.8%, respectively. Importantly, the combinatory treatment induced a more pronounced inhibition of tumour growth by 52.4% (Figure 16A). Thus, ASA can enhance the efficacy of anti-angiogenic therapies in different breast cancer models.

In order to verify that the increased efficacy of anti-angiogenic drugs is due to the inhibition of Cox-2 by ASA, 4T1 tumour-bearing mice were treated with the specific Cox-2 inhibitor SC-236 either as a single treatment or in combination with sunitinib. Single treatment with sunitinib induced a tumour growth decrease by 33.8%, while the Cox-2 inhibitor SC-236 reduced the tumour size by 41.9%. Furthermore, the combination of

both sunitinib and SC-236 showed a significant additive effect and resulted in a tumour growth reduction of 59.9% (Figure 16B). Hence, the efficacy of anti-angiogenic drugs can be increased by the inhibition of Cox-2, which might represent a novel target to improve the outcome of anti-angiogenic therapies.

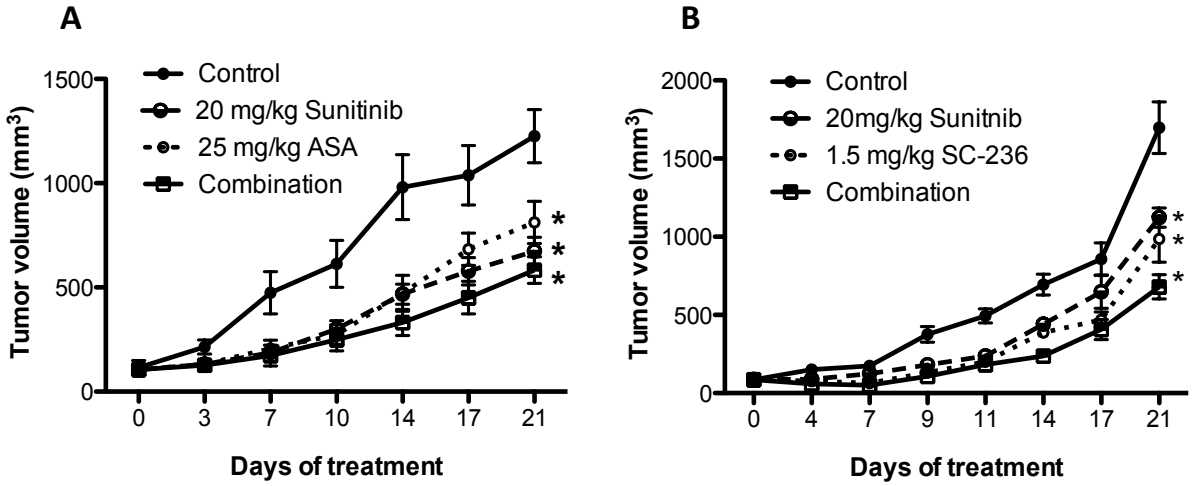

Figure 16: Additive effect of sunitinib upon Cox-2 blockade is reproducible in a different cancer model and could be attributed to Cox-2 inhibition. (A) 66cl4 tumour-bearing animals treated with 20mg/kg sunitinib or 25mg/kg ASA exhibited a tumour growth reduction by 45.1% and 33.8%, respectively. The combination of both inhibitors induced a significant additive inhibitory effect, leading to a tumour decrease of 52.4%; n=7; *P=0.01**. (B)** 4T1 tumour-bearing animals treated with 20mg/kg sunitinib or 1.5mg/kg of the Cox-2 specific inhibitor SC-236 exhibited a tumour growth reduction by 33.8% and 41.9%, respectively. The combination of sunitinib and SC-236 exhibited a significant additive inhibitory effect, leading to a tumour decrease by 59.9%; n=7; *P=0.0001. Experiments performed in collaboration with Dr. Isabel Ben Batalla.

In summary, the efficacy of anti-angiogenic drugs could be improved in combination with ASA. This effect can be explained due to the normalization of the PGE_2 levels, which were elevated upon single treatments with anti-angiogenic drugs. The normalization of PGE_2 levels observed upon treatments with ASA was due to reduction of Cox-2 levels in tumours treated with ASA. Moreover, the enhanced effects of anti-angiogenic drugs could be attributed to the inhibition of Cox-2, thereby indicating that this enzyme might be crucial for the efficacy of anti-angiogenic therapies. Importantly, additive anti-tumour effects of the combinatory treatment were reproducible in two different breast cancer models.

4.3. Concomitant inhibition of Cox-2 with anti-angiogenic therapies reduces tumour angiogenesis

Since PGE_2 can activate the pro-angiogenic PI3K/Akt or MAPK/Erk signalling pathways in ECs, the inhibition of Cox-2 might enhance the efficacy of VEGF-inhibiting

drugs such as sunitinib or DC101. To confirm this hypothesis, the effects of the treatments either with anti-angiogenic drugs or ASA as well as the combination of both on the microvessel density (MVD) were determined in 4T1 tumours. In order to determine the MVD, the well-described endothelial marker CD31 was quantified in immunohistochemical stainings. 4T1 tumours from animals treated with sunitinib or ASA showed a significant decrease of the MVD by 1.5 fold compared to tumours from the control group (Figure 17A). The combination of sunitinib and ASA induced a significant additive effect that resulted in a 3.2-fold decrease of tumoral blood vessels compared to 4T1 control tumours (Figure 17A). Hence, both single treatments were able to reduce the MVD in 4T1 tumours and this effect was significantly increased upon combinatorial treatment (Figure 17B).

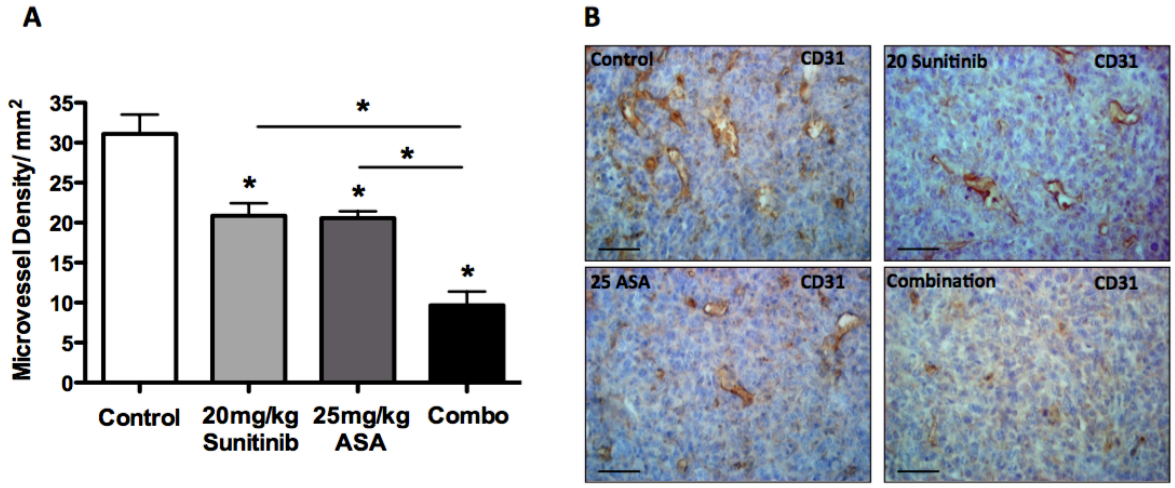

Figure 17: Combination of sunitinib and ASA elicits additive inhibitory anti-angiogenic effect. (A) MVD quantification in 4T1 tumour tissues showed a 1.5-fold significant decrease upon treatments with 20mg/kg sunitinib or 25mg/kg ASA. The combination of both inhibitors resulted in a 3.2-fold additive reduction of the MVD; n=7; *P<0.003. **(B)** Representative images of 4T1 control tumours or tumours from animals treated with sunitinib, ASA, or the combination of both inhibitors. Images were acquired with a Zeiss Axioscope. Scale bar: 50µm.

Furthermore, the MVD was also determined in 4T1 tumours from animals treated with DC101 and ASA. The MVD was only slightly decreased upon single treatments either with DC101 or ASA by 1.2-fold in comparison to the number of vessels in 4T1 control tumours (Figure 18A). Upon combinatory treatments with both DC101 and ASA, the MVD significantly decreased by 3.2-fold, which is comparable to the observed effects in experiments with sunitinib and ASA (Figure 18A). Taken together, concomitant inhibition of the VEGF pathway and Cox-2 by DC101 and ASA was able to significantly reduce the MVD in 4T1 tumours (Figure 18B).

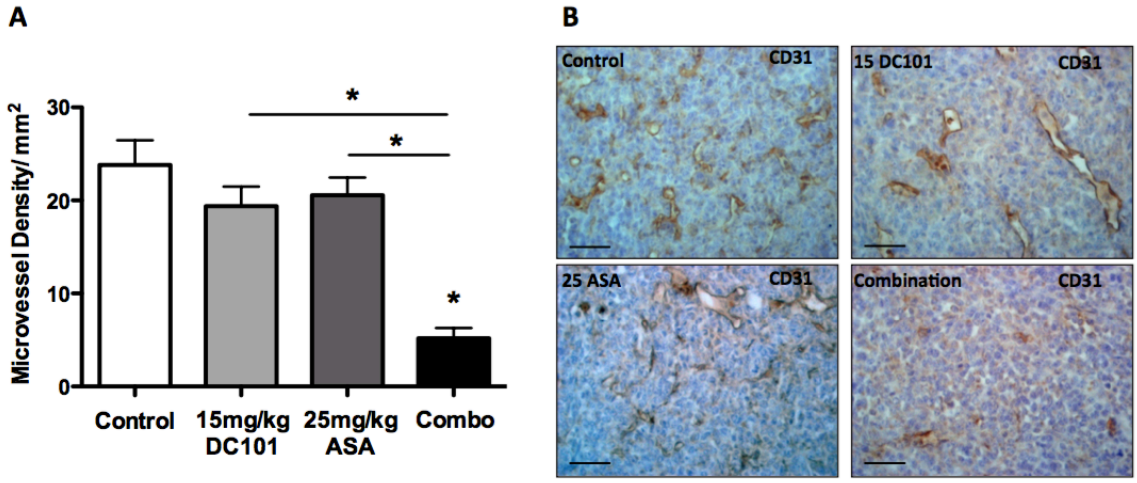

Figure 18: Combination of DC101 and ASA elicits additive inhibitory anti-angiogenic effect. (A) MVD quantification in 4T1 tumour tissues showed a 1.2-fold slight decrease upon treatments with 15mg/kg DC101 or 25mg/kg ASA. The combination of both inhibitors resulted in a 3.2-fold additive reduction of the MVD; n=7; *P<0.003. **(B)** Representative images of 4T1 control tumours or tumours from animals treated with DC101, ASA, or the combination of both inhibitors. Images were acquired with a Zeiss Axioscope. Scale bar: 50µm.

In conclusion, ASA exerts an additive anti-angiogenic effect when combined with drugs such as sunitinib or DC101. Moreover, the additive inhibitory effect on the tumour growth observed upon combination of anti-angiogenic drugs and ASA might be a consequence of impaired angiogenesis.

4.4. Anti-angiogenic drugs and concomitant inhibition of Cox-2 affect the tumour infiltration with cancer associated fibroblasts

CAFs are among the several stromal cell types that might be able to infiltrate the tumour and promote tumour angiogenesis by secreting different pro-angiogenic factors. These stromal cells represent a main population within the tumour, which are able to mediate resistance against anti-angiogenic therapies through mechanisms that include the secretion pro-angiogenic mediators besides the VEGF-axis and the suppression of the host anti-tumour immune response (Luo et al. 2015). The concomitant inhibition of Cox-2 in 4T1 tumour-bearing animals treated with anti-angiogenic therapies showed an additive effect in regard to the reduction of tumour-associated blood vessels. This reduction can be due to blockade of Cox-2-mediated pro-angiogenic effects but also by a reduced number of infiltrating stromal cells that might result from the inhibition of Cox-2. Therefore, concomitant treatments with ASA might prevent the rescue of tumour angiogenesis by interfering with the infiltration of stromal cells into the tumour. In order to study the effects of the inhibition of Cox-2 on the infiltration with CAFs, 4T1 tumours from animals treated with sunitinib or ASA, as well as the combination of both

inhibitors, were analysed by means of immunofluorescence staining of vimentin, a well-established marker for fibroblasts. The analyses of tumour tissues showed a significant reduction by 60.8% of the total number of CAFs upon single treatment with sunitinib and by 70.8% upon treatment with ASA (Figure 19A). Animals treated simultaneously with sunitinib and ASA displayed an additive reduction by 84.2% compared to 4T1 control tumours (Figure 19A). Thus, these results indicated that a reduction of the total number of CAFs occurs upon treatments with sunitinib or ASA, while the combination of both drugs potentiated this effect (Figure 19B).

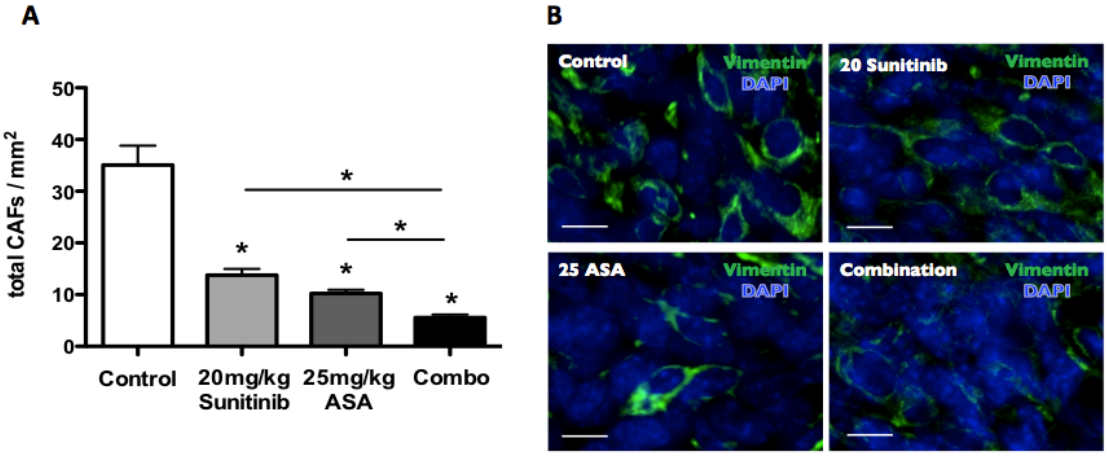

Figure 19: Sunitinib and ASA elicit additive inhibitory effect on infiltration with CAFs. (A) Quantification of total CAFs showed a significant decrease by 60.8% and 70.8% in 4T1 tumours upon treatment with 20mg/kg sunitinib or 25mg/kg ASA, respectively. An additive decrease by 84.2% was observed in tumours from animals treated with both sunitinib and ASA; n=7; *P<0.0001. **(B)** Representative images of immunofluorescence stainings for vimentin (green) and nuclei (blue) of 4T1 control tumours or tumours from animals treated with sunitinib, ASA or the combination of both inhibitors. Scale bar: 10µm.

For treatments with DC101 and ASA, the quantification of total CAFs in 4T1 tumours showed a small decrease by 23% in response to treatments with DC101, while ASA reduced CAF infiltration significantly by 35.3% (Figure 20A). A decrease of the total number of CAFs was more pronounced upon combinatory treatment with both DC101 and ASA. Tumours from these animals showed an increased significant reduction by 65.4% in comparison to 4T1 control tumours (Figure 20A). Therefore, the combination of DC101 and ASA exerted additive effects in reducing the total number of CAFs in 4T1 tumours, similarly as it was observed using sunitinib and ASA (Figure 20B).

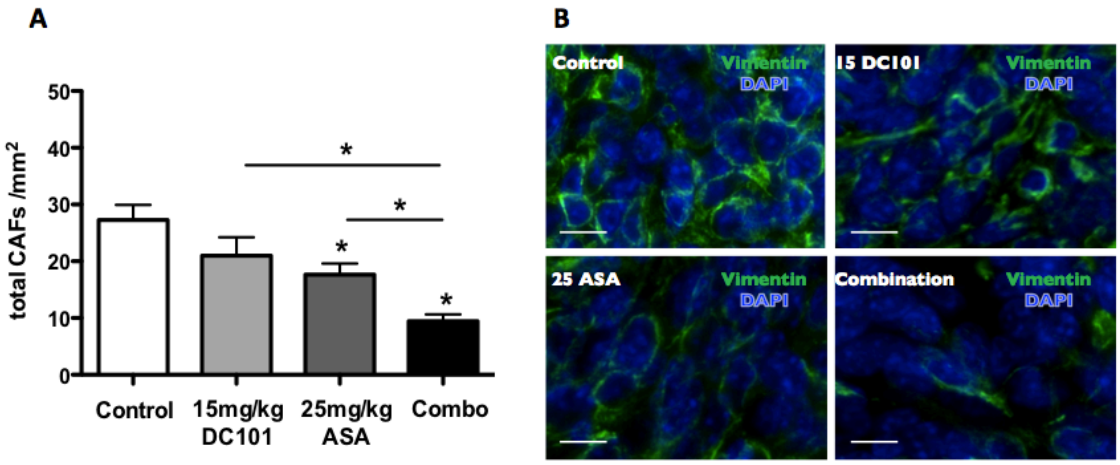

Figure 20: DC101 and ASA elicit additive inhibitory effect on infiltration with CAFs. (A) Quantification of total CAFs showed a slight decrease by 23% in 4T1 tumours upon treatment with 15mg/kg DC101. Treatment with 25mg/kg ASA reduced this number significantly by 35.3%. Combination of DC101 and ASA potentiated this effect, thus reducing the total number of CAFs by 65.4%; n=7; *P<0.0001. **(B)** Representative images of immunofluorescence stainings for vimentin (green) and nuclei (blue) of 4T1 control tumours or tumours from animals treated with DC101, ASA or the combination of both inhibitors. Scale bar: 10µm.

Taken together, treatment with ASA significantly reduced the total number of CAFs in 4T1 tumours. This reduction was enhanced when animals were treated simultaneously with ASA and either sunitinib or DC101. Therefore, the reduced number of infiltrating CAFs might be an explanation for the enhanced efficacy of anti-angiogenic therapies upon inhibition of Cox-2, since this cell population is able to rescue tumour vascularization in response to anti-angiogenic therapeutics such as sunitinib or DC101.

4.5. Anti-angiogenic drugs and ASA inhibit the activation of cancer associated fibroblasts

The role of tumour-infiltrating CAFs in sustaining tumour progression depends on their activation, which is induced by tumour-secreted factors (Cirri & Chiarugi 2011). Therefore, it was important to determine the fraction of CAFs with an activated phenotype from the previously determined total number of CAFs. To demonstrate this phenotype, co-immunofluorescence stainings with one of the main activation markers of CAFs, α-SMA, was performed in combination with vimentin. The quantification of activated CAFs showed a minor decrease by 19.1% upon treatment with sunitinib (Figure 21A-B). Treatment with ASA led to a significant reduction of activated CAFs by 69.1% compared to 4T1 control tumours (Figure 21A-B). The combination of sunitinib and ASA had an additive inhibitory effect of the activation of CAFs and induced a significant reduction of activated CAFs by 90.8% (Figure 21A-B).

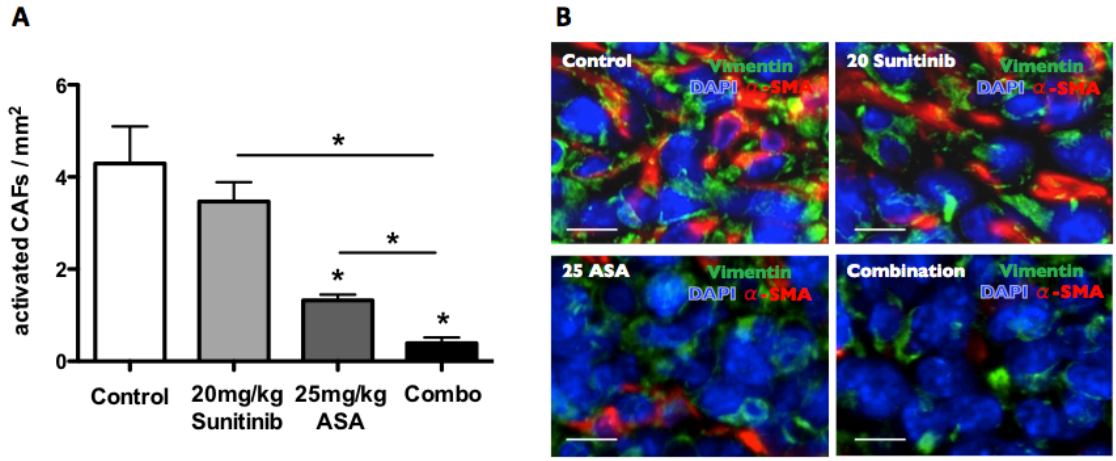

Figure 21: Sunitinib and ASA inhibit activation of infiltrating CAFs. (A) Quantification of activated CAFs within 4T1 tumours showed a slight decrease by 19.1% in the activation of CAFs upon treatment with 20mg/kg sunitinib. Treatment with 25mg/kg ASA inhibited significantly the activation of CAFs by 69.1%, while the combination of both inhibitors significantly reduced its activation by 90.8%; n=7; *P<0.001. **(B)** Representative images of immunofluorescence stainings for vimentin (green), α-SMA (red) and nuclei (blue) in 4T1 control tumours and tumours from animals treated with sunitinib, ASA or the combination of both inhibitors. Scale bar: 10μm.

In conclusion, sunitinib slightly decreased the activation of CAFs in 4T1 tumours, whereas ASA was much more effective in inhibiting CAF activation. However, this effect was more pronounced upon combinatory treatments with sunitinib and ASA. Remarkably, single treatments with sunitinib at higher dose levels were able to decrease the number of CAFs (36.7% and 74.5% for 40 and 60mg/kg sunitinib, respectively) as well as their activation (71.4% and 82.1% for 40 and 60mg/kg sunitinib, respectively) indicating that these effects are dose-dependent (Figure 22).

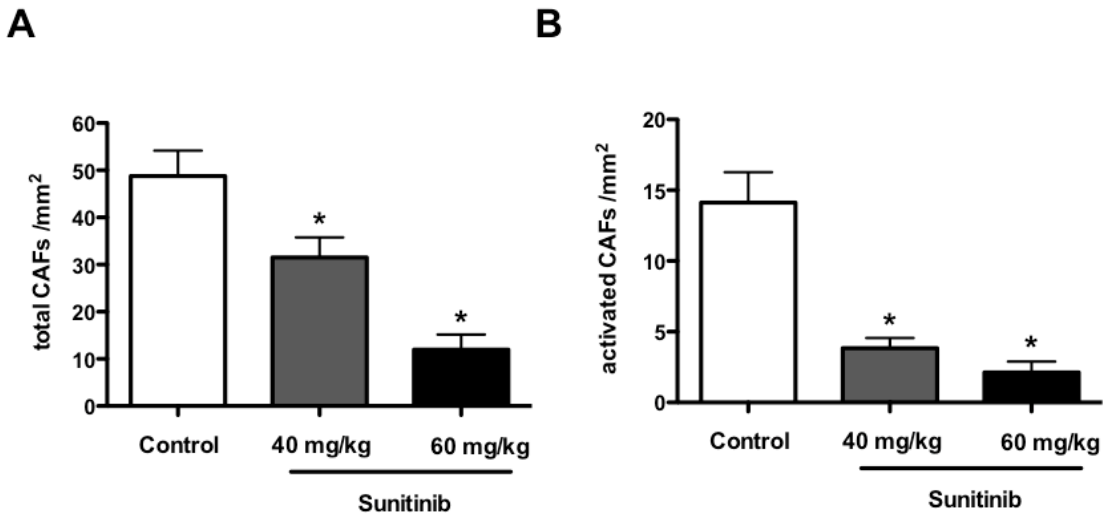

Figure 22: High dose levels of sunitinib inhibit infiltration and activation of CAFs. (A) Quantification of total CAFs showed significant decreases by 36.7% and 74.5% in 4T1 tumours upon treatments with 40mg/kg or 60mg/kg sunitinib, respectively; n=7; *P<0.05. **(B)** Quantification of activated CAFs showed significant decreases by 71.4% and 82.1% in the activation of CAFs in 4T1 tumours upon treatments with 40mg/kg or 60mg/kg sunitinib, respectively; n=7; *P<0.0001.

Similar to the single treatment with 20mg/kg sunitinib, 4T1 tumour-bearing animals treated with DC101 showed a decrease by 33.2% in the activation of CAFs, while treatments with ASA inhibited it significantly by 57.9% (Figure 23A). The combination of DC101 and ASA elicited a significant additive inhibitory effect on the activation of CAFs by 84% (Figure 23A). In conclusion, DC101 also slightly inhibited the activation of CAFs, while ASA induced a significant inhibition of the activation. As observed in the experiments with sunitinib, this effect was potentiated when animals were treated with the combination of DC101 and ASA (Figure 23B).

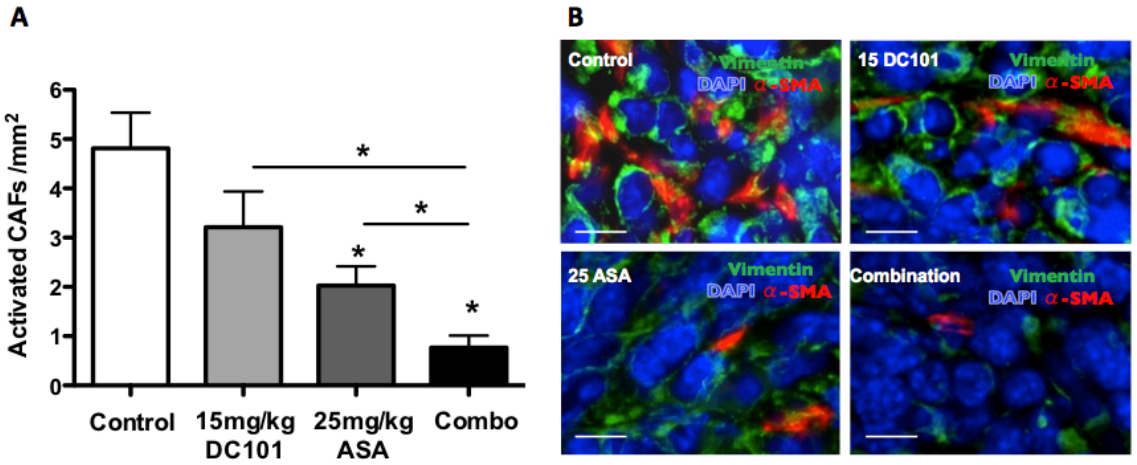

Figure 23: DC101 and ASA inhibit activation of infiltrating CAFs. (A) Quantification of activated CAFs within 4T1 tumours showed a slight decrease by 33.2% of the activation of CAFs upon treatment with 15mg/kg DC101, while treatment with 25mg/kg ASA reduced significantly the activation of CAFs by 57.9%. The combination of both inhibitors significantly reduced the activation of CAFs by 84%; n=7; *P<0.001. **(B)** Representative images of immunofluorescence stainings for vimentin (green), α-SMA (red) and nuclei (blue) in 4T1 control tumours and tumours from animals treated with DC101, ASA or the combination of both inhibitors. Scale bar: 10µm.

In order to confirm the observations that the inhibition of Cox-2 in combination with anti-angiogenic therapies results in an additive reduction of the activation of CAFs, *in vitro* experiments with primary CAFs were performed using ASA or SC-236 for the inhibition of Cox-2. The activation of primary CAFs was determined by measuring mRNA expression levels of the activation markers α-SMA and fibroblast activation protein (FAP) via qRT-PCR. These experiments revealed that the mRNA expression of α-SMA in primary CAFs was significantly reduced 1.7-fold by ASA and 3.9-fold by SC-236 compared to primary CAFs cultured in the absence of both inhibitors (Figure 24A). Furthermore, the culture of primary CAFs in the presence of ASA and SC-236 also induced a down regulation of the mRNA expression of FAP in primary CAFs (Figure 24B). These *in vitro* experiments showed that activation of primary CAFs could be reduced upon inhibition of Cox-2.

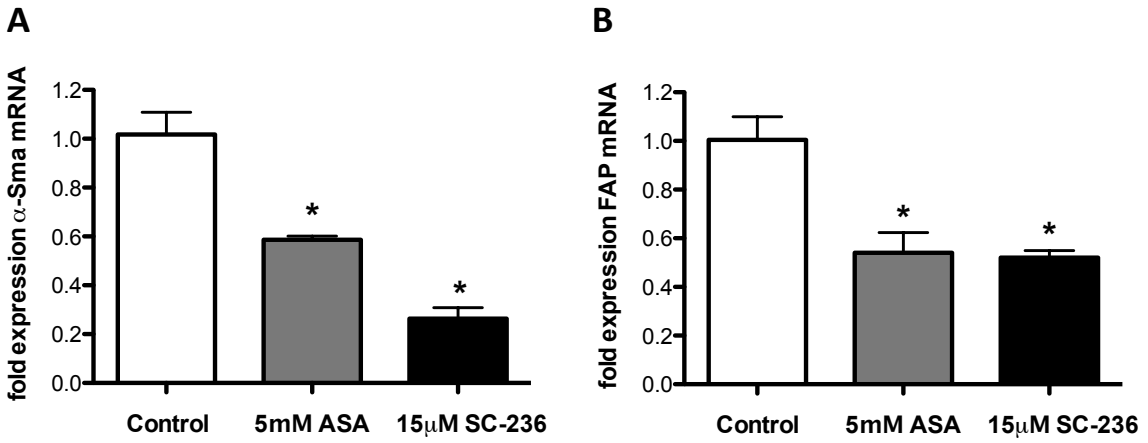

Figure 24: Inhibition of Cox-2 reduces activation of primary CAFs *in vitro*. Primary CAFs isolated from lung tumour tissues were cultivated with ASA (5mM) or SC-236 (15μM) for the inhibition of Cox-2. **(A)** mRNA expression levels of the activation marker α-SMA showed a 1.7 and 3.9-fold decrease upon incubation with ASA or SC-236, respectively, when compared to untreated primary CAFs; n=3; *P<0.05. **(B)** mRNA expression levels of the activation marker FAP showed a 1.9-fold decrease for both inhibitors compared to control primary CAFs; n=3; *P<0.01.

In conclusion, single therapies with sunitinib or DC101 were able to induce a slight inhibition of CAF activation in 4T1 tumours *in vivo*. Moreover, ASA alone significantly inhibited the activation of CAFs. Importantly, combinatorial administration of ASA with sunitinib or DC101 further reduced the number of intratumoral, activated CAFs. Additionally, *in vitro* experiments with primary CAFs incubated either with ASA or the specific Cox-2 inhibitor SC-236 showed a significant decrease of the mRNA expression levels of the fibroblast activation markers α-SMA and FAP, thereby indicating that Cox-2 might play a role in the activation of CAFs. CAFs are considered an important source of diverse pro-angiogenic factors in addition to VEGF and especially those with an activated phenotype secrete higher levels of these cytokines. Therefore, the reduced activation of CAFs might result in a decrease of secreted pro-angiogenic factors and the improvement of the efficacy of anti-angiogenic therapies.

4.6. Concomitant inhibition of angiogenesis and Cox-2 reduces the levels of pro-angiogenic cytokines

Among the variety of cytokines secreted by CAFs that are capable to promote tumour angiogenesis, many activate signal transduction pathways that cannot be blocked by VEGF pathway inhibitors (Luo et al. 2015). In order to investigate whether pro-angiogenic mediators besides the VEGF axis were reduced upon treatment with ASA, mRNA expression of different pro-angiogenic cytokines was analysed in 4T1 tumours by qRT-PCR. Several pro-angiogenic factors secreted by CAFs showed

differences in the expression pattern in response to treatment including transforming growth factor beta (TGFβ), basic fibroblast growth factor (FGF2), interleukin 6 (IL-6) and hepatocyte growth factor (HGF). Treatment with high doses of sunitinib (60mg/kg) was able to significantly reduce the mRNA expression levels of FGF2 and TGFβ by 2.5- and 3.8-fold, respectively (Figure 25A-B). Expression levels of other cytokines such as IL-6 and HGF showed a reduction by 2.3 and 1.5-fold, respectively (Figure 25C-D).

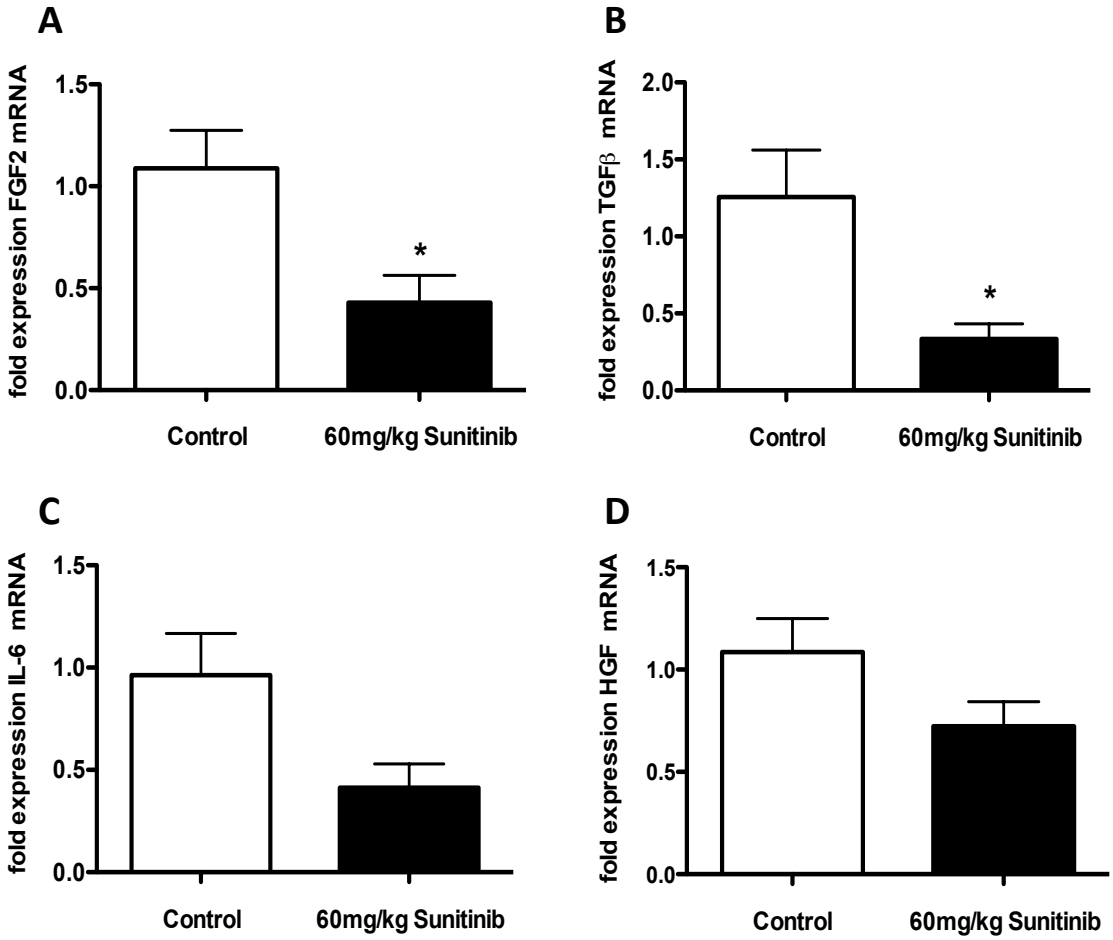

Figure 25: High doses of sunitinib reduce expression of pro-angiogenic cytokines in 4T1 tumours. Upon treatment with 60mg/kg sunitinib, mRNA expression levels of FGF2 were significantly decreased 2.5-fold compared to control tumours (A). Similarly, the TGFβ mRNA expression levels were significantly decreased 3.8-fold (B). mRNA expression levels of IL-6 were reduced 2.3- fold (C) and HGF 1-5 fold (D); n=3; *P<0.05 (A), *P<0.0001 (B).

This effect of sunitinib seemed to be dose dependent, as intermediate doses of sunitinib were not able to significantly reduce the mRNA expression levels of the tested pro-angiogenic cytokines (Figure 26). However, treatments with ASA induced a significant decrease of the mRNA expression levels of TGFβ (1.4-fold), HGF (1.6-fold) and IL-6 (1.7-fold), while for FGF2 the mRNA expression levels remained unaltered (Figure 26). The combination of sunitinib and ASA induced an additive reduction of the mRNA expression levels of the pro-angiogenic cytokines TGFβ, FGF2 and HGF, whose levels were

decreased 2.3, 1.9 and 3.5-fold, respectively (Figure 26). Therefore, these results demonstrate that the concomitant inhibition of Cox-2 and tumour angiogenesis has an additive inhibitory effect on the expression of pro-angiogenic cytokines that activate alternative signalling pathways beside the VEGF axis.

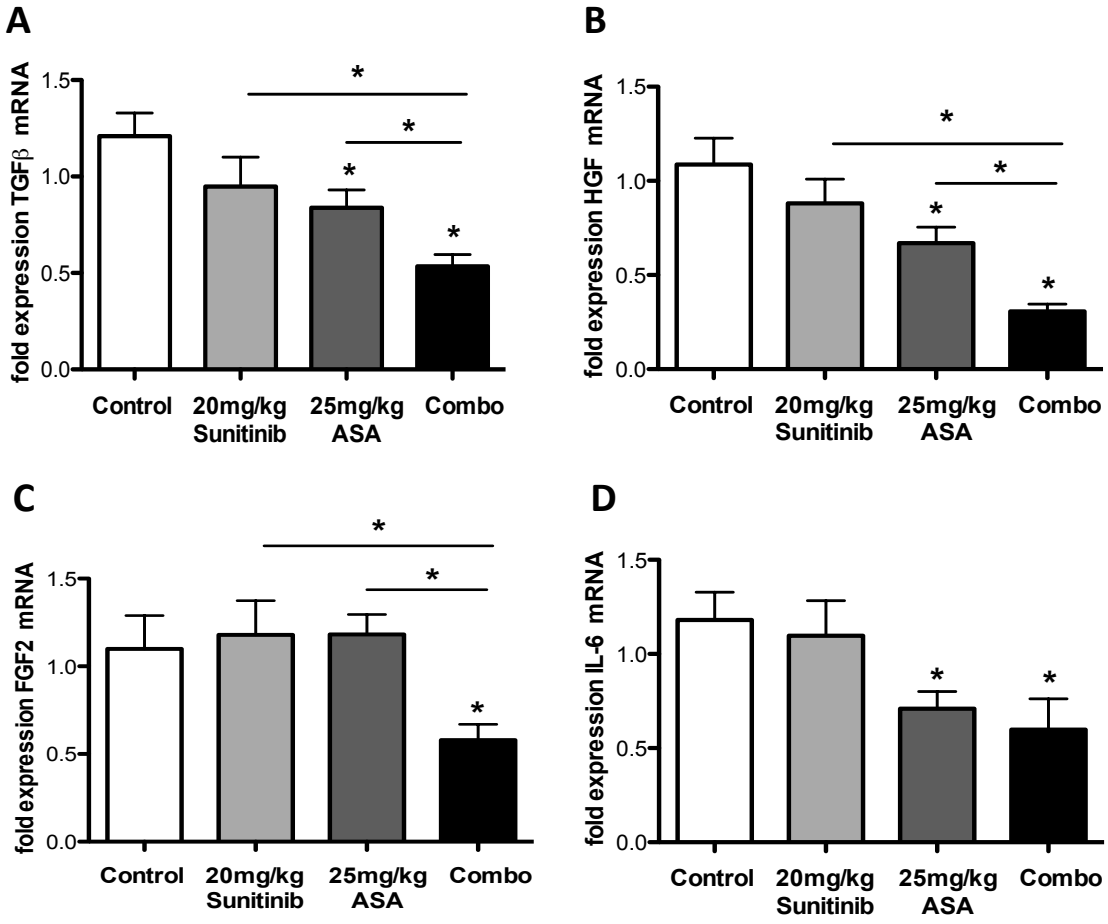

Figure 26: Sunitinib and ASA elicit additive inhibitory effects on mRNA expression of pro-angiogenic cytokines. mRNA expression levels of the pro-angiogenic cytokines TGFβ **(A)**, HGF **(B)**, FGF2 **(C)** and IL-6 **(D)** were not or minimal decreased upon treatment with 20mg/kg sunitinib. In contrast, treatment with 25mg/kg ASA reduced the expression levels of TGFβ (1.4-fold), HGF (1.6-fold) and IL-6 (1.7-fold). Combination of both inhibitors exerted an additive inhibitory effect on the mRNA expression of TGFβ (2.3-fold), FGF2 (1.9-fold) and HGF (3.5-fold); n=3; *P<0.05 (A), *P<0.05 (B), *P<0.05 (C) and *P<0.05 (D).

In order to study the influence of the inhibition of Cox-2 on the VEGF signalling pathway, the mRNA expression levels of VEGF-A were analysed in 4T1 tumours from animals treated either with ASA or SC-236 via qRT-PCR. This analysis revealed a 1.4-fold increase of the mRNA expression levels of VEGF-A in 4T1 tumours upon treatment with ASA, which was not significant (Figure 27A). Conversely, treatment with SC-236 elicited a 1.8-fold reduction of these levels, also not reaching statistical significance (Figure 27B). As a result, the additive effects observed from the inhibition of Cox-2 do not result

from an additional impairment of the VEGF signal pathway but rather from interfering with other signal transduction pathways that are important for angiogenesis.

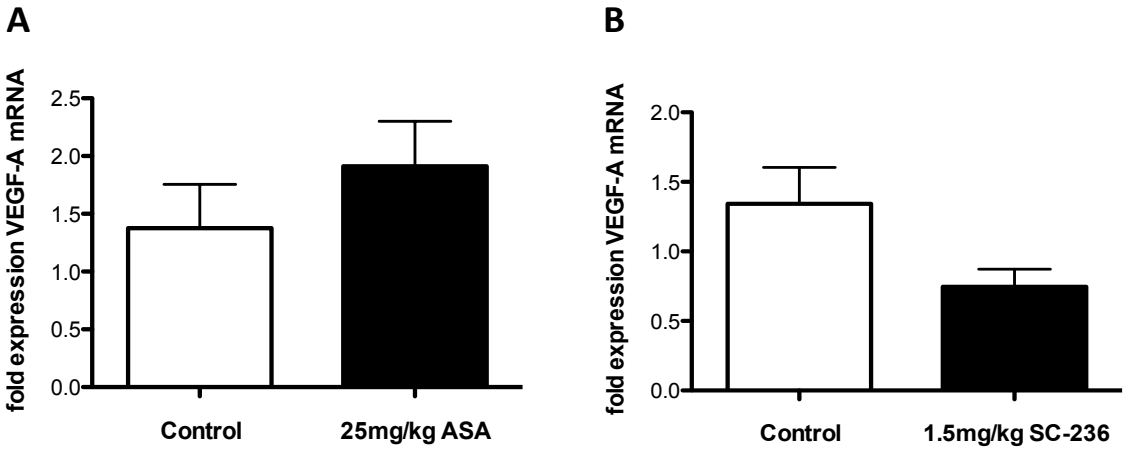

Figure 27: Inhibition of Cox-2 does not alter significantly VEGF-A mRNA expression levels. mRNA expression levels of VEGF-A were increased 1.4 fold upon treatment with 25mg/kg ASA (A). Inhibition of Cox-2 upon treatment with 1.5mg/kg SC-236 induced a 1.8-fold decrease of the VEGF-A mRNA expression levels (B). However, alterations of mRNA expression levels of VEGF-A upon treatments with both inhibitors showed no statistical significance; n=3; P=0.426 (A) and P=0.077 (B).

Taken together, sunitinib does not decrease the expression of pro-angiogenic cytokines in 4T1 tumours. However, combinatory treatment with sunitinib and ASA significantly decreased the expression of pro-angiogenic factors. Importantly, it could be shown that Cox-2 inhibition did not interfere significantly with the expression of VEGF-A, demonstrating that the concomitant inhibition of Cox-2 contributes to the effects of anti-angiogenic drugs in another way than interfering with the VEGF pathway.

4.7. Stroma cells express different pro-angiogenic cytokines

In order to identify cell populations responsible for the secretion of the pro-angiogenic cytokines mentioned above, stromal and tumour cells were isolated from 4T1 tumours of untreated animals via FACS sorting. These included besides 4T1 tumour cells CAFs, ECs, TAMs, gMDSCs and mMDSCs. Following cell sorting, expression levels of pro-angiogenic cytokines were measured by qRT-PCR. These analyses showed that pro-angiogenic cytokines such as HGF and FGF2 were predominantly expressed by ECs (Figure 28A, C) while 4T1 tumour cells exhibited higher expression levels of IL-6 and TGFβ (Figure 28B, D). However, CAFs represented an important source of cytokines such as HGF and IL-6 (Figure 28A-B). Furthermore, it could be demonstrated that FGF2 and TGFβ were also expressed by a wide range of cell types including TAMs, gMDSCs and mMDSCs (Figure 28C-D). As a consequence, the decreased expression of these pro-

angiogenic cytokines observed in 4T1 tumours upon treatments with sunitinib and ASA might not only result from a reduction of the number of CAFs or their activation but also due to the reduction of other intratumoral cell populations such as ECs, which are also altered in their quantity and quality upon treatments with anti-angiogenic drugs.

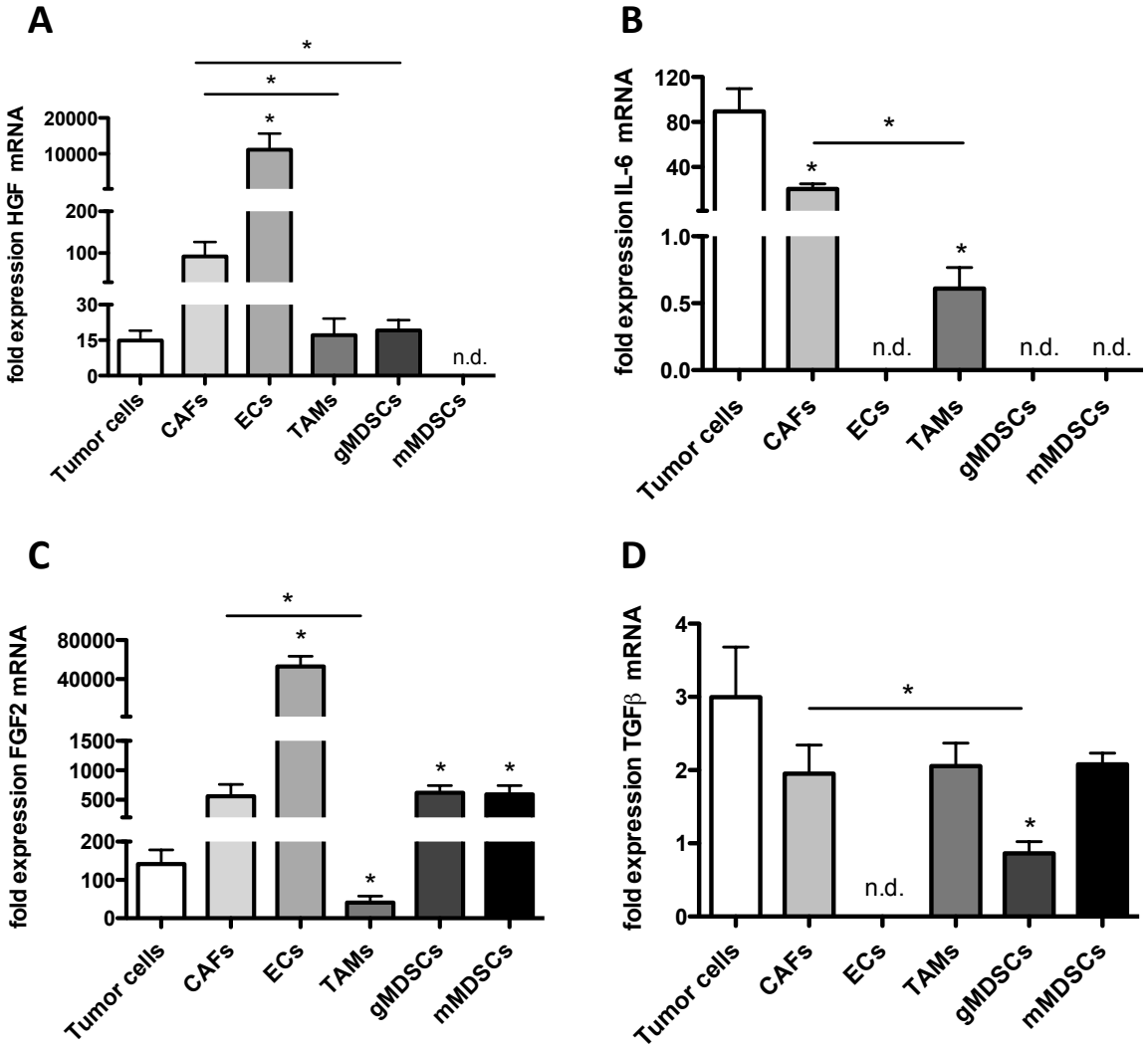

Figure 28: mRNA expression of pro-angiogenic cytokines from sorted 4T1 tumour and stromal cells. mRNA expression levels of the pro-angiogenic cytokines HGF **(A),** IL-6 **(B)**, FGF2 **(C)** and TGF β **(D)** were analysed in 4T1 tumour cells as well as different populations of stromal cells sorted from tumours of control animals. ECs were identified as main sources of HGF and FGF2 whereas CAFs expressed HGF and IL-6. Furthermore, tumour cells expressed mainly IL-6 and TGF β ; n=7; *P<0.05 (A), *P<0.001 (B), *P<005 (C), *P<0.05 (D).

4.8. Inhibition of Cox-2 blocks the proliferation and migration of CAFs

Based on the observed reduction of the number of tumour-infiltrating CAFs upon inhibition of Cox-2, further *in vitro* analyses were performed to determine its effects on the recruitment and proliferation of CAFs. This included the analysis of the proliferation of primary CAFs upon stimulation with PGE_2 and inhibition of Cox-2 by ASA or SC-236.

Primary CAFs stimulated with PGE_2 showed a significant increased viability in comparison to untreated primary CAFs, indicating a higher proliferation rate of 22.4% upon stimulation with PGE_2 (Figure 29A). In contrast, inhibition of Cox-2 by ASA or SC-236 decreased CAF proliferation by 26.3% (Figure 29A). Interestingly, the pro-proliferative effects of PGE_2 could be counteracted when Cox-2 was inhibited by ASA or SC-236 (Figure 29A). It has been reported that Cox-2 inhibitors can reduce the expression of Cox-2 in tumour cells leading to a reduced synthesis of PGE_2, which in turn reduces the proliferation of these cells (Sobolewski et al. 2010). Similarly to these findings, the analysis of Cox-2 expression in primary CAFs and in MRC-5 embryonic fibroblasts upon treatments with ASA or SC-236 revealed a significant reduction of the Cox-2 mRNA expression levels compared to untreated cells (Figure 29B). Furthermore, the inhibition of Cox-2 in primary CAFs as well as in MRC-5 cells resulted in reduced levels of secreted PGE_2 as determined by ELISA assays (Figure 29C). Taken together, both primary CAFs and MRC-5 cells might be able to secrete PGE_2, which stimulates their proliferation, thereby acting in an autocrine manner. Hence, the inhibition of Cox-2 by blocking PGE_2 secretion inhibits the proliferation of these cells.

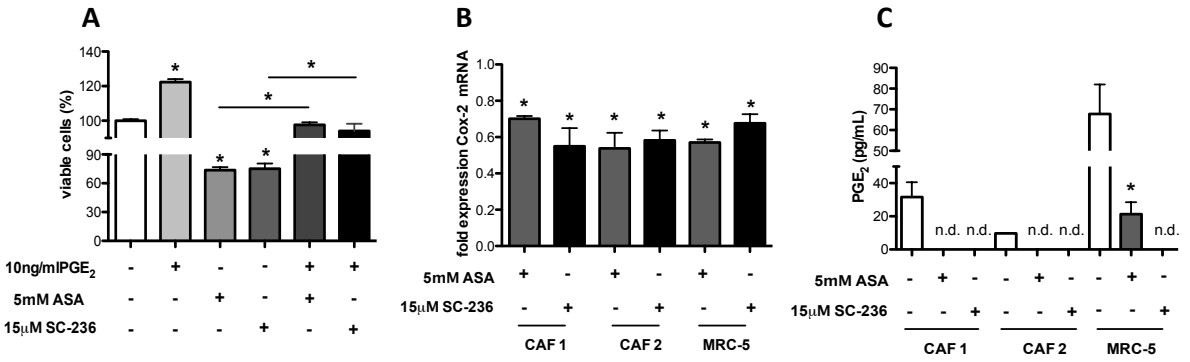

Figure 29: Inhibition of Cox-2 interferes with proliferation of primary CAFs and MRC-5 cells. (A) Proliferation of primary CAFs was increased by 22.4% upon treatment with 10ng/ml PGE_2. In contrast, Cox-2 blockade upon treatments with 5mM ASA or 15µM SC-236 elicited a reduction by 26.3%. Besides, both Cox-2 inhibitors counteracted the effects of PGE_2 on CAF proliferation; n=3; *P<0.05; experiment performed by Dr. Isabel Ben Batalla. **(B)** Treatments with both Cox-2 inhibitors elicited a reduction of the Cox-2 mRNA expression levels in primary CAFs and MRC-5 cells; n=3*P<0.05. **(C)** PGE_2 levels in primary CAFs and MRC-5 cells were decreased upon treatments with Cox-2 inhibitors; n.d. not detectable; n=3; *P=0.02.

Since the reduction of the Cox-2 mRNA expression levels and consequently the lower levels of PGE_2 are associated with a decrease of cell proliferation, Cox-2 might have a pivotal role in the activation of signalling pathways involved in cell proliferation and migration. These signalling pathways include among others the PI3K/Akt and MAPK/Erk pathways. To elucidate a potential involvement of Cox-2 in the activation of these pathways, the phosphorylation and thereby the activation of the kinases Akt and

Erk were analysed by western blotting. These analyses showed that neither the inhibition of Cox-2 by ASA or SC-236, nor the stimulation with PGE_2 in primary CAFs affected the phosphorylation of Erk (Figure 30A). Nevertheless, the phosphorylation of Akt in primary CAFs was increased upon stimulation with PGE_2, whereas the inhibition of Cox-2 by ASA or SC-236 decreased its phosphorylation (Figure 30B). Hence, it is possible that the inhibition of Cox-2 and consequently the decrease of PGE_2 levels may affect the proliferation of CAFs by inhibiting the Akt signalling pathway.

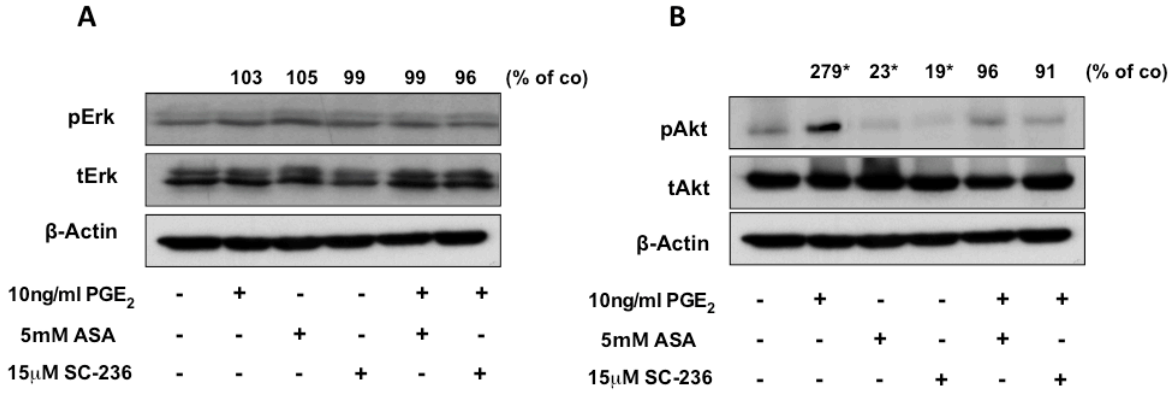

Figure 30: Inhibition of Cox-2 does not affect activation of MAPK/Erk pathway but interferes with activation of Akt pathway. (A) Stimulation with 10ng/ml PGE_2 and Cox-2 blockade with 5mM ASA or 15μM SC-236 did not show an increase of Erk phosphorylation in primary CAFs. **(B)** In contrast, primary CAFs stimulated with PGE_2 showed an increased Akt phosphorylation, which was blocked upon inhibition of Cox-2. Densitometric quantification was calculated as (phosphorylated Akt/ β-Actin)/(total Akt/ β-Actin); n=3; *P<0.01. Experiments performed by Isabel Ben Batalla.

In order to elucidate whether Cox-2 and PGE_2 activate the Akt signalling pathway, primary CAFs were cultured in the presence of PGE_2 or ASA and were additionally treated with the Akt inhibitor MK-2206. Primary CAFs were incubated with the Akt inhibitor MK-2206 at a dose of 7.5μM, as western blot analyses revealed an effective reduced phosphorylation of Akt using this dose compared to a lower dose of 5μM (Figure 31A). In this experiment, the stimulation with PGE_2 significantly induced CAF proliferation, whereas ASA and MK-2206 significantly suppressed the proliferation (Figure 31B). Furthermore, PGE_2-mediated proliferation of primary CAFs was counteracted upon Cox-2 and Akt inhibition. It is important to note that simultaneous inhibition of Cox-2 and Akt by ASA and MK-2206 did not induce an additive inhibitory effect on the proliferation of primary CAFs when compared to cells stimulated with ASA or MK-2206 alone. This indicates that Cox-2 mediates the proliferation of primary CAFs mainly through the Akt pathway (Figure 31B). Additionally, the stimulation with PGE_2 upon inhibition of both Cox-2 and Akt was not able to rescue cell proliferation, which remained at a similar rate to that observed upon inhibition of Akt and Cox-2 without

PGE_2 stimulation (Figure 31B). Hence, the inhibition of Cox-2 decreases the phosphorylation and activation of Akt and leads to a reduction of primary CAF proliferation, which could be mimicked by the administration of Akt inhibitors.

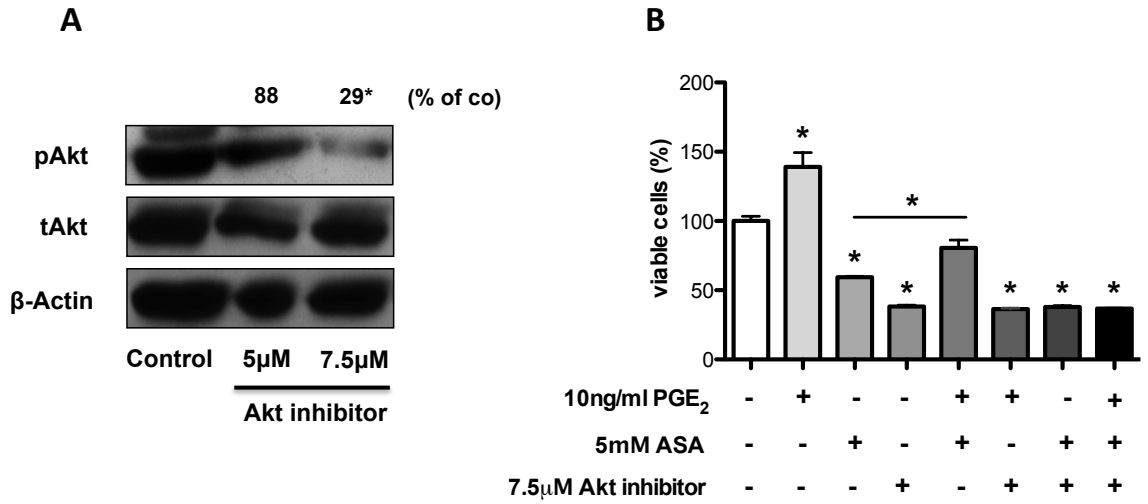

Figure 31: Akt Inhibition decreases proliferation of primary CAFs. (A) Treatment with 7.5μM MK-2206 showed a higher reduction of Akt phosphorylation in primary CAFs compared to treatment with 5μM MK-2206. Levels of total Akt and β-Actin remained unchanged upon treatments. Densitometric quantification was calculated as (phosphorylated Akt/ β-Actin)/(total Akt/ β-Actin); n=3; *P=0.0003. **(B)** Primary CAFs treated with 10ng/ml PGE_2 showed an increased proliferation, which was decreased upon treatment with 5mM ASA or 7.5 μM MK-2206. Both inhibitors counteracted the proliferation induced by PGE_2 and combination of both did not show an additive inhibitory effect; n=3; *P<0.01. Experiments performed by Dr. Isabel Ben Batalla.

As demonstrated previously, the proliferation of CAFs was reduced *in vitro* upon inhibition of Cox-2 by ASA. In order to study the effects of the Cox-2 inhibition on the proliferation of CAFs *in vivo*, immunofluorescence stainings of vimentin and BrdU, a nucleoside analogue of thymidine that was injected into the animals and incorporates into the DNA of dividing cells, was performed to quantify the number of proliferating CAFs in 4T1 tumours. Immunofluorescence stainings showed a 30% reduction of proliferating CAFs upon treatment with ASA in comparison to tumour tissues from control animals (Figure 32A). Therefore, it can be concluded that the proliferation of tumour infiltrating CAFs was also reduced *in vivo* upon treatment with ASA (Figure 32B).

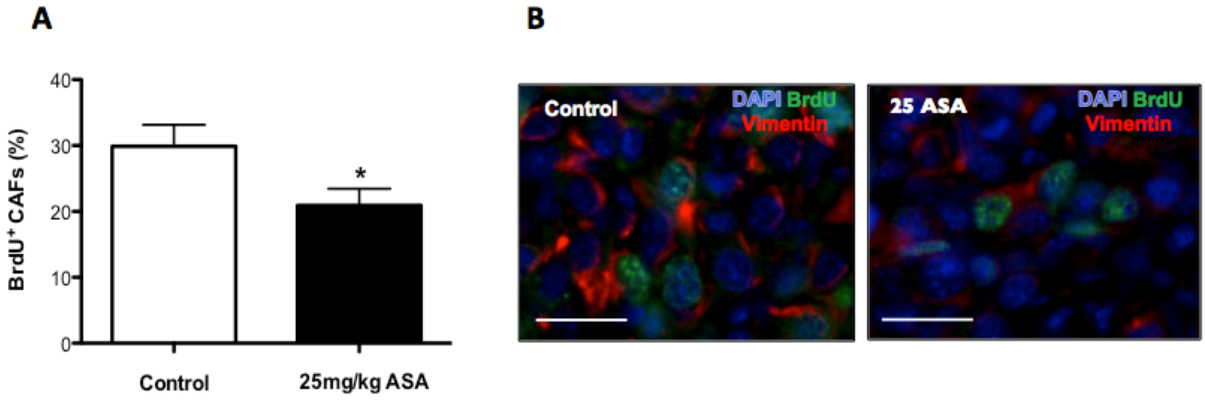

Figure 32: Inhibition of Cox-2 reduces proliferation of infiltrating CAFs in 4T1 tumours. (A) Morphometric analyses of proliferating CAFs in 4T1 tumours from animals treated with 25mg/kg ASA showed a reduction by 30% in comparison to 4T1 control tumours; n=7; *P<0.05 **(B)** Representative images of immunofluorescence stainings for vimentin (red), BrdU (green) and nuclei (blue) in 4T1 control tumours and tumours from animals treated with ASA. Scale bar: 25µm.

Taken together, ASA inhibits Cox-2 and the synthesis of PGE_2, thus decreasing the proliferation of CAFs by interfering with the activation of the Akt signalling pathway. Hence, these results explain the previously observed reduced number of CAFs in 4T1 tumours upon combinatory treatments with anti-angiogenic drugs and ASA.

Besides proliferation, migration of fibroblasts into the tumour represents an important step for tumour development. Hence, the analysis of the migration of primary CAFs upon Cox-2 inhibition or PGE_2 stimulation was analysed *in vitro* by performing migration assays using Boyden chambers. The quantification of migrating primary CAFs showed a 2.5-fold significant increase of the cell migration upon stimulation with PGE_2 compared to control primary CAFs (Figure 33A). Moreover, the inhibition of Cox-2 by ASA or SC-236 could decrease the migratory ability of primary CAFs by 35% (Figure 33A). Furthermore, the PGE_2-induced migration could be completely blocked upon treatment with ASA or SC-236, (Figure 33A).

Comparably to the proliferation of CAFs, it has been shown in other studies that the Akt signalling pathway also regulates the migration of CAFs (Al-Ansari et al. 2012). Therefore, primary CAFs were cultured with PGE_2 or ASA as well as with the Akt inhibitor MK-2206 in order to study the role of the Akt signalling pathway in the CAF migration. The stimulation with PGE_2 induced a 1.9-fold increase of the migratory capabilities of CAFs, while ASA or MK-2206 inhibited the migration of CAFs by 33% (Figure 33B). The inhibition of either Cox-2 or the Akt signalling pathway was also sufficient to block the migration of CAFs elicited upon stimulation with PGE_2 (Figure 33B). Furthermore, the simultaneous inhibition of Cox-2 by ASA and the Akt signalling

pathway by MK-2206 did not contribute to an additive effect on the reduction of CAF migration, neither in the presence nor in the absence of PGE_2 (Figure 33B). This indicates that the reduced migration of CAFs upon inhibition of Cox-2 is mainly regulated through the Akt signalling pathway.

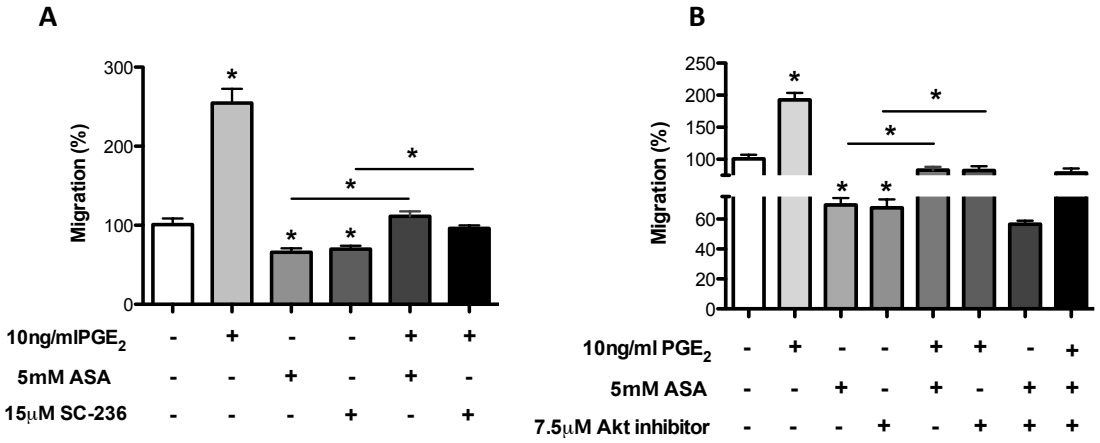

Figure 33: Inhibition of Cox-2 blocks migration of primary CAFs via Akt signalling pathway. (A) Upon treatment with 10ng/ml PGE_2, migration ability of primary CAFs was increased 2.5-fold compared to control primary CAFs. Migration of primary CAFs was decreased by 35% upon treatments with 5mM ASA or 15μM SC-236 and PGE_2-mediated migration was counteracted by both inhibitors; n=3; *P<0.005. **(B)** Primary CAFs showed a 1.9-fold increase of migration ability upon stimulation with PGE_2. The Akt inhibitor MK-2206 and ASA reduced the migration ability of primary CAFs by 33% and counteracted the effects of PGE_2. Combination of both inhibitors did not exert an additive inhibitory effect on migration of CAFs; n=3; *P<0.01. Experiments performed by Dr. Isabel Ben Batalla.

Additionally, the effects of the treatments with sunitinib and ASA on the mRNA expression levels of possible mediators involved in the recruitment of CAFs, such as TGFβ and PDGF-D, were evaluated in 4T1 tumour tissues by means of qRT-PCR. TGFβ was significantly decreased 2.3-fold in 4T1 tumours from animals treated with sunitinib and ASA (Figure 26A). The mRNA expression levels of the cytokine PDGF-D showed a 2.2-fold significant decrease upon treatment with ASA, and the combination of both sunitinib and ASA elicited an additive inhibitory effect that resulted in a 4.9-fold reduction of the PDGF-D levels (Figure 34). Therefore, the inhibition of Cox-2 together with anti-angiogenic therapies elicited a reduction of the levels of cytokines that mediate the recruitment of CAFs into the tumour, thus explaining the significant small number of CAFs observed in 4T1 tumours from animals treated with anti-angiogenic drugs and ASA.

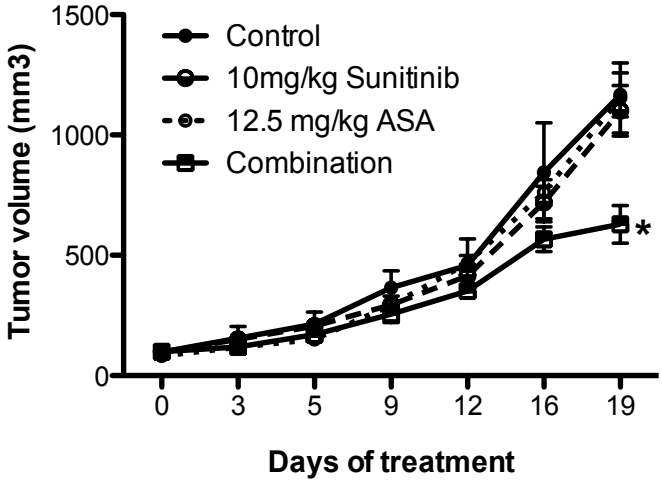

Figure 34: Inhibition of Cox-2 decreases the mRNA expression levels of PDGF-D. PDGF-D mediates the recruitment of CAFs and its mRNA expression levels were 2.2-fold decreased upon treatment with 25mg/kg ASA. Combination of sunitinib and ASA exerted an additive inhibitory reduction of 4.9-fold compared to 4T1 control tumours; n=7; *P<0.01.

Taken together, these *in vitro* and *in vivo* experiments demonstrate that Cox-2 elicits proliferation and migration of CAFs mainly through the activation of the Akt signalling pathway. Other important signalling pathways like the Erk signalling pathway seemed not to be regulated by Cox-2 or PGE_2. The inhibition of Cox-2 led to a reduced phosphorylation of Akt kinase, thus weakening its activation. Therefore, the previous observations of the significantly reduced number of CAFs in animals treated with anti-angiogenic therapies and ASA might be the consequence of the inhibition of Cox-2. This effect might explain the enhanced efficacy of anti-angiogenic drugs in combination with Cox-2 inhibition, since CAFs are important mediators of tumour angiogenesis. Additionally it could be shown that the inhibition of Cox-2 interfered with the regulation of cytokines that are important for the recruitment of fibroblasts into the tumours. Cytokines like TGF-β and PDGF-D were reduced in 4T1 tumours from animals treated with sunitinib and ASA, thus explaining the reduced number of CAFs in tumours when Cox-2 was inhibited.

5. Discussion

The effects of most current anti-angiogenic therapies are based on the interference with different steps of the VEGF signalling pathway, which is widely considered one of the most important signal transduction mechanisms implicated in tumour angiogenesis. However, despite the promising benefits that these therapies have shown in several pre-clinical and clinical studies, their effects during cancer treatment remain transient or are absent in some cases due to diverse resistance mechanisms that counteract these drugs (Bergers & Hanahan 2008; Welti et al. 2013).

Pre-clinical studies in several cancer models have shown that anti-angiogenic therapies induce hypoxia, which is characteristic in many tumours, and represents a condition that benefits the emergence of tumour resistance (Franco et al. 2006; Gaustad et al. 2012). In fact, hypoxia is a driving force for the recruitment of host-related cells that have tumour-protective functions, thereby counteracting the effects of diverse anti-cancer therapies (Loges et al. 2010). The presence of tumour hypoxia is considered as a selection mechanism that forces tumour cells to escape the prevalent hostile environment. This favours the expansion of tumour cells that are more tolerant to this condition or show enhanced invasive properties that facilitate the formation of tumour metastases (Loges et al. 2010). Treatment-induced hypoxia acts as a regulator of the expression of several genes that play a role in the resistance against anti-carcinogenic treatments. Among these genes is Cox-2, a key inflammatory mediator that is reportedly deregulated in many cancers such as colorectal and breast cancer (Yoshimura et al. 2004; Glynn et al. 2010).

The present work showed an upregulation of Cox-2 mRNA and PGE_2 levels upon administration of the anti-angiogenic drugs DC101 and sunitinib in a 4T1 breast cancer model (Figure 9-10). This upregulation correlated with intratumoral hypoxia (Figure 11). Interestingly, sunitinib induced more strongly the production of PGE_2 compared to DC101. Although Cox-2 mRNA expression levels did not show major changes between treatments with DC101 or sunitinib, these must not be necessary proportional to the levels of PGE_2, since the functionality of Cox-2 might depend on many other factors like post-transcriptional and post-translational modifications that could affect its enzymatic activity (Hoellen et al. 2011; Alexanian et al. 2014). However, the exact mechanisms that

regulate these modifications and how they can be differently affected by DC101 and sunitinib still need further investigations.

Inhibition of Cox-2 in combination with anti-angiogenic drugs at standard doses exerted additive anti-carcinogenic effects in the 4T1 breast cancer model (Figure 12A). Remarkably, these effects prevailed also at lower than typical therapeutic dose levels of anti-angiogenic drugs (Figure 13). Cox-2 blockade might result in the inhibition of alternative pro-angiogenic signalling pathways that can be activated by PGE_2, thus contributing to the increased efficacy of anti-angiogenic drugs. Several studies have demonstrated the role of Cox-2 and especially of PGE_2 in promoting tumour progression and angiogenesis by mechanisms besides the VEGF axis. PGE_2 signals through four distinct G protein-coupled receptors (EP1-4) (Sugimoto & Narumiya 2007). It can also transactivate the EGF receptor, which activates signal transduction cascades such as MAPK, PI3K/Akt, STAT and phospholipase C signalling pathways, thus resulting in cell proliferation, differentiation, migration and survival (Sales et al. 2004; Oshima et al. 2011; H. Zhang et al. 2014). Furthermore, PGE_2-mediated EP2 receptor signalling in ECs regulates their cell motility and survival, and contributes to tumour angiogenesis *in vivo* and *in vitro* (Kamiyama et al. 2006; Zhao et al. 2012). Hence, PGE_2 might contribute to the development of tumour resistance in cancer patients and exert pro-carcinogenic effects by mechanisms that are different from those exerted via VEGF and its receptors.

The expression of Cox-2 in different cancer cells has been shown to induce the expression of diverse pro-angiogenic factors such as VEGF, FGF2, TGF-1, and PDGF (Tsujii et al. 1998; Tjiu et al. 2006; Hoellen et al. 2011). The inhibition of Cox-2 in combination with sunitinib or DC101 decreased the microvessel density significantly when compared to tumour tissues from control animals (Figure 17-18). It is possible that the inhibition of Cox-2 causes the observed additive inhibitory effects on tumour angiogenesis by decreasing the expression of different pro-angiogenic factors that can support angiogenesis upon VEGF blockade. Therefore, Cox-2 might be an interesting therapeutic target in order to enhance the efficacy of anti-angiogenic treatments that target the VEGF growth factor family and its receptors. In addition, dose reductions of anti-angiogenic drugs might not only prevent adverse effects but also hypoxia, which can contribute to resistance against anti-angiogenic drugs by mechanisms such as the upregulation of Cox-2 expression and PGE_2 levels.

A previous pre-clinical study in human renal cell carcinoma models showed that sunitinib treatments increased the expression levels of Cox-2 in areas of tumour hypoxia and the effectiveness of the treatment could be enhanced upon inhibition of Cox-2 by the specific inhibitor celecoxib (X. Wang et al. 2013). However, no additive anti-angiogenic effects upon combination of celecoxib with sunitinib were detected and the mechanisms responsible for the increased efficacy of sunitinib were not elucidated in these renal cancer models. Hence, it is possible that Cox-2 inhibitors such as celecoxib act distinctly according to the tumour entity. The present work aimed to elucidate these mechanisms using a breast cancer model and the angiogenesis inhibitor DC101 besides sunitinib.

In order to reveal potential mechanisms for the additive effects of anti-angiogenic drugs at lower dose levels upon Cox-2 inhibition at which PGE_2 levels were unaffected, the tumour microenvironment was analysed. Hypoxia induces the secretion of several factors by tumour cells that mediate the recruitment and activation of distinct host-derived stromal cells such as TAMs, CAFs or MDSCs. For instance, tumour-cell derived TGFβ has been largely acknowledged to be one of the major activators of CAFs, together with other cytokines including PDGF, FGF2 or IL-6 (Cirri & Chiarugi 2011). Tumour cells also promote the recruitment and polarization of TAMs towards a M2 pro-tumorigenic phenotype by secreting oncostatin M and eotaxin (Tripathi et al. 2014). Similarly, MDSCs can be recruited by pro-inflammatory cytokines including Cxcl1 and Cxcl5. All these stromal cells play an important role in the rescue of tumour angiogenesis in response to anti-VEGF inhibitory agents through the secretion of a wide range of pro-angiogenic factors.

Cox-2 blockade as well as treatments with sunitinib reduced the number of CAFs and combinatorial treatments exerted additive effects on CAF reduction (Figure 19-20). In addition, ASA reduced the activation of CAFs and combinatorial treatments exerted additive effects on CAF activation (Figure 21, 23). The inhibition of recruitment and activation of CAFs may lead to a reduction of pro-angiogenic factors secreted by these cells that trigger angiogenesis, thus explaining the anti-carcinogenic and anti-angiogenic effects observed upon Cox-2 blockade. In fact, diverse studies have reported about the role of CAFs in supporting tumorigenesis by stimulating angiogenesis, cancer cell proliferation and invasion through the secretion of pro-inflammatory and pro-angiogenic factors (Orimo et al. 2005; Erez et al. 2013). Besides, many reports indicate

that CAFs are associated with a poor prognosis in different tumours types (Matsuoka et al. 2014; Cheng et al. 2015).

In contrast to sunitinib, DC101 slightly decreased the number of CAFs when administered alone (Figure 19-20). A possible explanation of the higher efficacy of sunitinib in the inhibition of the CAF infiltration could be the multi-target properties that this small molecule has for inhibiting different RTKs, including members of the platelet-derived growth factor receptor (PDGFR) family, which according to other publications are crucial for the proliferation and recruitment of CAFs (L. Mueller et al. 2007). DC101 acts only inhibiting the activity of the murine VEGFR-2 and perhaps the simple inhibition of this receptor was not enough to interfere with the recruitment of CAFs into the tumour.

A more efficient inhibition of infiltration and activation of CAFs was observed upon single treatments with higher dose levels of sunitinib (40mg/kg and 60mg/kg) (Figure 22). However, it is of clinical interest to maintain or increase the efficacy of this anti-angiogenic drug at lower doses since a higher dosage may imply a more aggressive development of tumour resistance. The use of 40mg/kg/d of sunitinib in the present mouse model corresponds already to the maximum tolerated dose of 75mg/d used in the clinics (Kollmannsberger et al. 2011). Due to the toxicity that these drugs may generate during the treatment, partial or complete interruptions are mandatory (Loges et al. 2010). The present work showed that the combination of 25mg/kg ASA, which corresponds to a daily dose of 150mg in humans (Jeong et al. 2013), together with sunitinib at a lower dose (20mg/kg) than the normal maximum tolerated dose evokes additive anti-carcinogenic and anti-angiogenic effects. This enhanced efficacy of anti-angiogenic drugs might result from the inhibition of CAF infiltration and activation elicited by ASA. Therefore, the effectiveness of anti-angiogenic drugs would be increased, while at the same time the risks of adverse side effects that these drugs can usually cause when administered at higher dose levels would be reduced.

Single treatments with ASA elicited a reduction of intratumoral cytokines including IL-6 and HGF (Figure 26). Nilsson et al reported that ovarian cancer cells express the highly angiogenic cytokine IL-6 *in vivo* and *in vitro*, which acts directly on ECs and enhance their cell migration (Nilsson 2005). HGF is expressed in oesophagus cancer cells and also contribute to angiogenesis (Xu et al. 2013). Remarkably, the mRNA expression

levels of both cytokines were not altered upon treatment with different doses of sunitinib, thereby indicating that their regulation occurs independently of the VEGF axis (Figure 26). In addition, the simultaneous inhibition of Cox-2 and VEGF-signalling pathway was able to decrease the mRNA expression levels of angiogenic cytokines such as TGFβ and FGF2 (Figure 26). Identification of stromal components in 4T1 tumours and the analysis of their cytokine expression profile showed that IL-6 mRNA was predominantly expressed in tumour cells as well as in CAFs. Expression of HGF mRNA was also detected in tumour cells although this cytokine was predominantly expressed in other stromal populations including ECs and CAFs. Other pro-angiogenic factors such as FGF2 and TGFβ were expressed by diverse cellular components of the tumour including TAMs and MDSCs. Taken together, the observed reduction of the mRNA expression levels of these pro-angiogenic factors did not seem to be only the result of a decreased infiltration or activation of CAFs. Instead, the effects of the inhibition of Cox-2 and VEGF signalling pathway could affect other cellular components in the tumour stroma that might be responsible for sustaining tumour angiogenesis through the secretion of other pro-angiogenic factors and are not the target of VEGF-inhibitors.

IL-6 and HGF have been shown to induce angiogenesis when VEGF inhibitors were applied and consequently this could reduce the efficacy of anti-angiogenic drugs (Shojaei et al. 2010). Since Cox-2 inhibition showed a decrease of the mRNA expression of IL-6 and HGF, this might explain the additive effects observed after combination of ASA with anti-angiogenic drugs. Moreover, it is possible that the additive anti-carcinogenic and anti-angiogenic effects of the combination of ASA with sunitinib or DC101 could be due to simultaneous interference with several signalling pathways apart from the VEGF signalling pathway in both tumour and host-derived stromal cells (Aparicio-Gallego et al. 2011; Alfonso et al. 2014). These signalling pathways could act alternatively to the VEGF signalling pathway and promote besides tumour angiogenesis other modes of vascularization like vessel co-option, growth by intussusception, vascular mimicry or vasculogenesis (Schneider 2005). Basu et al hypothesized that Cox-2 overexpressing invasive human breast cancer cells have the ability to differentiate into vascular channels without involvement of ECs (Basu et al. 2005). This alternative mechanism to angiogenesis is referred to as vascular mimicry and acts independently of the VEGF signalling pathway. Administration of Cox-2 inhibitors could counteract this mechanism by inhibiting Cox-2 overexpressing tumour cells that might be able to build a

vasculature and sustain tumour survival. Nevertheless, the mechanisms underlying this and other different modes of vascularization, and the signalling pathways responsible for their regulation still need to be investigated in more detail. In addition, the biology of breast cancer might be also an important criterion for the outcome of the treatment with both therapies. This work was performed using the 4T1 cell line, which do not express progesteron receptor, estrogen receptor or the gene for human epidermal growth factor receptor 2 (Her2) (Kaur et al. 2012). Therefore, further studies of the effects of both treatments need to be done in breast cancer models that are hormone receptor and/or HER-2 positive.

Several studies have reported that Cox-2 inhibition leads to a decrease of the mRNA expression levels of VEGF (Leung et al. 2003; Yoshinaka et al. 2006). However, treatments with Cox-2 inhibitors did not show in this work any interference with the mRNA expression of VEGF (Figure 27), indicating that the additive effects of the treatments with Cox-2 and VEGF-inhibitors are not necessarily a consequence of a possible decrease of VEGF expression upon Cox-2 blockade. These findings indicate that the effects of Cox-2 inhibition on the VEGF expression could be cancer-type specific. Nevertheless, any possible fluctuation of the VEGF expression upon Cox-2 inhibition may not have any impact on the observed additive effect of the combinatory therapy, since the pan-VEGFR inhibitor sunitinib fully blocks the signal cascade mediated by all members of the VEGFR family (Aparicio-Gallego et al. 2011). Concerning DC101, a possible decrease of VEGF expression levels might still have implications in the response elicited by other VEGF receptors like VEGFR-1, since it is not specifically inhibited by DC101. However, the signal transduction mediated by VEGFR-1 is weak compared to VEGFR-2 (Kerbel 2008; Patel-Hett & DAmore 2011; Welti et al. 2013).

The reduction of the number of CAFs as well as the inhibition of their activation upon combinatory treatments might be the result of anti-proliferative and anti-migratory effects exerted by ASA. *In vitro* experiments with primary CAFs and MRC-5 embryonic fibroblasts showed an increased proliferation and cell motility in response to PGE_2, which was inhibited upon Cox-2 blockade (Figure 29, 33A). In absence of PGE_2, inhibition of Cox-2 was still able to block the proliferation and migration of CAFs, thus indicating that the proliferative and migratory capabilities of these cells might only be regulated in part by PGE_2. Analyses of the mechanisms responsible for the proliferation of CAFs in response to PGE_2 showed that the Akt signalling pathway is involved in this

process but not the MAPK/Erk pathway (Figure 30). The inhibition of Cox-2 led to a decrease in Akt phosphorylation, which in contrast, could be increased upon stimulation of CAFs with PGE_2. Concomitant inhibition of Cox-2 and Akt did not show any additive effect on the proliferation and migration of CAFs demonstrating that the observed inhibition of CAF proliferation is mainly mediated by Cox-2 through interference with the Akt signalling pathway. In contrast to these results, other studies have reported that PGE_2 can have inhibitory effects on lung fibroblasts, thus indicating that the effects of PGE_2 on the proliferation of fibroblast might vary depending on tissue of origin (Huang et al. 2006). Hence, the inhibition of Cox-2 might also elicit a dual effect on the proliferation and migration of fibroblasts, which would be dependent on the cancer entity or the origin of the fibroblasts. However, further investigations of the mechanisms of action of Cox-2 are necessary to be performed in other cancer models in order to have a better insight of the role that this enzyme has in the biology of CAFs. Moreover, another important aspect that might be responsible for the reduction of CAFs observed upon treatments with Cox-2 inhibitors is the decrease of the expression of two factors capable of inducing their tumour recruitment, i.e. TGFβ and PDGF-D (Cirri & Chiarugi 2011; Cadamuro et al. 2013) (Figure 26, 34).

In summary, this work showed the benefits of the combining treatments that target the activity of Cox-2 and the VEGF signalling pathway simultaneously. These benefits consisted primarily of the enhanced, additive anti-carcinogenic and anti-angiogenic effects that both treatments have at a lower dosage than the common therapeutic dosage. This might be valuable for patients with breast cancer treatment since higher doses of anti-angiogenic therapies are known to induce intratumoral hypoxia, which is a major driving force in the development of a variety of tumour resistance mechanisms against these therapies. Furthermore, administration of lower doses of anti-angiogenic drugs could diminish the appearance of adverse effects that can lead to the interruption of treatment with these drugs. Moreover, the role of CAFs in the resistance against anti-angiogenic drugs was highlighted. In conclusion, these cells as well as other tumour stroma components could be the key that explains how tumours might evade these therapies. Hence, the combination of Cox-2 and anti-angiogenic inhibitors represents a novel, clinically applicable approach to increase the efficacy of anti-angiogenic drugs.

6. Summary

Conventional anti-angiogenic therapies used in treatment of different cancers act by interfering with the VEGF pathway, which is the main regulator of physiologic and pathologic angiogenesis. The efficacy of these therapies faces limitations due to the appearance of tumour resistance, as it occurs in several cancers including breast cancer.

In the present work, anti-angiogenic therapies were shown to induce the expression of cyclooxygenase-2 (Cox-2) in the murine breast cancer 4T1 tumour model. As a consequence, the levels of prostaglandin E_2 (PGE_2), the main product of Cox-2, were also increased.

The inhibition of Cox-2 using acetyl salicylic acid (ASA) resulted in the normalization of intratumoral PGE_2 levels. Interestingly, the combination of ASA either with the anti-vascular endothelial growth factor receptor-2 antibody (anti-VEGFR-2) DC101 or the small molecule receptor tyrosine kinase inhibitor (RTKI) sunitinib elicited additive anti-carcinogenic and anti-angiogenic effects.

Analyses of tumour tissues from animals treated with a combination of DC101 or sunitinib with ASA revealed a reduced infiltration of cancer-associated fibroblasts (CAFs) into the tumour. Activated CAFs have been described in several studies as a pro-tumorigenic component of the tumour stroma that can secrete pro-angiogenic cytokines besides the VEGF axis. Besides reducing CAF infiltration, this combinatory treatment was also able to decrease the activation of CAFs. Moreover, *in vitro* analyses showed that PGE_2 promotes the proliferation and migration of CAFs through the activation of the Akt signalling pathway, which could be blocked upon Cox-2 inhibition.

Therefore, combinatory therapies with angiogenesis and Cox-2 inhibitors might be considered as a novel clinical approach for the treatment of cancer that can enhance the efficacy of anti-angiogenic treatments.

Zusammenfassung

Anti-angiogene Therapeutika sind in den letzten Jahren zu einem wichtigen Bestandteil der modernen Krebsbehandlung geworden. Diese eingesetzten anti-angiogenen Therapien basieren auf der Inhibierung des VEGF-Signaltransduktionsweges, des zentralen Regulators von physiologischer als auch pathologischer Angiogenese. Die Wirksamkeit dieser Therapien ist jedoch aufgrund der Entstehung von Tumor-Resistenz begrenzt, die vor allem bei Brustkrebspatienten häufig vorkommen.

In dieser Arbeit wurde gezeigt, dass anti-angiogene Therapien die Expression von Cyclooxygenase-2 (Cox-2) im Brustkrebs 4T1 Mausmodell induzieren. Diese Induktion der Cox-2 Expression resultierte auch in einer erhöhten enzymatischen Aktivität, die zu einem Anstieg der Prostaglandin E_2 (PGE_2) Spiegel führte.

Die Inhibierung von Cox-2 durch Acetylsalicylsäure (ASA) führte zur Normalisierung der intratumoralen PGE_2 Werte. Interessanterweise resultierte die Kombination von ASA mit dem Antikörper DC101, der gegen den vascular endothelial growth factor Rezeptor-2 (VEGFR-2) gerichtet ist, oder mit dem Rezeptor-Tyrosinkinase-Inhibitor (RTKI) Sunitinib in stärkeren anti-karzinogenen und anti-angiogenen Effekten.

Analysen von Tumorgeweben nach kombinatorischer Behandlung von DC101 oder sunitinib mit ASA zeigten eine reduzierte Infiltrierung von tumorassoziierten Fibroblasten (CAFs). Aktivierte CAFs wurden in zahlreichen, kürzlich erschienen Arbeiten als pro-tumorigene Faktoren des Tumormikromilieus beschrieben. Unter anderem sekretieren aktivierte CAFs pro-angiogene Zytokine, die abseits der VEGF-Achse die Tumorangiogenese fördern. Die hier angewendete Kombinationstherapie konnte nicht nur die Anzahl der CAFs, sondern auch deren Aktivierung reduzieren. Des Weiteren zeigten *in vitro* Analysen, dass PGE_2 die Proliferation und Migration von CAFs durch die Aktivierung des Akt-Signalweges induziert, welcher durch die Inhibierung von Cox-2 blockiert wurde.

Daher stellt der kombinatorische Einsatz von anti-angiogenen Therapeutika mit Cox-2 Inhibitoren einen neuen klinischen Ansatz für die Behandlung von Krebspatienten dar, mit dem die Wirksamkeit der anti-angiogenen Therapien erhöht werde kann.

7. Abbreviations

α-SMA	alpha smooth muscle actin
Ang2	angiopoetin 2
AP	alkaline phosphatase
ASA	acetylsalicylic acid
BrdU	bromodeoxyuridine
CAF	cancer-associated fibroblast
cDNA	complementary DNA
CMC	carboxymethylcellulose
Cox-1	cyclooxygenase 1
Cox-2	cyclooxygenase 2
COXIB	Cox-2-specific inhibitor
CSC	cancer stem cell
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
EC	endothelial cell
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbent assay
EMA	European Medicines Agency
FACS	fluorescence activated cell sorting
FAP	fibroblast-activation protein
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF2	basic fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
gMDSC	granulocytic myeloid-derived suppressor cell
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
i.e.	lat. *id est* (it is)
IHC	immunohistochemistry
IF	immunofluorescence
Ig	immunoglobulin

IL-6	interleukin 6
IL-8	interleukin 8
i.p.	intraperitoneal
MAPK	mitogen-activated protein kinase
MDSC	myeloid-derived suppressor cell
mMDSC	monocytic myeloid-derived suppressor cell
mRNA	messenger RNA
MVD	microvessel density
NSAIDs	non-steroidal anti-inflammatory drug
PBS	phosphate buffered saline
PC	pericyte
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PlGF	placental growth factor
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the means
TAM	tumour-associated macrophage
TBS	Tris-buffered saline
TGFβ	transforming growth factor beta
TMB	tetramethylbenzidine
TNF- α	tumour necrosis factor alpha
TKI	tyrosine kinase inhibitor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

8. Literature

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9. Publications

The content presented in this work has been published:

Cyclooxygenase-2 blockade can improve efficacy of VEGF-targeting drugs

Ben-Batalla I, Cubas-Cordova M, Udonta F, Wroblewski M, Waizenegger JS, Janning M, Sawall S, Gensch V, Zhao L, Martinez-Zubiaurre I, Riecken K, Fehse B, Pantel K, Bokemeyer C, Loges S.

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11. Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hamburg, March 2016

Miguel Cubas Cordova

12. Confirmation of linguistic correctness

I hereby declare, that I have read the doctoral thesis from Mr. Miguel Cubas Cordova titled "**Cyclooxygenase-2 blockade can improve efficacy of VEGF-targeting drugs**" and I confirm its linguistic correctness in English.

Hamburg, March 2016

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