The Growth Factor PDGF and its Signaling Pathways in Colorectal Cancer

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der

Julius-Maximilians-Universität Würzburg

vorgelegt von

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Würzburg 2016

Eingereicht bei der Fakultät für Chemie und Pharmazie am

_____

Gutachter der schriftlichen Arbeit

1. Gutachter: _____

2. Gutachter: _____

Prüfer des öffentlichen Promotionskolloquiums

1. Prüfer: _____

2. Prüfer: _____

3. Prüfer: _____

Datum des öffentlichen Promotionskolloquiums

_____

Doktorurkunde ausgehändigt am

_____

Die vorliegende Arbeit wurde auf Anregung und unter Anleitung von

Frau Prof. Dr. Ana Maria Waaga-Gasser

an der Chirurgischen Klinik und Poliklinik für Allgemein-,
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des Universitätsklinikums Würzburg angefertigt.

Die vorliegende Arbeit wurde von

Frau Prof. Dr. Petra Högger

vom Lehrstuhl für Pharmazeutische Chemie
des Instituts für Pharmazie und Lebensmittelchemie
der Julius-Maximilians-Universität Würzburg betreut.

Jeder ist seines Glückes Schmied

Appius Claudius Caecus, 340-273 v. Chr.

In Gedenken an Eva-Maria Moll

Publikationen:

Grimmig T, Matthes N, Hoeland K, Moench R, et al: TLR7 and TLR8 expression increases tumor cell proliferation and promotes chemoresistance in human pancreatic cancer. Int J Oncol 47:857-66, 2015

Kannen V, Garcia SB, Silva WA, Jr., Mönch R, et al: Oncostatic effects of fluoxetine in experimental colon cancer models. Cell Signal 27:1781-8, 2015

Artikel unter Review (die hier präsentierten Daten wurden teilweise im folgenden Artikel verwenden):

Moench R, Grimmig T, Kannen V, et al: Exclusive inhibition of PI3K/Akt/mTOR signaling is not sufficient to prevent the PDGF-mediated effects on glycolysis and proliferation in colorectal cancer. Oncotarget

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B. Introduction

1. Colorectal Cancer (CRC)

1.1 Epidemiology and carcinogenesis

Cancer is the second leading cause of death after heart diseases even before accidental death in the United States.[1] As the third most common cancer, the colorectal cancer plays a pivotal role in tumor associated deaths in the United States,[1-3] Germany and other industrial nations.[4]

In Germany the incidence of colorectal cancer was 33,740 in men and 28,490 in women in 2012. Although the therapeutic options with chemoradiation and small molecules improved tumor related survival with a median age at diagnosis of 72 years in men and 75 years in women. The relative survival rate of only 63% illustrates the necessity of new therapeutic strategies.[4]

1.2 Risk factors and prevention

Several known factors influencing CRC are associated with nutritional components. Individuals with high fiber and vegetable intake, who consume low amounts of red and processed meat and alcohol, are less affected from CRC. An appropriate body weight, sufficient physical activity as well as the absence of tobacco products have a further positive influence on intestinal health.[5]

CRC is not only a matter of lifestyle but additionally possesses a hereditary component. The probability of developing CRC early in life is much higher in presence of inherited diseases such as the Lynch syndrome (HNPCC) or the familial adenomatous polyposis (FAP).[6,7] Crohn's disease and ulcerative colitis, both chronic inflammatory bowel diseases, also represent risk factors for CRC.

Colorectal cancer screening represents the most important detection and prevention strategy against CRC. The colonoscopy can detect existing early stage cancers, as well as detect and remove precancerous polyps. Another prevention procedure utilizes the detection of occult blood in the stool, released by the tumor or large polyps (fecal occult blood test).[7]

1.3 Staging and therapy

The median age of diagnosis was 71 years/men and 74 years/women and supports the adenoma carcinoma sequence model proposed by Fearon and Vogelstein that suggested multistep genetic alterations of tumor suppressor genes and oncogenes. This multistep process can take several decades to complete tumor development and therefore the main incidence of colorectal cancer occurs in later life.[8-11]

The stage classification of CRC tumors is determined according to the Union for International Cancer Control (UICC) and this TNM-system represents the basic guideline for tumor prognosis and therapy (Table 1).[12-14] T describes the size of the primary tumor, N shows the spreading to regional lymph nodes, and M indicates the presence of distant metastases.

Table 1: UICC staging[15]

UICC-stage	Dukes	TNM	Definition
0		Tis N0 Mo	Carcinoma in situ
I	A	T1 N0 M0	Invasion of submucosa
		T2 N0 M0	Invasion into muscularis propria
II	B	T3 N0 M0	Invasion into subserosa
		T4 N0 M0	Invasion of adjacent structures
III	C	Any T, N1-3, M0	Regional infiltration
IV	D	Any T, any N, M1	Distant metastases

T= size of the primary tumor; N= spreading to regional lymph nodes; M0= no distant metastases; M1= distant metastases

CRC therapy normally comprises tumor resection and if applicable, treatment is continued with radiotherapy and/or chemotherapy.

The most frequent first line chemotherapy in CRC consists of a combination of 5-Fluorouracil (5-FU), Folinic acid, and Oxaliplatin (FOLFOX-regime) or the combination of Irinotecan, 5-FU, and Folinic acid (FOLFIRI-regime).[14,16]

In recent years new insights in underlying mechanisms led new treatment strategies in colorectal cancer. Primarily VEGF vascular endothelial growth factor (VEGF),[17,18] its receptors, and the epidermal growth factor (EGF) and its receptors [19,20] were screened for new therapeutic targets. Both monoclonal antibodies and tyrosine kinase inhibitors exhibit new therapy possibilities for colorectal cancer patients by preventing VEGF and EGF signaling.[21] Cetuximab,[19,22] and Panitumumab as EGFR inhibitors,[23-25] as well as Bevacizumab and Aflibercept (both are VEGF-A inhibitors),[17,18,26] and Regorafenib and Ramucirumab (both are VEGFR-2 inhibitors)[21,27] are already approved and well known targeted therapies in CRC.[28]

Inhibition of the growth factors VEGF/EGF and their receptors decreases the activity of subsequent pathways, e.g. the PI3K/Akt/mTOR pathway, with consequences for cell proliferation and metabolism.[29] For example, the monoclonal antibody Bevacizumab intercepts VEGF before binding to VEGF receptors and thus inhibits the activation of intracellular signaling and angiogenesis. Not only VEGF but also the platelet-derived growth factor (PDGF) is able to influence tumor cell viability and proliferation.

2. Growth factors and their receptors in CRC

2.1 PDGF and the PDGF receptors

The platelet derived growth factor (PDGF) family consists of 5 isoforms (PDGF-AA, -AB, -BB, -CC, -DD)[30] that have the ability to bind to the PDGF receptor α and β, both members of the receptor tyrosine kinase family, with varying affinity. PDGF-BB is the compatible mitogen for both PDGF-receptor α and β[31] and plays a pivotal role for example in embryonic development, migration, organogenesis, and angiogenesis.[32,33] PDGF-BB binds to two receptors simultaneously and causes dimerization and autophosphorylation of these receptors. Hence signaling pathways get activated and carry the stimulating signal into the cell (Figure 1). After signal

transmission, the receptor is internalized, the binding between PDGF and the receptor dissolves and the receptor recovers to the cell membrane or is degraded.

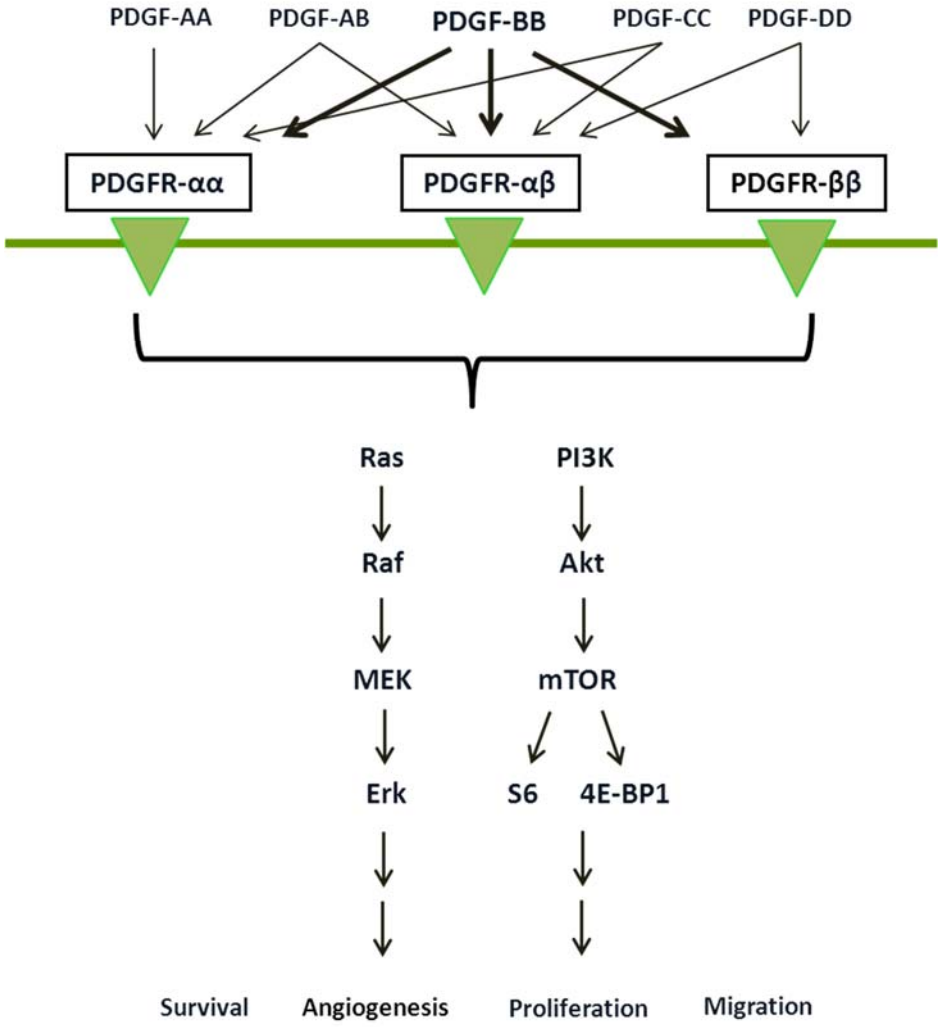

Figure 1: PDGF receptors, their binding ligands and signaling pathways with diverse intracellular effects.

The most important signaling pathways in CRC are the PI3K/Akt/mTOR (phosphoinositide 3-kinase) and the MAPK (mitogen-activated protein kinase) pathways with versatile effects on cells.[30,31,34,35]

PDGF and its receptors are expressed on colon cancer cells and surrounding tissue.[32,36,37] This fact implies the possibility that in tumor cells not only paracrine but also autocrine PDGF stimulation may occur, which can boost the tumor supporting effects of PDGF on tumor cells.[38]

Recent results indicated that PDGF-BB is associated with a progression of CRC and with a poor prognosis, but the molecular influence of PDGF on CRC cells is still poorly understood.[39]

2.2 VEGF and the VEGF receptors

The vascular endothelial growth factor (VEGF) was discovered by Senger *et al.* in 1982 in a guinea-pig cell line.[40] It works as a key regulator of angiogenesis and lymphangiogenesis.[41] VEGF also mediates proliferation, migration, differentiation, and cell survival.[42,43] Subsequent investigations of VEGF revealed five isoforms in mammals, VEGF-A, -B, -C, -D, and PlGF (placenta growth factor), which bind to the VEGF receptors (Figure 2).[44-46] VEGF-A exists in 4 splicing variants, of which VEGF165 shows the highest biological activity.[47,48]

The isoforms of VEGF bind to the receptor tyrosine kinases VEGFR-1, -2, and -3, with varying affinity. The two important receptors in CRC are VEGFR-1 and -2, which can be activated by VEGF-A. VEGFR-3 is predominantly located on the lymphatic endothelium.[45,49,50] The activation mechanism of VEGF is similar to PDGF/PDGFR activation. VEGF binds to VEGFR monomers and induces receptor homo- and hetero-dimerization and subsequent phosphorylation and activation of downstream signaling cascades.[43,51] Activated VEGF receptors activate the PI3K/Akt/mTOR and the MAPK pathway, comparable with the PDGF signaling.[42,51,52]

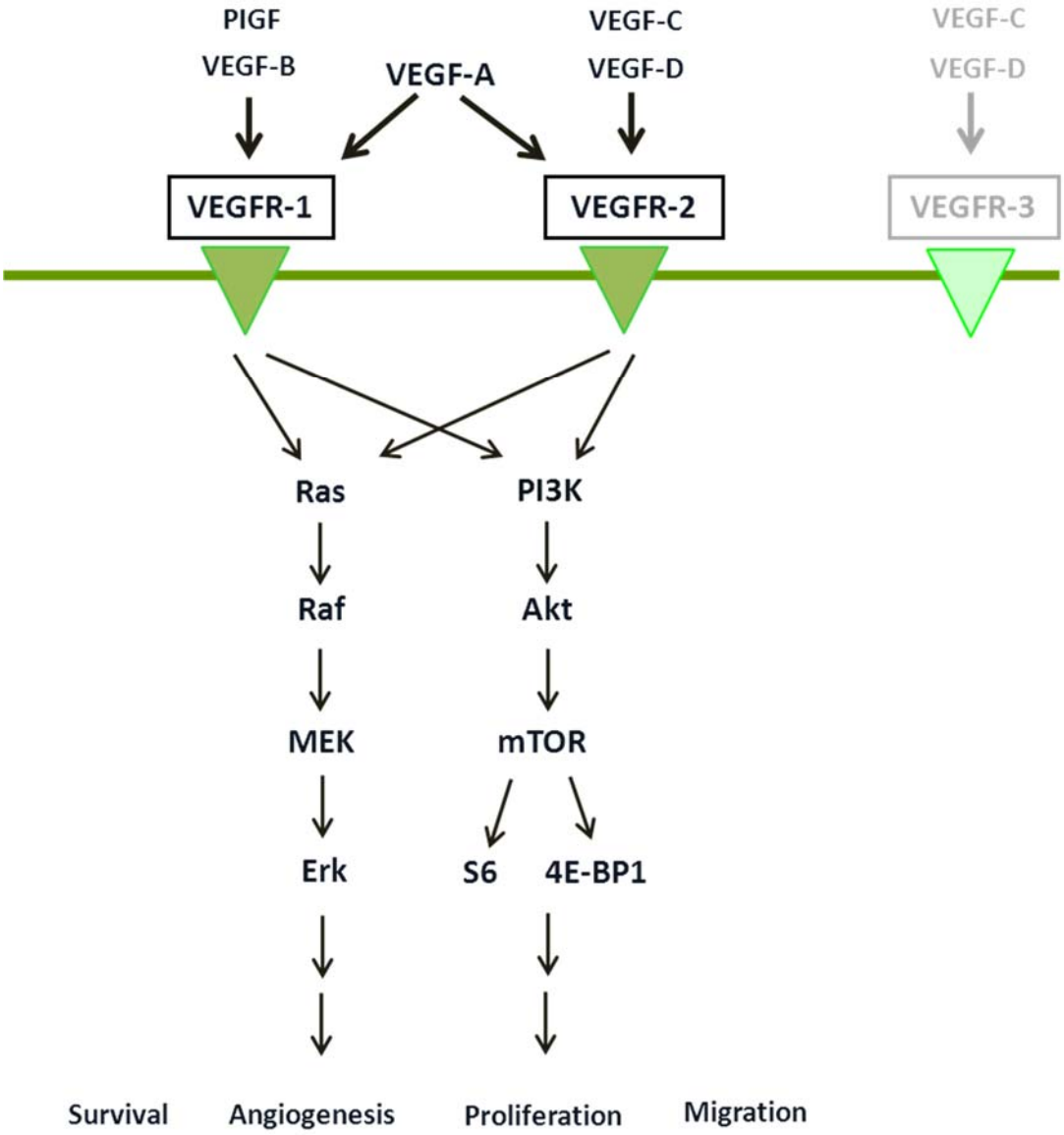

Figure 2: VEGF receptors, their binding ligands, and signaling pathways with diverse intracellular effects.

3. PDGF and VEGF downstream signaling

3.1 PI3K/Akt/mTOR pathway

Possible downstream signaling pathways targeted by PDGF are the PI3K/Akt/mTOR (phosphoinositide 3-kinase) pathway[31] and the MAPK pathway,[53,54] that maintain diverse crosstalk points with each other.[55-57]

A PDGF mediated activation of the PI3K/Akt/mTOR pathway can not only influence growth, proliferation and survival but also mediates shifts in energy metabolism, apoptosis, biosynthesis of macromolecules, and cell cycle progression.[58-60]

When the extensive PI3K/Akt/mTOR pathway (Figure 3) is activated by PDGF or VEGF-binding to their receptors (receptor tyrosine kinase), it launches a multistep process. PI3K (phosphoinositide 3-kinase) is recruited to the autophosphorylated intracellular domain of the receptor and phosphorylates the membrane bound PIP_2 (phosphatidylinositol (3,4)-bis-phosphate) to PIP_3 (phosphatidylinositol (3,4,5)-tris-phosphate). PIP_3 activates PDK1 (phosphoinositide-dependent kiniase-1) and thus the serine-threonine protein kinase Akt (protein kinase B) gets phosphorylated and activated. Akt, key molecule in the PI3K/Akt/mTOR pathway, influences more than 100 substrates, including mTOR (mammalian target of rapamycin). The serine-threonine kinase mTOR mediates phosphorylation of S6 (S6 ribosomal protein) and the translation regulation protein 4E-BP1 (eukaryotic translation initiation factor 4E binding protein 1, 4E-BP1 dephosphorylation mediates inactivation), both influencing protein synthesis.[59-63] This signal branch was investigated in the thesis.

3.2 MAPK pathway

Effects like growth, survival, and differentiation were also triggered by Erk (extracellular-signal-regulated kinase) activation.[64,65]

Receptor tyrosine kinases, like PDGFR and VEGFR, activate the GTPase (guanosine triphosphate) Ras (rat sarcoma), which transfers the signal to the serine-threonine kinase Raf (rapidly accelerated fibrosarcoma). Raf activates MEK (mitogen-activated protein kinase), and in turn activates Erk (extracellular signal-regulated kinase) (Figure 3). Erk phosphorylation and hence activation triggers numerous processes like proliferation, differentiation, migration, survival, and angiogenesis.[54,64,66]

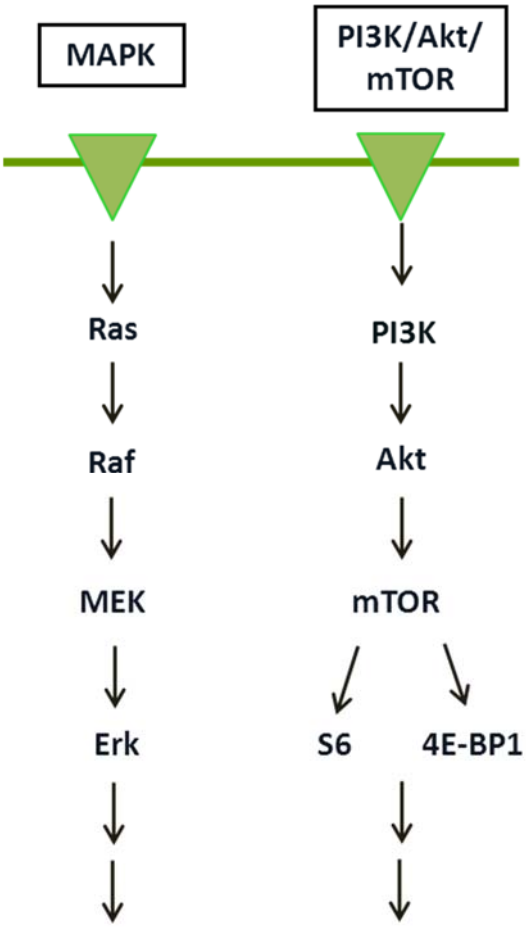

Figure 3: The PI3K/Akt/mTOR and MAPK downstream signaling.

4. Genetic alterations

The formation of CRC is based on accumulation of genetic alterations. Mutations in oncogenes and tumor suppressor genes can be found in most colorectal cancers. Common mutations exist in the genes APC (adenomatous polyposis coli) and MMR (mismatch repair gene), which are responsible for the familial adenomatous polyposis (FAP) and the Lynch syndrome.

In addition to hereditary genetic changes, alterations in oncogenes (KRAS (Kirstein rat sarcoma), c-Myc or BRAF[67]), and tumor suppressor genes (Retinoblastoma Rb) are very common in CRC.[68-70] These alterations also influence different intracellular pathways like the PI3K/Akt/mTOR and the MAPK pathway.

Knowledge of the mutational events of the tumor is very important for the success of an individualized patient therapy. For example, an effective treatment with the EGFR-inhibitor Cetuximab shows only an effect when the tumor is wild-type KRAS.[71]

4.1 Tumor suppressor Rb

In the malignant retinoblastoma tumor the Retinoblastoma (Rb) gene was first identified. Further studies demonstrated that the tumor suppressor Rb controls the transition of the G1-phase in the S-phase in the cell cycle. Hypophosphorylated Rb encloses the transcription factor E2F. Only free E2F is able to lead the cell cycle from the G1-phase into the S2-phase. Via phosphorylation of Rb, E2F is released which results in cell cycle progression and thus in increased proliferation.[72,73]

4.2 Tumor suppressor p53

The transcription factor p53 is a well-known tumor suppressor and demonstrates an exceedingly complex effectiveness in cells. Wild-type p53 controls the transition from the G2-phase into the M-phase in the cell cycle and also supervises DNA repair, apoptosis,[74] promotes oxidative phosphorylation.[75] p53 suppresses glycolysis via PI3K inhibition,[76-79] and decreases glucose uptake by GLUT1 inhibition.[75,80] Phosphorylation of p53 at Ser15 is mainly mediated by DNA damage and mTOR activation.[81]

In many tumor cells, however, p53 exhibits mutations, mainly point mutations that provoke a loss of function or even a gain of function. p53 mutations are very common in CRC. HT29 for example is a known p53 positive cell line, where p53 possesses oncogenic action.[74,82,83] The divergent p53-mutations, even in the same tumor entity, impede the anticancer therapy against p53.[84] But precisely this heterogeneity seems to generate new therapeutic opportunities.[85,86]

4.3 Oncogene c-Myc

The transcription factor c-Myc plays an important role as a potential oncogene in proliferation, cell cycle, and cellular metabolism. c-Myc is overexpressed in the majority of colon cancers[87-89] and the human HT29 cancer cell line is known to be myc-positive. As c-Myc exhibits a short half life of about 30 minutes,[90] phosphorylation at Serine 62 by Erk stabilizes c-Myc, whereas further Akt mediated phosphorylation at Threonine 58 initiates c-Myc degradation (Figure 4).[91-93]

Experiments with rat fibroblasts have shown that c-Myc influences LDHA (Lactate dehydrogenase A) and GLUT1 (Glucose transporter 1). C-Myc activates GLUT1, which leads to increased glucose uptake. The c-Myc provoked activation of LDHA[94] causes higher lactate generation and release by tumor cells.[95]

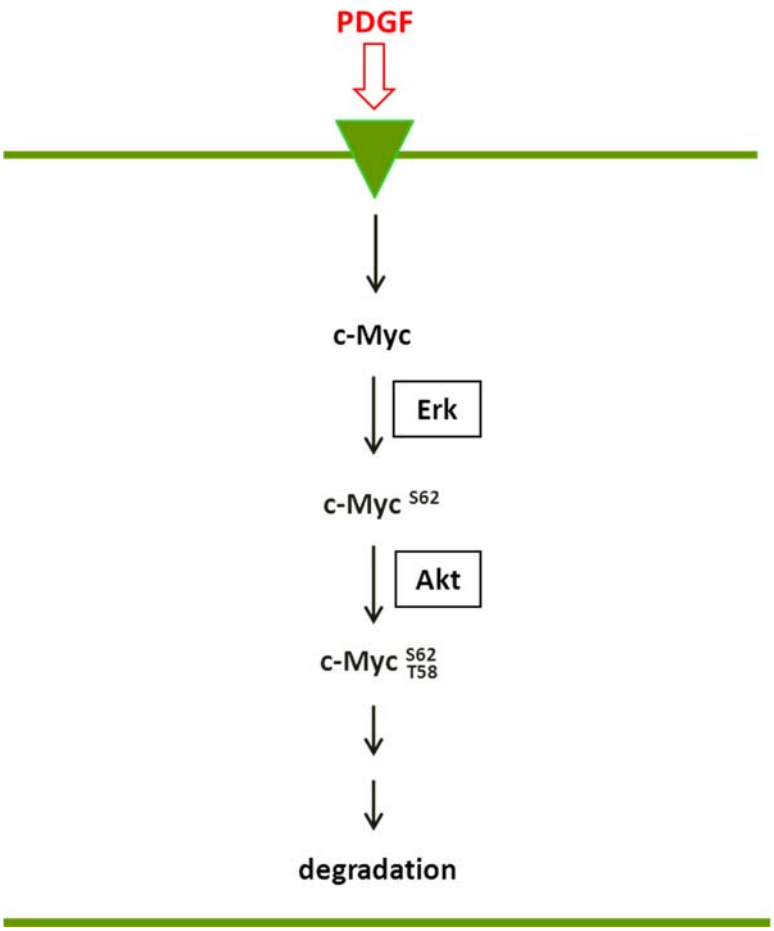

Figure 4: Phosphorylation events of c-Myc and the influence of PI3K/Akt/mTOR and MAPK signaling on c-Myc.[91-93]

5. HIF1α

The hypoxia-inducible-factor 1α (HIF1α), an important transcription factor in cells, plays a crucial role in the upregulation of glycolytic enzymes and transporters like GLUT1,[78] LDHA,[96,97] and MCT4 (monocarboxylate transporter 4).[98] HIF1α is stabilized during hypoxia and supports the Warburg effect.[78,99] In tumor cells it can be activated not only during hypoxia, but also under normoxic conditions ("pseudohypoxia").[100-102]

PI3K/Akt/mTOR can activate HIF1α with influence on glucose metabolism.[97,103] HIF1α triggers both VEGF secretion for increased angiogenesis[104] and the expression of glycolytic markers that exert influence on the glycolytic rate in tumor cells, with the subsequent enforcement of the synthesis of macromolecules leading to an increased tumor cell proliferation (Warburg effect).[78,105]

HIF1α inhibits c-Myc activity in a non-malignant environment.[106,107] In tumors however, HIF1α and c-Myc can collaborate with each other and promote metabolism and cell cycle changes in tumor cells.[88,97,108]

6. Warburg effect

Otto Warburg discovered a special metabolic behavior of cancer cells in the early 20th century. Tumor cells mainly use glycolysis to metabolize glucose[109] 2 molecules of ATP (Adenosine-5'-triphosphate), even in presence of oxygen, although glycolysis is energetically unfavorable in comparison to the oxidative phosphorylation (OXPHOS) (36 molecules of ATP).[110] Aerobic glycolysis supplies proliferating cancer cells with inevitable metabolites for enhanced proliferation and thus accelerates tumor growth.[111] A side effect of aerobic glycolysis is the increased accumulation of lactate, because pyruvate is not metabolized in the TCA cycle/mitochondria, but is converted to lactate by LDHA. Glucose uptake into the cell is also pronounced and a vast amount of lactate is excreted to the surrounding tissue by the tumor cells.[78,112-114]

7. Proliferation, cell cycle, and metabolism

Weinberg and Hanahan postulated the six hallmarks of cancer in 2000, which include continued proliferation.[115] Tumor cells can achieve this increased proliferation by self-production of growth factors and a potential autocrine stimulation (e.g. PDGF and VEGF),[38] a switch-off of tumor suppressors like Rb, and an activation of oncogenes (e.g. c-Myc).[116] For instance, by annulation of cell cycle checkpoints, tumor cells can accelerate the cell cycle and thus proliferation.[117]

The self-produced growth factors stimulate respective receptors, e.g. the PDGF- and VEGF-receptors, and subsequent signaling cascades react to this positive stimulation with increased proliferative signaling.[115,116]

Proliferation is also connected with tumor cell metabolism. The growth factors (PDGF and VEGF) activate intracellular signaling like the PI3K/Akt/mTOR pathway. This pathway can influence tumor cell metabolism, especially glycolysis, in a direct way. HIF1α and c-Myc influence glycolysis and thus the Warburg effect positively.[78,114]

The effects of the growth factors on tumor cells are highly complex and require further investigation.

8. Aim of studies

The purpose of this thesis was to elucidate the influence of PDGF on intracellular signaling, proliferation, cell cycle, and metabolism in CRC. By investigating the specific role of PDGF, new therapeutic intervention strategies in CRC might be developed to improve the prognosis for CRC patients.

C. Materials and Methods

1. Materials

1.1 Human colon cancer cell lines

The human colon cancer cell lines HT29, Caco-2, HCT116, and SW480, and the appropriate cell culture medium (Table 2) were obtained from ATCC (Manassas, VA). Penicillin/streptomycin was purchased from Life Technologies and cultivated as described in 2.1.2.

Table 2: Used human colon cancer cell lines

Cell line	HT29	Caco-2	HCT 116	SW480
ATCC® Number	ATCC® HTB-38™	ATCC® HTB-37™	ATCC® CCL 247™	ATCC® CCL-228™
Tissue	Colon	Colon	Colon	Colon
Disease	colorectal adeno-carcinoma	colorectal adenocarcinoma	colorectal carcinoma	colorectal adenocarcinoma
Morpho-logy	epithelial	epithelial	epithelial	epithelial
Medium	McCoy's 5A Medium, 10% (v/v) fetal bovine serum and 1% (v/v), penicillin/streptomycin	Eagle's Minimum Essential Medium, 20% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin	McCoy's 5A Medium, ATCC, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin	RPMI Medium 1640, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin

1.2 Patients and controls

1.2.1 Ethics Statement

The Human Research Ethics Committee of the University of Wuerzburg gave ethical approval (reference 8/11) for this research. An informed consent was obtained from all patients before tissues were collected.

1.2.2 Human colon cancer tissue samples

Colon cancer tissue samples were obtained from 46 patients (5 adenoma, 19 patients at UICC I/II stage, 22 patients at UICCIII/IV stage) undergoing curative surgical resection in our surgical department between 09/2009 and 05/2013. The stage classification of the tumor tissue was determined according to the Union Internationale Contre le Cancer (UICC) system. All tissues were instantly shock frozen in liquid nitrogen, using Tissue Tek (Sakura (Torrance, CA) and suitable cryo molds, and stored at -80 °C until analyzed.

1.3 Animals

Balb/c nude mice were maintained under defined conditions in accordance with institutional guidelines and experiments were performed according to approved experimental protocols.

1.4 Consumption items

Table 3: Consumption items

Product	Manufacturer
Cell culture flask Cell Star (25 cm^2, 75 cm^2, 175cm^2)	Greiner Bio-One (Frickenhausen, Germany)
Cellometer SD100 Counting Chamber	Peqlab (Erlangen, Germany)
Cell scratcher	Sarstedt AG & Co. (Nuembrecht, Germany)
Combitips advanced (2, 5, 10 mL)	Eppendorf (Hamburg, Germany)
Cryo vials	Greiner Bio-One (Frickenhausen, Germany)

Digital timer	Hartenstein GmbH (Wuerzburg, Germany)
Falcon tubes Cell Star (15, 50 mL)	Greiner Bio-One (Frickenhausen, Germany)
Fuji Medical X-Ray Film 100 NIF	Fujifilm Cooperation (Tokyo, Japan)
Hard-Shell® Low Profile 96-Well Semi-Skirted PCR Plates	Biorad (Hercules, CA, USA)
iBlot Gel Transfer Stacks	Life Technologies (Carlsbad, CA, USA)
Microscope cover slips	Paul Marienfeld GmbH (Lauda-Koenigshofen, Germany)
Microscope slides	Paul Marienfeld GmbH (Lauda-Koenigshofen, Germany)
Microseal® 'C' optical seals	Biorad (Hercules, CA, USA)
NuPAGE Novex 4-12% Bis-Tris Gel	Life Technologies GIBCO (Carlsbad, CA, USA)
NuPAGE Novex 3-8% Tris-Acetate Gel	Life Technologies GIBCO (Carlsbad, CA, USA)
Pipette filter tips (10, 200 µL)	Sarstedt AG & Co. (Nuembrecht, Germany)
Pipette filter tips (1000 µL)	Biozym Scientific GmbH (Hessisch Oldendorf, Germany)
Safeseal micro tubes (1.5, 2 mL)	Sarstedt AG & Co. (Nuembrecht, Germany)
Scalpel blade	PFM medical (Koeln, Germany)
Serological pipettes (2, 5, 10, 25 ml)	Greiner Bio-One (Frickenhausen, Germany)
TC flask, Cell+, vented cap (75 cm^2, 175$cm^{2)}$	Sarstedt AG & Co. (Nuembrecht, Germany)
Tissue culture test plates 96-well	TPP (Trasadingen, Switzerland)
UV cuvette micro	Brand (Wertheim, Germany)
6 Well cell culture plate	Greiner Bio-One (Frickenhausen, Germany)

1.5 Media, buffers, solutions

1.5.1 Self-made buffers, reaction solutions and reagents

1.5.1.1 RIPA Buffer

- 20 mM Tris-HCL (pH 7.5)
- 150 mM NaCl
- 1 mM EDTA
- 1% NP-40
- 1% Na-Deoxycholat
- 0.1% SDS
- 5 mM NaF
- Proteaseinhibitor cocktail (Calbiochem, Merck, Darmstadt, Germany)
- Phosphatase inhibitor cocktail (Calbiochem, Merck, Darmstadt, Germany)
- Dithiothreitol (DTT)

1.5.1.2 Blocking solutions

- 5% (m/v) nonfat dry milk in TBS-Tween 0.1% or
- 5% (m/v) BSA (bovine serum albumin) in TBS-Tween 0.1%

1.5.1.3 Glucose-free buffer, pH7.4

- 110 mM NaCl
- 5 mM KCl
- 1 mM $MgCL_2$
- 4 mM Na_2PO_4
- 50 mM Na-HEPES

1.5.2 Ready-to-use buffers, reaction solutions and reagents

Table 4: Ready-to-use buffers, reaction solutions and reagents

Product	Manufacturer
Acetone	Sigma-Aldrich (Munich, Germany)
Antibody diluent	Dako (Glostrup, Denmark)
cDNA - Human cDNA human adult normal tissue: Colon	Biochain Institute, Inc. (Newark, CA, USA)
Cell titer®AQueous one-Solution Cell Proliferation Assay	Promega (Mannheim, Germany)
Dako Power Block	Dako (Glostrup, Denmark)
3,39-Diaminobenzidine (DAB)	Biogenex (San Ramon, CA, USA)
49,6-Diamidino-2-phenylindoldihydrochlorid (DAPI)	Sigma-Aldrich (Steinheim, Germany)
Dimethylsulfoxide (DMSO)	Sigma-Aldrich (Munich, Germany)
Disodium phosphate (Na_2PO_4)	Sigma-Aldrich (Munich, Germany)
Dithiothreitol (DTT)	Fluka Analytical (St. Gallen, Switzerland)
EnVision K1395 double-stain Block	Dako (Glostrup, Denmark)
Ethanol	Sigma-Aldrich (Munich, Germany)
Ethylenediamine tetraacetic acid·Na2-salt, analytical grade (EDTA)	Serva Electrophoresis GmbH (Heidelberg, Germany)
Glucose Transporter Inhibitor IV, WZB117	Calbiochem, Merck (Darmstadt, Germany)
HiMark™ Prestained High Molecular Weight Protein Standard	Life Technologies GIBCO (Carlsbad, CA, USA)
Human PDGF-BB, research grade	Miltenyi Biotec (Bergisch Gladbach, Germany)
Hydrogen peroxide (H_2O_2)	Merck (Darmstadt, Germany)
InSolution™ Akt Inhibitor IV	Calbiochem, Merck (Darmstadt, Germany)
Magnesium chloride ($MgCL_2$)	Sigma-Aldrich (Munich, Germany)
Methanol	Sigma-Aldrich (Munich, Germany)
Mounting media	Dianova GmbH (Hamburg, Germany)
Na-HEPES pH 7.4	Calbiochem, Merck (Darmstadt, Germany)
Nonfat dry milk powder	AppliChem (Darmstadt, Germany)
NP-40	Sigma-Aldrich (Munich, Germany)
NuPAGE® Antioxidant	Life Technologies GIBCO (Carlsbad, CA,

	USA)
NuPage® LDS Sample Buffer (4x)	Life Technologies GIBCO (Carlsbad, CA, USA)
NuPage® MOPS SDS Running Buffer (20x)	Life Technologies GIBCO (Carlsbad, CA, USA)
NuPAGE® Sample Reducing Agent (10x)	Life Technologies GIBCO (Carlsbad, CA, USA)
NuPAGE® Tris-Acetat SDS Running Buffer (20x)	Life Technologies GIBCO (Carlsbad, CA, USA)
PageRuler Prestained Protein Ladder	Thermo Scientific (Waltham, MA, USA)
Phenylmethylsulfonyl fluoride (PMSF)	AppliChem (Darmstadt, Germany)
Phosphataseinhibitor Cocktail III, Animal-free	Calbiochem, Merck (Darmstadt, Germany)
PI3-K/mTOR Inhibitor III, PKI-179	Calbiochem, Merck (Darmstadt, Germany)
Polyvinyl-alcohol mounting medium	(Sigma-Aldrich (Munich, Germany)
Ponceau S 0.1% (w/v) in 5% acetic acid	Sigma-Aldrich (Munich, Germany)
Potassium chloride (KCl)	Sigma-Aldrich (Munich, Germany)
Proteaseinhibitor Cocktail V, 50x	Calbiochem, Merck (Darmstadt, Germany)
Recombinant Human VEGF 165	R&D Systems (Minneapolis, MN, USA)
RNAlater	Sigma-Aldrich (Munich, Germany)
Roti®-Quant	Carl Roth (Karlsruhe, Germany)
Sodium chloride (NaCl)	Sigma-Aldrich (Munich, Germany)
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (Munich, Germany)
Sodium deoxycholate (NaDOC)	Sigma-Aldrich (Munich, Germany)
Sodium fluoride (NaF)	Sigma-Aldrich (Munich, Germany)
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific (Waltham, MA, USA)
SuperSignal West Femto Chemiluminescent Substrate	Thermo Scientific (Waltham, MA, USA)
Taqman Gene Expression Mastermix	Life Technologies (Carlsbad, CA, USA)
Tissue-Tek	Sakura (Torrance, CA)
Tris Buffered Saline (TBS) 10x	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Tris-HCL (pH 7.5)	AppliChem (Darmstadt, Germany)
Trypsin/EDTA solution (0.05%/0.02%)	Biochrom AG (Berlin, Germany)
Tween-20	AppliChem (Darmstadt, Germany)
UltraPure™ DNase/RNase-Free	Life Technologies GIBCO (Carlsbad, CA,

Distilled Water	USA)
ZM 323881 hydrochloride	Santa Cruz Biotechnology (Dallas, TX, USA)

1.5.3 Kits

Table 5: Kits

Product	Manufacturer
D-Lactate Assay Kit (Colorimetric)	Abcam (Cambridge, UK)
EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse	Dako (Glostrup, Denmark)
Glucose Assay Kit	Abcam (Cambridge, UK)
Human OXPHOS Magnetic Bead Panel	Merck (Darmstadt, Germany)
ImProm-II™ Reverse Transcription System	Promega (Mannheim, Germany)
11-Plex Akt/mTOR Phospho MAG Bead Panel	Merck (Darmstadt, Germany)
9-Plex Multi-Pathway MAG Panel	Merck (Darmstadt, Germany)
RNeasy Mini Kit	Qiagen (Hilden, Germany)

1.6 Cell culture media, solutions and reagents

Table 6: Cell culture media, solutions and reagents

Product	Manufacturer
Accutase	Sigma-Aldrich (Munich, Germany)
Bovine serum albumin (BSA)	Life Technologies (Carlsbad, CA, USA)
Dulbecco's Phosphate Buffered Saline (DPBS)	Life Technologies (Carlsbad, CA, USA)
Eagle's Minimum Essential Medium (EMEM)	ATCC (Manassas, VA, USA)
McCoy’s 5a Medium	ATCC (Manassas, VA, USA)
Penicillin/streptomycin	Biochrom AG (Berlin, Germany)

RPMI 1640 Medium	ATCC (Manassas, VA, USA)
Trypanblue solution (0.4%)	Sigma-Aldrich (Munich, Germany)
Trypsin/EDTA solution (0.05%/0.02%)	Biochrom AG (Berlin, Germany)

1.7 Antibodies

1.7.1 Primary antibodies

Table 7: Primary antibodies

Antibodies	Manufacturer	Application
β-Actin (13E5) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Akt Antibody	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Anti-cmyc (phospho S62) (33A12E10)	abcam (Cambridge, UK)	Western Blot
Anti-cmyc (phospho T58)	abcam (Cambridge, UK)	Western Blot
Anti-Glucose Transporter GLUT1 antibody (SPM498)	abcam (Cambridge, UK)	Western Blot
Anti-SLC16A3 (MCT4) antibody	abcam (Cambridge, UK)	Western Blot
Anti-Vimentin (EPR3776)	abcam (Cambridge, UK)	Immunohistochemistry
Cofilin (D3F9) XP® Rabbit mAb (HRP Conjugate)	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
mTOR (7C10) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Pan-Cytokeratin (H-240)	Santa Cruz Biotechnology (Dallas, TX, USA)	Immunohistochemistry
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
PDGF-B (F-3)	Santa Cruz Biotechnology (Dallas, TX, USA)	Immunohistochemistry, Western Blot
PDGF Receptor α (D1E1E) XP® Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
PDGF Receptor β (28E1) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-Akt (Ser473)	Cell Signaling Technology,	Western Blot

(D9E) XP® Rabbit mAb	Inc. (Danvers, MA, USA)	
Phospho-c-Raf (Ser338) (56A6) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-4E-BP1 (Thr37/46) (236B4) Rabbbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-GSK-3α/β (Ser21/9) (D17D2) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-MEK1/2 (Ser217/221) (41G9) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-mTOR (Ser2448) (D9C2) XP® Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-p53 (Ser15) Ab	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-Rb (Ser807/811) Ab	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho–Rb (Ser795) Ab	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
VEGF (C-1)	Santa Cruz Biotechnology (Dallas, TX, USA)	Immunohistochemistry, Western Blot
VEGF Receptor 1 Ab	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot
VEGF Receptor 2 (55B11) Rabbit mAb	Cell Signaling Technology, Inc. (Danvers, MA, USA)	Western Blot

1.7.2 Secondary antibodies

Table 8: Secondary antibodies

Secondary antibodies	Conjugate	Manufacturer
Donkey-anti- mouse-Cy3	Cy3	Dianova GmbH (Hamburg, Germany)

goat anti-mouse IgG	HRP	Santa Cruz Biotechnology (Dallas, TX, USA)
goat anti-rabbit IgG	HRP	Santa Cruz Biotechnology (Dallas, TX, USA)
Goat-anti-rabbit-Alexa 488	Alexa Fluor 488	Dianova GmbH (Hamburg, Germany)

1.7.3 TaqMan gene expression assays

Table 9: TaqMan gene expression assays (all assays were purchased from Life Technologies, Carlsbad, CA, USA)

Target	Assay ID
ACTB	Hs01060665_g1
FLT1	Hs01052961_m1
KDR	Hs00911700_m1
LDHA	Hs00855332_g1
HIF1A	Hs00153153_m1
Ki67	HS01032443_m1
SLC16A4	Hs01006127_m1
SLC2A1	Hs00892681_m1
18S	HS03928895_g1
PDGFB	Hs00966522_m1
PDGFRA	Hs00998018_m1
PDGFRB	Hs01019589_m1
RPLP0	Hs00420895_gH
VEGFA	Hs00900055_m1

1.8 Lab devices

Table 10: Lab devices

Device	Manufacturer
Accu Jet pro	Brand GmbH (Wertheim, Germany)

BioPhotometer	Eppendorf (Hamburg, Germany)
Biorad CFX96 Touch Real-Time PCR Detection System	Biorad (Hercules, CA, USA)
BX51 Microscope	Olympus (Tokyo, Japan)
Cellometer Auto T4 Plus	Peqlab (Erlangen, Germany)
Centrifuge Biofuge fresco	Kendro (Langenselbold, Germany)
Centrifuge Multifuge 1 S-R	Thermo Scientific (Waltham, MA, USA)
Cryostat Leica CM3050 S	Leica Biosystems (Wetzlar, Germany)
Cytospin4 Cytocentrifuge	Thermo Scientific (Waltham, MA, USA)
Dynatech MRX microplate reader	Dynatech (Houston, TX, USA)
Elektophoresis Power Supply E835	Consort (Turnhout, Belgium)
ELISA Reader Thermo Max Mikroplate Reader	MWG Biotech (Ebersberg, Germany)
Eppendorf Research® pipette: 0,5-10 µl, 10-100 µl, 100-1000 µl, 0,5-10 µl 8-channel	Eppendorf AG (Hamburg, Germany)
FluorChem FC2	Alpha Innotech/ProteinSimple (San Jose, CA, USA)
iBlot dry blotting system	Life Technologies (Carlsbad, CA, USA)
Incubator Function Line	Thermo Scientific (Waltham, MA, USA)
Inverted microscope Axiovert 40C	Zeiss (Jena, Germany)
Laminar flow hood Hera Save	Kendro (Langenselbold, Germany)
MagPix System	Merck (Darmstadt, Germany)
Mastercycler Gradient	Eppendorf (Hamburg, Germany)
Multipette plus	Eppendorf (Hamburg, Germany)
Nanodrop 2000c	Thermo Scientific (Waltham, MA, USA)
QIAcube	Qiagen (Hilden, Germany)
QIAshredder	Qiagen (Hilden, Germany)
Scale CP 4201	Sartorius (Goettingen, Germany)

Scanner CanonScan9000F	Canon (Tokyo, Japan)
Shaker Unimax 1010	Heidolph Instruments GmbH (Schwabach, Germany)
Thermo Max Mikrolate Reader	MWG Biotech (Ebersberg, Germany)
Thermomixer Comfort	Eppendorf (Hamburg, Germany)
Vortex Mixer Gene 2	Scientific Instruments (West Palm Beach, FL, USA)
Water bath	Memmert (Schwabach, Germany)
XCell Sure Lock chamber	Life Technologies (Carlsbad, CA, USA)
x-ray cassette	Dr. Goos–Suprema GmbH (Heidelberg, Germany)

1.9 Software

Table 11: Software

Software	Manufacturer
Biorad CFX manager analysis software	Biorad (Hercules, CA, USA)
Cellometer Auto	Peqlab (Erlangen, Germany)
Cell Sens Dimension	Olympus (Tokyo, Japan)
Canon EP Navigator EX	Canon (Tokyo, Japan)
FluorChem SP	Alpha Innotech/ProteinSimple (San Jose, CA, USA)
Graph Pad Prism 5	Graph Pad Software, Inc. (La Jolla, CA, USA)
ImageJ	National Institute of Health (Bethesda, MD, USA)
Image Studio Lite Ver 3.1	LI-COR Biotechnology (Bad Homburg, Germany)
Microsoft Office 2010	Microsoft Corporation (Redmond, WA, USA)
Nanodrop 2000/2000C	Thermo Scientific (Waltham, MA, USA)

Oxygraph Plus	Hansatech Instruments (Norfolk, UK)
Softmax Pro 4.8	Molecular Devices (Sunnyvale, CA, USA)
xPonent 4.2	Merck (Darmstadt, Germany)

2. Methods

2.1 Cell culture

For all cell culture procedures proper aseptic techniques were applied in a Hera Save laminar flow hood (Kendro, Langenselbold, Germany).

2.1.1 Thawing frozen cells

Frozen cells were thawed gently and diluted slowly, using pre-warmed growth medium and transferred to a 50 ml falcon tube. A centrifugation step at 300 x g was performed for 10 minutes to spin cells down. The supernatant was discarded; the pellet was resuspended in 1000 µl of pre-warmed growth medium and transferred to a T75 culture flask containing 15 ml of pre-warmed growth medium.

2.1.2 Passaging and maintaining cells

Cells were cultured at 37 °C in 5% CO_2. SW480 and SW620 cells were cultured with Leibovitz's L-15 Medium, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. The HCT116 and the HT29 cell line were cultured in McCoy's 5A Medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Caco-2 cells were cultured in Eagle's Minimum Essential Medium supplemented with 20% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin.

For cell passaging, growth medium was removed, cells were washed with 10 ml of DPBS and incubated with 5 ml of trypsin/EDTA at 37 °C. After cell detachment, 10 ml of medium were added, cells were transferred to a 50 ml falcon tube and spun down for 10 minutes at 300 x g. Subsequently supernatant was discarded, the pellet

was resuspended in 1000 µl of pre-warmed growth medium and filled to a total volume of 10 ml. To seed a determined number of cells trypan blue staining and counting was performed as described in B.2.1.4

For Caco-2 cell passaging the medium was taken off, cells were washed with 10 ml of DPBS and incubated for 8 to 12 minutes at 37 °C with 5 ml of PBS/EDTA 1%. PBS/EDTA was discarded and cells were incubated with trypsin/EDTA for 1 to 2 minutes. Afterwards 10 ml of medium were added, cells were transferred to a 50 ml falcon tube and spun down for 10 minutes at 300 x g. Subsequently supernatant was discarded, the pellet was resuspended in 1000 µl of pre-warmed growth medium and filled to a total volume of 15 ml in Sarstedt TC flasks. To seed a determined number of cells, trypan blue staining and counting was performed as described in B.2.1.4.

2.1.3 Cryopreservation of cells

Cells were trypsinized and centrifuged as described in B.2.1.2. After removing the supernatant the pellet was resuspended by adding 1500 µl of freezing medium (growth medium supplemented with 10% DMSO). Cells were transferred to a 2000 µl cryovial and put at -80 °C overnight. The following day vials were transferred to liquid nitrogen for long-term storage.

2.1.4 Counting and trypan blue staining of cells

In order to assess cell viability, cells were detached and washed with DPBS, then cells were mixed 1:1 with trypan blue solution and 20 µl of the mix were transferred to a Cellometer SD100 Counting Chamber. A Cellometer Auto T4 Plus device was used for cell counting. Viable cells exclude trypan blue, while dead cells exhibit were blue staining due to trypan blue uptake.

2.2 HT29/SW480/ Balb/c nude mice-xenogeneic colon carcinoma model

For *in vivo* growth studies, HT29 or SW480 cells were injected subcutaneously (2 x 10^6 cells; HT29: n=6; SW480: n=3) into both flanks or intraportally (1,5 x 10^6 cells; HT29: n=6; SW480: n=2) of recipient Balb/c nude mice. 37 days post tumor cell

inoculation the mice were sacrificed, tumor growth was documented and tumor volume was determined.

2.3 Immunological methods

2.3.1 Cryosections

After surgical removal, the colon tumor tissue samples and normal human colon tissue samples from the patients were snap-frozen instantly in liquid nitrogen using Tissue Tek and suitable cryo molds. Samples were stored at -80 °C until further analysis. Normal colon tissues of healthy individuals were prepared as controls. For serial cryostat sections (5 mm) a Cryostat Leica CM3050 S was used. The sections were mounted on microscope glass slides.

2.3.2 Cytospin preparations of adherent cells

For cytospin preparations HT29 colon cancer cells were cultured in short-term primary cultures. The human colon cancer cells were harvested at an exponential growth phase using Accutase (Sigma-Aldrich, Munich, Germany) solution. After washing cells with DPBS, they were adjusted at a final concentration of 2 x 10^5 cells/ml. Cytospin preparations were performed with 50 µl of cell suspension at 550 rpm for one minute in a Cytospin4 cytocentrifuge.

2.3.3 Staining procedures

2.3.3.1 Immunofluorescent staining

The colon cancer tissue slides were fixed in acetone and the antibodies bound during the first staining step over night were covered and fixed with Dako Doublestaining system, K1395 (Dako, Glostrup, Denmark), according to the manufacturer's instructions.

The immunofluorescence double staining (coexpression) was detected with VEGF and PDGF, panCytokeratin monoclonal antibodies (all purchased from Santa Cruz; dilution: 1:100), and Vimentin (from abcam; dilution: 1:100). Rabbit anti-mouse

Cy3, and goat anti-rabbit Alexa488 secondary antibodies were purchased from Dianova, Hamburg, Germany. An Olympus BX51 microscope and the CellSens Dimension software were used for visualization.

2.3.3.2 Immunohistochemical staining

The slides were incubated with a primary antibody (antibody dilution: PDGF 1:50; VEGF 1: 150) or isotype control antibody. After incubation with the primary antibody, HRP-conjugated AffiniPure Donkey anti-mouse or a Donkey anti-rabbit or a Donkey anti-goat IgG were used as secondary antibodies. Slides were subsequently incubated in DAB, counterstained with Haemalaun and mounted with Glycergel.

2.4 MTS cell proliferation assay

2500 cells were seeded in each of the 96 wells, cultured with McCoy's 5A Medium, supplemented with FCS. After a recovering time of 72 hours the cells were washed with PBS and given medium without FCS. After another 24 hours, cells were treated with VEGF (100 ng/ml), PDGF (100 ng/ml), VEGF and PDGF (100 ng/ml respectively), and/or InSolution Akt Inhibitor IV (10 µM) or PKI-179 (80 nM) for 30 minutes, 60 minutes, and 24 hours or 24, 48, and 72 hours. After treatment, cells were given 100 µl of fresh medium and immediately 20µl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) -reagent (Promega, CellTiter 96® AQueous One Solution Cell Proliferation Assay) was added in each well. After incubation for 1 hour at 37 °C the 96 well plates were measured with 490 nm wavelength using an ELISA reader according to the manufacturer's instructions.

2.5 Gene expression analysis

2.5.1 Extraction of total RNA from adherent cells

Cells were washed with DPBS and lysed with the RLT lysis buffer, a component of the RNeasy Mini Kit. RNA extraction was conducted with the QiaCube and the RNeasy Mini Kit according to the manufacturer's instructions.

2.5.2 Extraction of total RNA from tissue

After thawing, the cancer tissues, stored in RNAlater, were cut into small pieces. These samples were homogenized on a Tissue Lyser in Buffer RLT (component of RNeasy Mini Kit) and centrifugated for 3 minutes at full speed and 4 °C. RNA extraction was performed with a QIACube device (Qiagen, Hilden, Germany) using a RNeasy Mini Kit according to the manufacturer's instruction.

2.5.3 Real-time quantitative reverse transcription-PCR (RT-qPCR)

Gene expression of PDGF, VEGF, PDGFRα and β, VEGFR1 and 2, HIF1α, GLUT1, MCT4, LDHA, and Ki67 was analyzed in colon cancer specimens by real time PCR. Cell culture probes were produced in three independent experiments and were analyzed. Complementary DNA (cDNA) synthesis was performed according to the manufacturer's instructions using the ImProm-II reverse transcriptase system (Promega, WI, USA) and an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) The TaqMan gene expression assays were purchased from Life Technologies (Carlsbad, CA, USA). All samples were assayed in duplicate according to the manufacturer's instructions and normalized during data analysis. The housekeeping genes β-Actin, 18S, and RPLP0 were used for relative quantification. The relative quantification value was expressed as $2^{-\Delta\Delta Cq}$. Results from the human colon cancer tissues samples and the Balb/c nude mice-xenogeneic colon carcinoma model samples were normalized to colon normal tissue purchased from Biochain (Hayward, CA, USA). PCR reactions were conducted with a BioRad CFX96 Touch real-time PCR detection system.

2.6 Western blot

2.6.1 Preparation of whole cell extracts

Cells were washed with DPBS twice and directly lysed in RIPA buffer. Before using the RIPA buffer, phosphataseinhibitor cocktail, proteaseinhibitor cocktail, and DTT were added to the RIPA buffer. The cells were left for 30 minutes at 4 °C with

gently shaking for better lysis result. Then the cells were sonificated, centrifuged at a speed of 13.000 x g for 15 minutes and at 4 °C; the supernatant was stored at -80 °C.

2.6.2 SDS polyacrylamide gelelectrophoresis (SDS-PAGE)

Quantification of the protein amount in each probe was detected by Bradford measurement using Roti-Quant solution. For protein detection, protein extracts were run on precast NuPAGE® Novex® 4–12% Bis-Tris Gels or 3-8% Tris-Acetate Gels (both Invitrogen/Life Technologies, Carlsbad, CA, USA), dependent on the protein weight. SDS polyacrylamide gelelectrophoresis was performed as described in the NuPAGE® Technical Guide (Invitrogen, Life technologies) at 4 °C, 200 V, and 100 W for 60-80 minutes with an appropriate protein ladder as reference.

2.6.3 Western blotting and immunodetection

After SDS-PAGE the protein extracts were transferred to nitrocellulose membranes with iBlot Dry Blotting System (Invitrogen, Life technologies). Ponceau S staining was conducted to survey the successful protein transfer and subsequently nitrocellulose membranes were destained with TBS-T. Membranes were blocked with 5% non-fat dry milk powder or 5% BSA in TBS-T 0.1% for 60 minutes at room temperature, and were incubated overnight at 4 °C with primary antibodies. After incubation, membranes were washed 3 x 15 minutes with TBS/T. Secondary antibodies were incubated for 1h at room temperature. After another three washing steps bands on membranes were detected by using SuperSignal West Pico or Femto Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) and quantified with Image Studio Lite Ver 3.1.

2.7 Single stimulation and combined stimulation with PDGF and/or VEGF

HT29 and Caco-2 cells (1.5 x 10^6) were seeded in 75 cm^2 flasks. After 24 hours cells were stimulated for 24, 48, and 72 hours with new stimulation every 24 hours:

1. Ctrl
2. VEGF 100 ng/ml
3. PDGF 100 ng/ml
4. PDGF + VEGF (100 ng/ml respectively)

After stimulation time, whole cell extracts and RNA were produced.

2.8 Pathway inhibition

For PI3K/Akt/mTOR pathway inhibition the following experiment designs were conducted with HT29 cells:

1. Ctrl
2. PDGF (100 ng/ml)
3. Inhibitor
4. PDGF + inhibitor

Treatment was applied for 30 minutes, 60 minutes, and 24 hours and WCE were prepared for Western Blot analysis. InSolution Akt Inhibitor IV (10 µM) was used for Akt inhibition, and PKI-179 (80 nM) (both from Calbiochem-Merck, Darmstadt) was used for PI3K inhibition.

2.8.1 Akt inhibition and synchronous stimulation

HT29 cells (3 x 10^6) were seeded in 75 cm^2 flasks. After 24 hours cells were treated with the Akt Inhibitor IV (10 µM), with PDGF (100 ng/ml) or with Akt Inhibitor IV and PDGF together as shown in 2.8. After treatment cells were harvested and whole cell extracts were prepared for Western Blot examination.

2.8.2 PI3K inhibition and synchronous stimulation

Cells were treated like described for the Akt inhibition procedure but instead of Akt Inhibitor IV the PI3K inhibitor PKI-179 (80 nM) was used.

2.9 Glucose uptake and lactate release

HT29 cells were seeded in flasks, after reaching 60% confluency cells were stimulated with PDGF (100 ng/ml) for 24 hours. One untreated flask served as control. After 24 hours, cells were washed with PBS, harvested and incubated in glucose-free buffer for 10 minutes. Then cells were washed twice with the glucose-free buffer. 1 x 10^6 cells were given into a 1.5 ml cap with 250 µl of glucose-free buffer. Glucose (20 mM) and/or GLUT1 inhibitor (10 µM; WZB, 117) were added according to the scheme below:

1. glucose (Ctrl)
2. glucose + WZB117
3. glucose + PDGF
4. glucose + WZB117 + PDGF

After 15 min of incubation, the cells were collected, centrifuged and supernatant was collected and used for measurements with the Lactate assay kit (abcam ab83429) according to the manufacturer's instructions.

The centrifuged cells were treated with 100 µl of glucose assay buffer (Glucose assay kit, abcam ab102517), centrifuged and the resulting supernatant was measured with the Glucose assay kit according to the manufacturer's instructions.

2.10 MILLIPLEX assay for human oxidative phosphorylation

Tumor protein extraction and procedure was performed according to the manufacturer's instructions (MILLIPLEX® human oxidative phosphorylation (OXPHOS) magnetic bead panel; Cat.# H0XPSMAG-16K, purchased from Merck Millipore, Darmstadt, Germany). The protein volume of HT29 probes used in the assay was 10 µg; sample preparations and data collection were performed according to the manufacturer's instructions. Two independent probes were analyzed; values were given as median fluorescence intensity (MFI) collected from replicates. The measurements were obtained from the MagPix device and results were evaluated with the Xponent software.

2.11 Oxygen measurements

Oxygen consumption of HT29 and Caco-2 cells was measured with the Oxytherm system (Hansatech Instruments). The Oxytherm system was used in accordance with the manufacturer's instructions. After calibration was conducted, 10 million treated or untreated cells in one ml of medium were measured with the Oxytherm device:

1. Ctrl
2. VEGF 100ng/ml
3. PDGF 100ng/ml
4. PDGF + VEGF (respectively 100ng/ml)

Results were evaluated with the Oxygraph plus software.

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (Graph Pad Software Inc., San Diego CA, USA). A two-way ANOVA with the Bonferroni post hoc test or a one-way ANOVA with post hoc tests, chosen according to sample distribution, were used. Data were presented as mean ± standard deviation (SD). $P < 0.05$ was considered to be statistically significant.

Quantification of Western Blot bands were analysed with Image Studio Lite. The relative densities of the bands were expressed as percentage of the control.

D. Results

1. PDGFRα and β, and VEGFR1 and 2 expression in human colon cancer tissues and colon cancer cell lines

To elucidate the impact of PDGF in CRC, the presence of PDGFRα, PDGFRβ and VEGFR1 and 2 in human colorectal cancer tissues were investigated in a first step. PDGFRα expression analysis exhibited no increase in stage UICCI/II and III/IV patient tumors, but a significantly higher gene expression of PDGFRβ in stage UICCI/II, and III/IV tumors ($p < 0.001$) (Figure 5A, and B). Compared to adenomas ($p < 0.01$) all UICC stages tumors showed a significantly higher expression of PDGFRβ. Both VEGFR1 and 2 showed a significantly higher gene expression compared to the controls ($p < 0.001$) (Figure 5C, and D).

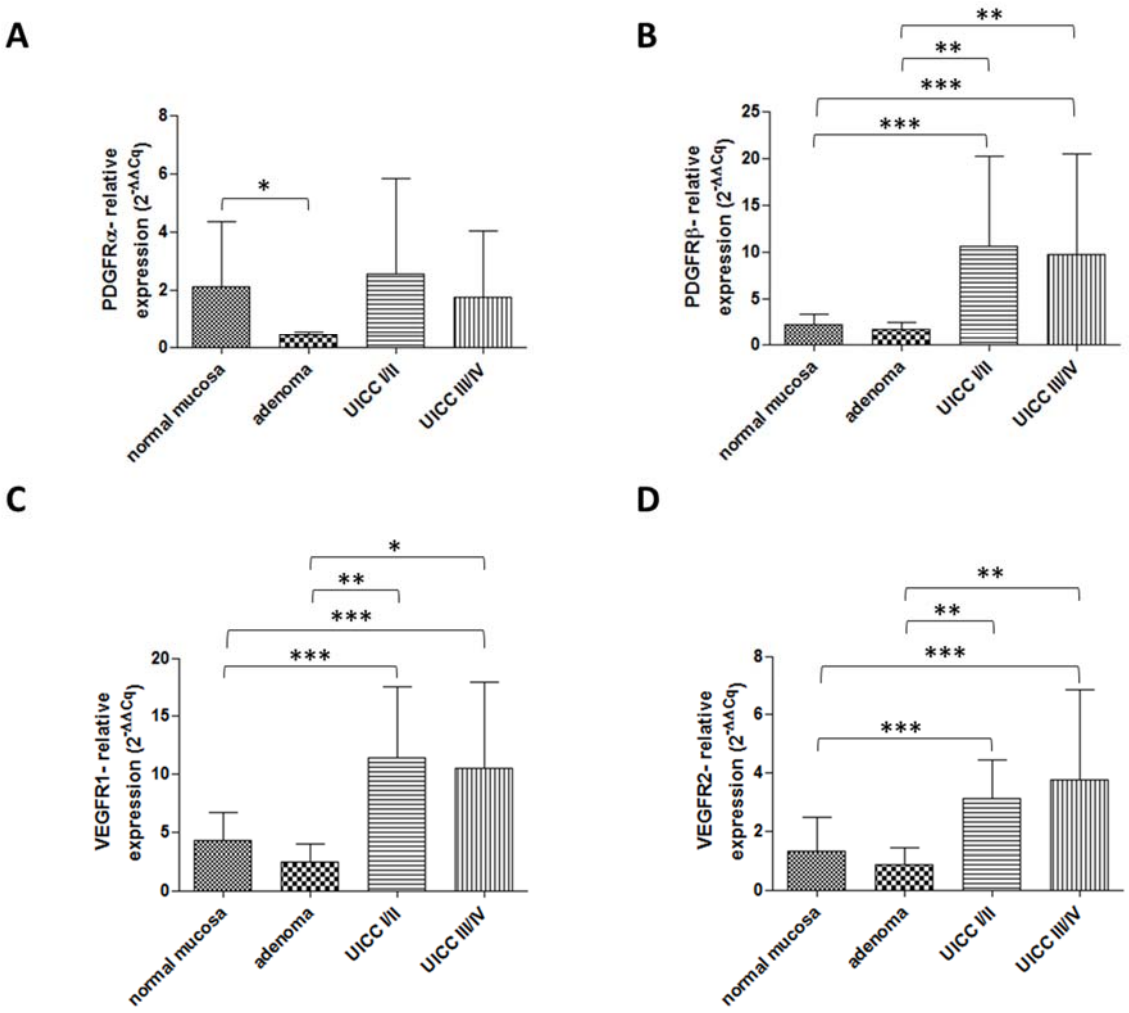

Figure 5: Receptor expression in human colon cancer probes

PDGFRα, β and VEGFR1, 2 gene expression in human colon cancer probes. Increased gene expression of PDGFRα **(A)** and significantly increased gene expression of PDGFRβ **(B)** and VEGFR1 and 2 **(C and D)** in human colon cancer probes in UICCI/II and III/IV. The normalization was performed with normal $2^{-\Delta\Delta Cq}$. Results are presented as ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

Investigation of the human colon cancer cell lines HT29 and Caco-2 showed a different receptor expression. HT29 colon cancer cells expressed only VEGFR1 and 2 while PDGFRα and β were not expressed. The Caco-2 cells showed positivity for both, PDGFRα, β and VEGFR1, 2 on protein level (Figure 6A). Cytospin staining of HT29 cells (Figure 6B) and Caco-2 cells (Figure 6C) confirmed these results.

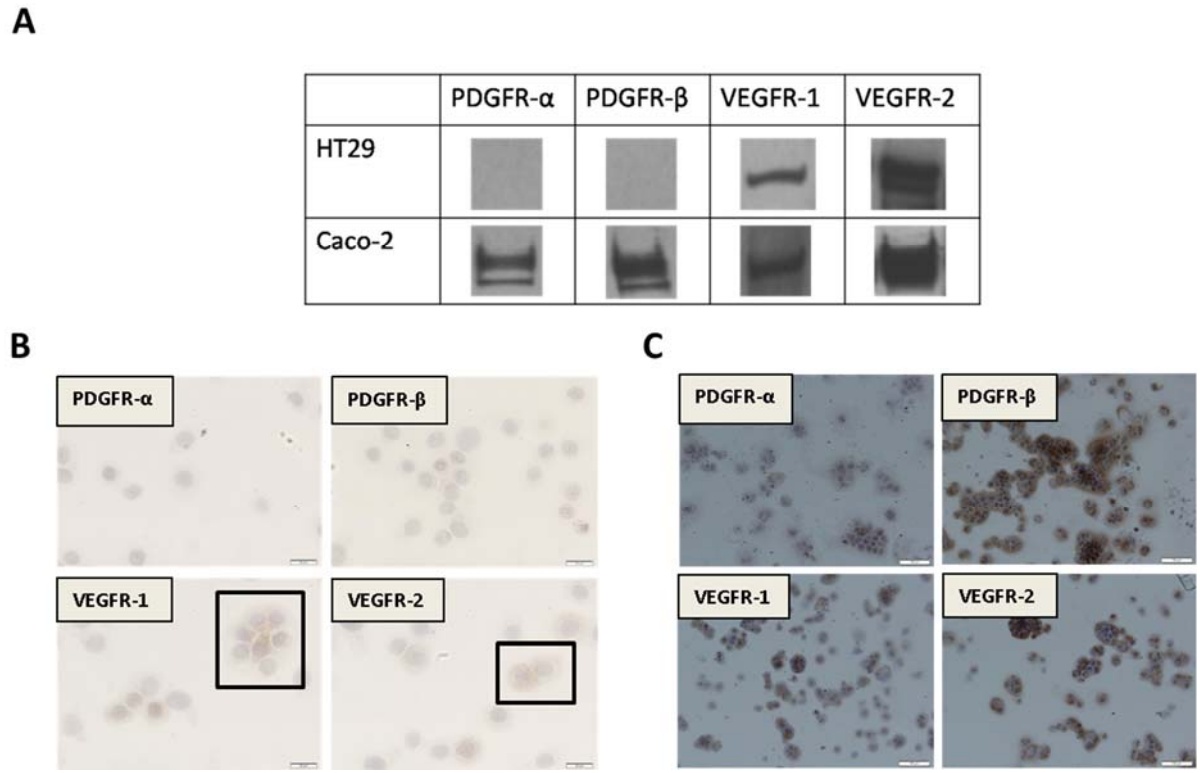

Figure 6: PDGFR, VEGFR expression on protein level in HT29 and Caco-2

(A) Western blot investigations showed a positive VEGFR1 and 2 expression in HT29 cells and a positive VEGFR1, 2 and PDGFRα, β expression in Caco-2 CRC cells (β-Actin as loading control). (B) Cytospin staining of PDGFRα, β and VEGFR1, 2 in HT29 **(B)** and Caco-2 **(C)** cells showed the same results.

When Caco-2 cells were stimulated with PDGF, VEGF or both PDGF and VEGF, receptor gene expression was increased. PDGF predominantly affected the PDGF receptors, whereas the VEGF receptors were mainly influenced by VEGF (Figure 7A-D).

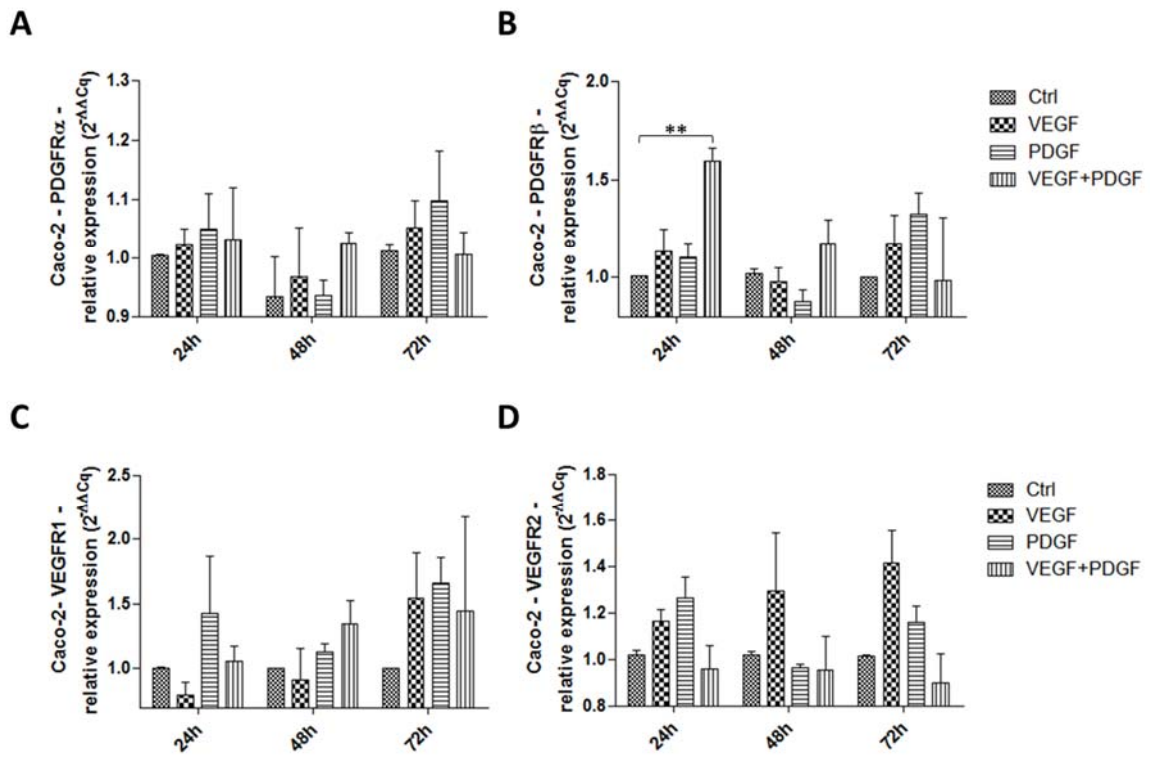

Figure 7: Gene expression of the PDGF- and VEGF-receptors in the Caco-2 cell line

PDGFRα **(A)**, β **(B),** and VEGFR1 **(C)** and 2 **(D)** expression were increased during stimulation with PDGF and VEGF. Normalization was performed with normal $2^{-\Delta\Delta Cq}$. Results are presented as ± SD. **p < 0.01, n=3.

2. PDGF and VEGF expression in colon cancer cell lines and in human colon cancer tissue

2.1 Expression in human colon cancer tissue and xenograft model

To examine PDGF and VEGF expression, investigations of both colon cancer tissue and colon cancer cell lines were conducted. Gene expression of PDGF and VEGF in tissues of patients with colorectal cancer at stages UICC I/II and III/IV were significantly upregulated compared to normal mucosa (p < 0.01: PDGF UICC III/IV, and p < 0.001: PDGF UICC I/II, VEGF UICC I/II and III/IV) (Figure 8A, and B). For PDGF a stage dependent increase in gene expression was observed (UICC III/IV vs. UICC I/II). Investigation of subcutaneous or liver tumors from HT29 or SW480 cells,

removed from BALB/c nude mice, showed an increased gene expression of PDGF and VEGF compared to normal colon tissue controls (Figure 8C, and D).

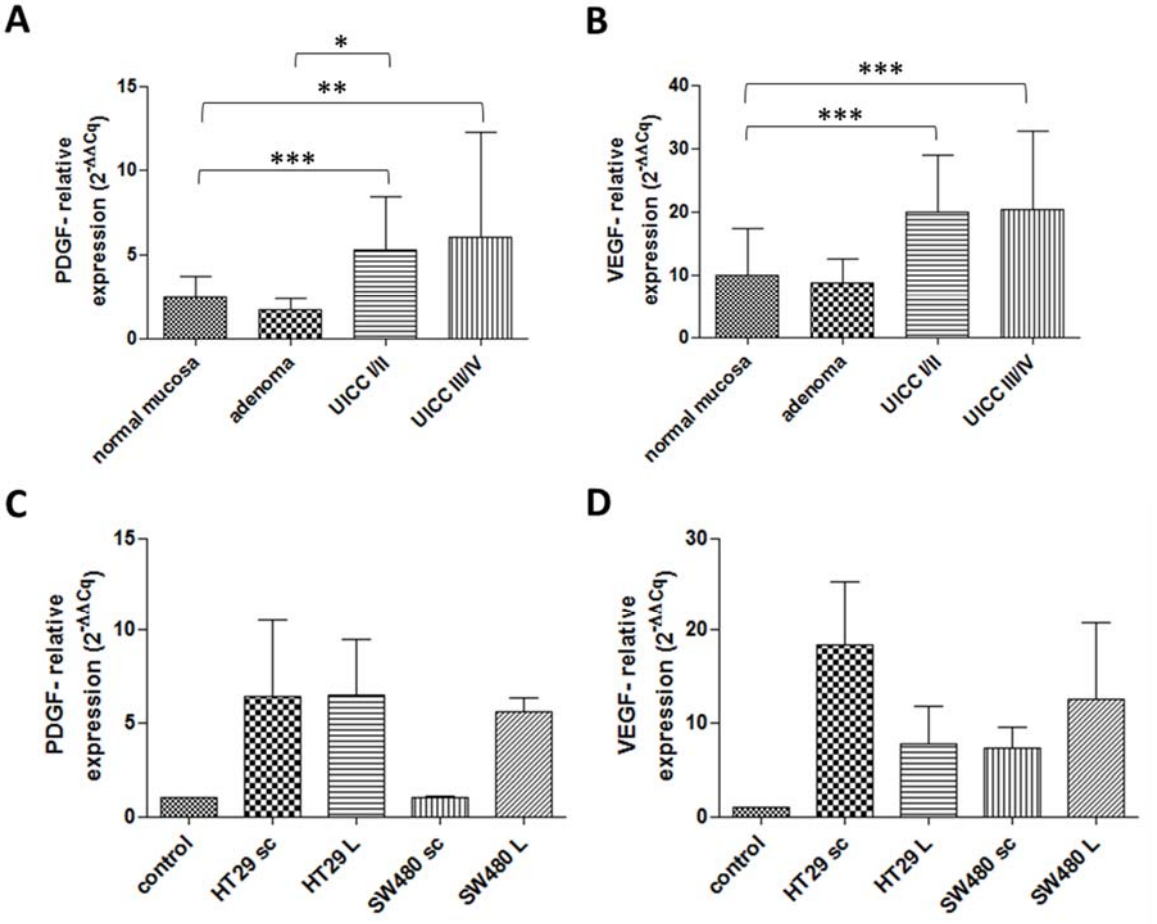

Figure 8: PDGF and VEGF expression in human colon cancer probes

Significantly increased gene expression of PDGF **(A)** and VEGF **(B)** in human colon cancer probes in UICCI/II and III/IV. Normalization was performed with normal $2^{-\Delta\Delta Cq}$. Results are presented as ± SD, *p < 0.05, **p < 0.01, ***p < 0.001. Increased PDGF **(C)** and VEGF **(D)** gene expression in the xenogeneic carcinoma model with subcutaneous (sc) or intraportal (L) injection of HT29 and SW480 cells.

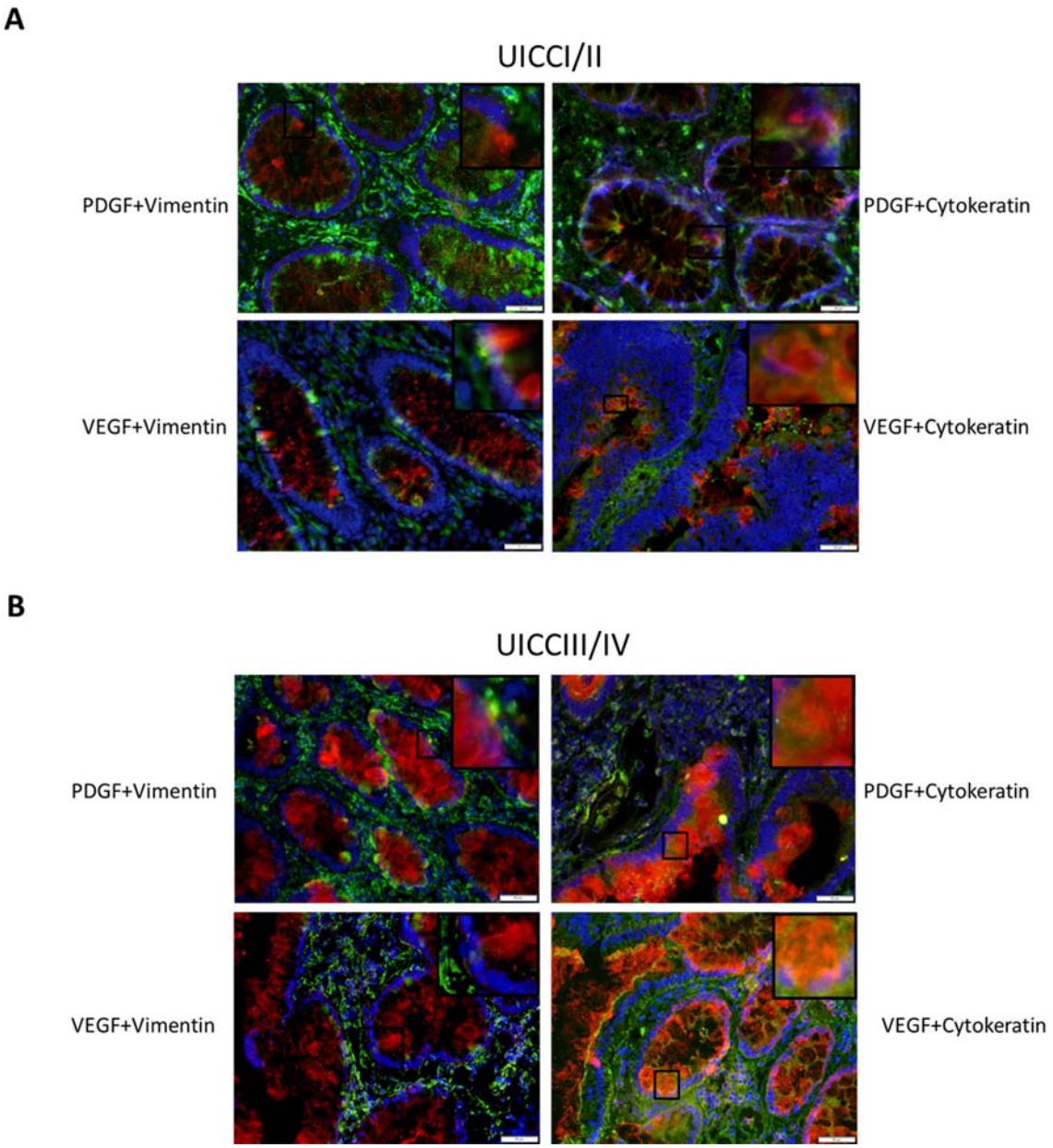

Figure 9: Immunofluorescence double staining of colon cancer tissue

UICC I/II and - III/IV exhibited a positive expression of PDGF (Cy3 red) and VEGF (Cy3 red) in epithelial (Cytokeratin, Alexa 488 green) cancer tissue but either no or occasionally low expression of PDGF or VEGF in stromal regions (Vimentin, Alexa 488 green). Magnification x20; DAPI blue (nuclear counterstaining).

Immunofluorescence double staining showed a stage dependent increased PDGF and VEGF expression in epithelial regions of UICC stages I/II (Figure 9A) and III/IV tumors (Figure 9B). In the stromal regions PDGF and VEGF expression was

either marginally detectable or absent. Interestingly, the expression intensity of PDGF was comparable to the expression intensity of VEGF (Figure 9A, and B).

2.2 Expression in colon cancer cell lines

Next, the significance of PDGF in human colon cancer cell lines the HT29 and Caco-2 CRC cell line were investigated. Stimulation of HT29 and Caco-2 cells with PDGF and VEGF showed different PDGF and VEGF expression patterns. PDGF stimulation in HT29 cells increased PDGF expression after 72 hours ($p < 0.05$) (Figure 10A). VEGF stimulation, however, produced an increased gene expression of VEGF after 48 hours (Figure 10B).

Stimulation of Caco-2 cells with PDGF and VEGF resulted in an unchanged PDGF expression during PDGF or VEGF stimulation (Figure 10C), and only a moderate VEGF expression were detectable during PDGF stimulation (Figure 10D).

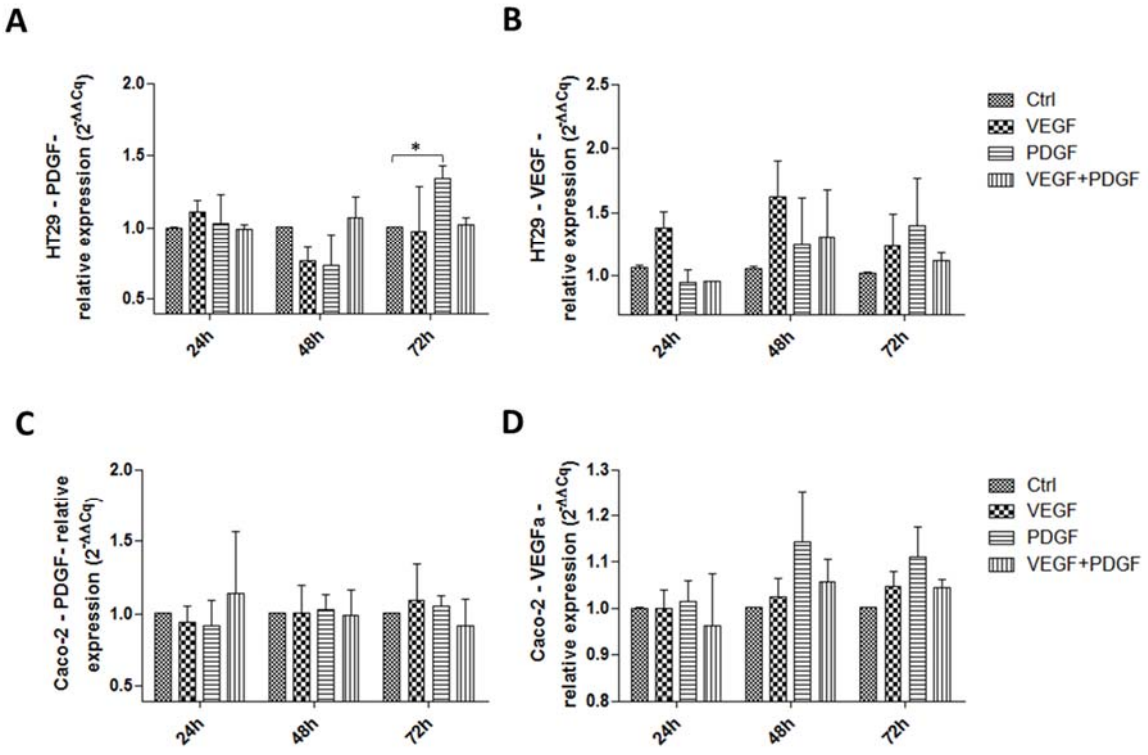

Figure 10: PDGF and VEGF expression in HT29 and Caco-2

Increased gene expression of PDGF **(A)** and VEGF **(B)** in HT29 and Caco-2 **(C and D)** cells. Normalization was performed with normal $2^{-\Delta\Delta Cq}$. *$p < 0.05$.

HT29 cells expressed PDGF and VEGF on protein level, which was detectable in both cytospin staining (Figure 11A) and Western Blot immunodetection (Figure 11B).

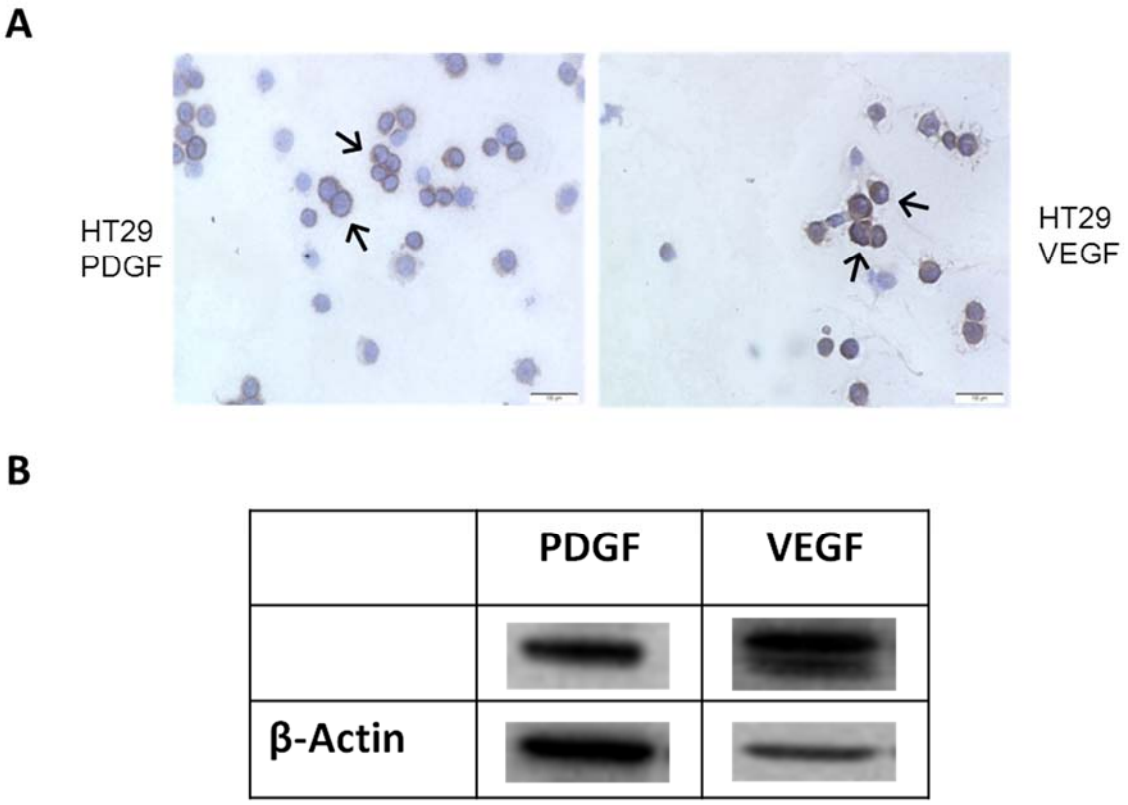

Figure 11: PDGF and VEGF expression on protein level in HT29 cells

Positive cytospin staining of PDGF and VEGF **(A)**. Positive western blot expression of PDGF and VEGF **(B)** in HT29 cells.

3. Binding behavior and rate of binding of PDGF to Caco-2 cells

In order to gain essential information for further experiments by identifying the binding rate of PDGF to tumor cells, Caco-2 cells were incubated with PDGF (100 ng/ml) in a time dependent manner. Cell staining with an anti-PDGF antibody revealed that a minimal incubation time of 5-10 minutes was required for PDGF attachment (Figure 12).

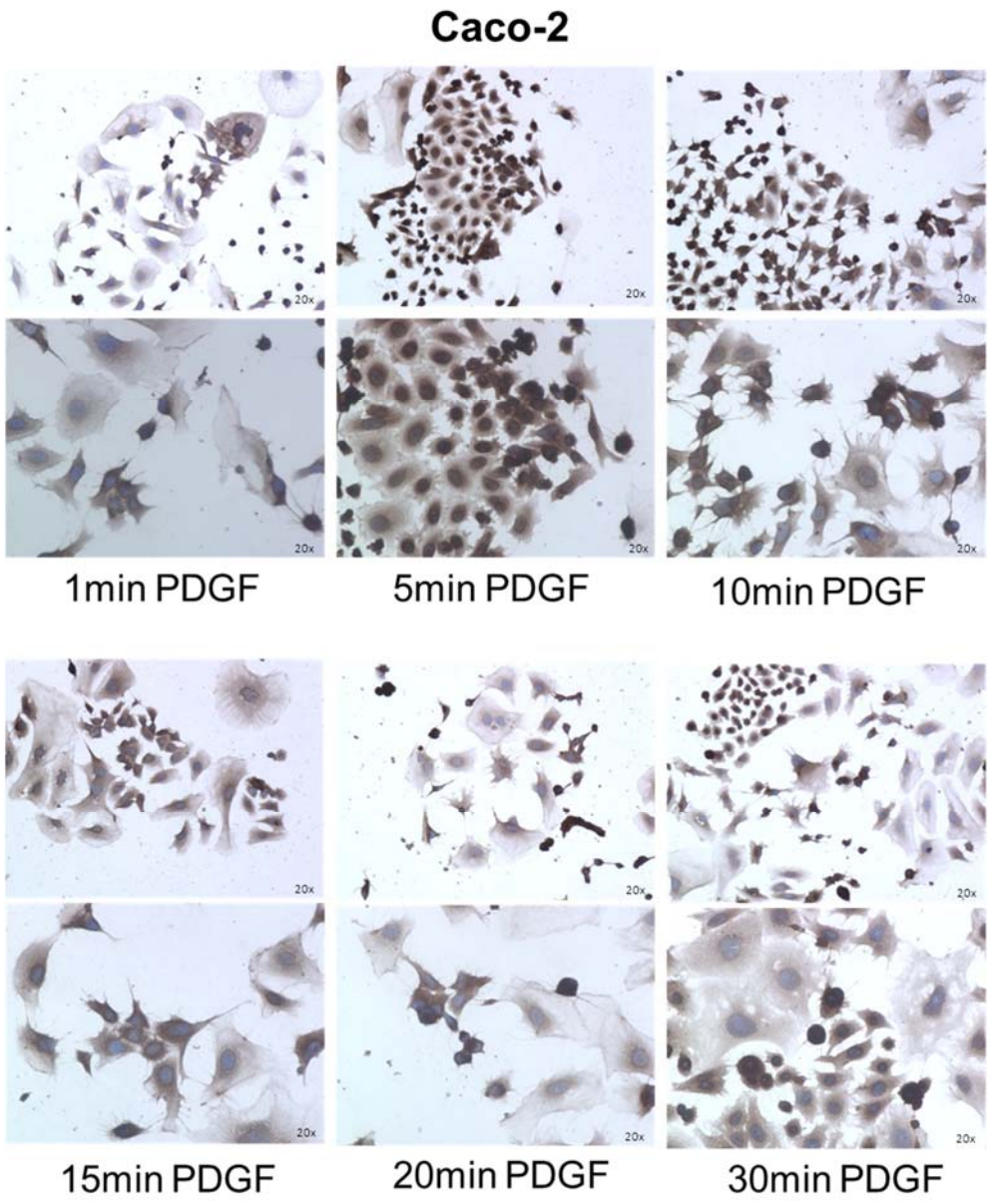

Figure 12: PDGF-binding rate in Caco-2 cells

PDGF needed at least 5 minutes of incubation to bind to Caco-2 cells (1 x 10^5 Caco-2 cells were seeded on a slide, which were coated with collagen for 1 hour).

4. Proliferation during stimulation in colon cancer cells

The influence of PDGF on tumor cell proliferation was initially analyzed by counting of HT29 cells after PDGF- and VEGF-stimulation. After 72 hours of stimulation, cells treated with VEGF or with PDGF showed a higher proliferation, compared to the unstimulated controls (Figure 13A). The following MTS proliferation

assay showed an increased proliferative effect of PDGF- and VEGF-stimulation in HT29 cells ($p < 0.05$: 24 hours VEGF treatment). However, combined stimulation with PDGF and VEGF showed no synergistic impact (Figure 13B). Comparable results were obtained by gene analysis of the proliferation marker Ki67. It showed an increased proliferative effect of PDGF and VEGF, primarily after a 24 hours period of stimulation ($p < 0.01$: after 24 hours of VEGF treatment; $p < 0.05$: after 24 hours of PDGF treatment) (Figure 13C).

The MTS proliferation assay with Caco-2 cells showed an increased proliferation rate after stimulation with PDGF and VEGF. VEGF provoked in Caco-2 cells a significant increase in proliferation ($p < 0.01$: after 24 and 48 hours; $p < 0.001$: after 72 hours) (Figure 13D). Ki67 was upregulated by PDGF and VEGF stimulation in the Caco-2 cells (Figure 13E). Proliferative effects of PDGF and VEGF were also elicited in SW480 ($p < 0.001$: after 24 and 48 hours of VEGF treatment, $p < 0.01$: after 72 hours of VEGF treatment; $p < 0.05$: after 48 hours of PDGF treatment; $p < 0.01$: after 72 hours of PDGF treatment) (Figure 13F) and HCT116 ($p < 0.05$: after 24 and 48 hours of VEGF treatment; $p < 0.01$: after 72 hours of VEGF treatment) (Figure 13G) colon cancer cells. The well characterized late UICC stage cancer cell line, the HT29 colon cancer cell line, was used for further experiments. The Caco-2 colon cancer cell line, which was originated from early UICC stages, was also used for most of the further experiments.

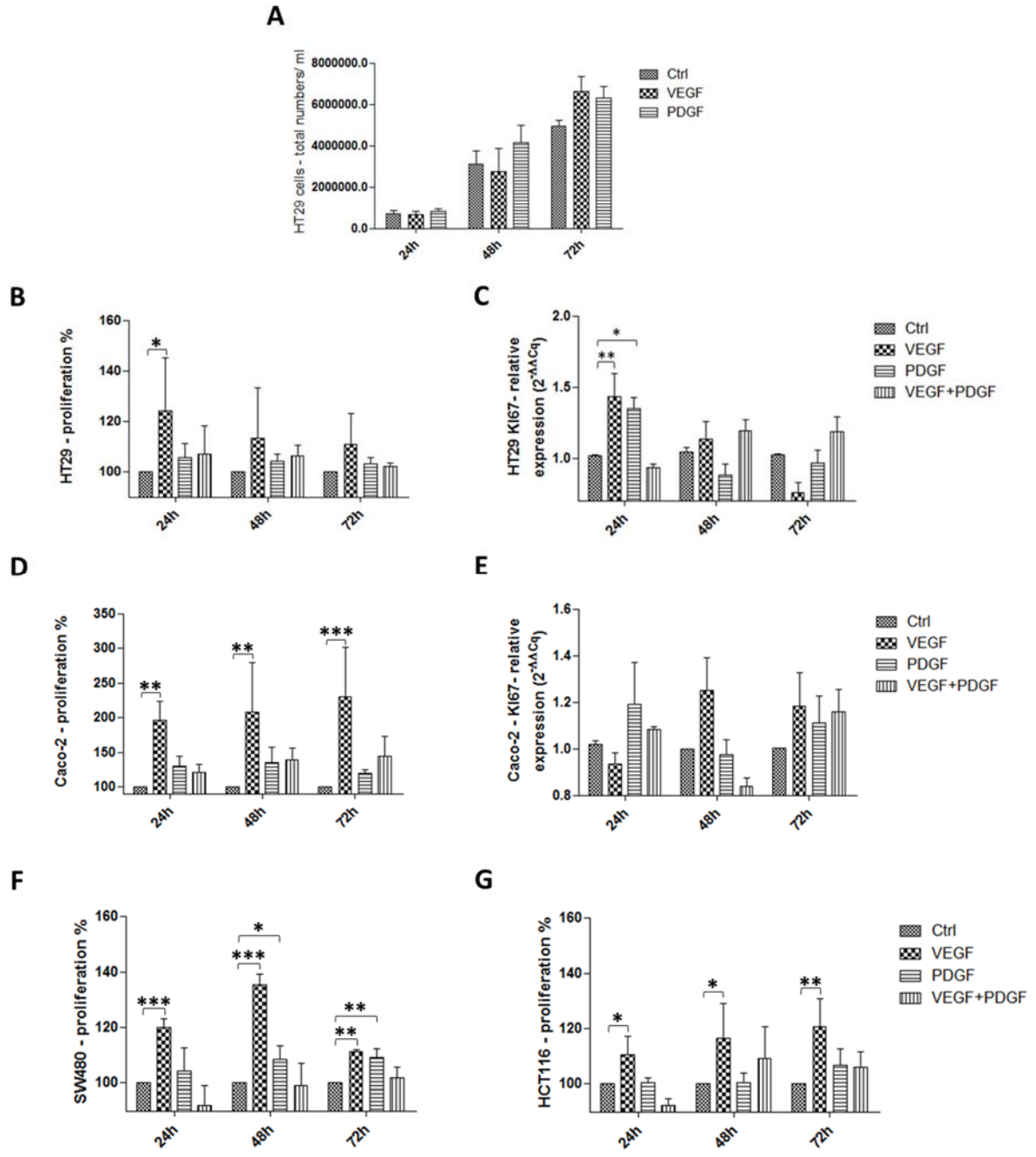

Figure 13: Influence of PDGF stimulation on proliferation in HT29, Caco-2, SW480, and HCT116 cell lines

(A) Increased cell number after PDGF and VEGF stimulation (PDGF and VEGF stimulation 100 ng/ml, new stimulation every 24 hours). PDGF and VEGF stimulation enhanced cell proliferation in the MTS assay in HT 29 **(B)**, Caco-2 **(D)**, SW480 **(F),** and HCT116 cells **(G)**. The proliferation marker Ki67 was upregulated on gene level during PDGF and VEGF stimulation in both HT29 **(C)** and Caco-2 **(E)** cells. Results are presented as ± SD.*p < 0.05, **p < 0.01, ***p < 0.001, n=3.

5. Influence of PDGF and VEGF-stimulation and PI3K/Akt/mTOR pathway inhibition on proliferation

To analyze the influence of the PI3K/Akt/mTOR pathway on proliferation, MTS proliferation assays were performed with the Akt Inhibitor IV and PI3K inhibitor. Incubation with the Akt Inhibitor IV demonstrated a reduced cell proliferation ($p < 0.001$: after 24 and 72 hours of Akt inhibition, $p < 0.01$ and $p < 0.05$: after 48 hours of Akt inhibition) by inhibition of the serine/threonine kinase Akt, a key component of the PI3K/Akt/mTOR pathway. After Akt inhibition, PDGF and VEGF weakened the anti-proliferative effect caused by the Akt inhibitor. After 24 hours, neither PDGF nor VEGF were able to deregulate the inhibitory effect on cell proliferation (Figure 14A). The visible alterations in cell morphology pointed to severe damage induced by the Akt inhibitor (Figure 14B). However, the PI3K inhibitor showed a substantially lower anti-proliferative effect in the HT29 cells compared with the Akt inhibitor (Figure 14C).

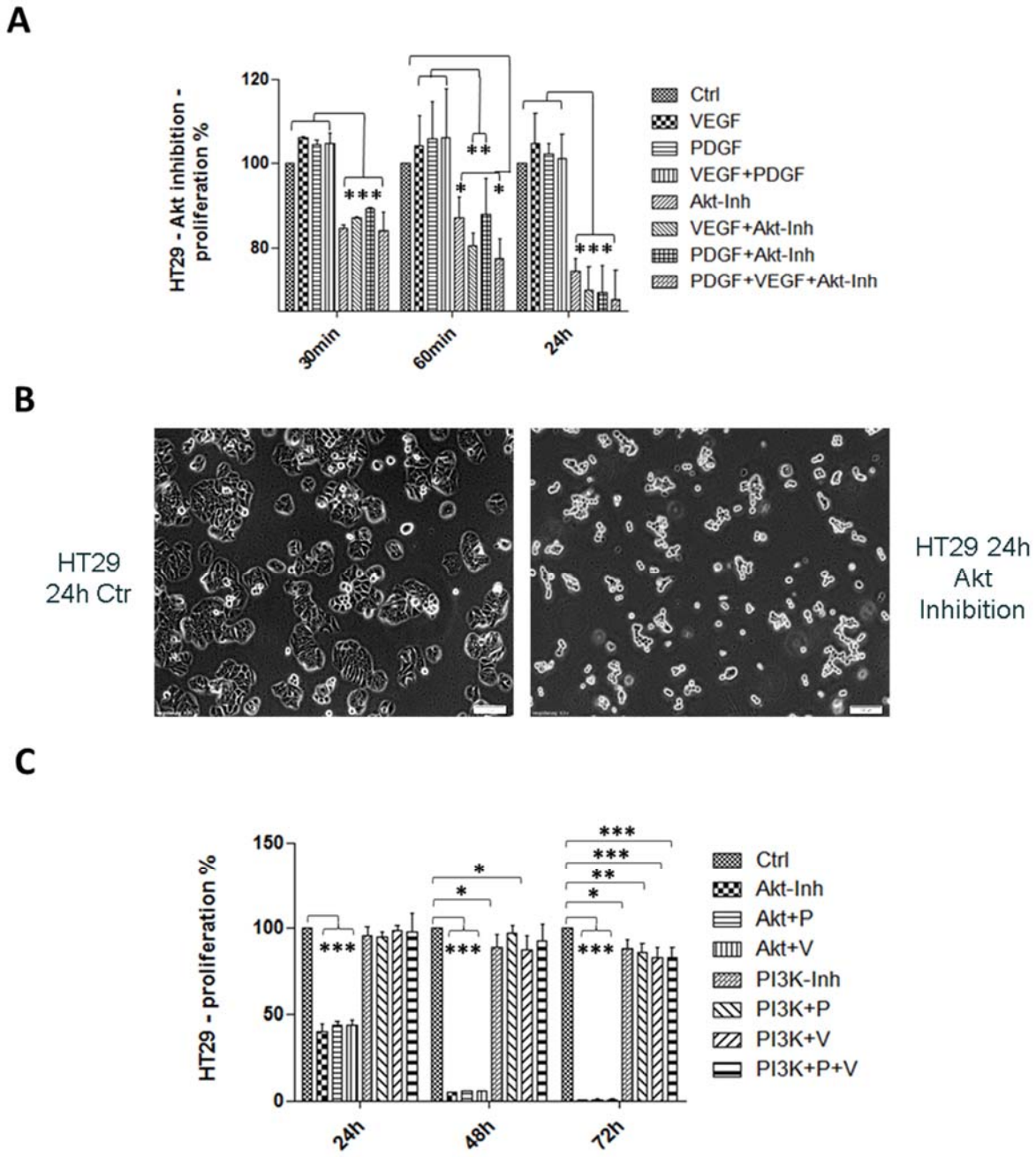

Figure 14: Influence of PDGF stimulation or PI3K/Akt/mTOR inhibition on HT29 cell proliferation

Inhibition of Akt resulted in a decreased proliferative response and stimulation with PDGF and VEGF in an increased proliferation after 24 hours in the MTS assay in HT29 cells **(A)**. Anti-proliferative effect of the Akt inhibition compared to the untreated control cells in the HT29 cell morphology **(B)**. Comparison of the strong anti-proliferative effect of the Akt inhibitor and the weak anti-proliferative effect of the PI3K inhibitor **(C)**. Results are presented as ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, n=3.

6. Effects of PDGF stimulation and Akt inhibition on the PI3K/Akt/mTOR and MAPK pathway in HT29 cells

The Akt inhibitor IV was also used to analyze the effect of PDGF signaling downstream in HT29 colon cancer cells via PI3K/Akt/mTOR. Whereas stimulation with PDGF alone increased Akt protein expression vigorously even combined stimulation with PDGF and inhibition with the Akt inhibitor IV resulted in an increased Akt protein expression compared to controls (Figure 15A). Phosphorylated and thereby activated Akt (pAkt) showed comparable results after a 30-minute treatment. pAkt was downregulated during Akt inhibition, and upregulated during PDGF stimulation. However, after 60 minutes of treatment a reversed effect was observed. pAkt was significantly upregulated after 60 minutes of Akt inhibitor influence ($p < 0.001$) (Figure 15B). Stimulation with PDGF decreased its expression level by means of an active PI3K/Akt/mTOR signaling. Combined Akt inhibition and stimulation of HT29 cells with PDGF resulted in an increased activity of pAkt, compared to control and single PDGF stimulation, but decreased activity compared to exclusive Akt inhibiton. PDGF was able to mitigate the Akt inhibition and increase PI3K/Akt/mTOR pathway activity; pAkt dephosphorylation and thus pAkt reactivation occurred faster than without influence of PDGF (Figure 15B). mTOR (Mammalian Target of Rapamycin), S6 (pS6), and 4E-BP1 are downstream targets of Akt. pmTOR and pS6 were inhibited during Akt inhibition (pmTOR: $p < 0.05$ after 30 min; pS6: $p < 0.05$ after 30minutes, 60 minutes, and 24 hours), and activated after stimulation with PDGF (pmTOR: $p < 0.001$ after 30 minutes and 24 hours, $p < 0.01$ after 60 minutes; pS6: $p < 0.01$ after 30minutes, $p < 0.05$ after 24 hours) (Figure 15C and D). 4E-BP1 was dephosphorylated and thus translation was inactivated by Akt inhibition, but stimulation with PDGF increased the inactive phosphorylated version of 4E-BP1 ($p < 0.05$ after 30 minutes) (Figure 15E). Combined inhibition and activation showed a higher activity of pS6 and of p4E-BP1 and a significantly increased activity of pmTOR ($p < 0.001$ after 30 minutes, $p < 0.05$ after 60 minutes, $p < 0.01$ after 24 hours) compared to solely Akt inhibition in HT29 colon cancer cells (Figure 15C-D).

pErk, a downstream target of the MAPK pathway,[54] was also significantly downregulated by Akt inhibition after 24 hours ($p < 0.001$). Stimulation with PDGF did not show an activating effect on pErk (Figure 15F).

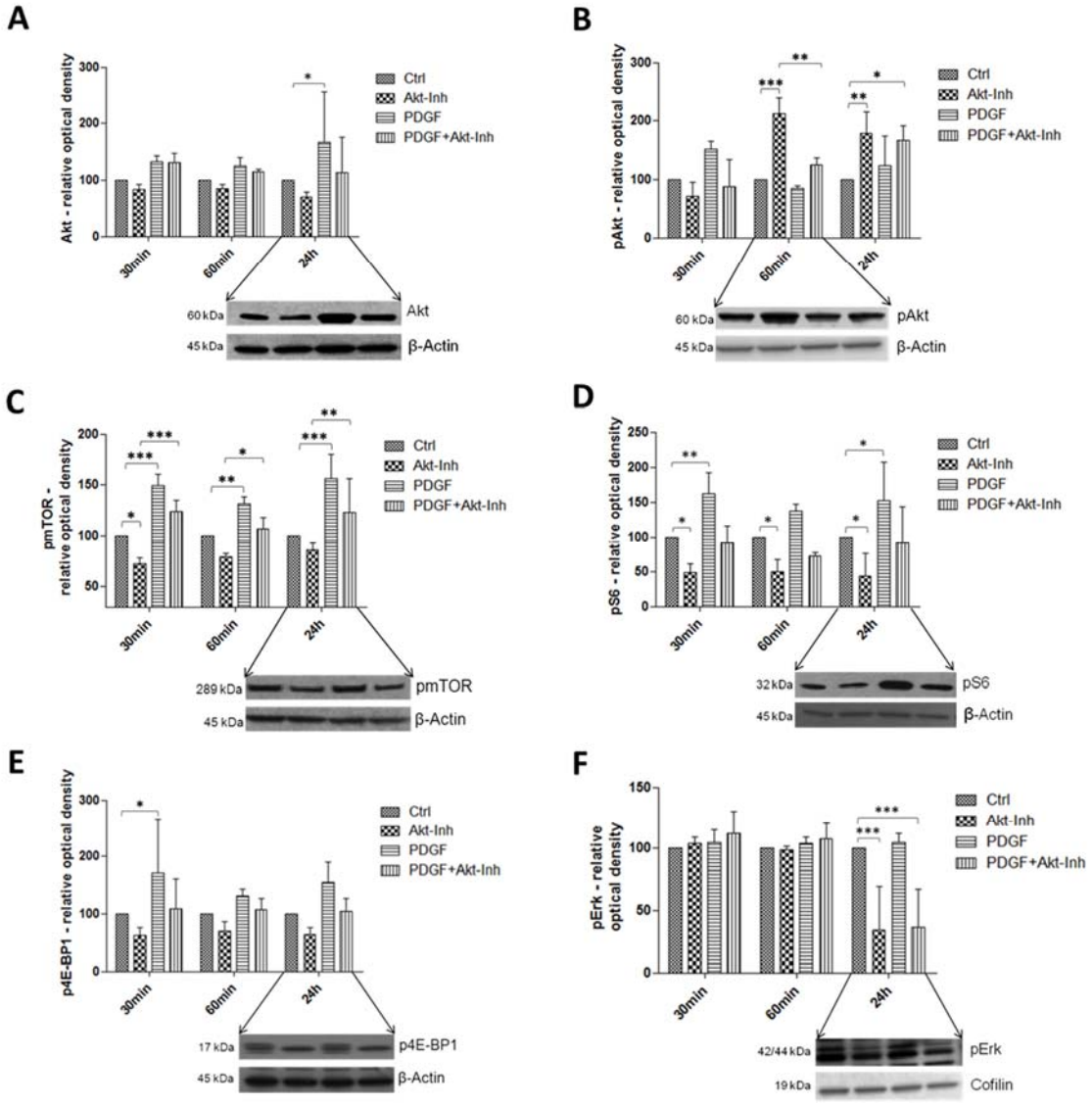

Figure 15: Effect of stimulation with PDGF and Akt inhibition of PI3K/Akt/mTOR and MAPK pathway in HT29 colon cancer cells

The lower panels show the representative western blots; the upper panels demonstrate the quantification of three independent experiments of Akt **(A)**, pAkt **(B)**, pmTOR **(C)**, pS6 **(D)**, p4E-BP1 **(E**), and pErk **(F)** expression. Cells were treated with the Akt Inhibitor IV (10 µM) or PDGF (100 ng/ml) and both Akt inhibitor and PDGF. Both the PI3K/Akt/mTOR and the MAPK pathway seemed to be inhibited by the Akt inhibitor. PDGF increased PI3K/Akt/mTOR pathway activity but not MAPK pathway activity. Results are presented as ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, n=3.

pcRaf and pMEK, also part of the MAPK signaling, but upstream of Erk, remained unaffected by both PDGF and the Akt inhibitor (Figure 16A and B). Both GLUT1 and MCT4 activity were reduced during Akt inhibition (Figure 16C and D).

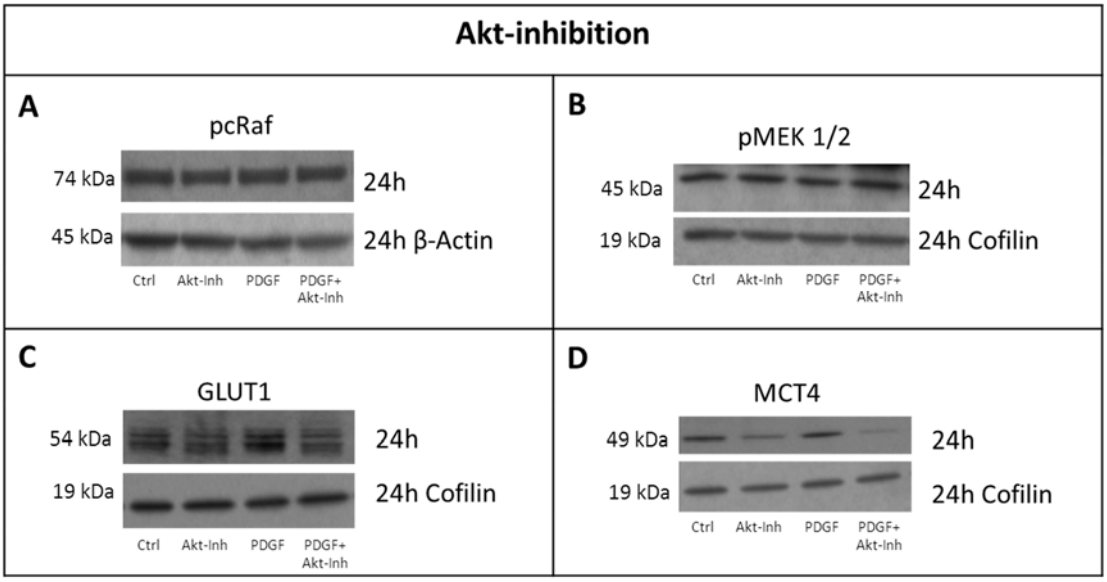

Figure 16: Analysis of the MAPK pathway and glycolysis marker after stimulation with PDGF and Akt inhibition

Representative western blots of pcRaf **(A)**, pMEK 1/2 **(B)**, GLUT1 **(C),** and MCT4 **(D)** during Akt inhibition (loading control β-Actin or Cofilin). Cells were treated with Akt Inhibitor IV (10 µM) or PDGF (100 ng/ml) or with both Akt inhibitor and PDGF, n=3. pcRaf and pMEK 1/2 were not affected by PDGF or the Akt inhibitor while GLUT1 and MCT4 activity were reduced during Akt inhibition.

7. Influence of PDGF and Akt pathway inhibition on tumor suppressor Retinoblastoma (Rb) and p53

The tumor suppressors Rb and p53 were investigated to check the influence of PDGF on tumor cell cycle. Stimulation with PDGF provoked an increased phosphorylation and thus inactivation of Rb [$p < 0.01$ (60 minutes) and $p < 0.001$ (30 minutes and 24 hours) for pRb807/811, and $p < 0.05$ (24 hours) and $p < 0.01$ (30 minutes) for pRb795]. Inhibition of Akt showed a pronounced decline in the phosphorylation status of Rb [$p < 0.05$ (60 minutes) and $p < 0.001$ (24 hours) for pRb807/811 and $p < 0.001$ (24 hours) for pRb795] and therefore an increase Rb activity, which resulted in a more effective cell cycle control. PDGF was also able to decrease the effect of the Akt inhibitor ($p < 0.001$ (30 minutes) and $p < 0.01$ (60 minutes for pRb807/811) during the combined treatment, and to increase the phosphorylation and hence inactivation of Rb (Figure 17A and B).

p53 expression analysis exhibited an activation after stimulation with PDGF, while the Akt inhibition attenuated the p53 activity in HT29 cancer cells after 24 hours of treatment (Figure 17C).

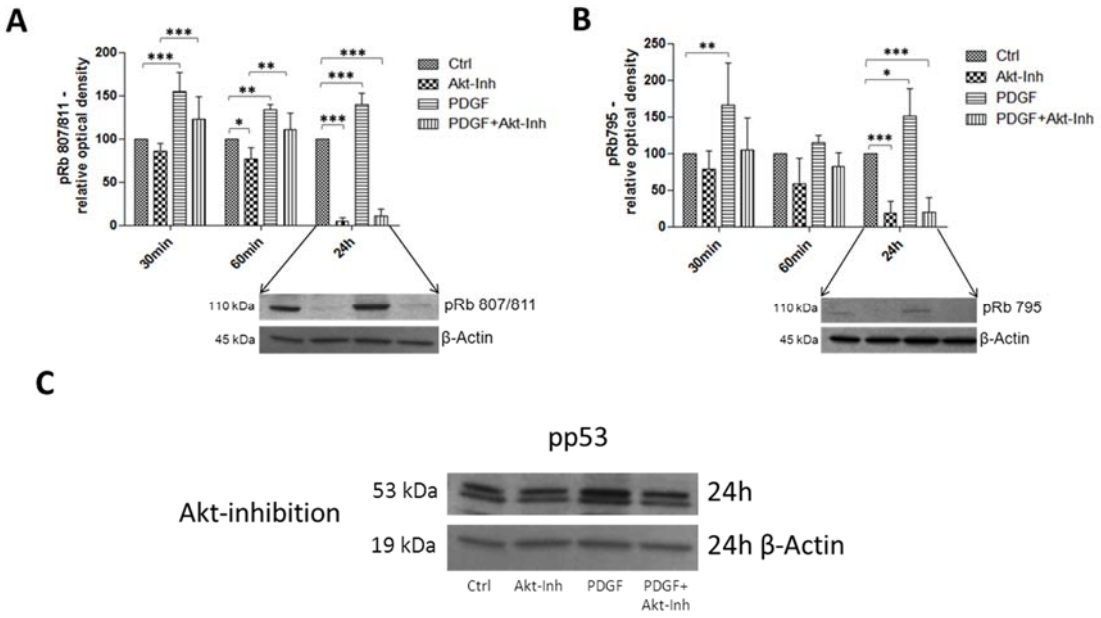

Figure 17: Investigation of changes of the cell cycle checkpoints after stimulation with PDGF and/or Akt inhibition

Representative western blots of pRb (Ser 807/811) **(A)**, pRb (Ser 795) **(B),** and pp53 **(C).** pRb was normalized to Actin loading control. Cells were treated with Akt Inhibitor IV (10 µM) or PDGF (100 ng/ml) and with both Akt inhibitor and PDGF. Results are presented as ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, n=3. PDGF increased the amount of phosphorylated and thus inactivated Rb; the Akt inhibitor showed the opposite effect: Rb was activated. p53, however, was activated during stimulation with PDGF and inhibited during Akt inhbition.

8. Effects of stimulation with PDGF and/or PI3K inhibition on the PI3K/Akt/mTOR pathway and the MAPK pathway in HT29 colon cancer cells and human colon cancer

The second used PI3K/Akt/mTor inhibitor, besides the Akt inhibitor of chapter C.6, targets PI3K, which is localized upstream of Akt (Figure 3). pAkt was significantly downregulated after 30 minutes ($p < 0.01$) and further on after 60 minutes ($p < 0.01$) by the PI3K inhibitor (Figure 18A). The downstream targets of Akt, pS6 and p4E-BP1 were likewise downregulated, particularly after 30 minutes and 60

minutes respectively (pS6: $p < 0.05$ after 30 minutes and 24 hours; $p < 0.01$ after 60 minutes) (Figure 18B and C). In contrast pS6 and p4E-BP1 were activated after stimulation with PDGF (pAkt: $p < 0.01$ after 30 minutes; pS6: $p < 0.05$ after 60 minutes). The combined treatment with the PI3K inhibitor and PDGF resulted in a more profound expression of pS6 and p4E-BP1 compared to incubation with the PI3K inhibitor alone.

pErk was not affected by PDGF stimulation, but interestingly pErk was significantly activated by PI3K inhibition ($p < 0.01$ after 30 minutes, and $p < 0.001$ after 60 minutes) (Figure 18D), a contrary result compared to that after inhibition of Akt (Figure 15F).

pcRaf and pMEK, upstream targets of Erk, were not modified by PDGF or after PI3K inhibition (Figure 18E).

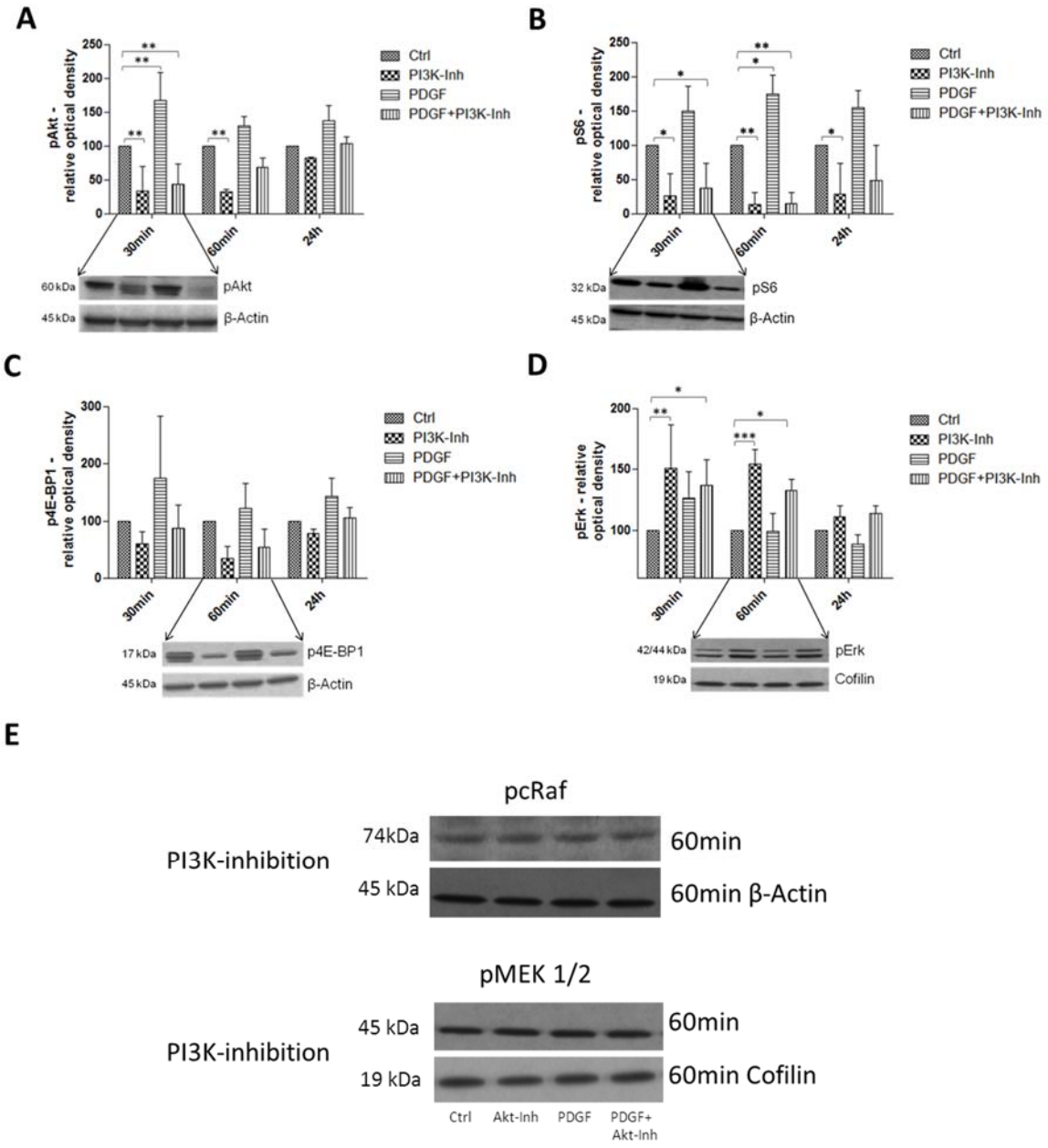

Figure 18: Effects of stimulation with PDGF and/or PI3K inhibition on the PI3K/Akt/mTOR pathway and the MAPK pathway in HT29 colon cancer cells

The lower panels show representative western blots and the upper panels demonstrate the quantification of three independent western blot experiments of pAkt **(A)**, pS6 **(B)**, p4E-BP1 **(C)**, and pErk **(D),** normalized to β-Actin or Cofilin loading control expression. Cells were treated with PI3K Inhibitor (80 nM) or PDGF (100 ng/ml) and with both PI3K inhibitor and PDGF. Results are presented as ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. **(E)** Representative western blot of pcRaf and pMEK 1/2 during PI3K inhibition (loading control β-Actin and Cofilin). While the PI3K/Akt/mTOR pathway was inhibited during PI3K inhibition, the MAPK pathway was activated from Erk.

9. Tumor cell metabolism and glycolysis

Changes in cell proliferation are often linked to an altered cell metabolism. To analyze the impact of PDGF on tumor cell metabolism, glycolysis, mitochondrial activity, and oxygen consumption was investigated.

9.1 GLUT1, LDHA, and MCT4 gene expression in human colon cancer tissues

The three glycolysis marker GLUT1, LDHA, and MCT4 were investigated to elucidate the importance of glycolysis in CRC. In primary colon cancer, a constitutively increased activity of GLUT1, LDHA, and MCT4 was observed. GLUT1 was highly significantly upregulated in all tumor stages ($p < 0.001$) (Figure 19A). LDH analysis showed also a higher expression in all stages ($p < 0.05$ UICCI/II) (Figure 19B). MCT4 exhibited a significantly higher expression, particularly in advanced stages ($p < 0.05$ UICCI/II; $p < 0.001$ UICC III/IV) compared to the controls and to patients with colon adenomas (Figure 19C).

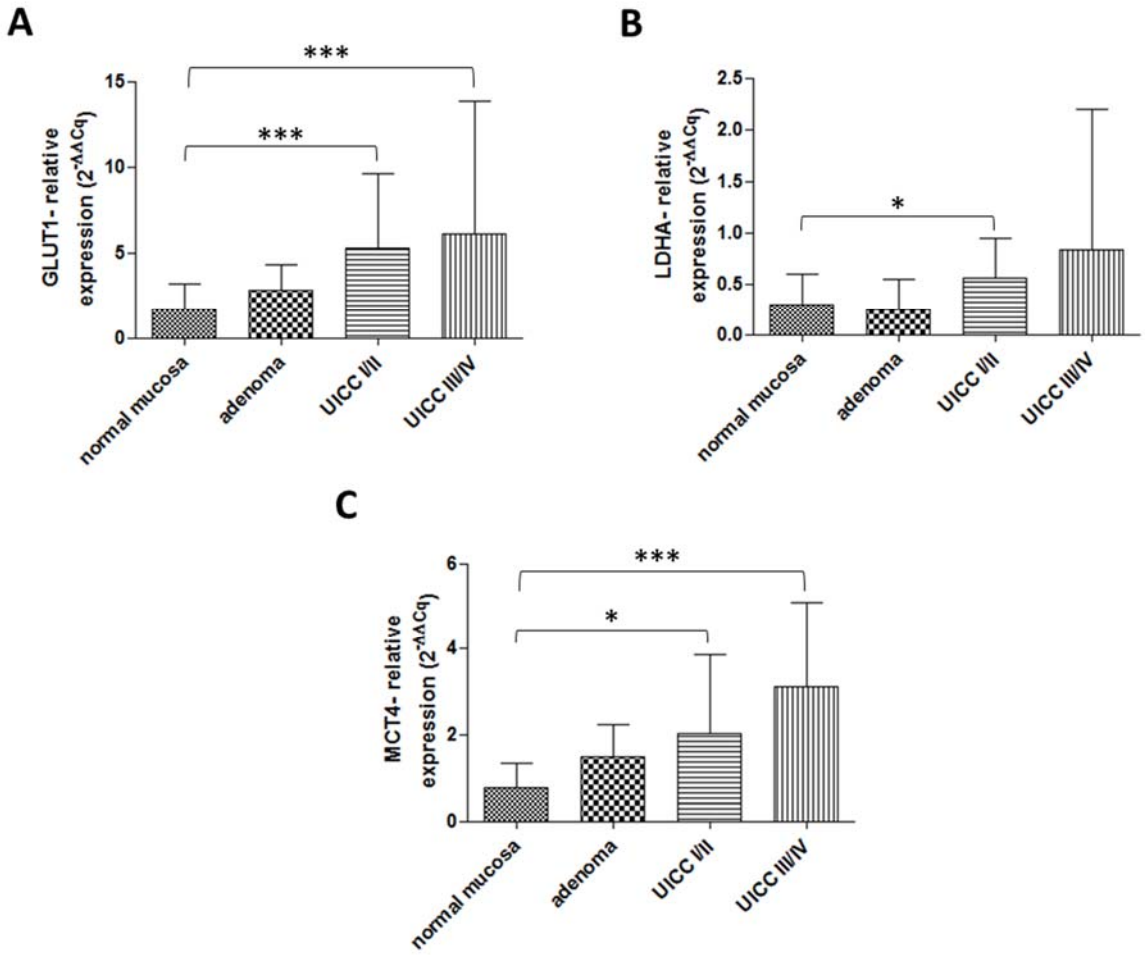

Figure 19: Influence of PDGF on glycolysis

Analysis of glycolysis markers in primary colon cancers: GLUT1 **(A)**, LDHA **(B)**, and MCT4 **(C)** gene expression was upregulated at early and late UICC stages. The normalization was performed with normal $2^{-\Delta\Delta Cq}$. Results were presented as ± SD. *$p < 0.05$, ***$p < 0.001$.

9.2 Influence of PDGF and VEGF on glycolysis in colon cancer

9.2.1 GLUT1, LDHA, and MCT4 gene expression in HT29 colon cancer cells

After 24 hours of stimulation with PDGF, GLUT1 gene expression was increased in HT29 cells ($p < 0.05$) (Figure 20A). Upregulated LDHA and MCT4 gene expression followed after 48 hours and 72 hours of stimulation with PDGF (Figure 20B and C). Stimulation with VEGF provoked a higher expression of GLUT1, LDHA and MCT4, compared to controls, but only after 72 hours of stimulation.

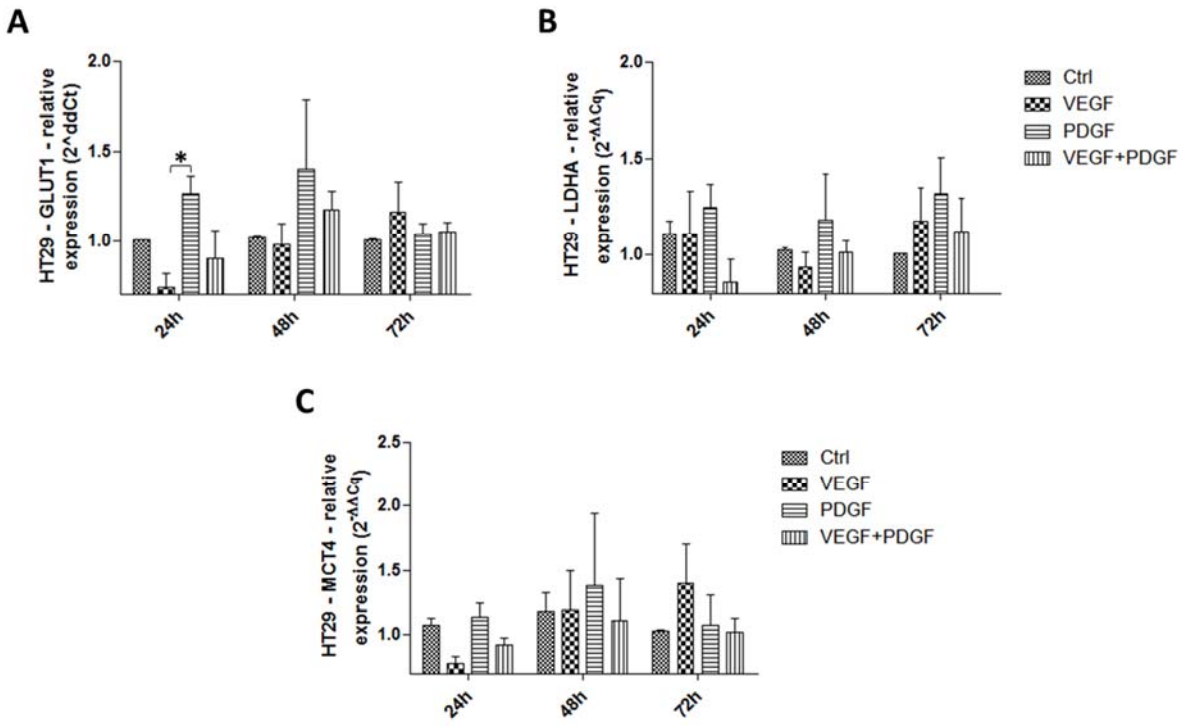

Figure 20: Gene expression of glycolysis markers in HT29 cells

RT-qPCR analysis of glycolysis in HT29 colon cancer cells after stimulation with PDGF and/or VEGF (100 ng/ml, respectively) for 24 hours, 48 hours, and 72 hours. The glycolysis marker GLUT1 **(A)**, LDHA **(B)**, and MCT4 **(C)** were upregulated after stimulation with PDGF and also after stimulation with VEGF. Normalization was performed with $2^{-\Delta\Delta Cq}$, n=3.

9.2.2 GLUT1, LDHA, and MCT4 gene expression in Caco-2 cells

The activating glycolytic effect of PDGF on Caco-2 cells was less pronounced than in HT29 colon cancer cells. None of the glycolytic markers showed an increase in gene activity, neither after stimulation with PDGF nor stimulation with VEGF (Figure 21A-C). Therefore, the HT29 colon cancer cell line was used for further metabolism experiments.

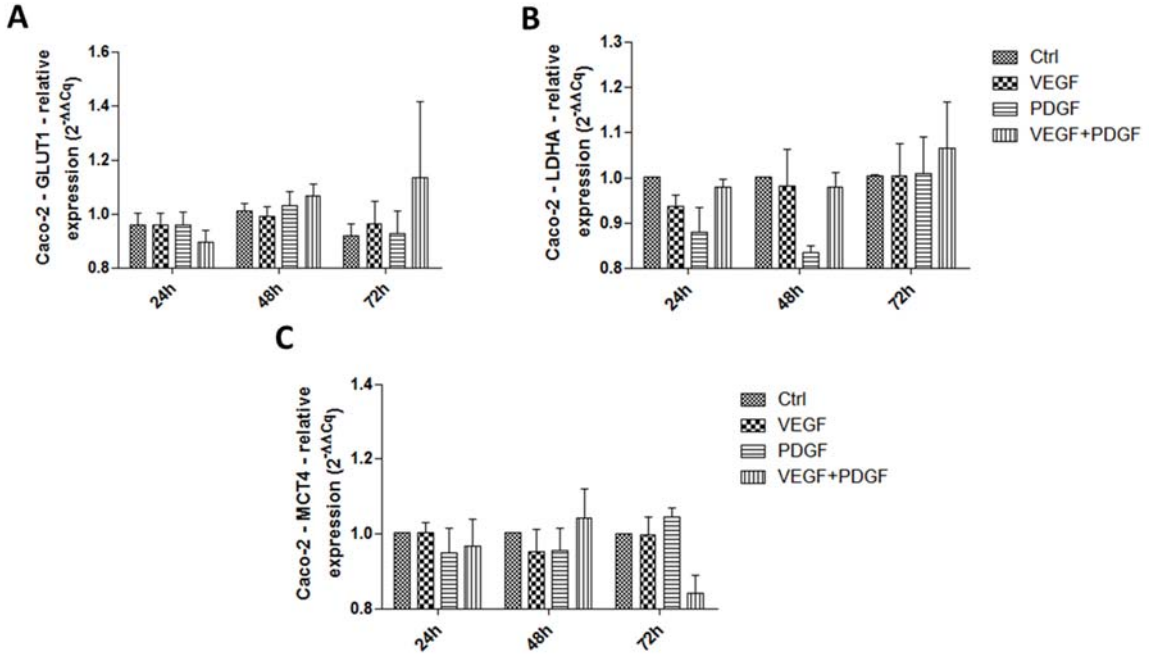

Figure 21: Gene expression of glycolysis markers in Caco-2 cells

RT-qPCR analysis of glycolysis in Caco-2 colon cancer cells after stimulation with PDGF and/or VEGF (100 ng/ml, respectively) for 24 hours, 48 hours, and 72 hours. The glycolysis marker GLUT1 **(A)**, LDHA **(B)**, and MCT4 **(C)** showed no significant change in gene expression during stimulation with PDGF and VEGF. Normalization was performed with $2^{-\Delta\Delta Cq}$, n=3.

9.3 Influence of PDGF on glucose and lactate metabolism

To investigate the influence of PDGF on glucose uptake and lactate release in HT29 colon cancer cells the glucose transporter 1 (GLUT1) inhibitor IV, WZB117, was used. The GLUT1 inhbitor resulted in a decreased glucose uptake, whereas a higher glucose influx was detected after stimulation with PDGF. PDGF abrogated the inhibiting effect of the GLUT1 inhibitor. As a consequence glucose uptake in the tumor cells was increased, even in the presence of the GLUT1 inhibitor. Furthermore, the release of lactate as a product of the glycolysis was reduced during GLUT1 inhibiton and enhanced after stimulation with PDGF. Stimulation with PDGF modified the glucose uptake and lactate release in HT29 colon cancer cells. Stimulation with PDGF also caused a higher glucose uptake into the cancer cells and an increased

lactate release after 15 minutes of treatment. PDGF can also reduce the inhibitory effect of the glucose transporter 1 inhibitor (Figure 22A and B).

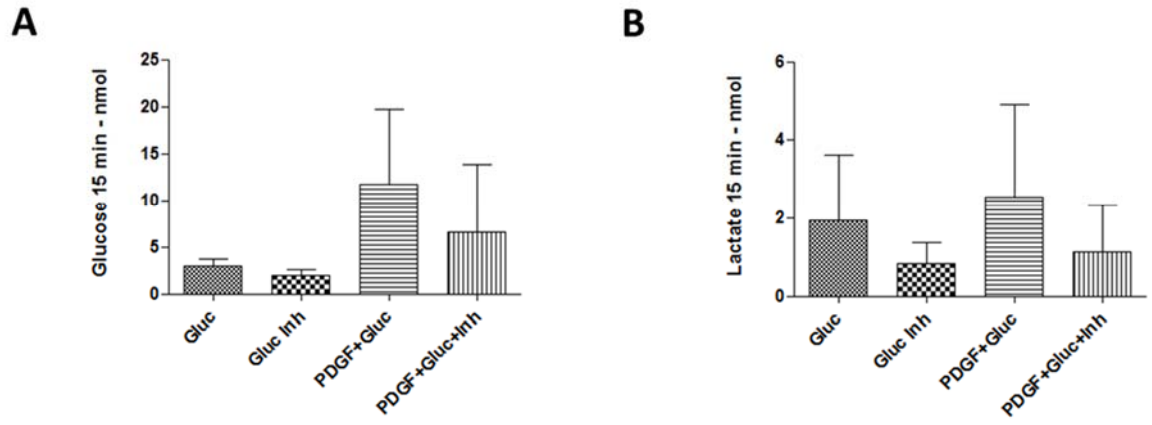

Figure 22: Analysis of the glucose and lactate metabolism in HT29 colon cancer cells

Cancer cells treated with PDGF showed an upregulated glucose uptake **(A)** and lactate release **(B)** even in presence of the glucose transporter 1 inhibitor, n=3.

9.4 Influence of PDGF on oxygen consumption in HT29 colon cancer cells

The level of oxygen consumption in the tumor cells provides an indication of the activity of the oxidative phosphorylation. The oxygen consumption during stimulation with PDGF and VEGF remained unchanged in the HT29 colon cancer cells (Figure 23).

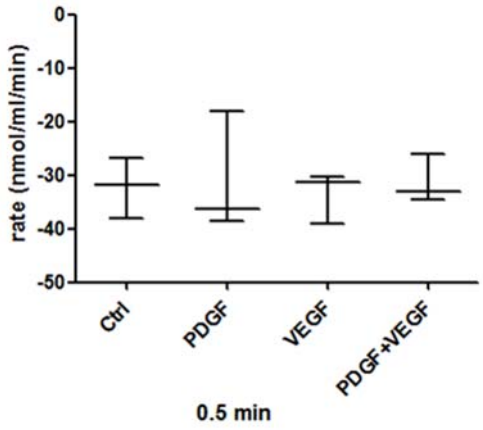

Figure 23: Oxygen consumption analysis in HT29 colon cancer cells after stimulation with PDGF and/or VEGF

Oxygen consumption remained constant during stimulation with PDGF and/or VEGF. Measurements were conducted with a Oxytherm system (Hansatech Instruments). Results are presented as ± SD; n=3.

9.5 Influence of PDGF on mitochondrial complexes in HT29 colon cancer cells

Measurement of the activity of the mitochondrial complexes I-V showed a loss of activity after stimulation with PDGF and VEGF compared tounstimulated cells, whereas PDGF possesed a stronger inhibitory effect than VEGF (Figure 24A-F).

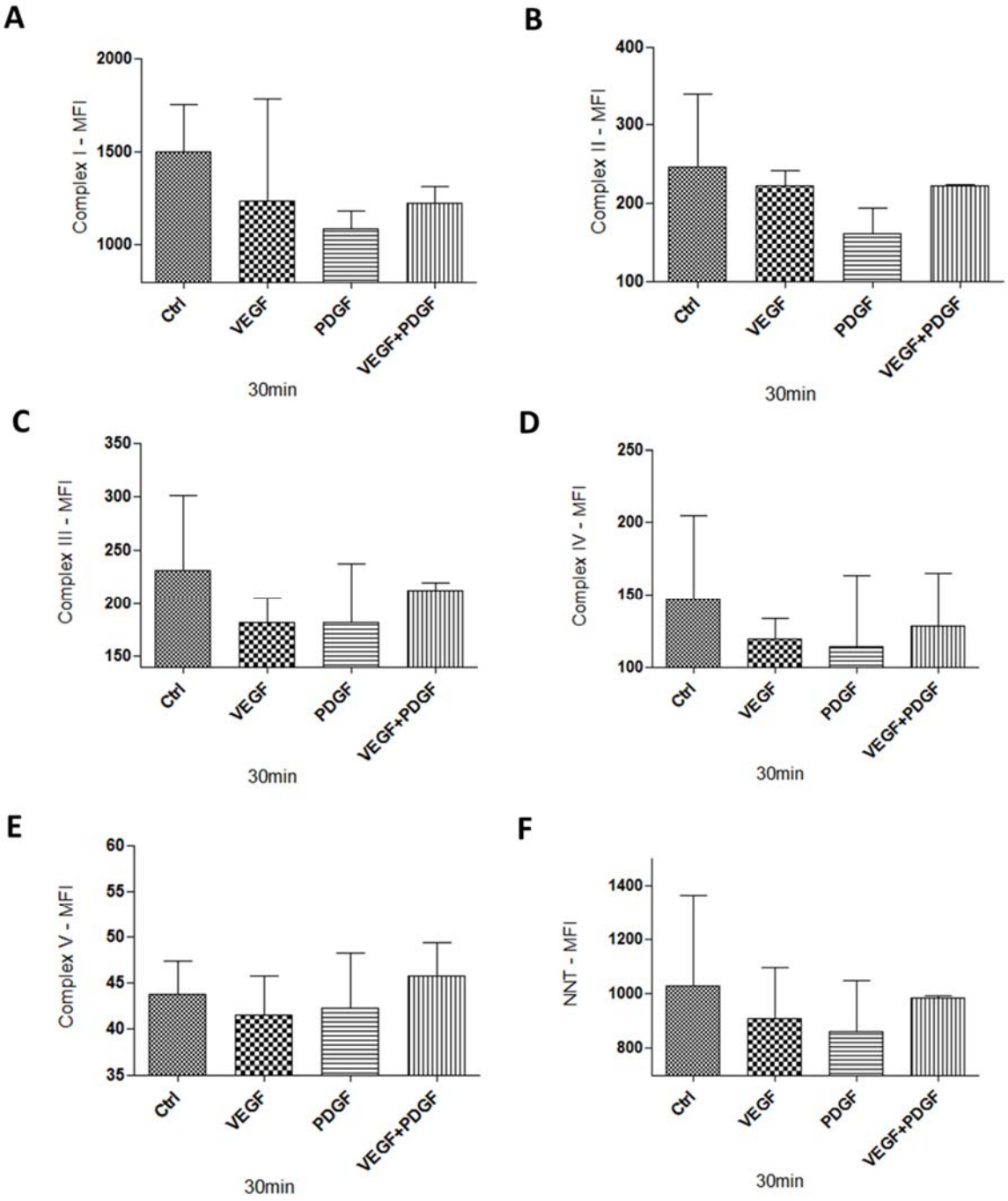

Figure 24: Analysis of the mitochondrial complexes activity in HT29 colon cancer cells after stimulation with PDGF and/or VEGF

Stimulation with PDGF and/or VEGF showed a decreased activity of the mitochondrial complexes. Measurements were conducted with the MagPix device. Results are presented as ± SD; n=2.

10. Investigation of HIF1α in human colon cancer and in HT29 colon cancer cells

HIF1α plays an important role in glycolysis regulation and thus in proliferation. In primary colon cancers, a significantly higher gene expression of HIF1α was observed compared to normal mucosa (UICC I/II: $p < 0.01$; UICC III/IV: $p < 0.001$) (Figure 25A).

Stimulation with PDGF resulted in an increased HIF1α expression in HT29 colon cancer cells which was further intensified after stimulation with VEGF ($p < 0.05$) after 48 hours (Figure 25B). Stimulation in Caco-2 cells exhibited only moderate activating effects on HIF1α expression (Figure 25C).

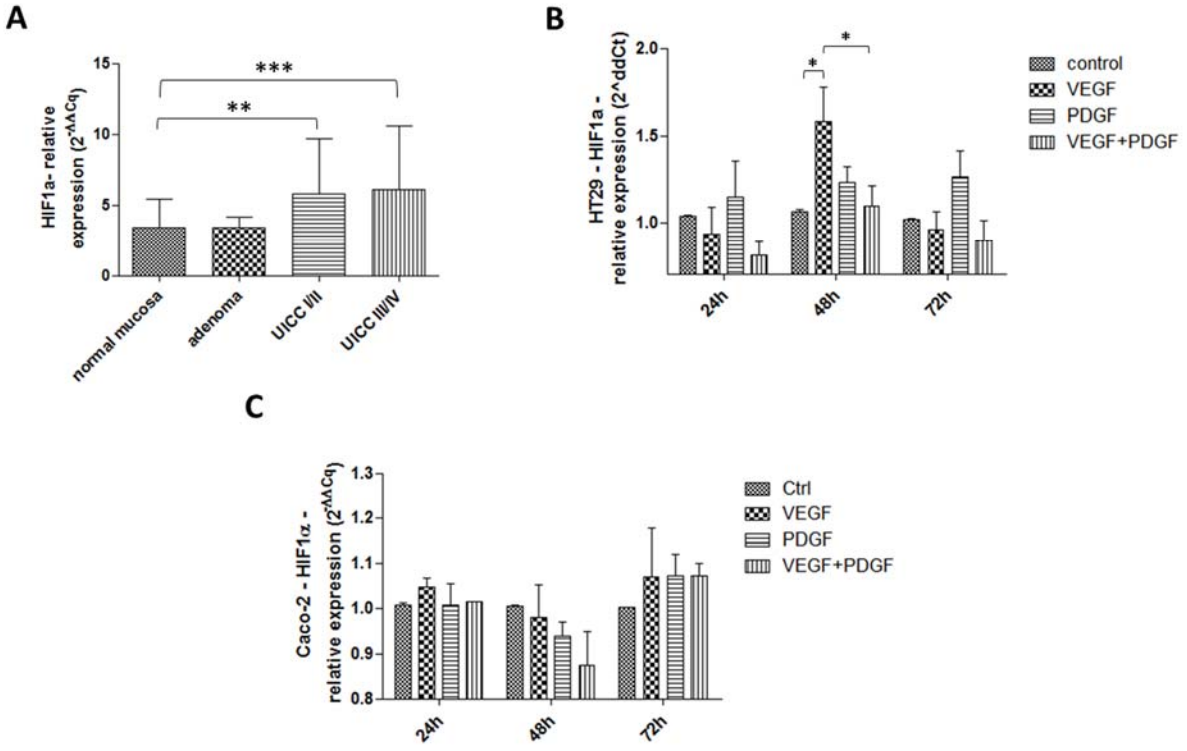

Figure 25: Investigation of HIF1α gene expression (RTq-PCR) in human colon cancer, HT29 colon cancer cells, and Caco-2 colon cancer cells

HIF1α gene expression was upregulated at stage UICC I/II ($p < 0.01$) and III/IV ($p < 0.001$) in human colon cancer **(A)**, and after stimulation with PDGF or VEGF (100 ng/ml, respectively) in HT29 cells **(B)**. The Caco-2 cells showed only slight effects caused by stimulation **(C)**.

11. Investigation of c-Myc in HT29 colon cancer cells and human colon cancer

The oncogene c-Myc also influences glycolysis and regulates tumor cell proliferation. Activated c-Myc (pcmyc S62) was highly significantly supressed after Akt inhibition ($p < 0.001$) in HT29 cancer cells (after 60 minutes and 24 hours of incubation time). PDGF significantly increased the c-Myc activity after 24 hours of stimulation ($p < 0.01$) (Figure 26A). Inhibition of PI3K resulted in a similar decreased but weakened effect than inhibition of Akt (Figure 26B). pcmyc T58 was also inhibited after Akt inhibition ($p < 0.01$ after 60 minutes; $p < 0.001$ after 24 hours) and significantly activated after stimulation with PDGF ($p < 0.05$ after 60 minutes; $p < 0.01$ after 24 hours) (Figure 26C). After inhibition of PI3K no inhibiting effect was visible (Figure 26D).

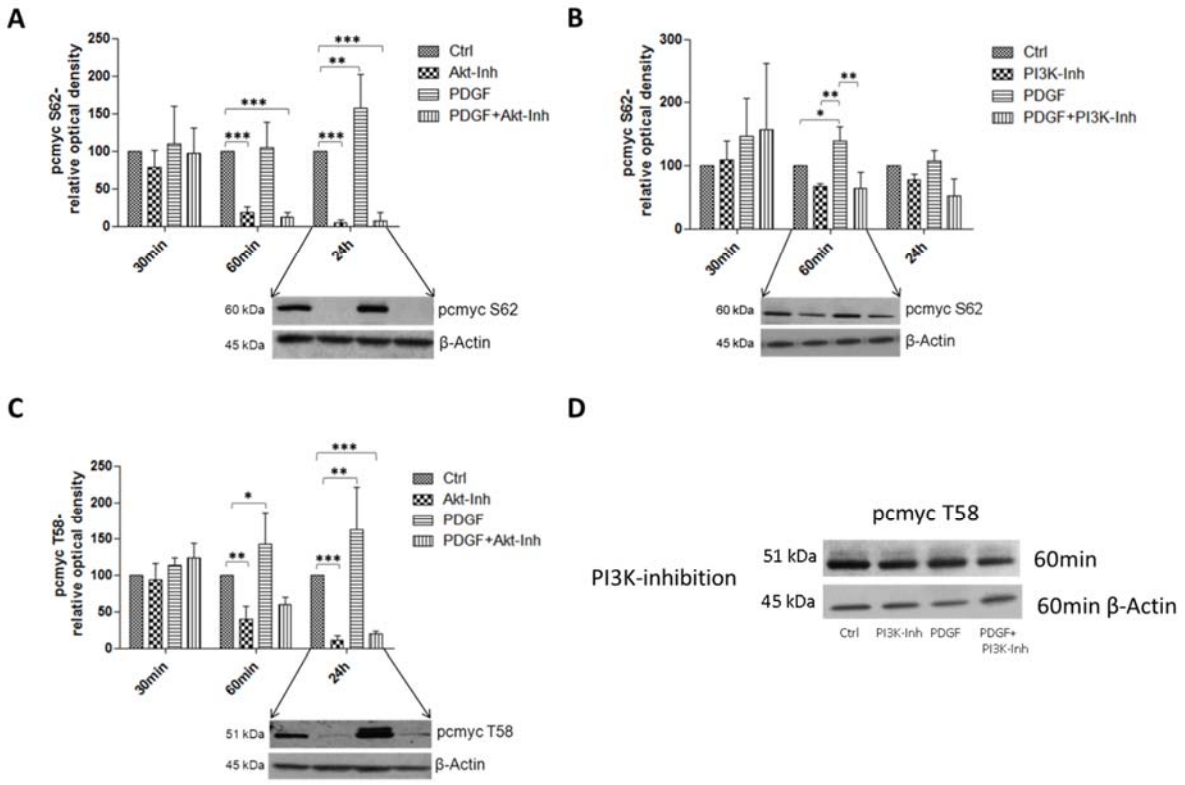

Figure 26: Effects of stimulation with PDGF and/or Akt or PI3K inhibition on c-Myc in HT29 colon cancer cells

The lower panels show representative western blots and the upper panels demonstrate the quantification of three independent western blot experiments of pc-Myc (S62) and pc-Myc (T58) normalized to β-Actin loading control expression. Cells were treated with PDGF (100 ng/ml), Akt Inhibitor IV (10 µM) **(A and C)** or PI3K Inhibitor (80 nM) **(B and D)**. Results are presented as ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n=3. The Akt inhibition caused an inactivation of pc-Myc (S62) (A) and

pc-Myc (T58) (C) while the PI3K inhibitor showed a lower inactivation of pc-Myc (S62) (B) and no inactivation of pc-Myc (T58) (D). PDGF activated both pc-Myc (S62) (A) and pc-Myc (T58).

E. Discussion

1. The expression of PDGF, VEGF, PDGFRα and β, and VEGFR1 and 2, in CRC

In clinical practice, it has been shown that monoclonal antibodies and tyrosine kinase inhibitors against VEGF, or the VEG receptors, can be successfully used as therapeutic agents for CRC.[118] However, recent studies have emphasized the increased importance of PDGF in colorectal cancer, although its influence appears to be multifactorial and is not yet fully understood.[39,119] Consequently, PDGF expression in human colon cancer was analyzed and its complex role investigated. Whenever required, the impact of both PDGF and VEGF were examined and compared in experiments to highlight the significance of both growth factors, although, as yet, only VEGF is an established clinical target.

As described in C.1, analyses of PDGF and VEGF-binding partners (PDGFRα and β, VEGFR1 and 2) showed a significantly increased gene expression of PDGFRβ, and VEGFR1 and 2, in human UICC stage I-IV tumors, compared to normal mucosa. In contrast, PDGFRα exhibited only a moderate increase in gene expression level in tumorigenic material.

As demonstrated, HT29 colon cancer cells were positive for VEGFR1 and 2-expression only. By contrast, the human colon cancer cell line, Caco-2, expressed all four receptors. Exposure to PDGF and VEGF resulted in increased gene expression of the PDGF and VEGF receptors in Caco-2 cells, which provided evidence of a productive interaction between the ligands and their receptors.

In human colon cancer tissue, PDGF and VEGF were expressed to significant levels at all stages of disease (UICC I-IV), suggesting the involvement of both ligands in tumor progression. Immunofluorescence double staining (Figure 9) showed that the observed secretion of PDGF and VEGF in human colon cancer tissue was due to epithelial colon cancer cells, while PDGF expression was considerably lower or even absent in stromal regions. VEGF exhibited the same, enhanced expression in colon cancer tissue. Additionally, *in vitro*, both the HT29 and Caco-2 cancer cells secreted

PDGF and VEGF, as demonstrated in C.2. These data suggest a possible autocrine stimulation of cancer cells.[38,120,121] Based on these findings, a precise investigation of the role of PDGF on colon cancer cells was undertaken.

2. The influence of PDGF on CRC cell proliferation

Given that PDGF expression appeared to be advantageous to colorectal tumor cells, its influence on proliferation and thus tumor growth was investigated.

Data from MTS proliferation assays showed increased proliferation for the HT29, Caco-2, SW480, and HCT116 colorectal cell lines after stimulation with PDGF, and especially VEGF. Despite the absence of any expression for PDGF receptors on HT29 cells (Figure 6), which is consistent with observations made by Kitadai *et al.*[122] and McCarty *et al*.[123], growth, and metabolic stimulation following exposure to PDGF were still evident. One possible explanation for these responses could be some residual PDGFR expression at the cell surface, which may still be sufficient to activate signal transduction following ligand exposure. Additionally, Ball *et al.* showed that VEGF could bind and promote signaling via the PDGFR.[124] Alternatively, PDGF may also bind other receptors/structures on the cell surface to promote signal transduction.[125]

Combined stimulation with PDGF and VEGF failed to reveal any synergistic effects, suggesting a competitive mechanism at the cell surface.[126] Furthermore, the significant effect of PDGF on proliferation would appear to underline its importance as a plausible target in colon cancer therapy.

2.1 The influence of PDGF on the PI3K/Akt/mTOR and MAPK pathways

By analyzing the PDGF signal transduction pathway in tumor cells, new therapeutically useful targets to prevent PDGF-induced proliferation might be found. To assess the PDGF-mediated influence on the PI3K/Akt/mTOR pathway, two pathway inhibitors were administered to HT29 cells. While Akt inhibition showed a strong anti-proliferative effect, the PI3K inhibitor was inconsequential in terms of tumor cell proliferation. Further investigation of the increased proliferation mediated

by PDGF revealed the activating potential of the PI3K/Akt/mTOR pathway. The MAPK pathway appeared to remain unaffected given that PDGF stimulation had no effect on either cRaf or MEK1/2, two components of the MAPK pathway, as assessed by western blot (Figure 16 and 18). These data would agree with previous research.[127]

Interestingly, the two inhibitors, Akt inhibitor IV (Akt inhibitor) and PKI-179 (PI3K inhibitor), provoked different effects in terms of HT29 cancer cell proliferation. The Akt inhibitor induced a dramatic anti-proliferative effect, whereas tumor cell proliferation was largely unaltered by PI3K inhibition. A likely explanation for these observations is that the PI3K/Akt/mTOR and MAPK pathways are subject to a newly discovered inhibitory crosstalk. Mendoza *et al.* and others have already described crosstalk between these pathways,[55,56,128] but not the inhibitory connection between Akt and Erk that is described here. This inhibitory crosstalk is based on the interaction between Akt, in the PI3K/Akt/mTOR pathway, and Erk in the MAPK pathway. As demonstrated in chapters C.6 and C.8, the results of protein analyses following Akt inhibition show an increased activation of pAkt, together with down regulation of the downstream targets of Akt, including pmTOR, pS6, and p4E-BP1.[60,63] These data would suggest that Akt inhibition is implemented downstream of Akt, causing the accumulation of phosphorylated Akt after 60 minutes. Because downstream signaling was blocked, pmTOR, pS6, and p4E-BP1 protein expression were all down regulated after 24 hours (the translation repressor 4E-BP1 binds to eIF-4E and inhibits translation, protein synthesis, and proliferation; phosphorylation mediated by mTOR inactivates 4E-BP1[129-131]).

In addition to the effects on the downstream targets of Akt, down regulation of pErk in the MAPK pathway (shown in western blot analyses in Figures 15 and 18) was caused by inhibitory crosstalk due to increased Akt phosphorylation. This indicated a simultaneous inhibition of both the PI3K/Akt/mTOR and MAPK pathways by the Akt inhibitor, and a subsequent reduction of cancer cell proliferation (Figure 27).

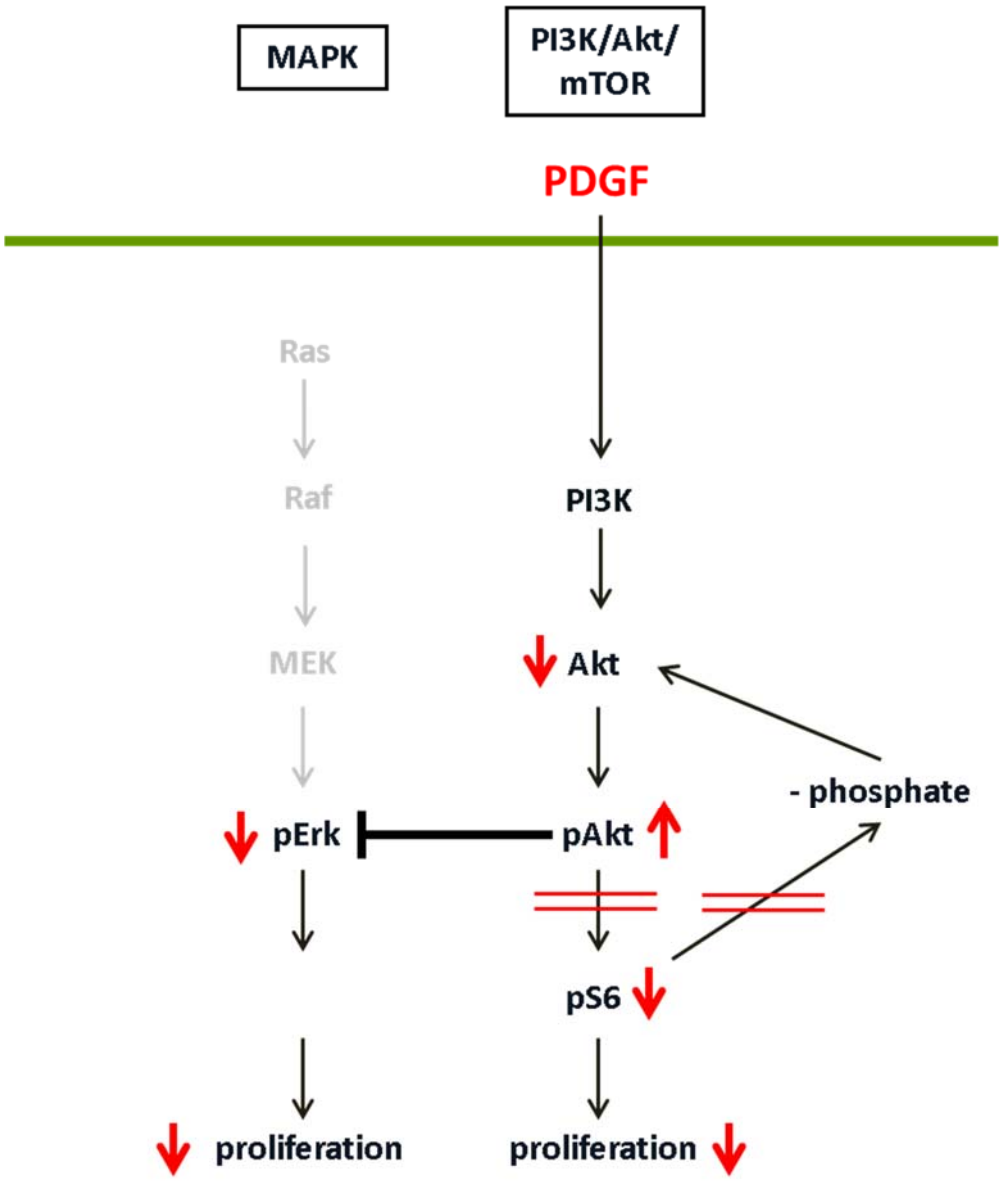

Figure 27: Schematic to show the inhibitory crosstalk between the PI3K/Akt/mTOR and MAPK pathways during Akt inhibition

The Akt inhibitor repressed both the PI3K/Akt/mTOR and MAPK pathways by activation of a newly discovered inhibitory crosstalk, which suppressed proliferation in HT29 cells.

PI3K inhibition, which disrupts the proximal terminus of the PI3K/Akt/mTOR pathway (Figure 28), resulted in the expected downregulation of pAkt and increased activity of pErk, due to the prevention of inhibitory crosstalk inhibition mediated by pAkt. PDGF and PI3K inhibitor did not influence the upstream MAPK targets cRaf and MEK1/2. While reduced PI3K/Akt/mTOR signaling would diminish proliferation, this impact is abrogated by increased activity in the MAPK pathway that also imposes

its proliferative effects. The existence of these antagonistic mechanisms resulted in cell proliferation that remained largely unaltered by PI3K inhibition (Figure 28). These data support the necessity to inhibit both the PI3K/Akt/mTOR and MAPK pathways to effectively target tumor cell proliferation and viability. If a single pathway is to be inhibited, then the PI3K/Akt/mTOR pathway should be chosen given that inhibition of both pathways may provoke undesirable clinical side effects. Therefore, future studies should focus on the development of a PI3K/Akt/mTOR-specific inhibitor, which also prevents the compensatory crosstalk with the MAPK pathway.

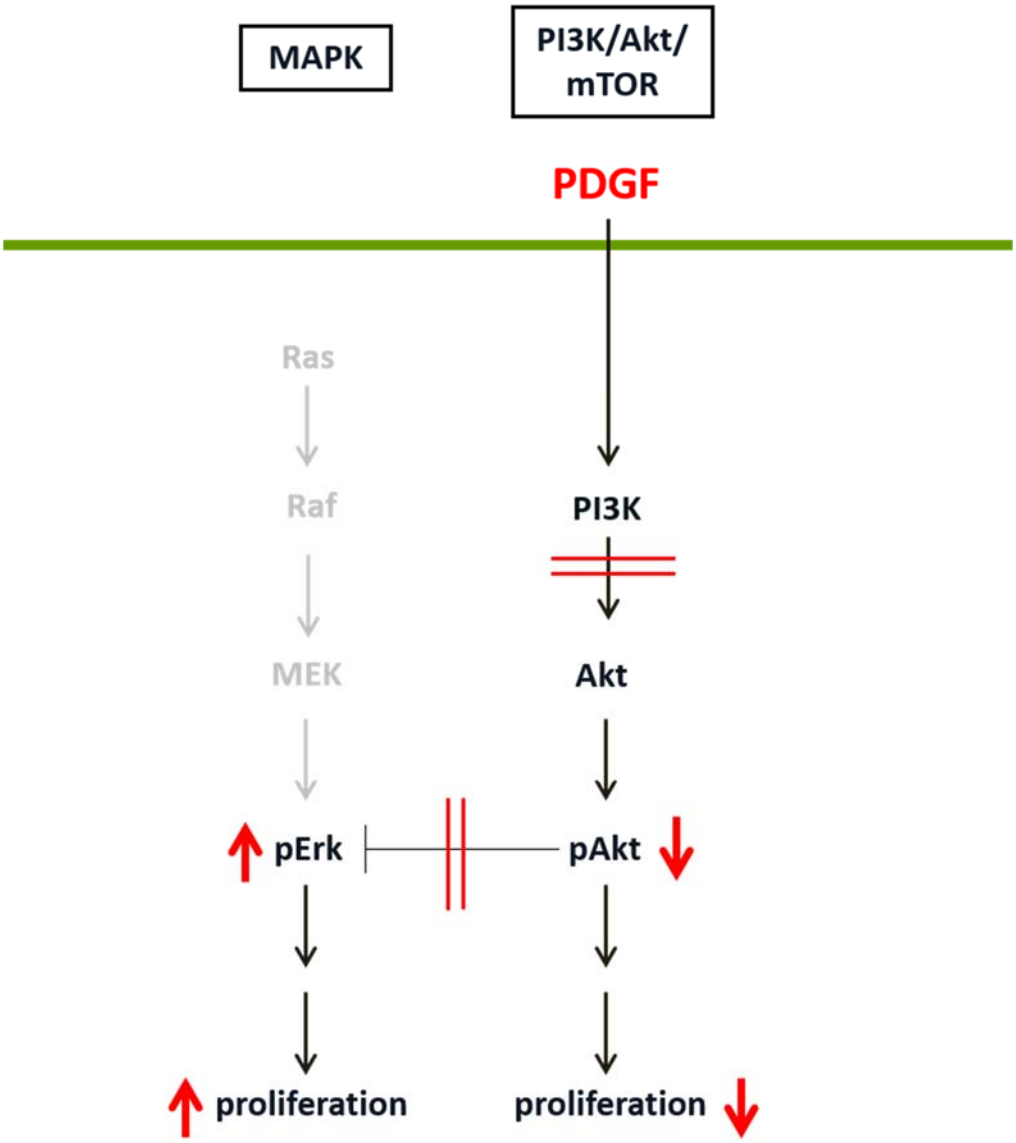

Figure 28: Schematic to illustrate inhibitory crosstalk between the PI3K/Akt/mTOR and MAPK pathways during PI3K inhibition

Proliferation remained largely unaffected given the activation of Erk due to the inhibitory crosstalk between Akt and Erk.

2.2 Inactivation of Rb by PDGF

An additional explanation for the influence of PDGF on proliferation was its supplementary inhibition of the tumor suppressor protein, retinoblastoma (Rb).

During the cell cycle, the tumor suppressor, Rb, controls the transition from G1 to S-phase (Figure 29). Rb captures the transcription factor E2F, which effectively prevents its promotion of S-phase entry (requiring free E2F). Phosphorylation of Rb is required for its release of E2F, which is then free to drive S-phase entry and cell cycle progression.[73,132-134]

As shown in chapter C.7, Akt inhibition resulted in the reduced phosphorylation of Rb and thereby increased levels of activated Rb with a reinforced tumor suppressive function, and thereby anti-proliferative effect. PDGF, on the other hand, led to increased phosphorylation, and hence inactivation of Rb, via PI3K/Akt/mTOR signaling, thus avoiding cell cycle arrest. By eliminating this cell cycle checkpoint, the tumor cells gained a proliferative advantage (Figure 30).[135]

2.3 Synergistic effects of PDGF and p53

Other than Rb, the tumor suppressor p53 also regulates cell cycle control. p53 restricts G2- to M-phase entry (Figure 29). Mutations of p53 are common events in the malignant development of CRC,[82,136] hence the p53-mutant HT29 cell line represents an appropriate model with which to investigate PDGF-mediated effects in a p53-abnormal ($p53^{mut)}$ environment.

In non-malignant cells, activation of Akt leads to a reduction in p53's tumor suppressive activity, which is reversed by low level Akt signaling.[134,137,138]

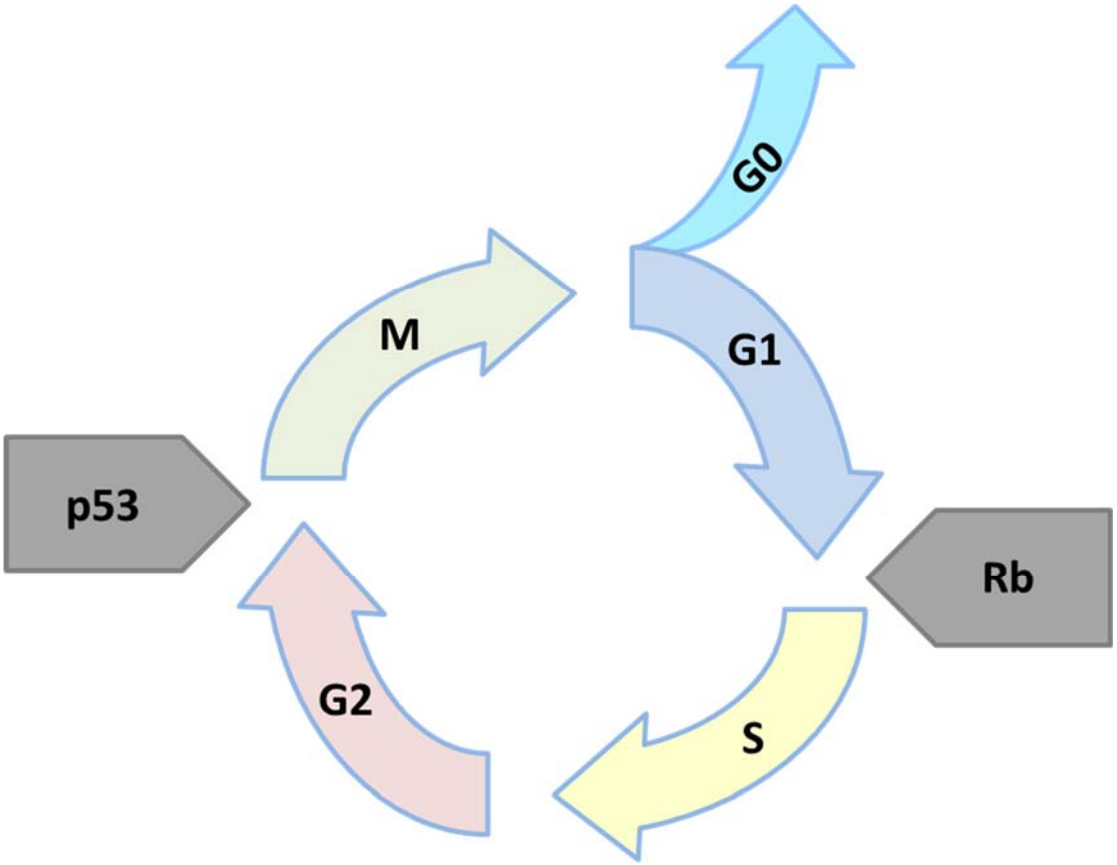

Figure 29: Schematic image of the cell cycle and checkpoints

The tumor suppressor Rb controls the transition from the G1 to S-phase; p53 controls the cell cycle at the G2/M transition.

However, in cancer cells, p53 frequently acquires diverse “gain of function” point mutations, even in cells of the same tumor.[84] Ordinarily, the mutation of p53 diminishes its tumor suppressive activity. But missense mutations can also result in active, tumor promoting effects which accelerate tumor progression.[139,140] In this fashion, PDGF, the PI3K/Akt/mTOR pathway, and $p53^{mut}$, can act in a synergistic manner to accelerate the transition through the G2/M cell cycle checkpoint[141] and promote proliferation. As shown in Figure 17C, PDGF enhanced $p53^{mut}$ activity; accordingly inhibition of the PI3K/Akt/mTOR pathway leads to decreased $p53^{mut}$ activity, which is indicated by the positive connection between the pathway and former tumor suppressor p53 (Figure 30).

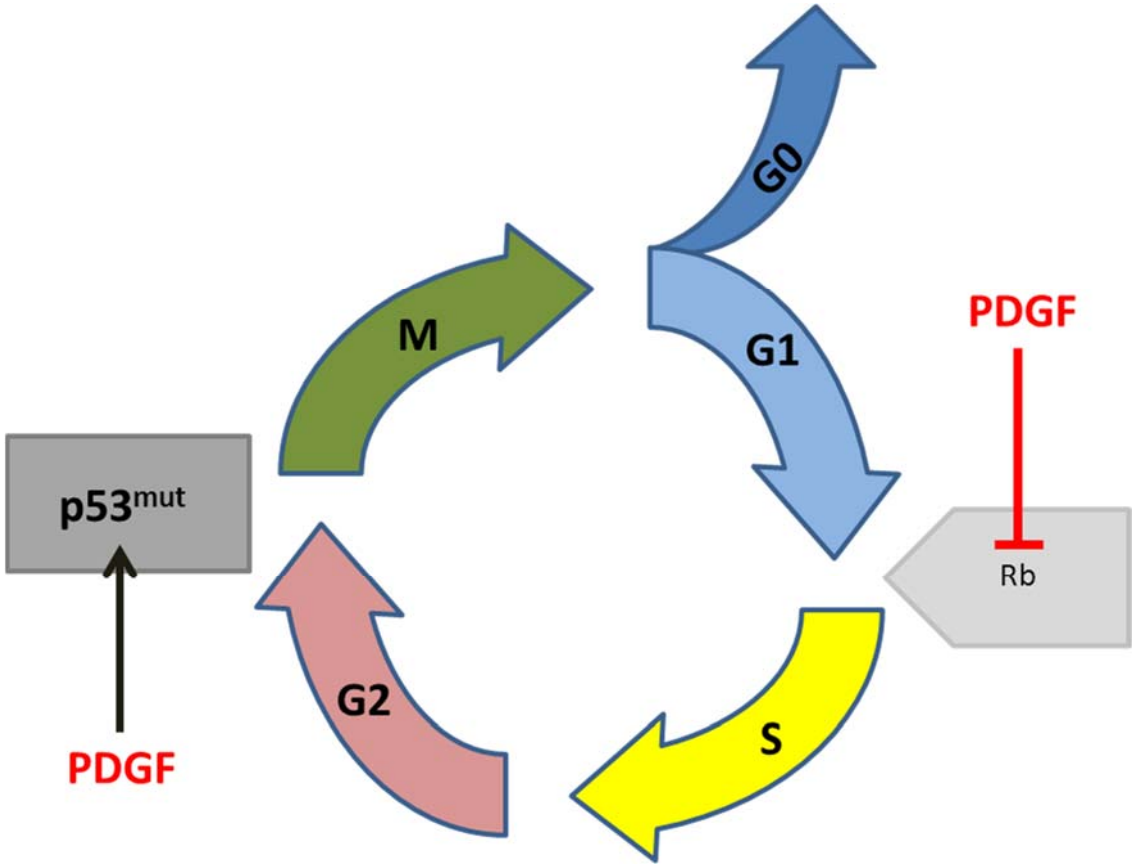

Figure 30: Schematic to illustrate the influence of PDGF on the cell cycle and the checkpoints in HT29 cells during PDGF stimulation

The tumor suppressor Rb was inhibited while $p53^{mut}$ activity was further increased, thus accelerating the cell cycle.

2.4 PDGF and HIF1α

The activity of HIF1α, an important cellular transcription factor, can be altered by activation of the PI3K/Akt/mTOR pathway under normoxic conditions.[76,78,100,102,103,142] An investigation of a human colorectal cancer patient cohort described in chapter C.11, showed increased HIF1α gene expression at all tumor stages (UICC I-IV), with augmented HIF1α expression appearing to correlate with a poorer prognosis.[143] In HT29 cancer cells, HIF1α was cumulatively upregulated during stimulation with PDGF and VEGF, whereas in Caco-2 cells, an early UICC stage CRC cell line, joint stimulation with both ligands resulted in no change to HIF1α activity. PDGF and VEGF may, collectively, be able to influence

HIF1α more efficiently in HT29 cells. Alternatively, synergy between the pathways may only be evident in later stage tumors. These data highlight the important role and boosting effect of PDGF on HIF1α activity in CRC.

2.5 The influence of PDGF on c-Myc

Ordinarily, the life cycle of the oncogene c-Myc involves two important phosphorylation events. Erk-mediated c-Myc-phosphorylation at serine 62 (S62) stabilizes and further activates the short-lived c-Myc protein.[91] Phosphorylation at threonine 58 (T58) of c-Myc by GSK3β (glycogen synthase kinase 3β) provokes its ubiquitination and proteasomal degradation. Interestingly, T58 phosphorylation can also be driven by Erk,[91,144,145] which implies a previous S62 phosphorylation event. c-Myc T58 phosphorylation, and thus degradation, can be prevented by activation of the PI3K/Akt/mTOR pathway, following GSK3 β inhibition.[91,92,145,146] c-Myc activity affects both proliferation and apoptosis,[147,148] but its exact regulatory mechanisms are still to be elucidated.

Furthermore, c-Myc and the PI3K/Akt/mTOR signaling pathway can cooperate to increase cell proliferation in different cell types.[149,150]

2.5.1 Akt inhibition

The western blot data presented in chapter C.12 demonstrated that the phosphorylation of c-Myc S62 and T58 were more pronounced during exposure to PDGF, with inhibition of Akt resulting in a decrease in pc-Myc. c-Myc S62 was almost entirely inhibited during Akt inhibition, which may also contribute to tumor cell proliferation. The significant increase in T58 phosphorylated c-Myc during PDGF stimulation was, however, surprising. Normally, phosphorylation at T58 results in the degradation of c-Myc, and would be unfavorable for tumor progression.[91,92] The predicted increase in c-Myc T58-phosphorylation and degradation during Akt pathway inhibition, due to cooperation between c-Myc and the PI3K/Akt/mTOR pathway in the tumor, did not arise; instead, the opposite effect (results from Figure 26C) was observed.

Among others, data from Okuyama *et al.* indicate that degradation of c-Myc via T58 phosphorylation can suppress tumor cell apoptosis and thus supports tumor progression.[90,151] This would explain our seemingly paradoxical results. The Erk signaling pathway would also have been suppressed during Akt inhibition by the still active inhibitory crosstalk mechanism (described in D.2.1). Should c-Myc S62 and T58 remain dephosphorylated by both Akt and Erk inhibition, then the apoptosis-prevention aspects of phorphorylated c-Myc would be negated, resulting in accelerated c-Myc degradation and decreased tumor cell proliferation. However, during PDGF stimulation, an increase in c-Myc T58 phosphorylation was evident, presumably via the PI3K/Akt/mTOR pathway, indicating a stronger anti-apoptotic effect (Figure 31).

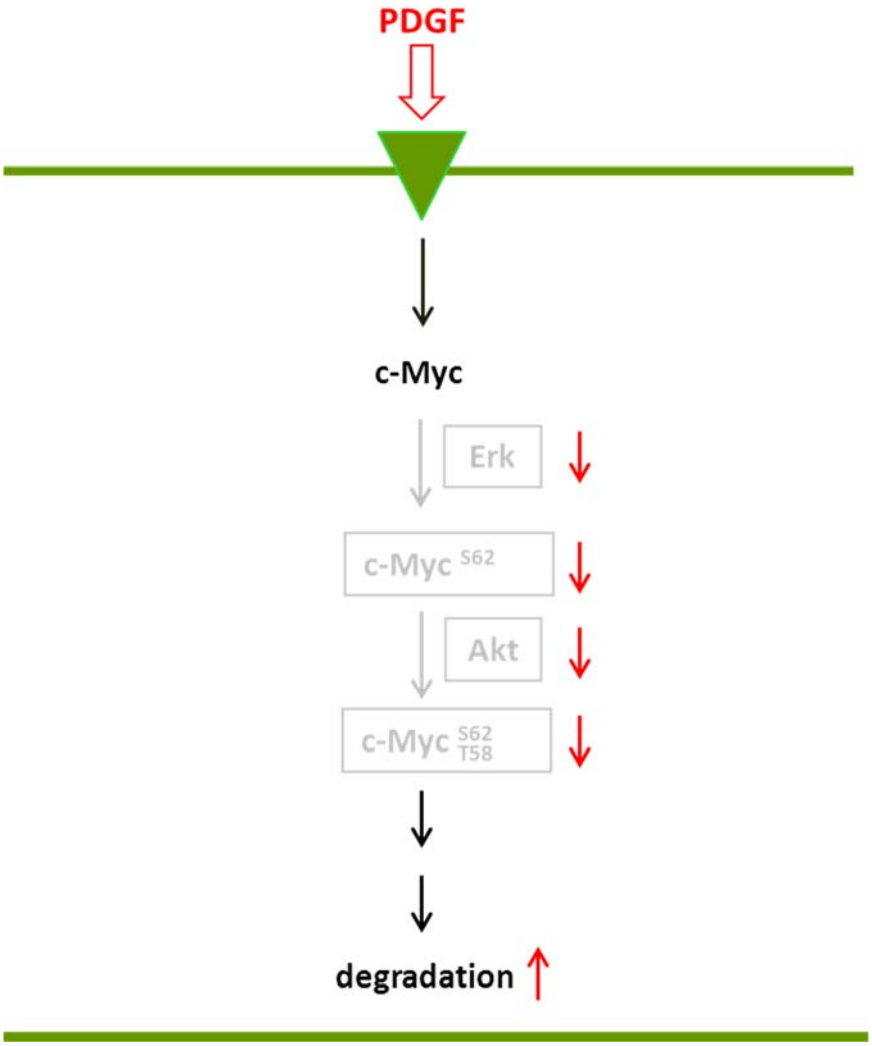

Figure 31: Accelerated c-Myc degradation during Akt inhibition

c-Myc stabilization mediated by S62 phosphorylation, and apoptosis-prevention mediated by T58 phosphorylation, were both inhibited by Akt inhibition.

2.5.2 PI3K inhibition

As demonstrated in chapter C.12, the PI3K inhibitor showed only a moderate inhibition of c-Myc S62 generation, and no effect on T58. This could be attributed to the activation of the MAPK pathway due to the reverse inhibitory crosstalk between this and the PI3K/Akt/mTOR pathway (discussed in D.2.1). c-Myc phosphorylation at serine 62, and hence at threonine 58, can be driven, in this instance, by Erk. c-Myc activation at S62 mediates its increased activity with phosphorylation at T58, preventing tumor cells from undergoing apoptosis (Figure 32), which is an additional explanation for increased cell survival during PI3K inhibition (compare the results from Figure 14C). During PI3K inhibition, the MAPK pathway remained active, which cushioned the effects of Akt inactivation and its anti-proliferative effects.

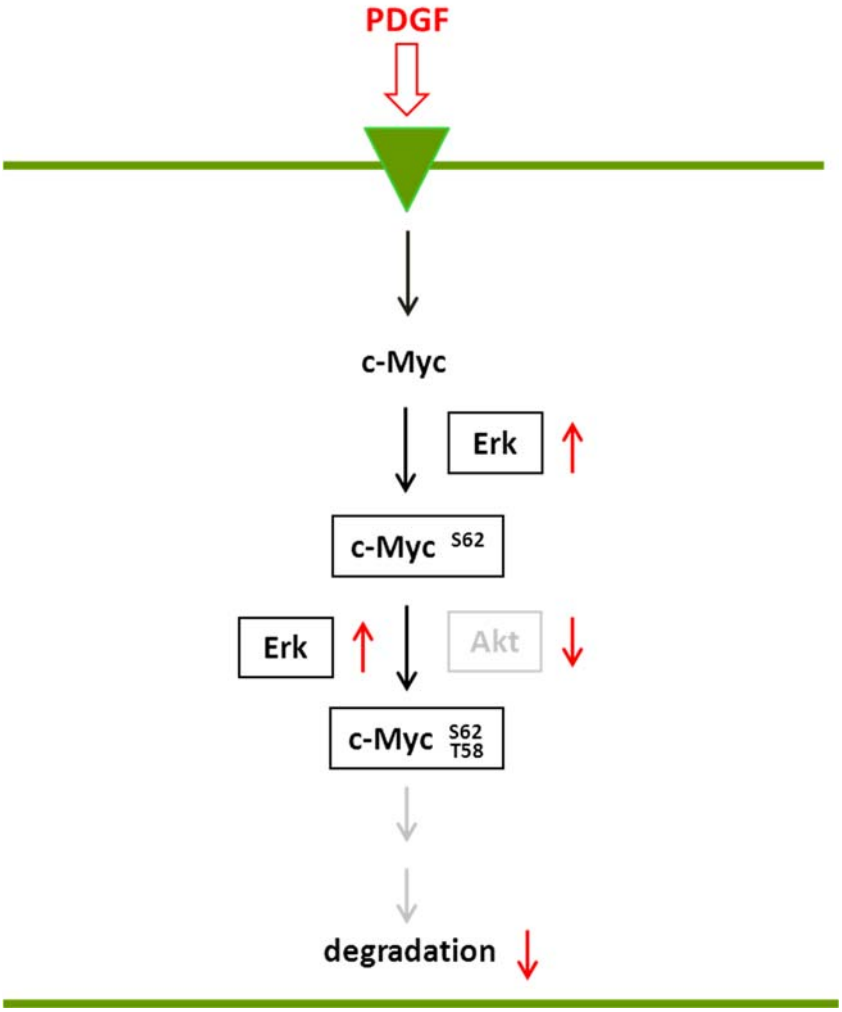

Figure 32: Erk activation during PI3K inhibition bypasses the Akt inhibitory effect on c-Myc

c-Myc stabilization, mediated by S62 phosphorylation, and the apoptosis-preventative effect of T58 phosphorylation, were mediated by Erk.

3. Changes in glucose metabolism mediated by PDGF

Stimulation with PDGF provoked not only changes in tumor proliferation, but also in the metabolism of colon cancer cells, principally by activation of the PI3K/Akt/mTOR signaling pathway.[78]

To characterize the PDGF-mediated effects on tumor cell metabolism, three glycolytic parameters were initially investigated; these were GLUT1, LDHA, and MCT4. The glucose transporter 1 (GLUT1) regulates glucose uptake,[76,78,152] while lactate dehydrogenase A (LDHA) converts pyruvate to lactate.[76,78,153,154] Monocarboxylate transporter 4 (MCT4) controls the release of lactate from tumor cells to the surrounding tumor cell environment.[76,78,155] Many tumor cells actively engage in a metabolic transition which encourages production of the biosynthetic intermediates required to support increased cell proliferation and hence tumor growth.[78,156] This often involves a glycolytic shift towards “oxidative glycolysis” instead of metabolizing glucose by oxidative phosphorylation, with the assistance of oxygen (Warburg effect).[76,114,157,158] As shown in C.10.1, in the investigated human colon cancer tissues, all three targets (GLUT1, LDHA and MCT4) were significantly upregulated, particularly at late UICC stages. In addition, HT29 colon cancer cells exhibited, during PDGF stimulation, increased GLUT1, LDHA, and MCT4 expression, increased glucose uptake, and lactate release, decreased mitochondrial activity, but no increased oxygen consumption, despite the absence of PDGF receptors (as already discussed in D.1). Conversely, Caco-2 cells, which are an early UICC cell line, exhibited only a weak or no glycolytic shift during PDGF and VEGF stimulation. The influence of PDGF on tumor cells would therefore appear to increase with tumor malignancy. These data indicate a higher glycolytic rate during PDGF stimulation, even in the presence of oxygen, since oxygen consumption remained constant. The colon cancer cells seem to be capable of taking advantage of their mutant status to regulate energy metabolism, which also involves the surrounding stroma,[159] and more flexibly so in the presence of PDGF. Unlike proliferation, where the VEGF growth factor took the dominant role,[155] PDGF instead seems to play a greater role in the conversion to a malignant metabolism.

Figure 16C and D demonstrated that the inhibition of Akt negatively affected GLUT1 and MCT4 activity, and thus glycolysis. This appears to be the critical

influence of PDGF on the PI3K/Akt/mTOR signaling pathway and metabolism: PDGF influences colon cancer cells in an Akt-promoting, glycolysis dependent manner. Due to this synergy, any influence on glycolysis might be used as a promising therapeutic strategy[160] with which to target human colon cancer. Possibly, inhibition of PDGF, and thus inhibition of the PI3K/Akt/mTOR pathway, may render any use of specific glycolysis inhibiting substances unnecessary.

4. Synergistic connections between PDGF, c-Myc, Rb, p53^{mut}, HIF1α, PI3K/Akt/mTOR, and metabolism

While the oncogenic potential of the "triad" of transcription factors p53^{mut}, c-Myc, and HIF1α, and their interplay, is of great importance in influencing tumor metabolism and proliferation,[161,162] PDGF also exerts its influence via Rb and the PI3K/Akt/mTOR pathway.

4.1.1 c-Myc and p53^{mut}

Smith *et al.* discovered that c-Myc deregulation leads to cell apoptosis in CRC patient probes, but not in the presence of a p53 point mutation; c-Myc therefore influences metabolism, especially in mutated cells. It is able to accelerate glycolysis via LDHA and GLUT1 mobilization,[87,95,163,164] and furthermore, it plays a role in p53 regulation.[165,166] The association between c-Myc and p53^{mut} synergistically prevents apoptosis, and induces tumor progression in CRC.[167] Okuyama *et al.* discovered the downregulation of c-Myc as a favorable event in cancer cells during hypoxia and/or starving conditions.[90] Our data additionally suggest an overall tumor promoting and anti-apoptotic effect mediated through c-Myc downregulation in a p53^{mut} environment during the exposure to PDGF.

4.1.2 p53^{mut}, Rb, and HIF1α

The c-Myc-p53^{mut} axis did not appear to be the only synergistic association in CRC. High levels of wild-type p53 results in the degradation of HIF protein,[162] while

$p53^{mut}$ prevents HIF1 ubiquitination and degradation, and thus accelerates energy metabolism and angiogenesis.[103,168]

The mutation of p53 also prevents its inhibitory influence on cellular glycolysis (e.g. inhibition of GLUT1),[74-76,79,169] which negates an important control mechanism for the cell. This integration with metabolism implies the necessity of in-depth metabolic investigations when assessing the influence of PDGF. Interestingly, as demonstrated in C.14, Caco-2 cells did not show increased HIF1α expression during stimulation. This oxidative glycolysis-supporting effect therefore seemed much more pronounced in late stage UICC as PDGF had a stronger HIF-promoting effect in HT29 cells (a late UICC stage cell line) than in early stage Caco-2 cells. These divergent HIF1α expression pattern among the CRC cell lines illustrates the complexity of HIF1α as a therapeutic target.[103]

The data presented in chapter C.7 showed the $p53^{mut}$ activating and Rb suppressing influence of PDGF (via the PI3K/Akt/mTOR pathway), with tumor supporting effects which underline its importance in altering CRC expression.

4.1.3 HIF1α and c-Myc

In non-tumor cells, HIF1α and c-Myc are antagonists.[88,106,170] In tumor cells however, HIF1α and c-Myc can collaborate to increase target gene expression (e.g. HK2 (Hexokinase 2), PDK1 (pyruvate dehydrogenase kinase isozyme 1), and VEGF-A),[171] primarily when c-Myc is overactive, as is the case in HT29 cells and many late stage CRC patients.[171,172] c-Myc and Akt stabilize HIF1α during hypoxia, but also during normoxia.[97] This c-Myc-HIF1α cooperation accelerates glycolysis (GLUT1,[95] LDHA,[163] and MCT4 activation) and thus the Warburg effect, and leads to a poor patient prognosis.[87,97,108,164,170,172-174] The data presented in C.14 and C.15 suggest that PDGF has a continuing ability to trigger c-Myc and HIF1α activity, with a beneficial influence on glycolysis.

More precise investigations of PDGF will be needed to decipher its precise impact on c-Myc and HIF1α in CRC, before we can realistically consider the c-Myc-HIF1α axis as a therapeutic target.

4.1.4 Rb/E2F and c-Myc

The Rb-E2F-complex mediates changes not only in the cell cycle but also in metabolism. Active E2F is able to regulate the switch from oxidative phosphorylation to oxidative glycolysis, depending on cellular needs during tumor cell proliferation.[175,176] By inhibiting Rb, and thus activating E2F, PDGF also exerted its metabolic influence through this complex. During exposure to PDGF, tumor cells show an increased plasticity in switching their oxidative phosphorylation and glycolytic needs in accordance with cellular demands.[176] HT29 tumor cells exemplify this ability to switch from oxidative phosphorylation to glycolysis. During exposure to PDGF, tumor cells can react even more flexibly to metabolic changes and adapt their metabolism more effectively. The increased glucose uptake in HT29 cells during PDGF stimulation seems to further activate the phosphorylation, and thus inactivation of Rb (chapter C.7), and, as a result, the activation of E2F, which provides an additional glycolysis-promoting effect.

c-Myc can also cooperate with pRb and E2F, mediating changes in the cell cycle, and also changes in metabolism, by regulating genes involved in glycolysis, and the inhibition of oxidative metabolism.[175-178]

4.1.5 The influences of PDGF in CRC: conclusions

Modulation of the complex and aberrant network of oncogenes, tumor suppressors, and intracellular signaling pathways (PDGF, Akt, Erk, HIF1α, c-Myc, Rb, and $p53^{mut}$) active in colon cancer cells increases the mutagenic potential of these cells. By these changes, especially in glucose metabolism (Figure 33),[78,158] tumor cells can create their own ideal growth environment and take full advantage of their mutagenic potential for accelerated tumor progression.[178] The results reported here indicate that VEGF has a stronger influence on proliferation, whereas PDGF exerts a comparatively greater control on metabolism; both mitogens seem to cooperate in a synergistic manner to accelerate CRC progression.

The existing crosstalk between Akt and Erk (discussed in D.2.1) assists the tumor cell to respond rapidly and effectively to various cancer therapies. Possible autocrine PDGF stimulation in colon cancer cells emphasizes the importance of PDGF in CRC, and the structures influenced by PDGF serve as potential targets with which to

intervene in future tumor therapies. Therefore, further research should be undertaken to clarify the relationship between these actors in CRC, and to investigate possible alternative activation partners of PDGF, besides PDGF receptors.

Oxidative glucose metabolism seems to be increased at later UICC stages, due to increased malignancy, in part due to the greater number of mutations in CRC cells. PDGF cumulatively catalyzes this glycolysis-promoting process through the PI3K/Akt/mTOR pathway. While this influence may be small, its collective effects constitute a significant promotion in tumor progression.

In conclusion, the data show that PDGF is able to activate the PI3K/Akt/mTOR pathway (even in the presence of an Akt inhibitor), HIF1α, $p53^{mut}$, c-Myc, and supresses the cell cycle checkpoint and tumor suppressor Rb (Figure 33).

Additively, these effects alter tumor metabolism and boost proliferation, and thus tumor progression. The newly discovered inhibitory crosstalk between Akt and Erk, indicates that more in-depth investigations of the crosstalk mechanisms that operate between better known pathways (i.e. PI3K/Akt/mTOR), need to be performed, not least because a targeted approach to therapy needs to be highly selective in order to block possible escape mechanisms. These data also highlight the importance of the PDGF-PI3K/Akt/mTOR pathway-axis and its potential as a target in CRC.

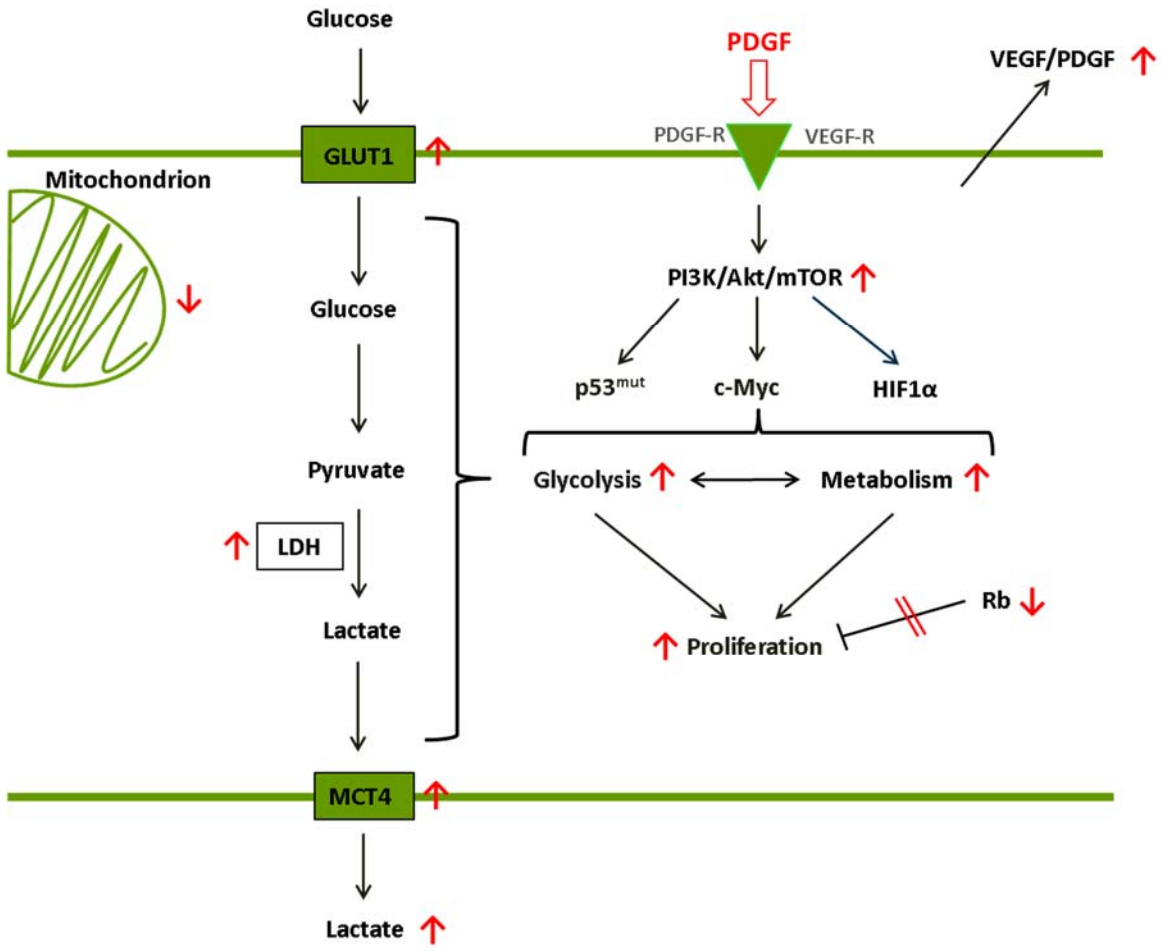

Figure 33: PDGF-mediated effects in CRC

PDGF promotes proliferation by increased glycolysis, supported by PI3K/Akt/mTOR pathway activation and subsequent $p53^{mut}$, c-Myc, and HIF1α activation. Additional inactivation of Rb accelerates cell cycle progression and proliferation.

5. Targeting PDGF for tumor therapy

The approval of monoclonal antibodies and tyrosine kinase inhibitors against the VEGF/R and the EGF/R improved the second line treatment for metastatic colorectal cancer (mCRC). The combination of the anti-angiogenic agent Bevacizumab with FOLFOX (5-FU, Folinic acid, and Oxaliplatin) also improved overall survival.[18] Aflibercept in combination with FOLFIRI (Irinotecan, 5-FU, and Folinic acid) improves survival,[26] with both agents targeting VEGF. The small-molecule tyrosine kinase inhibitor Regorafenib, and the monoclonal antibody, Ramucirumab, as VEGFR inhibitors, were also able to improve survival when given

as a monotherapy,[21] or in combination with FOLFIRI.[27] These data show that inhibition of VEGF, and the VEGF receptors, show considerable potential for CRC therapy.

Imatinib, Sunitinib, Sorafenib, and Pazopanip, principally target the VEGF receptors or Abl, but also the PDGF receptors,[35] and are approved in chronic myelogenous leukemia,[179] unresectable hepatocellular carcinoma,[180] and renal cell carcinoma.[181,182] However, there are no approved inhibitors directed against PDGF or its receptors in CRC. Therefore, this work investigated whether PDGF, and his downstream targets, also represent potential targets in CRC.

Due to the importance of glycolysis in tumor progression, there are currently experimental efforts to use inhibitors against GLUT1, LDHA, and MCT4 *in vitro*.[183] Fasentin, a GLUT1 inhibitor, was promising when tested in prostate cancer cells.[184] WZB117, the GLUT1 inhibitor used in experiment C.10.3, was also used by Liu *et al.* to down-regulate glycolysis in lung cancer cells.[185] The LDHA inhbitors FX11 and N-hydroxyindole-based inhibitors were able to inhibit tumor progression in pancreatic and lymphoma cells,[186] as well as in other cancers.[187] In glioblastoma, α-cyano-4-hydroxy-cinnamic (MCT inhibitor) acid reduced invasion and induced necrosis.[188] However, these agents also inhibit glycolysis in non-tumor cells. The challenge of future research will be to find tumor-specific isoforms for therapy with which to minimize side effects.

By virtue of its oncogenic activity, a c-Myc activator seems less promising for CRC therapy at present,[90] and the correlation between c-Myc phosphorylation, apoptosis, and metabolism requires closer investigation. Effective c-Myc inhibitors exist *in vitro*, but we await their *in vivo* transfer. Consequently, the investigation of downstream c-Myc targets may be more fruitful.[189]

Future research is needed to evaluate whether the inhibition of PDGF, or its downstream targets, can result in an improved CRC therapy. Further, it should be confirmed whether specific inhibition of PDGF by monoclonal antibodies is preferable to a less specific inhibition effected by multi-tyrosine kinase inhibitors, in terms of minimizing the clinical spectrum of side effects.

By identifying alternative binding partners for PDGF at the cell surface, new therapeutic targets could be identified, and thus possible escape mechanisms averted. This would be of relevance not only in CRC, but also in other tumors. Should

the PDGF-PI3K/Akt/mTOR pathway-axis be selectively inhibited, the development of the tumor cells could be negatively affected and treatment-related side effects minimized. Thus the regulation of PDGF signaling, as with VEGF, could be used to support existing cancer therapy.

F. Summary

A successful therapy for colorectal cancer (CRC), one of the most common malignancies worldwide, requires the greatest possible research effort. Of critical importance is an understanding of the relevant intracellular networks of signaling cascades, their activation, and the resulting cellular changes that are a prerequisite for a more successful CRC therapy. Vascular endothelial growth factor (VEGF) and the appropriate VEGF receptors represent molecular targets that have already been successfully implemented in the clinic (i.e. using monoclonal antibodies, tyrosine kinase inhibitors). However, for platelet derived growth factor (PDGF) and the relevant PDGF receptors, there are currently no clinically approved molecular therapeutics available. However, there are preliminary data to show that PDGF and its associated signaling pathways play an important role in CRC progression. In particular, the PI3K/Akt/mTOR pathway is emerging as an important intracellular partner of PDGF with which to control proliferation, migration, and angiogenesis in tumor cells.

Therefore it was the objective of this work to investigate the multifactorial influence of PDGF on proliferation and metabolism, depending on CRC mutation status. The intention was to identify new therapeutic targets for future cancer therapy through analyses of PDGF-induced intracellular changes.

For this purpose two human colorectal cancer cell lines were analyzed at gene and/or protein level for components of the PI3K/Akt/mTOR and MAPK signaling pathway, c-Myc, p53, and HIF1α (hypoxia-inducible-factor 1α). Changes in proliferation and metabolism, either during stimulation with PDGF and/or PI3K/Akt/mTOR inhibition, were also investigated. Experiments conducted at protein level during PDGF stimulation and/or PI3K/Akt/mTOR inhibition revealed changes in

signaling pathways and crosstalk. The influence of the tumor suppressors (retinoblastoma, Rb), oncogenes (c-Myc, $p53^{mut}$), and HIF1α during stimulation with PDGF, and their interactions in the tumor cell with respect to proliferation and glycolysis warrant further examination in terms of clinical treatment options. Investigations at the gene level of ex vivo samples (UICC I-IV) complete the study with regards to the clinical relevance of PDGF.

PDGF stimulation increases tumor cell proliferation in HT29 cells via the PI3K/Akt/mTOR pathway rather than the MAPK pathway. However, if the PI3K/Akt/mTOR pathway is pharmacologically blocked, PDGF stimulation is mediated by inhibitory crosstalk through the MAPK pathway. Further analyses revealed that specific Akt inhibition impedes tumor cell growth, while PI3K inhibition had little effect on proliferation. Inhibitory crosstalk was found to be responsible for these different effects. Careful intervention strategies are therefore required if future therapies intend to make use of these specific signaling pathways. One aim of future research should be to gain a better understanding of the crosstalk between these signaling pathways. In this fashion, “over-inhibition” of the signal pathways, which would result in additional clinical side effects for patients, could be prevented.

In late stage UICC, more mutation events occur, with tumorigenicity promoted by an increased mutation rate. Given that PDGF is increasingly expressed in the late UICC stages, our data would indicate that PDGF's effects are amplified with increasing malignancy. The activating effect of PDGF on the PI3K/Akt/mTOR pathway and subsequent changes in the activity of $p53^{mut}$, Rb, c-Myc, and HIF1α, lead to an unfavorable prognosis for colon cancer patients. PDGF acts on colon cancer cells in an Akt-activating, glycolysis-dependent manner. PDGF increases glycolysis and the ability of CRC cells to adjust their energy metabolism. These activities should be taken as possible starting points with which to design therapeutic interventions for CRC therapy.

PDGF, as another representative of the growth factor family, seems to play a similar role to VEGF in CRC. The data from this study underline the importance of the PDGF - PI3K/Akt/mTOR pathway-axis and its potential as a possible target in colorectal cancer. Thus PDGF represents an attractive therapeutic target, besides the VEGF/EGFR-based therapies already used in CRC.

G. Zusammenfassung

Die erfolgreiche Therapie von Darmkrebs, eine der häufigsten malignen Erkrankungen weltweit, erfordert den größtmöglichen Forschungsaufwand. Von entscheidender Bedeutung ist das Verständnis der maßgeblichen intrazellulären Vernetzungen der Signalkaskaden, deren Aktivierung und die daraus resultierenden zellulären Veränderungen als Vorrausetzung einer erfolgreicheren Darmkrebstherapie. Der Vascular Endothelial Growth Factor (VEGF) und die entsprechenden VEGF Rezeptoren stellen molekulare Ziele dar, die im Kolorektalkarzinom bereits erfolgreich in die Klinik implementiert wurden (wie zum Beispiel monoklonale Antikörper oder Tyrosinkinaseinhibitoren). Für den Wachstumsfaktor Platelet Derived Growth Factor (PDGF) und den entsprechenden PDGF Rezeptoren stehen jedoch noch keine klinisch zugelassenen molekularen Therapeutika zu Verfügung. Allerdings zeigen erste Daten, dass PDGF und seine zugehörigen Signalwege auch eine wichtige Rolle in der Tumorprogression spielen. Insbesondere der PI3K/Akt/mTOR Signalweg kristallisiert sich als wichtiger intrazellulärer Partner von PDGF heraus, um die Proliferation, Migration und Angiogenese in Tumorzellen zu steuern.

Deshalb war es Ziel dieser Arbeit, den multifaktoriellen Einfluss von PDGF auf die Proliferation und den Metabolismus, abhängig vom Mutationsstatus, im CRC näher zu untersuchen. Neue therapeutische Angriffsziele für eine zukünftige Tumortherapie sollen durch Analyse der PDGF-bedingten intrazellulären Veränderungen gefunden werden.

Hierfür wurden zwei humane Kolorektalkarzinom Zelllinien auf Gen- und/oder Proteinebene auf Komponenten des PI3K/Akt/mTOR- und des MAPK-Signalwegs, auf c-Myc, p53 und HIF1α (Hypoxia-inducible-factor 1α) analysiert. Ferner wurden Änderungen der Proliferation und des Metabolismus jeweils unter Stimulation mit PDGF bzw. PI3K/Akt/mTOR Inhibition untersucht. Durchgeführte Proteinuntersuchungen unter PDGF Stimulation bzw. PI3K/Akt/mTOR Inhibition offenbarten Veränderungen in den Signalwegen und des Crosstalks. Auch die Beeinflussung der Tumor Suppressoren (Retinoblastoma, Rb), Onkogenen (c-Myc, $p53^{mut}$) und HIF1α unter PDGF Stimulation und deren Zusammenspiel in der

Tumorzelle hinsichtlich Proliferation und Glykolyse rechtfertigen im Hinblick auf klinische Therapiemöglichkeiten weitere Untersuchungen. Die Genuntersuchung von ex vivo Gewebeproben (UICC I-IV) komplettieren die Untersuchung unter Beachtung der klinischen Relevanz von PDGF.

Die PDGF Stimulation steigert die Tumorzellproliferation in den HT29 Kolonkarzinom Zellen über den PI3K/Akt/mTOR Signalweg anstatt über den MAPK Signalweg. Allerdings wird die Stimulation mit PDGF durch den inhibitorischen Crosstalk über den MAPK Signalweg vermittelt, sollte der PI3K/Akt/mTOR Signalweg durch pharmakologische Inhibition blockiert sein. Die weitergehende Analyse hat gezeigt, dass eine spezifische Akt Inhibition das Tumorzellwachstum hemmt, während eine PI3K Inhibition kaum Einfluss auf die Proliferation besitzt. Der inhibitorische Crosstalk ist für diese unterschiedlichen Effekte verantwortlich. Sorgfältige Interventionsstrategien sind daher erforderlich, wenn man diese spezifischen Signalwege zukünftig therapeutisch ausnutzen möchte. Daher sollte ein besseres Verständnis der Crosstalks zwischen diesen Signalwegen Ziel zukünftiger Forschung sein. Auf diese Weise könnte auch eine „Über-Inhibition“ der Signalwege verhindert werden, die zusätzliche klinische Nebenwirkungen für die Patienten zur Folge hätte.

In den späten UICC Stadien treten vermehrt Mutationen auf, die die Kanzerogenität mit steigender Mutationsrate fördert. Angesichts der Tatsache, dass PDGF in den späten UICC Stadien verstärkt exprimiert wird, deuten unsere Daten darauf hin, dass die Effekte von PDGF die erhöhte Malignität verstärken. Der aktivierende Effekt von PDGF auf den PI3K/Akt/mTOR Signalweg und nachfolgende Aktivitätsänderungen von $p53^{mut}$, Rb, c-Myc und HIF1α führen zu einem ungünstigen Verlauf bei Patienten mit Kolorektalkarzinom. PDGF wirkt auf Darmkrebszellen in einer Akt- aktivierenden, Glykolyse-abhängigen Art und Weise. PDGF steigert die Glykolyse und die Fähigkeit der kolorektalen Karzinomzellen, ihren Energiemetabolismus unter PDGF Stimulation anzupassen. Diese Aktivitäten sollten als mögliche therapeutisch nutzbare Ausgangspunkte verwendet werden, um Therapieansätze für das kolorektale Karzinom zu entwerfen.

PDGF, als weiterer Vertreter aus der Familie der Wachstumsfaktoren, scheint eine vergleichbare Rolle wie VEGF im kolorektalen Karzinom zu spielen. Die Daten dieser Studie unterstreichen die Wichtigkeit der PDGF - PI3K/Akt/mTOR

Signalwegsachse und deren Potential für ein mögliches Angriffsziel im kolorektalen Karzinom. PDGF stellt somit ein interessantes therapeutisches Ziel neben der bereits genutzten VEGF/EGFR – basierten Therapie im kolorektalen Karzinom dar.

H. Abbreviations

Abl	Abelson murine leukemia viral oncogene homolog
(p)Akt	(phospho) Ppotein kinase B
ATP	adenosine-5’-triphosphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
(p)cmyc	protein of avian myelocytomatosis virus MC29 oncogene
CRC	colorectal Cancer
Ctrl	control
DAB	3,39-diaminobenzidine
DAPI	49,6-diamidino-2-phenylindoldihydrochlorid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	dulbecco's Phosphate Buffered Saline
DTT	dithiothreitol
(p)4E-BP1	(phospho) eukaryotic translation initiation factor 4E binding protein 1
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF-4E	eukaryotic translation initiation factor 4E
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle’s minimum essential medium
(p)Erk	(phospho) extracellular-signal-regulated kinase
FAP	familial adenomatous polyposis

FLT1	vascular endothelial growth factor receptor 1
5-FU	5-fluorouracil
GLUT1	glucose transporter 1
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HIF1a	hypoxia-inducible-factor 1α
HK 2	hexokinase 2
HNPCC	hereditary non-polyposis colorectal cancer (Lynch syndrome)
HRP	horseradish peroxidase
IgG	immunoglobulin G
KCl	potassium chloride
KDR	vascular endothelial growth factor receptor 2
KRAS	Kirstein rat sarcoma
LDHA	lactate dehydrogenase A
MAPK	mitogen-activated protein kinase
mCRC	metastatic colorectal cancer
MCT4	monocarboxylate transporter 4
(p)MEK 1/2	(phospho) mitogen-activated protein kinase kinase 1/2
MFI	median fluorescence intensity
$MgCl_2$	magnesium chloride
(p)mTOR	(phospho) mammalian target of rapamycin
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]
Na	sodium
NaCl	sodium chloride
NaDOC	sodium deoxycholat
NaF	sodium fluoride
Na-HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt
Na_2PO_4	disodium phosphate

OXPHOS	human oxidative phosphorylation
(p)p53	(phospho) p53 protein
PAGE	polyacrylamide gelelectrophoresis
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor receptor α
PDGFRβ	platelet-derived growth factor receptor β
PlGF	placenta growth factor
PI3K	phosphoinositide 3-kinase
PDK1	pyruvate dehydrogenase kinase isozyme 1
PIP_2	phosphatidylinositol (3,4)-bis-phosphate
PIP_3	phosphatidylinositol (3,4,5)-tris-phosphate
PMSF	phenylmethylsulfonyl fluoride
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
(p)Raf	(phospho) rapidly accelerated fibrosarcoma
Rb	retinoblastoma
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	real-time quantitative reverse transcription-polymerase chain reaction
(p)S6	(phospho) S6 ribosomal protein
SD	standard deviation
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TCA cycle	tricarboxylic acid cycle
UICC	Union for International Cancer Control
USA	United States of America
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
WB	Western blotting

I. Figures

J. Tables

K. References

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Danksagung

Allen voran danke ich Frau Prof. Dr. Ana Maria Waaga-Gasser für die freundliche Aufnahme in Ihre Arbeitsgruppe und die Betreuung während der Doktorarbeit. Das mir entgegengebrachte Vertrauen, die fachlichen Diskussionen und ihr alles andere als „verhaltener Optimismus“ in kritischen Situationen haben diese Doktorarbeit maßgeblich mitgestaltet und voran gebracht. Vielen Dank für die wunderbare und lehrreiche Zeit!

Besonderer Dank gilt Frau Prof. Dr. Petra Högger für die sehr unkomplizierte und professionelle Betreuung.

Prof. Stopper danke ich herzlich für Ihre Bereitschaft, als Prüfer zu fungieren.

Herrn Prof. Dr. Martin Gasser danke ich sehr herzlich für viele angeregte Diskussionen und die enorme fachliche Unterstützung.

Herrn Prof. Dr. Christoph-Thomas Germer, Direktor der Chirurgischen Klinik und Poliklinik für Allgemein-, Viszeral-, Gefäß- und Kinderchirurgie (Chirurgie I) des Universitätsklinikums Würzburg, danke ich für die Bereitstellung des Arbeitsplatzes und die Möglichkeit, diese Promotionsarbeit zu erstellen.

Mein außerordentlicher Dank gilt der gesamten Arbeitsgruppe Molekulare Onkoimmunologie. Die freundschaftliche und professionelle Arbeitsatmosphäre und die gute Kommunikation haben das Arbeiten sehr angenehm gestaltet. Tanja Grimmig, Nadine Rhodes, Mariola Dragan, Ingrid Strauß und Sabine Müller-Morath danke ich darüber hinaus für die exzellente fachliche Unterstützung.

Dr. Vinicius Kannen danke ich für den Einblick in seine Sicht auf die Wissenschaft.

Dr. Stefanie und Dr. Sebastian Sommer danke ich für die Einführung in die Sauerstoffmessung.

Herzlichen Dank an Katharina Fenkner und Anja Hesse für ihr kritisches Auge.

Meine tiefste Dankbarkeit spreche ich Tanja und Nadine für die wunderbare Freundschaft, für viele emotionale Diskussionen, für die mentale Unterstützung usw. aus. Kurz gesagt: vielen Dank, dass es euch gibt!

Ich danke meinen vier Mädels für ihre Energie und das unendliche Vertrauen!

Schließlich danke ich meinen engsten Freunden und meiner Familie für die Zuversicht und die nicht endende Unterstützung.

Rosi, vielen Dank für alles!

Zu guter Letzt danke ich meiner Mutter Ute und Robin für die Hilfe und Unterstützung über all die Jahre.