Aus der Klinik für Gynäkologie mit Brustzentrum
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DISSERTATION

A novel role of CDK5 in tumor growth, migration and proliferation of breast cancer cell lines MDA-MB-231 and BT-474

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von

Negin Karimian

aus Berlin

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Zusammenfassung

Zielsetzung

Obwohl sich das Gesamtüberleben und das rezidivfreie Überleben von Patienten mit Brustkrebs in den letzten Jahrzehnten verbessert haben, sind die therapeutischen Optionen, insbesondere für den tripelnegativen und HER2-positiven Brustkrebs, limitiert. Vorausgehende Studien belegen die wichtige Rolle der zyklinabhängigen Kinasen für die Entstehung von multiplen Karzinomen. In dieser Studie wurde der karzinogene Effekt von der zyklinabhängigen Kinase 5 (CDK5) untersucht, um ihre Bedeutung für das Tumorwachstum, die Proliferation und die Migration von malignen Brustkrebszellen zu verstehen.

Methoden

Das Gesamtüberleben (n = 1402) und das rezidivfreie Überleben (n = 3951) von Patientinnen mit Brustkrebs wurde in Abhängigkeit von der CDK5-Expression unter Verwendung der Software *KM plotter* analysiert. Mithilfe der The-Genome-Cancer-Atlas-Daten von Patientinnen mit Brustkrebs (n = 536) wurde die Expression von CDK5 im Tumorgewebe im Vergleich zu gesundem Gewebe untersucht. Die Expressionslevel von CDK5 wurden in multiplen Brustkrebszelllinien in vitro mithilfe von Western Blots detektiert. Wir führten die nachfolgenden Untersuchungen anhand der CDK5-überexprimierenden Zellen MDA-MB-231 und BT-474 durch. Hierfür erfolgten Soft-Agar-, Sulforhodamine-B- und Wundheilungsassays, um die Bedeutung der CDK5 für das Tumorwachstum, die Proliferation und Migration zu bestimmen. Die Experimente wurden jeweils in drei Behandlungsgruppen durchgeführt (Gruppe 1: CDK5-Überexpression mittels Plasmid DNA, Gruppe 2: spezifische CDK5-Inhibition mittels siRNA, Gruppe 3: unspezifische Inhibition von CDKs mittels Roscovitin). Mittels Western Blot erfolgte zudem die Untersuchung der Beziehung zwischen CDK5 und dem bekannten karzinogenen mTOR-Signalweg sowie Integrin β4.

Ergebnisse

Eine erhöhte Expression von CDK5 in Patientinnen mit Brustkrebs korreliert signifikant mit einem kürzeren Gesamtüberleben und rezidivfreien Überleben. Die Analyse der TCGA-Daten deckte höhere Level von CDK5 in Brustkrebsgewebe im Vergleich zu gesundem Gewebe auf. Die Ergebnisse der Western blots weisen auf eine Überexprimierung in den Brustkrebszelllinien MDA-MB-231, BT-474 und ZR-75 hin. Es zeigt sich zudem, dass die Überexpression von CDK5 das Tumorwachstum, die Proliferation und Migration von malignen Brustzellen in vitro signifikant

erhöht. Die spezifische und unspezifische Inhibition von CDK5 führt hingegen zu einer Reduktion des Tumorwachstums, der Proliferation und der Migration im Vergleich zur CDK5-Überexpression. Zusätzlich wurde gezeigt, dass CDK5 den mTOR-Signalweg aktiviert und Integrin β4 heraufreguliert.

Fazit

Zusammengefasst legen unsere Ergebnisse eine neue Bedeutung der CDK5 und ihrer klinischen Relevanz in der Progression von Brustkrebs dar. Zusätzlich scheint Integrin β4 eine karzinogene Rolle im Brustkrebs zu spielen und wird möglicherweise von CDK5 reguliert. Dies macht CDK5 zu einem potentiellen neuen Zielprotein, insbesondere im triplenegativen und HER2-positiven Brustkrebs.

Abstract

Aim of study

Although the overall survival and relapse-free survival of breast cancer patients has increased in the past few decades due to novel treatment methods, the therapeutic options especially for the triple-negative and HER2-positive subtypes are limited. Multiple studies identified an important role of cyclin-dependent kinases in different cancer types. In this study we investigated the pro-cancer effect of cyclin-dependent kinase 5 (CDK5) to understand its possible role in the tumor growth, migration and proliferation of breast cancer cell lines.

Methods

The overall survival (n = 1402) and the relapse-free survival (n = 3951) of patients with breast cancer depending on the expression of CDK5 was analyzed using the software *KM plotter*. To evaluate the CDK5 expression of tumor tissue compared to non-tumor tissue, The Cancer Genome Atlas (TCGA) data set of breast cancer patients (n = 536) was investigated. Additionally, western blotting was used to identify the expression levels of CDK5 in different breast cancer cell lines *in vitro*. We then focused on the CDK5 overexpressing cell lines MDA-MB-231 and BT-474 to investigate the role of CDK5 in tumor growth, proliferation and migration. For this, we used soft agar, sulforhodamine B and wound healing assays. These assays were performed in three different setups (group 1: CDK5 overexpression with plasmid; group 2: CDK5 silencing with siRNA; group 3: unspecific inhibition of several CDKs using Roscovitine). Through western blotting, connections between CDK5 and the oncogenic signaling mTOR pathway as well as integrin β4 were investigated.

Results

High expression of CDK5 correlates with a poorer overall survival and relapse-free survival rate of breast cancer patients. The analysis of the TCGA data set also reveals higher levels of CDK5 in breast cancer tissues compared to non-tumor tissues. The western blotting indicates an overexpression of CDK5 in breast cancer lines MDA-MB-231, BT-474 and ZR-75. The assays show that CDK5 overexpression leads to increased tumor growth, cell proliferation and migration *in vitro*. Specific and unspecific CDK5 inhibition show opposite effects compared to CDK5 overexpression. Additionally, we observed that CDK5 activates the mTOR pathway and upregulates integrin β4.

Conclusion

In summary, the results show a new role of CDK5 and its clinical relevance in breast cancer progression. Additionally, integrin β4 also seems to play an important role in breast cancer mediated by CDK5. This makes CDK5 a potentially new target in the treatment of breast cancer, especially for triple-negative and HER2-positive breast cancer subtypes.

1 Introduction

Cancer is one of the main health issues that humans have been facing for decades. In 2012, about 14.1 million people were diagnosed with cancer worldwide and in the same year 8.2 million cancer-related deaths were recorded (1). These numbers may rise even further to 20.3 million new cases of cancer and 13.2 million deaths by 2030, which makes cancer a major health problem that needs to be overcome (2,3).

Of these 14.1 million people, about 478,000 were diagnosed with cancer in Germany alone, including breast cancer as one of the most common types together with bowel, lung and prostate cancer (4). Although the understanding and treatment options of breast cancer have improved over the past few decades, the treatment of advanced and metastatic breast cancer is still challenging.

1.1 Breast cancer

Breast cancer is the most commonly diagnosed cancer type in women and second most common cancer diagnosed after lung cancer in women in developed countries (5). 1.7 million people worldwide were diagnosed with breast cancer in 2012, which accounts for 12 % of all new cancer cases and 25 % of all cancers in women (5). In Germany 70,000 women were diagnosed with breast cancer in 2012. One in eight women are diagnosed with breast cancer during their lifetime. The risk for men to develop breast cancer is about 1 in 1,000. They make less than 1 % of all breast cancer cases. The incidence of breast cancer has increased since 1990 in developed countries and the mortality rates have decreased, possibly due to advanced treatment and early detection (4).

Several modifiable and non-modifiable risk factors are associated with the development of breast cancer (6). Two of the main risk factors for breast cancer are female gender and age. 50 % of women are diagnosed with breast cancer between the age of 50 and 69 (7). Family history and genetic factors also increase the risk of breast cancer, but only 5 to 10 % of patients show genetic mutations. BRCA1 and BRCA2 mutations are identified in 3-8 % of all breast cancer patients. The tumor suppressors BRCA1 and BRCA2 are responsible for the repair of double-stranded DNA using homologous recombination (8). Breast cancer patients with BRCA1 mutation tend to develop high grade and triple-negative breast cancer. They also have a 40 % risk for ovarian cancer and a higher chance to develop colon, pancreatic and prostate cancer (6). BRCA2 mutations are also associated with high-grade tumors and develop estrogen receptor positive, progesterone receptor positive and HER2-negative tumors. They have a 10 % risk for ovarian cancer and a higher chance to develop pancreatic, prostate cancer, gastric and gallbladder cancer as well as

melanoma (6). More rare mutations associated with breast cancer are TP53, PTEN and CDH1. Hormonal factors play also an important role in developing breast cancer, especially including endogenous and exogenous estrogens. Lower age of menarche, higher age of menopause, late first pregnancy and nulliparity are associated with the development of breast cancer. In postmenopausal women the use of hormone replacement therapy correlates with the development of breast cancer. The use of oral contraceptives is associated with an increased risk of breast cancer, when used for more than 10 years (9). Additionally, lifestyle factors like smoking, alcohol, unhealthy diet and obesity are known to be involved in the development and prevention of breast cancer.

Benign breast diseases are grouped into proliferative and non-proliferative diseases. Proliferative lesions with atypia show a higher risk of turning into cancer. The density of breast tissue also might be associated with the development of breast cancer (6). Breast cancer subtypes are grouped into noninvasive (*in situ*) and invasive carcinomas. Most of them are adenocarcinomas and can be divided further into ductal and lobular carcinomas. Less common subtypes are mucinous, medullary, tubular and inflammatory breast cancer types. About 70% of patients diagnosed with breast cancer have an invasive ductal carcinoma (10). Breast tumors are detected by self-examination, routine mammography screening or ultrasound. Patients can show different symptoms, especially in advanced tumor stadiums like changing breast size or shape, retractions, redness, peau d´orange or ulcerations (10). The treatment of breast cancer depends on the stage, which is defined by tumor size, lymph node involvement and distant metastasis (10). Additionally, the identification of the molecular subtypes is becoming more and more important. The five different subtypes are clinically relevant and influence the prognosis as well as the treatment.

The treatment of breast cancer includes surgery, chemotherapy, radiation and targeted as well as hormone therapy (10). Although the overall survival and relapse-free survival rates increased in the past few decades due to the novel treatment methods, therapeutic options especially for advanced and the triple-negative as well as HER2-positive subtypes of breast cancer are not yet satisfying (11). Therefore, identification of new targets for breast cancer therapies is becoming more and more important. Currently, efforts are in place to develop personalized therapies and to improve surveillance strategies for groups at higher risk.

1.2 Cyclin-dependent kinases and cancer

Kinases are one of the most important targets for cancer treatment, since they are involved in different cellular processes (12). As enzymes, they phosphorylate proteins at different amino acid residues leading to enhanced activity or inactivity of proteins (13). So far a number of kinase groups have been identified such as serine/threonine kinases or tyrosine kinases (14).

Cyclin-dependent kinases (CDKs) belong to the group of proline-directed serine-threonine kinases. Their activity depends usually on their regulatory subunits, known as cyclins (15). There are about 26 genes in humans encoding 21 CDKs and five CDK-like kinases (CDKLs), a subgroup of the CDKs (16). In general, CDKs are grouped into the "classical" CDKs, proteins with a cyclin-binding site (PFTAIRE and PCTAIRE proteins) and proteins, which are related to the CDKs (like CDC2L or CCRK). CDKs form the foundation of our cell cycle clock by controlling the cellular G1, S, G2 and M phase. The so called interphase CDKs CDK2, CDK4 and CDK6 with Cyclins E/A and D as well as the mitotic CDK1 with Cyclins A/B regulate the cell cycle directly (17). Next to their well-known role in cell division and transcription, they are also involved in other cellular processes like DNA damage repair, proteolytic degradation, epigenetic regulation, stem cell self-renewal, metabolism regulation, spermatogenesis and neuronal functions (18). As multifunctional proteins, the deregulation and overexpression of CDKs and cyclins play a major role in the development and progression of cancer (19). Current investigations of these kinases are ongoing to identify new targets in cancer therapy (12).

Deregulation of CDKs can be caused by gene amplification, protein overexpression, alternative splicing and expression of abbreviated cyclins (20). Additionally, mistimed expression, mislocalization and inhibited inactivation of CDKs or cyclins can result in the development of cancer (21,22). Different inhibitors have been studied in the past few decades to treat cancer, including unspecific ones like flavopiridol, olomoucine and roscovitine as well as specific inhibitors such as fascaplysin, ryuvidine or purvalanol (23,24). The first generation CDK inhibitors like flavopiridol and other so called pan-CDK inhibitors with poor selectivity caused dose-limiting side effects like diarrhoea, myelosuppression, anaemia and nausea (25). Second generation CDK inhibitors were developed and tested in multiple studies, showing promising effects in different malignancies.

In glioma a combination of flavopiridol and temozolomide has been shown to lead to higher cytotoxic effects *in vitro* and *in vivo* (26). The first generation pan-CDK inhibitor dinaciclib also showed antiproliferative effects in human glioma cells and led to increased cell death in combination with the pan-Bcl-2 inhibitorABT-737 (27). In another preclinical study, dinaciclib

was shown to be efficient for the treatment of T-cell acute lymphoblastic leukemia (T-ALL) by decreasing levels of pro-survival proteins like survivin, cyclin T1 and c-MYC (28). Additionally, a beneficial role of CDK inhibitors was shown in different hematological diseases such as acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML); and different lymphoma like anaplastic large cell lymphoma, mantle cell lymphoma and B-cell lymphoma (29–31). Furthermore, the inhibition of CDKs in solid tumors is currently being investigated. The inhibition of CDK4 and CDK6 showed significant results in preclinical and clinical studies of bladder (32), colorectal (33), glioblastoma (34,35), hepatocellular (36), lung (37), melanoma (38), multiple myeloma (39), ovarian (40,41), pancreatic (42), prostate (43) and especially breast cancer (44).

Inhibitors of CDK4/CDK6 are currently under investigation for single and combined therapies in early and advanced breast cancer. Three of these selective inhibitors have been approved for treatment to date (45). In all the studies, hormone-receptor positive and HER2-negative breast cancer patients were included. In a phase II study PALOMA-1, postmenopausal women with locoregional relapse and metastatic breast cancer received palbociclib and the aromatase inhibitor letrozole, or letrozole only. The combination of palbociclib and letrozole showed a significant improvement of the progression-free survival and object response rate (46).

In the following phase III study PALOMA-2 the combination of palbocicib and letrozole showed similar results as first-line treatment in advanced breast cancer (47). The combination of the selective estrogen degrader fulvestrant and palbocicib in PALOMA-3 was identified as a new treatment option for patients with advanced breast cancer that showed a relapse or progression after or during their treatment (48).

The CDK4/CDK6 inhibitor abemaciclib was tested in different clinical studies, including the MONARCH trials. In the phase III study MONARCH 2, 669 patients received abemaciclib and fulvestrant or a placebo and fulvestrant. Patients selected for this study had been previously treated with neoadjuvant or adjuvant therapy and still showed progression of their disease or were receiving an endocrine therapy as first-line treatment. The combination of abemaciclib and fulvestrant significantly increased rates of progression-free survival and object response rates (49).

In the phase III study MONARCH 3, 493 postmenopausal hormone-receptor positive and HER2-negative breast cancer patients received either abemaciclib or placebo and either aromatase inhibitors anastrozole or letrozole as first-line treatment. The combination of abemaciclib with letrozole or anastrozole again improved progression-free survival and object response rates (50).

In the MONALEESA-2 study, ribociclib was used in patients as a first-line treatment for postmenopausal women with breast cancer. The combination of ribociclib with letrozole showed

similar results compared to other CDK4/6 inhibitors (51). In multiple other studies the new CDK4/6 inhibitors showed clinical benefits in estrogen receptor-positive/HER2-negative advanced breast cancer types as first- and second-line treatment.

1.3 Cyclin-dependent kinase 5

CDK5 is known as an unusual member of the CDK family, which is, unlike its other family members, not activated by cyclins or T-loop phoshorylation (15). Its kinase activity depends on the non-cyclin proteins p35 and p39 or their shortened counterparts p25 and p29 (52,53). Recently two new activators have been identified, Cyclin I and cyclin I-like (54,55). CDK5 is expressed in all human tissues, however it shows highest levels of expression and activity in neuronal tissue (56). It is mainly involved in regulating neuronal migration, axon guidance and synaptic transmission (57). In fact, CDK5 plays an important role for neuronal survival (58). Therefore, deregulation of CDK5 leads to different neurodegenerative diseases like Alzheimer´s disease, amyotrophic lateral sclerosis, Huntington´s disease and Parkinson (59–61).

Several studies revealed a new role of CDK5 in the development and progression of cancer. Gene amplification of CDK5 and its activators, as well as polymorphism in the CDK5 gene promoter region, were observed in cancer cells (62–65). Deregulation and increase of CDK5 is associated with a number of malignancies like head and neck squamous cell carcinoma (66) and hepatocellular carcinoma (67), as well as glioblastoma (68), breast (69–71), colorectal (72), lung (73–75), thyroid (76,77), ovarian (78), pancreatic (64,79,80) and prostate cancer (81–83). Additionally, increased levels of CDK5 were associated with poor clinical outcome in several tumors, including breast and lung cancer as well as pituitary adenoma (70,75,84). Surprisingly, lower levels of CDK5 implicate a poorer outcome in gastric cancer and longer metastasis-free survival in breast cancer patients (85–87).

A critical role of CDK5 in tumorigenesis was proposed in different studies (88). In medullary thyroid cancer CDK5 leads to cell proliferation by regulating the retinoblastoma (Rb)/E2F and the STAT3 pathway (89). The phosphorylation of STAT3 affects tumor growth in prostate cancer (83). CDK5 also phosphorylates the androgen receptor directly, thus stabilizing the receptor (81). It was shown that CDK5 is also involved in an angiogenic pathway targeting Notch-dependent endothelial cell proliferation (90). In hepatocellular carcinoma, CDK5 stabilizes hypoxia-inducible factor-1α (HIF-1 α). HIF-1 α is an important transcription factor involved in tumor angiogenesis, which is why the inhibition of CDK5 results in reduced angiogenesis (91). CDK5 is also involved in cell migration and invasion. CDK/p35 regulate migration in lung cancer through the human basic helix-loop-helix transcription factor achaete-scute homologue-1 (hASH1) (73).

In prostate cancer CDK5 was shown to be necessary for cell migration and invasion (82). CDK5 also affects cell migration in pancreatic cancer, where K-Ras enhances the activity of p25 and therefore CDK5 (64). CDK5 also influences epithelial-mesenchymal transition (EMT) by increasing TGF-β1 levels in breast cancer (70). EMT is a process in which cells modify their cell-cell-adhesion and their phenotype to invasive, mobile cells (92). Therefore, EMT plays a critical role in cancer progression and the metastatic behavior of cancer. Chemoresistance is another aspect influenced by CDK5. The upregulation of CDK5 and its activator cyclin I lead to cisplatin resistance in cervical cancer (93). Knockdown of CDK5 increased the sensitivity to paclitaxel in ovarian cancer, predominantly by modulating AKT and inducing a G1 arrest (78).

1.4 Integrin β4

Integrins are transmembrane glycoproteins which mediate cell-cell and cell-matrix interactions (94,95). They belong to the family of heterodimeric cell adhesion receptors and are composed of an α and β subunit (96). 18 α-subunits and 8 β-subunits have been identified. Integrins are regulators of cell proliferation, survival and migration (97). In addition to their role in physiological processes, they are also known to be involved in tumor initiation, cancer progression and metastasis (98,99).

Integrin β4 (ITGB4) is one of the subunits (100). It has a long cytoplasmic domain and forms a heterodimer with the α6 subunit. Integrin $\alpha_6\beta_4$ was shown to be involved in tumor progression by associating different pathways. Integrin $\alpha_6\beta_4$ and ErB-2 overexpression result in higher levels of cell proliferation and invasion (101). It was also shown to activate other growth factors like c-Met, Ron, LPA1, LPA2 (102–105) and regulates the activation of AKT/PKB in cells with mutated p53 (106). Furthermore, it interacts with the Ras-MAPK pathway through Shc (107). ITGB4 is also involved in EMT, probably indirectly by increasing levels of proteins that enhance EMT such as protein S100 (108,109).

ITGB4 itself was shown to be of importance in different malignancies. The overexpression of ITGB4 was described in several cancer types, like breast, bladder (110–112), cervical (113–115), head and neck (116–118), lung (119–122), thyroid (123–125) and pancreatic cancer (126–130). Higher levels of ITGB4 were found to be associated with poorer prognosis in breast cancer (131). ITGB4 might also play a role in the development of lung metastases in breast cancer, probably by adhesion to the chloride channel accessory protein human CLCA2, which is known as a key protein in epithelial differentiation (132). ITGB4 may also be a possible biomarker for

mesenchymal carcinoma cells in triple-negative breast cancer, which are known to be more resistant to cancer treatments (133).

1.5 PI3K/ AKT / mTOR signaling pathway

Molecular signaling pathways play an important role in the understanding of cancer progression. Specific signaling receptors are activated by external or internal stimuli that bind to them, leading to the phosphorylation of different downstream proteins. Different signaling pathways influence each other and regulate cell growth, proliferation, migration and survival.

One of the major pathways involved in cancer progression is the PI3K/ AKT/ mTOR pathway (134). For a better understanding of this complex pathway, an overview is shown in Figure 1. Different transmembrane tyrosine kinase growth factor receptors activate the PI3K/ AKT/ mTOR pathway by phosphorylation of phosphoinositide 3-kinase (PI3K) (135). PI3K phosphorylates the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) leading to the downstream activation. The inhibition of this phosphorylation is regulated through the phosphatase and tension homolog protein (PTEN), which is an upstream key regulator of the pathway (136). PTEN itself acts as a tumor suppressor and is known to be mutated in different cancer types (137). PIP3 can activate phosphoinositide-dependent kinase 1 (PDK1) and AKT directly (138,139). AKT, also called protein kinase B, is known to have three isoforms (Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ), which are expressed in different tissues (140,141). AKT has two phosphorylation sites: PDK1 phosphorylates AKT at a threonine residue at amino acid position 308, and mechanistic target for rapamycin complex 2 (mTORC2) phosphorylates AKT at a serine residue at position 473. Phosphorylation of both sides leads to activation of the TSC1/TSC2 (tuberous sclerosis complex 1 and 2) by AKT, which inactivates the Ras homolog enriched in brain protein (RHEB) by causing GTP hydrolysis (142). By phosphorylation of TSC2 through AKT this complex is inhibited, and therefore activates mTORC1 by making the phosphorylation of mTORC1 through RHEB possible. MTORC1 and mTORC2 are both multiprotein complexes and are activated by intracellular stimuli as described above and extracellular stimuli like energy status or oxidative stress (143–145). Both complexes have different functions. MTORC1 is involved in mRNA translation and protein synthesis. Synthesis is increased for a number of proteins by activation of mTORC1 like eukaryotic initiation factor 4E (4EBP1) and ribosomal S6 (S6) or HIF-alpha (146–148). MTORC2 on the other hand regulates cell survival, migration and metabolism (149,150).

An aberrant function of the PI3K/ AKT/mTOR pathway can not only lead to cancer, but can also cause obesity, diabetes and cardiovascular as well neurodegenerative diseases (151–153). New inhibitors are being developed to target different proteins of this pathway. PI3K-targeted drugs like the mTOR inhibitors deforolimus, everolismus or temsirolimus are under investigation and partially approved for renal cell carcinoma (154), neuroendocrine tumors (155), and giant cell astrocytoma (156), as well as pancreatic (157,158) and breast cancer (159). Other inhibitors of this pathway such as PI3K-inhibitors or AKT inhibitors were tested as single or combined treatment options in clinical studies to improve the survival rates of patients and identify their new role as potential anti-cancer therapeutics (160).

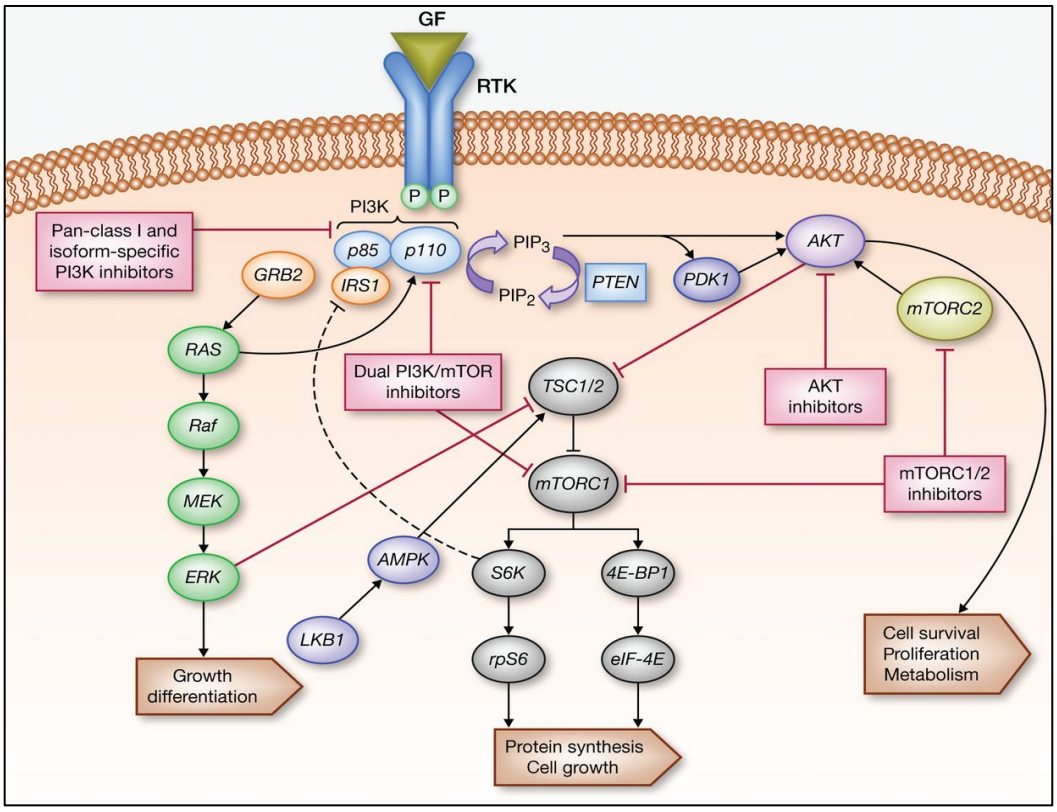

Figure 1: Overview of PI3K/AKT mTOR pathway. The overview shows different steps of the PI3K/AKT mTOR pathway and its possible inhibitors. Figure taken from: Rodrigo Dienstmann, Jordi Rodon, Violeta Serra and Josep Tabernero, Picking the Point of Inhibition: A Comparative Review of PI3K/AKT/mTOR Pathway Inhibitors, Molecular Cancer Therapeutics, 2014.

1.6 Aim of work

Fortunately, the development of new breast cancer drugs like Trastuzumab or Tamoxifen has decreased the mortality rate and increased the overall relapse-free survival of breast cancer patients. However, much about this elusive disease is still unknown. This is why identifying new targets in breast cancer and understanding the basics of the development of breast cancer is fundamental.

In this study we aimed to investigate the role of CDK5 in breast cancer. We analyzed the following areas in this study:

- Evaluating the effect of CDK5 on overall and relapse-free survival in breast cancer patients
- Expression levels of CDK5 in breast cancer cell lines
- Role of CDK5 in tumor growth, cell proliferation and migration
- Relationship between CDK5, integrin β4 and the AKT/mTOR pathway

2 Materials and methods

The experiments of this study were performed in the department of surgery and cancer at Imperial College London in the laboratory of professor Justin Stebbing.

2.1 Materials

The materials of this study can be found in Tables 1 to 17.

Table 1 Equipment

Equipment	Supplier
Beckmann Du 530 Life Science	Harlow Scientific
Cell incubator	Thermo Fischer Scientific
Centrifuge	Thermo Fischer Scientific
Digital Scale	Sartorius group
Gyro-rocker SSL3	Stuart
Heat blocker	Grant Instruments
Hemocytometer	Thermo Fischer Scientific
Hypercassette	GE Healthcare Life Sciences
Light Microscope	Optika
Multichannel pipette (ErgoOne®)	Starlab
OPTIMAX X-Ray Film Processor	Protec GmbH & Co.
Pipette controller	Swiftpet pro
Pipettes (Discovery comfort)	Starlab
Roller mixer SRT9	Stuart
Vortex machine – Genie 1	Scientific industries
VWR 3600 Orbital Shaker	Marshall Scientific LLC.
Quantification machine	Thermo Fischer Scientific
Tetra Vertical Electrophoresis Cell	Bio-Rad Laboratories GmbH
Transfer machine CS-500 V	Cleaver Scientific Ltd.

Table 2 Consumption items

Material	Supplier
6-well-cell-culture-cluster	Corning incorporated
96-well-cell-culture-cluster	Corning incorporated
Cell lifter	Corning incorporated
Cover slips	Corning incorporated
Eppendorfs	Star lab
Falcon (15 ml, 50 ml)	Thermo Fischer Scientific
Flasks (T75, T175)	Thermo Fischer Scientific
Filter tips (10 µl ,20 µl, 200 µl,1000 µl)	Star lab
Hybond ECL nitrocellulose membrane	GE Healthcare
Stripette (5 ml, 10 ml, 25 ml)	Corning Incorporated costar

Tubes	Thermo Fischer Scientific

Table 3 List of chemical products and reagents

Chemical products and reagents	Recipe/ supplier
Agarose	Sigma
30% (w/v) Acrylamide; 0,8% (w/v) Bis-Acrylamide Stock solution	
APS	Bio-Rad
Bradford Dye Reagent	Bio-Rad
β-Mercaptoethanol	Sigma
Dimethyl sulfoxide	Sigma
Dulbecco´s modified Eagle medium	Sigma
Distilled water	Sigma
Dried skimmed milk	Marvel original
ECL detection reagent	GE Healthcare
EDTA	Sigma
Ethanol	Marvel original
FCS	GE Healthcare
Glycin	Sigma
HiPerFect transfection reagent	Quiagen
L-glutamine	Thermo Fischer Scientific
Lipofectamine 2000 Reagent	Invitrogen Life Technologies Ltd.
Methanol	Sigma
Opti-Mem I reduced Serum Medium	Gibco
PBS	Sigma
Penicillin/ Streptomycin	Thermo Scientific
Phosphateinhibitor	Thermo Scientific
Proteininhibitor	Thermo Scientific
Radioimmunoprecipitation assay buffer	Sigma
Rainbow marker	Thermo Scientific
Roscovitine	Cell signalling, 9885
Trizol reagent	Invitrogen
TEMED	Thermo Scientific
Trypan blue	Thermo Scientific
Trypsin	Thermo Scientific

Table 4 List of buffers and solutions

Buffers and solutions	Recipe
Blocking buffer	Skimmed milk (5%), 10x TBS-Tween
Cell lysis buffer	RIPA buffer, proteininhibitor and phosphateinhibitor
1M Tris-HCl	60.5 g Tris in total of 500ml ddH20 and adjusted to desired pH with pure HCl
5x Loading buffer	0.25M Tris-HCl, pH 6.8; 15% SDS; 50% glycerol; 25% beta-mercaptoethanol; 0.01% bromophenol blue

10x SDS-PAGE Running buffer	10g SDS; 30.3g Tris, 144.1g glycine dissolved in 1l of ddH20
0,4% Sulforhodamine B	0.4% w/v SRB powder dissolved in 1% acetic acid
10x TBS	24.23g Trizma HCl; 80.06 g NaCl dissolved in 1l of ddH20 and adjusted pH to 7.6 with pure HCl
TBS-Tween	100ml of TBS 10x; 900ml ddH20; 1ml Tween® 20 (BDH)
10x Transfer buffer	Tris Base 5.8g; Glycine 2.9g; Methanol 200ml and make up to 1l with ddH20

Table 5 Mammalian cell lines

Cell line	Morphology	Classification	Immunoprofile
BT-474	Epithelial	Luminal B	ER+, PR+, HER2+
BT-549	Epithelial	Basal	ER-, PR-, HER2-
MCF-7	Epithelial	Luminal A	ER+, PR+, HER2-
MDA-MB-231	Mesenchymal	Basal	ER-, PR-, HER2-
SK-BR-3	Epithelial	HER2	ER-, PR-, HER2
T47D	Epithelial	Luminal A	ER+, PR+, HER2-
ZR75-1	Epithelial	Luminal A	ER+, PR+/-, HER2+

Table 6 Tumorigenicity of mammalian cell lines

Cell line	Tumorigenicity
BT-474	human cell line, derived from solid, ductal carcinoma
BT-549	human cell line, derived from solid, ductal carcinoma
MCF-7	human cell line, derived from metastatic site (pleura) of an adenocarcinoma
MDA-MB-231	human cell line, derived from metastatic site (pleura) of an adenocarcinoma
SK-BR-3	human cell line, derived from metastatic site (pleura) of an adenocarcinoma
T-47D	human cell line, derived from metastatic site (pleura) of an adenocarcinoma
ZR-75-1	human cell line, derived from metastatic site (ascites) of ductal carcinoma

Table 7 Normal growth, antibiotic-free and freezing medium

Medium	Recipe
Antibiotic-free medium	500 ml DMEM 10% FCS 1% L-glutamine (2mM)
Normal growth medium	500 ml DMEM 10% FCS 1% L-glutamine (2mM) 1% Penicillin (50 units/ ml) 1% Streptomycin (50 μg/ml)
Freezing medium	Culture growth medium 5% DMSO

Table 8 Transfection medium and reagents

Transfection medium	Transfection reagent
Antibiotic-free medium (recipe in Table 4)	HiPerfect Transfection Reagent
Opti-MEM I Reduced Serum Medium	Lipofectamine 2000 Reagent

Table 9 Plasmid and control

Gene	Supplier
Empty vector	Addgene
CDK5	Addgene

Table 10 siRNA and control

siRNA	Supplier
CDK5 siRNA 9	Quiagen, SI00604674
CDK5 siRNA 10	Quiagen, SI00604681
siControl	Quiagen, SI03650318

Table 11 Transfection recipe gene silencing

Reagent	6-well plate	10-cm dish
CDK5 siRNA (20nM)	4 µl	32 µl
Antibiotic-free medium (recipe in Table 4)	184 µl	1472 µl
Hiperfect Transfection reagent	12 µl	96 µl

Table 12 Transfection recipe overexpression

Reagent	6-well plate	10-cm dish
Plasmid	1 µg	5 µg
Opti-MEM I Reduced Serum Medium (Opti-MEM)	200 µl	1000 µl
Lipofectamine	3 µl	15 µl

Table 13 Roscovitine treatment

Reagent	6-well plate	10-cm dish
Roscovitine	1 µg	5 µg
DMSO	200 µl	1000 µl

Table 14 Recipe for Western blot gels

Gel	Components	Volume of components (ml) per gel mold volume of 5 ml for 10 % gel
Running gel (5 ml)	dH2O	2.3

	30% acryl-bisacrylamide mix	1.3
	1.5 M Tris (pH 8.8)	1.3
	10% SDS	0.05
	10% ammonium persulfate	0.05
	TEMED	0.003
Stacking gel (2 ml)	dH2O	1.4
	30% acryl-bisacrylamide mix	0.33
	1.5 M Tris (pH 8.8)	0.25
	10% SDS	0.02
	10% ammonium persulfate	0.02
	TEMED	0.002

Table 15 Primary antibodies

Antibody	Species	Concentration	Supplier
CDK5	rabbit	1:500	Cell signalling, 2506
Integrin-β4	rabbit	1:800	Cell signalling, 4707
mTOR	rabbit	1:500	Cell signalling, 2972
Phospho-AKT	Mouse	1:500	Cell signalling, 4051
Phospho-mTOR	rabbit	1:500	Cell signalling, 2971
Phospho-S6	rabbit	1:500	Cell signalling, 2211
S6	rabbit	1:500	Cell signalling, 2217
Tubulin	rabbit	1:3000	Abcam, 18251

Table 16 Secondary antibodies

Antibody	Concentration	Supplier
Polyclonal goat anti-mouse IgG/ HRP	1:3.000	DAKO (Cambridge, UK) P0447
Polyclonal goat anti-rabbit IgG/ HRP	1:3.000	DAKO (Cambridge UK, P0048

Table 17 Computer program

Name	Supplier
ImageJ	National Institutes of Health, Bethesda, USA
GraphPad Prism	GraphPad Software, La Jolla, USA
Microsoft plate reader	Microsoft Corporation, Redmond USA
Microsoft Office 365	Microsoft Corporation, Redmond USA

2.2 Methods

2.2.1 Culturing and passaging cells

Breast cancer cell lines were kept in a tissue culture room under aseptic conditions. To ensure a sterile environment, all the work with cancer cells was done under a laminar flow hood. The gloves, workspace and the flasks were regularly cleaned with 70% ethanol.

Human breast cancer cell lines were taken from a cryopreservation stock of the lab. All cells were incubated in T75 (10 ml) or T175 (20 ml) flasks in a humidified atmosphere with a controlled CO_2 level of 5% and 95% relative humidity at a stable temperature of 37 °C.

Cell lines were used until a maximum of thirty passages to prevent the cells from genetic drift. A microscope was used to check the confluency, vitality and possible contamination of the cells daily. Change of cell media was performed every second or third day.

The cells were split to maintain confluency of about 90%. The media and ethylenediamine tetraacetic acid (EDTA) supplemented with 10% Trypsin (EDTA-T) were heated to room temperature thirty minutes before use. To passage the cells media was aspirated and cells were washed two to three times with room temperature phosphate buffered saline (PBS).

Depending on the flask, 2 ml (T75) or 4 ml (T175) of EDTA-T was added to the cells. The cells were then placed in the incubator at 37 °C for five minutes in order to detach. After checking the cells under the microscope to make sure they were detached, the same amount of normal growth media as EDTA-T was added into the flasks. The cells were pipetted into a 15 ml falcon tube. The cells were centrifuged at 1000 rpm for three minutes. After the centrifugation, the supernatant was aspirated, and the pellet was resuspended with normal growth media. The cells were reseeded into a new flask and fresh media was added.

2.2.2 Counting and plating cells

For experiments, cells were prepared as described above. After adding media to EDTA-T, 10 µl of the suspension was added to an Eppendorf tube with 10 µl Trypane blue. 10 µl of this suspension was taken out and pipetted into the edge of the hemacytometer counting chamber. Cells in and touching the lines were counted in the four corners as well as the central square of the hemacytometer. The desired number of cells was added to each well of a 6-well plate. Media was filled up to 2 ml in the well.

2.2.3 Freezing and defrosting cells

For freezing cells, media was removed from flasks and cells were trypsinized as described in 2.2.1. Cells were transferred to a 15 ml falcon tube and were counted as described in 2.2.2. After centrifuging the cells, they were resuspended in freezing media creating a cell suspension of $1x10^6$ cells per ml. 1 ml of cells was added into storage vials and cells were transferred to -20 °C for one hour. The cells were stored at -80 °C for long-term storage.

2.2.4 Cell transfections and treatment

2.2.4.1 Gene silencing

For siRNA transfection, cells were plated in 6-well plates (150.000 cells/ well), 96-well plates (MDA-MB-232: 5 x 10^3 cells/ well; BT-474: 8 x 10^3 cells/ well) or 10 cm dishes (1,000,000/ dish). Cells were allowed to attach overnight in the incubator. On the following day, the specific CDK5 siRNA 9 or 10 (20 nM) was prepared in Opti-MEM and vortexed. After an incubation time of five minutes at room temperature Hiperfect transfection reagent was added to the solution and vortexed again. The resulting solution was incubated at room temperature for 15 minutes. The media of the cells was then switched to antibiotic-free medium. The siRNA solution was then added drop-wise to each well. The cells were transfected for 72 hours before being used for experiments.

2.2.4.2 Overexpression

Cells were seeded in 6-well plates or 10 cm dishes as described before. Plasmid DNA was prepared in Opti-MEM. At the same time, Lipofectamine 2000 was prepared with an equal amount of Opti-MEM. Each solution was vortexed and incubated at room temperature for five minutes. After that, the solutions were mixed to form a master mix and incubated again for 30 minutes at room temperature. The master mix was then added drop-wise to the cells and the plates were gently shaken. The cells were then incubated for 24 hours with transfection solution before proceeding to the experiments.

2.2.4.3 Cell treatment with roscovitine

For treatment with roscovitine, cells were plated in 6-well plates or 10 cm dishes as described before. Roscovitine was added to DMSO and then added drop-wise to the cells.

2.2.5 Western blotting

2.2.5.1 Extracting proteins

Cells were placed on ice and washed three times with pre-cooled PBS. The PBS was aspirated, 50 µl full RIPA buffer was added, and the cells were scraped from the plates. After this, the cells were transferred to cooled Eppendorf tubes, vortexed and left on ice for 15 minutes. They were then centrifuged at 4 °C for 20 minutes at 13,000 rpm. The supernatant was transferred into a new Eppendorf tube and the pellet was discarded.

2.2.5.2 Quantifying protein

A Bradford assay was used to quantify the protein amount in the sample. A concentration-dependent colour change in the solution used for this assay reports the presence of protein in the sample. 1 µl of each sample was added into an Eppendorf tube with 1000 µl of Bradford buffer and incubated for five minutes at room temperature. 200 µl of this solution was transferred in two wells of a 96-well-pate to measure duplicates. The pure Bradford buffer was used as a control. A spectrophotometer was then used to quantify the protein amount by measuring the absorbance at a wavelength of 595 nm.

2.2.5.3 Loading, transfer and blocking

Gels with 10% polyacrylamide were used for SDS-gel-electrophoresis (Table 14). 50 µg of total protein was loaded onto the SDS-Page gel as well as 5 µl of rainbow markers. The gels were run for 20 minutes at 100 V for the stacking, and then 40-60 minutes at 140 V for the separation of the proteins. Electrophoresis was performed in running buffer.

The protein in the gels was then transferred onto a nitrocellulose membrane using a transfer machine. The filter papers and the nitrocellulose membrane with the gel were put in the transfer buffer. Nitrocellulose membranes were placed between three filter papers on each side and protein transfer was performed at a voltage of 15 V and 800 mA. Then the membranes were blocked in 5% non-fat milk in 40 ml TBS-Tween (TBS-T) for 1 hour.

2.2.5.4 Antibody staining and detection

The membranes were incubated in the primary antibody overnight at 4 °C on a roller mixer. After this the membrane was washed three times for five minutes each round in TBS-T. The membrane was then incubated at room temperature on a roller mixer for one hour in rabbit or mouse secondary antibody. The antibody description can be found in Tables 15 and 16.
The membrane was set on a surface and the enhanced chemiluminescence (ECL) was prepared. 1 ml of ECL was set on the membrane. The liquid was drained, and the membrane was set in a hypercassette. Then the film was developed in a dark room with the OPTIMAX X-Ray Film Processor.

2.2.6 Sulforhodamine B assay

The Sulforhodamine B (SRB) assay is used to determine the proliferation of cells by quantifying the cellular protein content (161). Breast cancer cells were seeded into a 96-well plate with a density of 5 x 10^3 cells/ well (MDA-MB-231) or 8 x 10^3 cells/ well (BT-474). Breast cancer cell lines were seeded into 96-well plate and kept in the incubator for a certain amount of time. For each sample six replicates were used. To stop the plated cells, 50 µl of 40% TCA were added to the wells and the plates were incubated for 1 hour at 4 °C. Afterwards the plates were rinsed 10 x with water. Plates were dried overnight and 50 µl of 0,4% Sulphorhodamine B solution was added to each well. Cells were stained at RT for 30 minutes. The staining was removed and the plates were rinsed with 1% acetic acid. The plates were kept at RT until they were dry. 150 µl of Tris was added to each well and put on a plate shaker for 30 minutes. After this, cells were analyzed in a plate reader at wavelength of 540 nm.

2.2.7 Soft agar assay

Several 5, 10 and 20 ml stripettes were warmed in the incubator for five to ten minutes to prevent the agarose from solidifying in the stripette. The pre-made and autoclaved 3% 2-hydroxyethyl agarose solution was microwaved at mid temperature two to three times for 15 seconds till the agarose became fluid. The bottle of agarose was kept in a container with pre-warmed distilled water as well as a 50 ml falcon tube containing a culture media in the laminar flow hood. 6 ml of the agarose solution were transferred into a new 50 ml falcon tube with the pre-warmed stripettes and 24 ml pre-warmed culture media were added. The tubes were gently inverted, and 2 ml of the

agarose-media solution was added into each well of a 6-well plate. The 6-well plates were incubated for 1 hour at 4 Celsius until it solidified. While preparing the cells, the plate was put for 30 minutes into the incubator.

While the plates were in the incubator, the cells were trypsinized and diluted to a concentration of $4x10^4$ cells/ ml. 120,000 cells were prepared in 3 ml. The agarose was again prepared as described before. 2 ml of the 3% 2-hydroxyethyl-agarose were transferred again into a 50 ml falcon tube and 8 ml of pre-warmed media were added to the 50 ml falcon tube. The mixture was gently converted. 2 ml of the cells were mixed in a 1:1 dilution with the agarose-media mixture. The plate was taken out of the incubator and 1 ml of the cell-agarose mixture was added gently with a 1000 ml pipette onto the bottom layer of the 6-well plate (triplicates). The 6-well plate was then incubated for 20 minutes at 4 °C to allow the top layer to solidify. The soft agar plate was put into the incubator for at least two weeks. The plates were controlled under the microscope every three days. Pictures were taken at day 10 and day 17. Colony diameter was measured with Image J. Different scale lengths were adjusted. Cells were normalized to untreated or controls.

2.2.8 Wound healing assay

Cells were seeded into a plate and silencing, overexpression or roscovitine treatment was performed. Cells were kept in the incubator until they reached 100% confluency. After this, the plates were taken into the laminar flow hood and the media was aspirated. Three different scratches were made into the monolayer of each well with a 200 µl tip and were washed two times with PBS. 2 ml culture media was added into each well and pictures were taken with a microscope.

2.2.9 Statistical analysis

To understand the clinical relevance of CDK5 the patient data set from kmplot.com was used as well as a TCGA data set of 536 breast cancer patients (162).

The following criteria were selected at kmplot.com (163):

- Gene: CDK5
- Auto select: best cut off
- Survival: relapse-free survival (RFS) or overall survival (OS)

For the statistical analysis of the assays we used the GraphPad Prism software. For evaluation of the significance t-tests and analyses of variance (ANOVA) were performed. A p-value of $< 0,05$ was defined as significant.

3 Results

The main focus of this study was to investigate the role of CDK5 in cancer progression. The clinical relevance of CDK5 was analyzed and different assays were used to evaluate its effect on tumor growth, proliferation and migration. Additionally, western blots were performed to examine the possible role of CDK5 related pathways for cancer progression.

3.1 Higher levels of CDK5 are associated with poorer clinical outcome

Firstly, the clinical relevance of CDK5 was analyzed using patient data from kmplot.com as described in 2.2.9. The measurements for the overall survival rate are based on data of 1,402 patients (Figure 2A). For the relapse-free survival, analysis was performed on data of 3,951 breast cancer patients (Figure 2B). The plots show two groups of breast cancer patients: low CDK5 expression and high CDK5 expression. The Kaplan-Meier curves suggest that higher levels of CDK5 are associated with shorter overall survival ($p = 0.027$; Figure 2A) and relapse-free survival ($p = 0.00035$; Figure 2B). Statistical analysis of the TCGA data set additionally shows significant CDK5 overexpression in tumor tissues compared to non-tumor tissues (Figure 2C).
In order to further analyze CDK5 expression in cancer cells, common human breast cancer cell lines were screened by use of western blotting. BT-474, ZR-75 and MDA-MB-231 breast cancer cell lines were found to have increased levels of CDK5 compared to other breast cancer cell lines (Figure 2D). In the following, we focused on two of these CDK5 overexpressing cell lines namely BT-474 and MDA-MB-231.

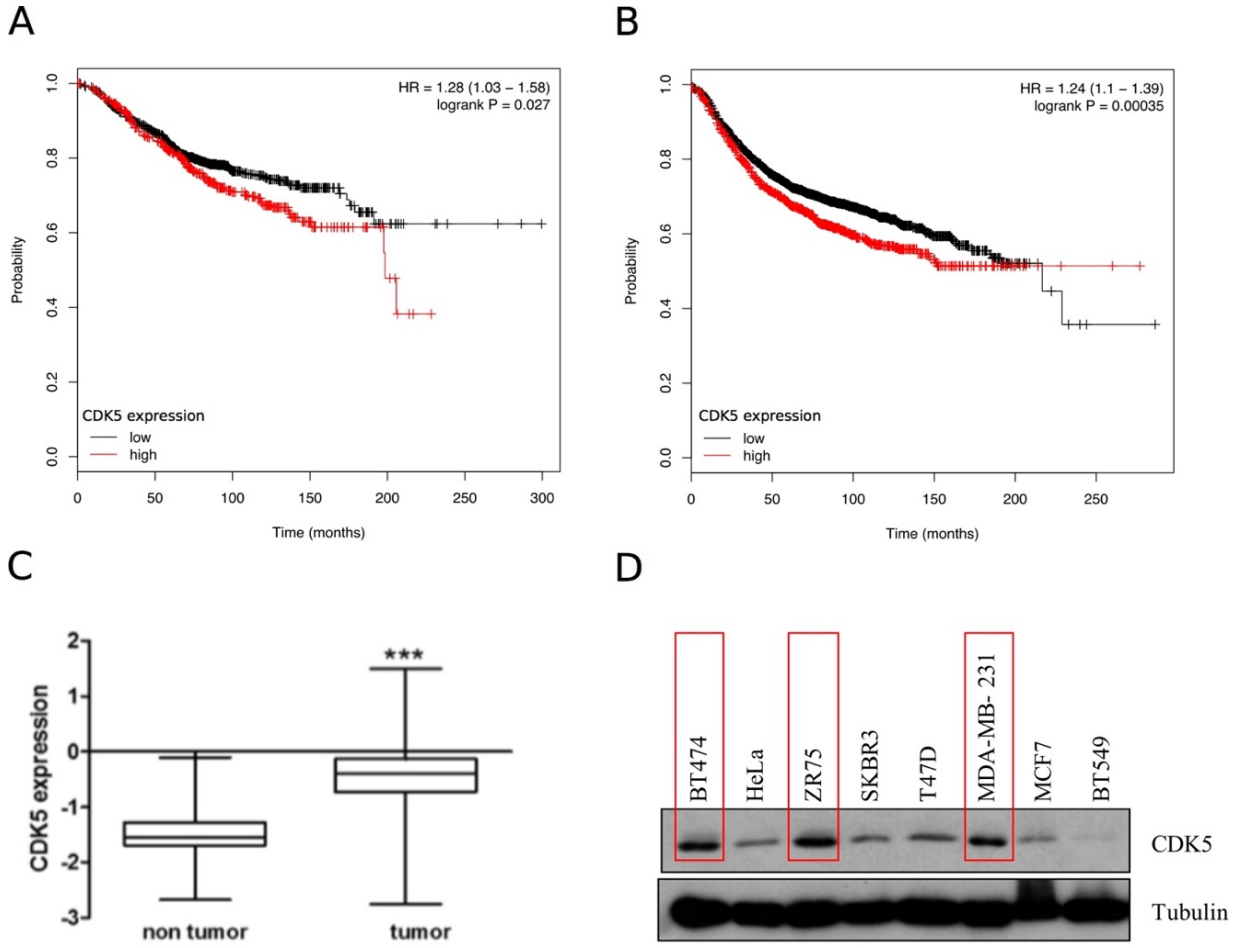

Figure 2: Kaplan-Meier analyses of overall and relapse-free survival. CDK5 is overexpressed in tumor tissue and in cell lines BT-474, MDA-MB-231 and ZR75. Analysis of patient data: Kaplan-Meier plots of overall **(A)** and relapse-free survival **(B)**. The survival rate as computed from the patient data is shown over a timespan of about 25 years. Patients were categorized into two groups, low and high CDK5 expression. **(C)** TCGA data set of 536 breast cancer patients comparing CDK5 expression levels between non-tumor and tumor tissue. **(D)** Human breast cancer cell lines were analyzed using western blotting. Increased levels of CDK5 are observed in BT-474, MDA-MB-231 and ZR-75 cells.

3.2 CDK5 is essential for tumor growth in vitro

To analyze the effect on tumor cell proliferation, soft agar and SRB assays were performed on BT-474 and MDA-MB-231 cells. Both cell lines were prepared in different CDK5 expressing setups. Firstly, CDK5 was overexpressed in the cells by using plasmid transfection. Overexpression of CDK5 was confirmed by subsequent western blot analysis. Secondly, CDK5 was silenced specifically with two different siRNAs. Lastly, the breast cancer cell lines were treated with

roscovitine, which is commonly used as a CDK5 inhibitor, but also inhibits CDK1, CDK2, CDK7 and CDK9 (21). Western blots for siRNA and roscovitine treated cells show consistent downregulation of CDK5.

3.2.1 Results of the soft agar assay

The soft agar assay is an important method to validate the anchorage-independent growth of cells on a solid surface. Cells were prepared as described in section 2.2.7. At the beginning and end of the soft agar assay, cell colonies were imaged, and colony sizes were measured. As can be seen in Figures 3-4, MDA-MB-231 and BT-474 cells with silenced CDK5 show consistently smaller colonies compared to the untreated cells. BT-474 cells treated with CDK5 siRNAs display greater growth reduction (ca. 50%) compared to BT-474 cells treated with roscovitine (only about 30%, see Figure 5-6). In MDA-MB-231 cells the opposite effect was observed, as roscovitine showed a greater reduction in tumor growth compared to the siRNA silencing of CDK5 (50% vs. 80%).
In the CDK5 overexpressing samples, larger cell colonies were observed compared to the control samples. An about fourfold increase in the diameter of the tumorsphere was observed in the CDK5 vector treated samples for both cell lines (see Figure 7-8).

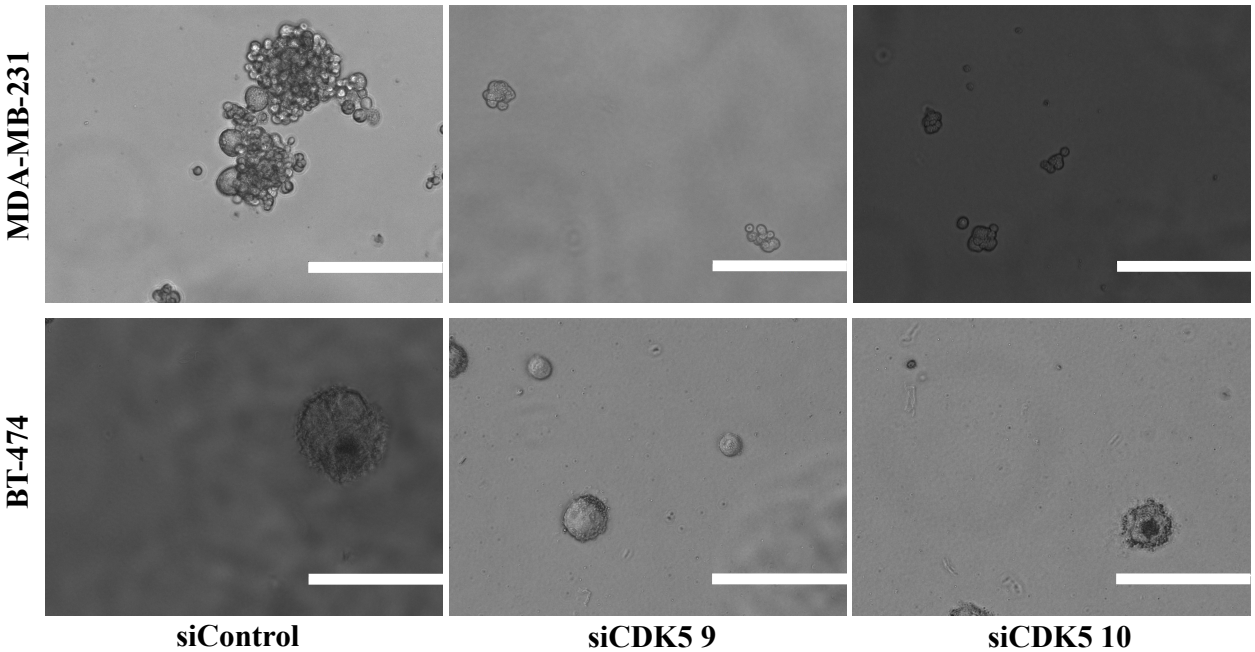

Figure 3: Effect of CDK5 silencing on MDA-MB-231 and BT-474 cells in soft agar assays. MDA-MB-231 and BT-474 cells were transfected with either a control siRNA (siControl), siCDK5 9 or siCDK5 10. A noticeable reduction of the colony size is observed upon CDK5

silencing. Images of representative colonies are shown, scale bar MDA-MB-231: 400 µm, scale bar BT-474: 200 µm.

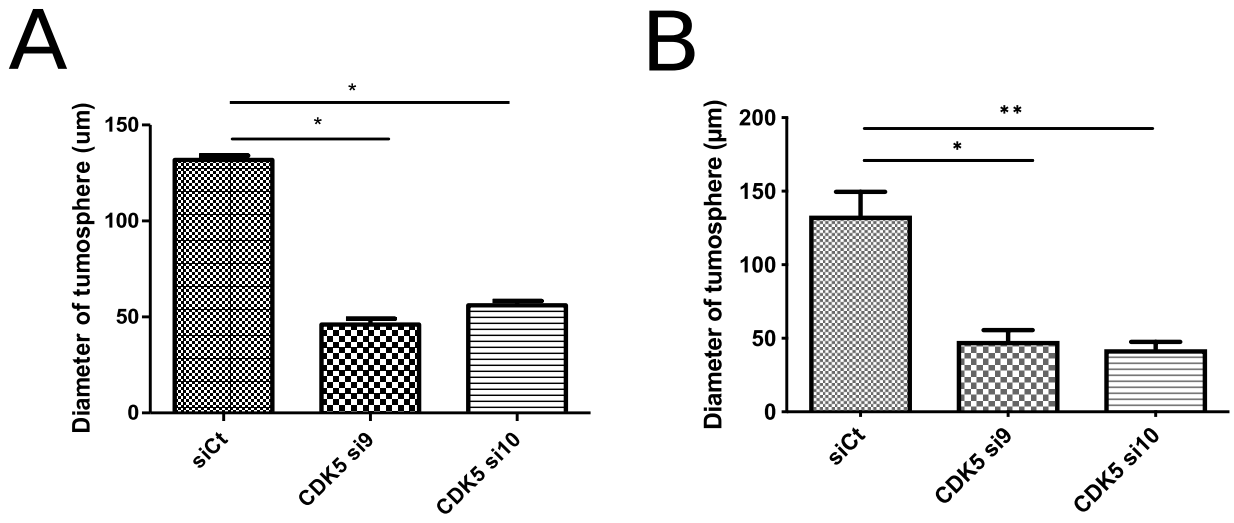

Figure 4: Bar charts of CDK5 silencing in MDA-MB-231 and BT-474 cells in soft agar assays. Diameters of colonies were measured via Image J and normalized to siControl. Bar charts show data of three different experiments of MDA-MB-231 **(A)** and BT-474 **(B)**. A decrease of about 50-60 % in the tumorsphere size is observed in both cell lines after treatment with CDK5 specific siRNAs. (* $p < 0.05$; ** $p < 0.01$)

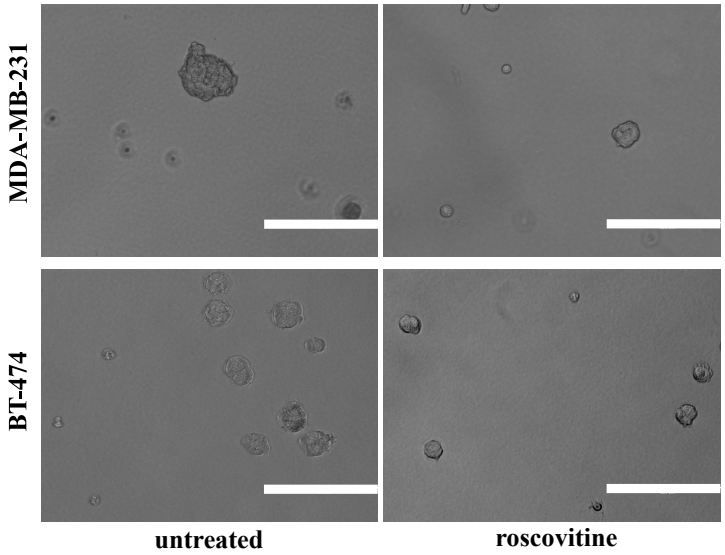

Figure 5: Effect of roscovitine (20 nM) on MDA-MB-231 and BT-474 cells in soft agar assays. MDA-MB-231 and BT-474 cells were treated with roscovitine for 24 hours. Colony sizes are reduced compared to the untreated samples. Images of representative colonies are shown, scale bar 200 µm.

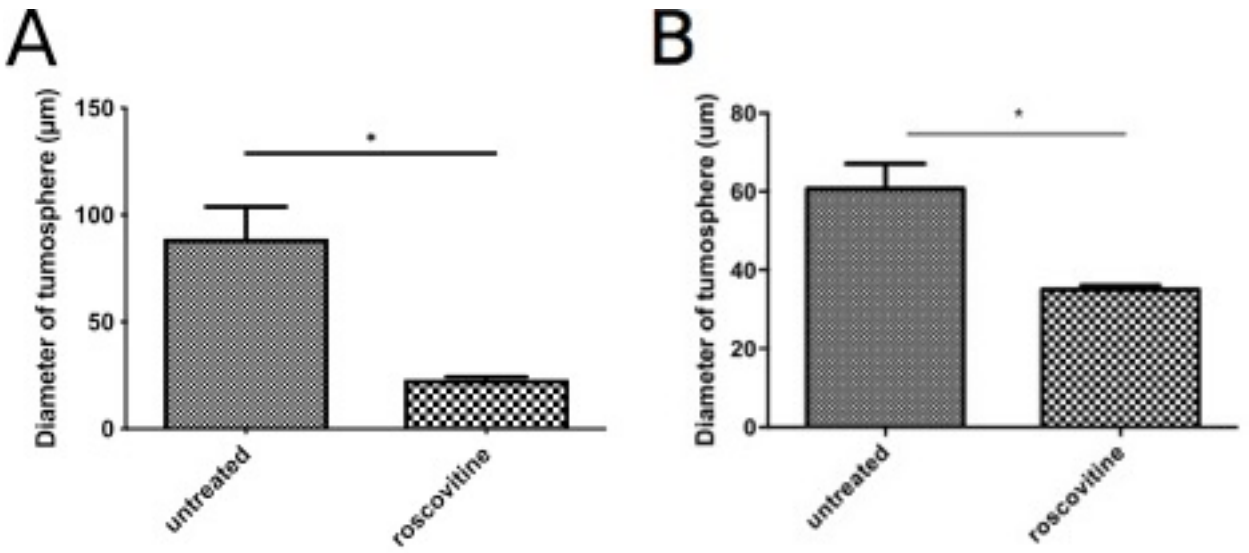

Figure 6: Tumosphere size after roscovotine treatment (20 nM) in MDA-MB-231 and BT-474 cells. Diameters of colonies were measured via Image J and normalized to untreated cells. Bar charts show data of three different experiments of MDA-MB-231 **(A)** and BT-474 **(B)**. For both cell lines, roscovitine treatment reduces the colony size. (* $p < 0.05$)

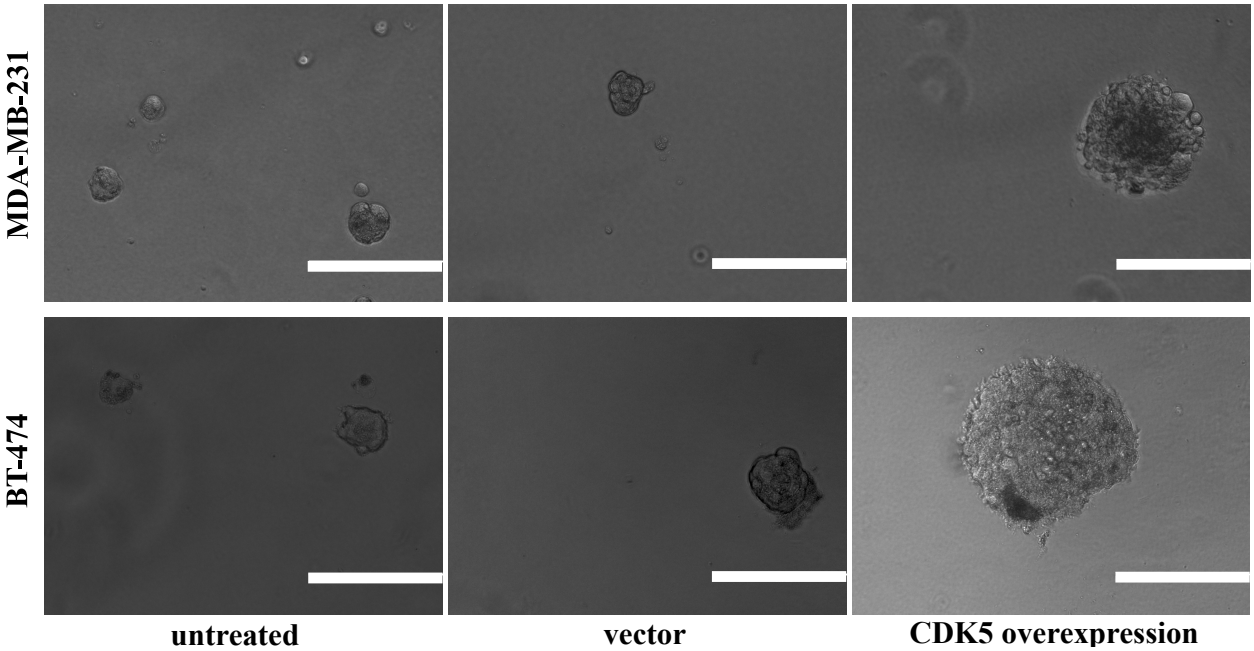

Figure 7: Effect of CDK5 overexpression on MDA-MB-231 and BT-474 cells in soft agar assays. MDA-MB-231 and BT-474 cells were transfected with CDK5 plasmid DNA or empty vector and incubated for 24 hours. A significant increase in tumosphere size is observed for CKD5 overexpressing cells. Diameters of colonies were measured via Image J. Images of representative colonies are shown, scale bar 200 µm.

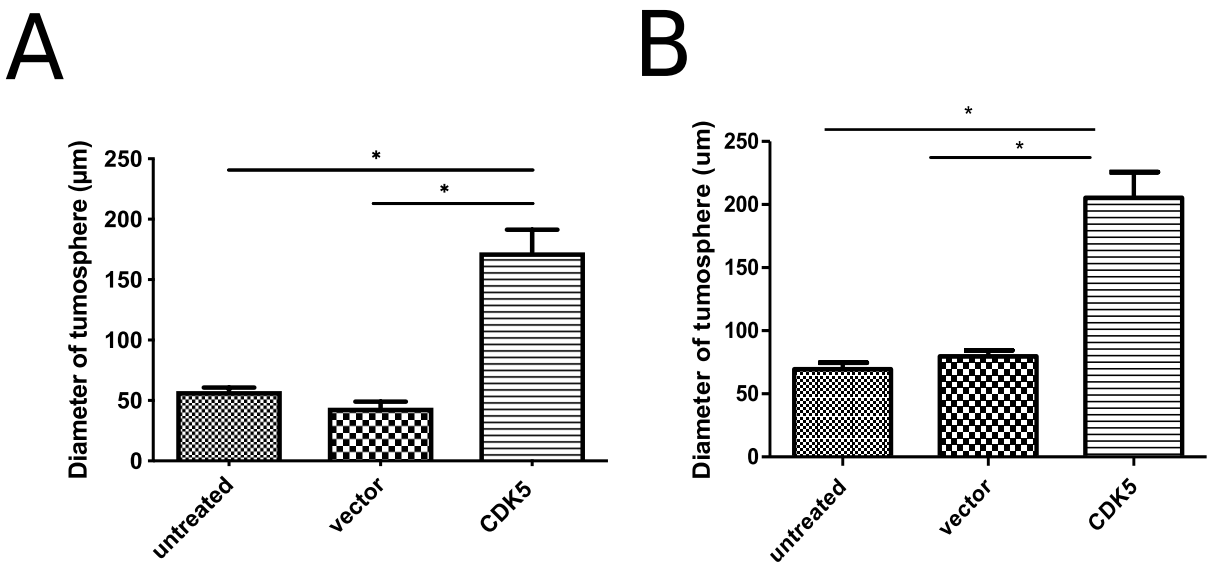

Figure 8: Effect of CDK5 overexpression in MDA-MB-231 and BT-474 cells. Diameters of cell colonies were measured via Image J and normalized to untreated cells. Bar charts show data of three different experiments of MDA-MB-231 **(A)** and BT-474 **(B)**. CDK5 overexpression increases the size of cell colonies drastically in both cell lines. (* $p < 0.05$)

3.2.2 Results of the SRB assay

SRB assays are a well-known method to investigate drug-induced cytotoxicity or cell proliferation. As shown in Figure 4, cell growth in both cell lines treated with CDK5 inhibiting siRNAs was significantly reduced compared to the control experiments. The SRB data suggests a slightly stronger effect in the BT-474 cells, however this trend was not confirmed in other experiments.

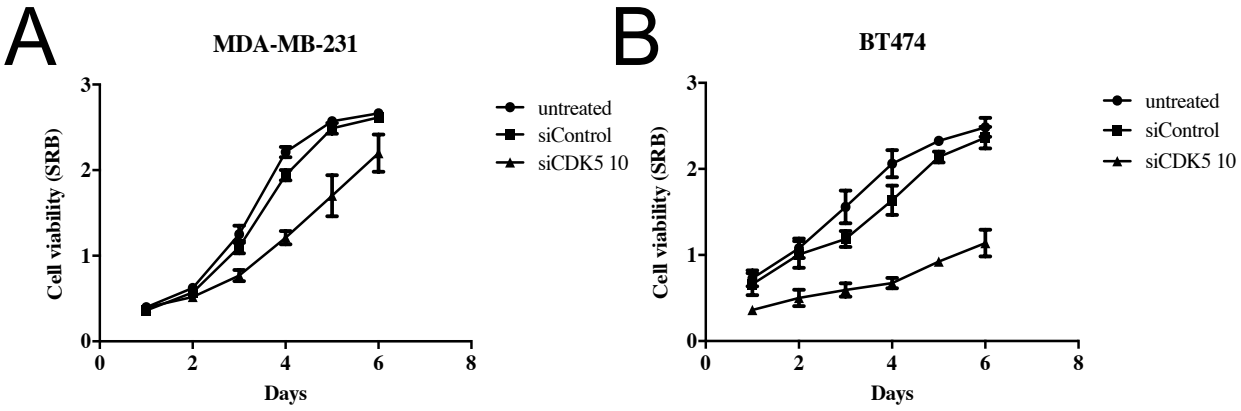

Figure 9: Effect of CDK5 silencing on cell proliferation in MDA-MB-231 and BT-474 cells. SRB assays were performed to identify the role of CDK5 in cell proliferation. MDA-MB-231 **(A)** and BT-474 **(B)** cells were transfected with siControl or siCDK5 10. A significant reduction in

cell proliferation is observed for both cell lines when treated with siRNAs. Error bars represent standard deviations over 3 independent experiments.

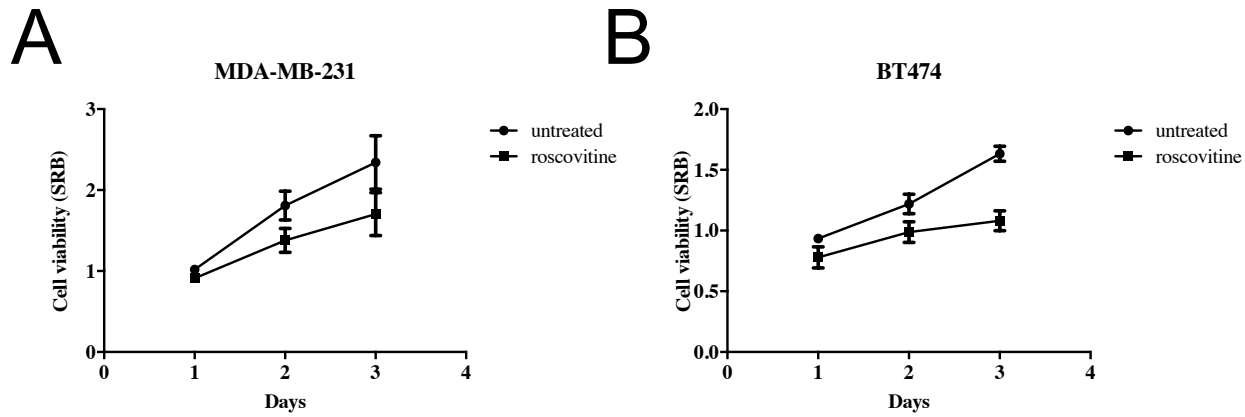

Figure 10: Effect of roscovitine treatment on cell proliferation in MDA-MB-231 and BT-474 cells. MDA-MB-231 **(A)** and BT-474 **(B)** cells were treated with roscovitine (20 nM) for 24 hours. Roscovitine treatment leads to a reduction in cell viability in both cell lines. Errors were computed from standard deviations over 3 independent experiments.

3.2.3 Conclusion of tumor growth and cell proliferation assays

The combined results of the two proliferation assays performed indicate a key role of CDK5 in tumor growth. CDK5 inhibition consistently results in reduced cell proliferation and overexpression of CDK5 increased cell growth in both cell lines.

3.3 CDK5 is important for cell migration

As described in section 2.2.8, a scratch is made on the confluent cell layer using a pipette tip to evaluate the healing, which is characterized by the migration and division of the cells to close this created wound (164). In order to analyze the relevance of CDK5 for cell migration, we performed wound healing assays while silencing or overexpressing CDK5 in both cell lines.

3.3.1 Wound healing assay with silenced cells

In the control experiments for MDA-MB-231 cells, the created wound was filled to a greater extend after 24h compared to samples with silenced CDK5 (Figure 11).

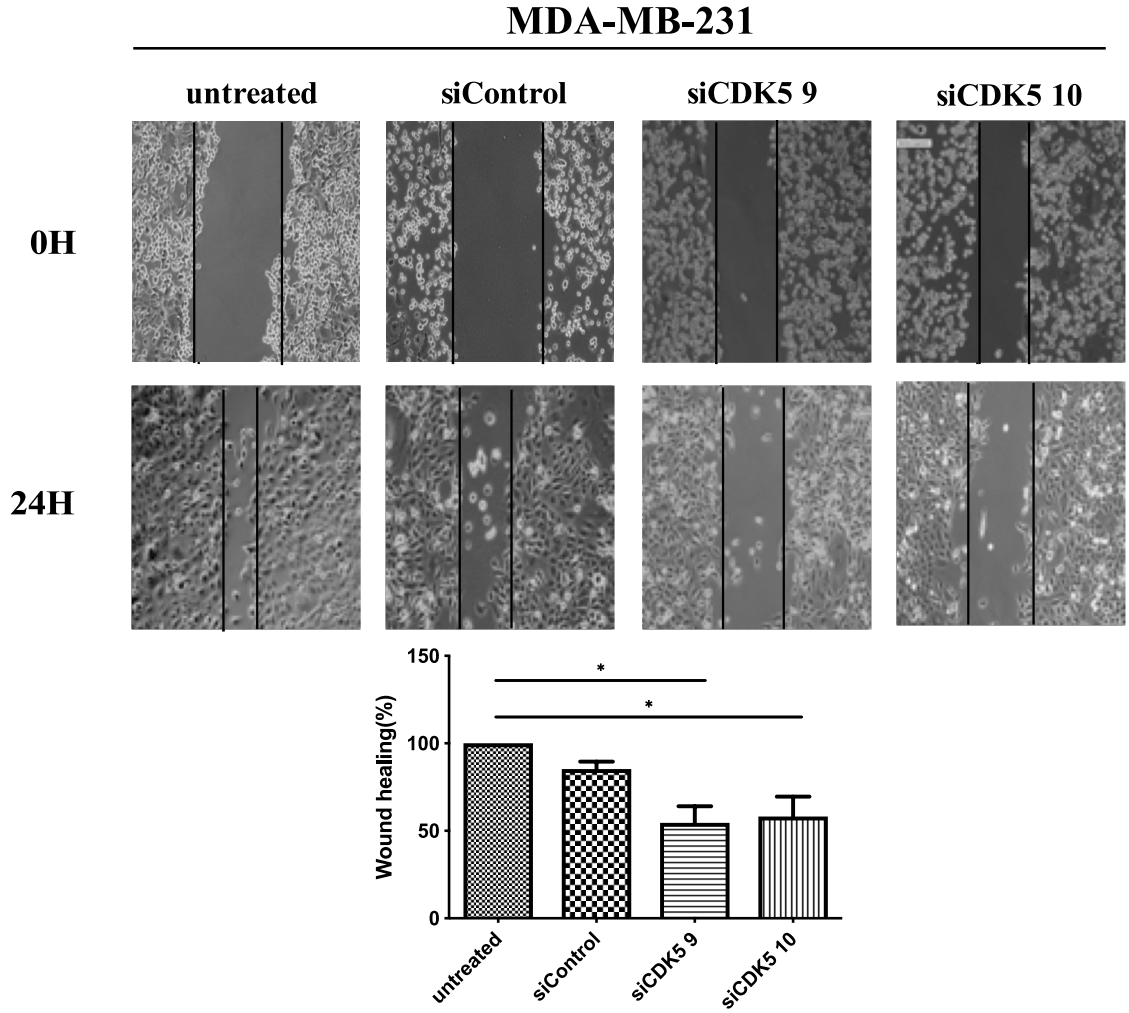

Figure 11: Effect of CDK5 silencing on cell migration in MDA-MB-231 cells in vitro. Cells were transfected with siControl, siCDK5 9 and siCDK5 10, or left untreated for 72 hours. Representative pictures are shown (upper panel). The wound diameter was analyzed with Image J and normalized to the untreated cells (lower panel). A reduction in wound healing is observed for cells in which CDK5 was inhibited. (* $p < 0.05$)

3.3.2 Wound healing assay with overexpressed cells

The two control experiments for MDA-MB-231 cells show a comparable degree of wound healing after 24h. Compared to this, the samples overexpressing CDK5 show an increased degree of wound healing (see Figure 12).

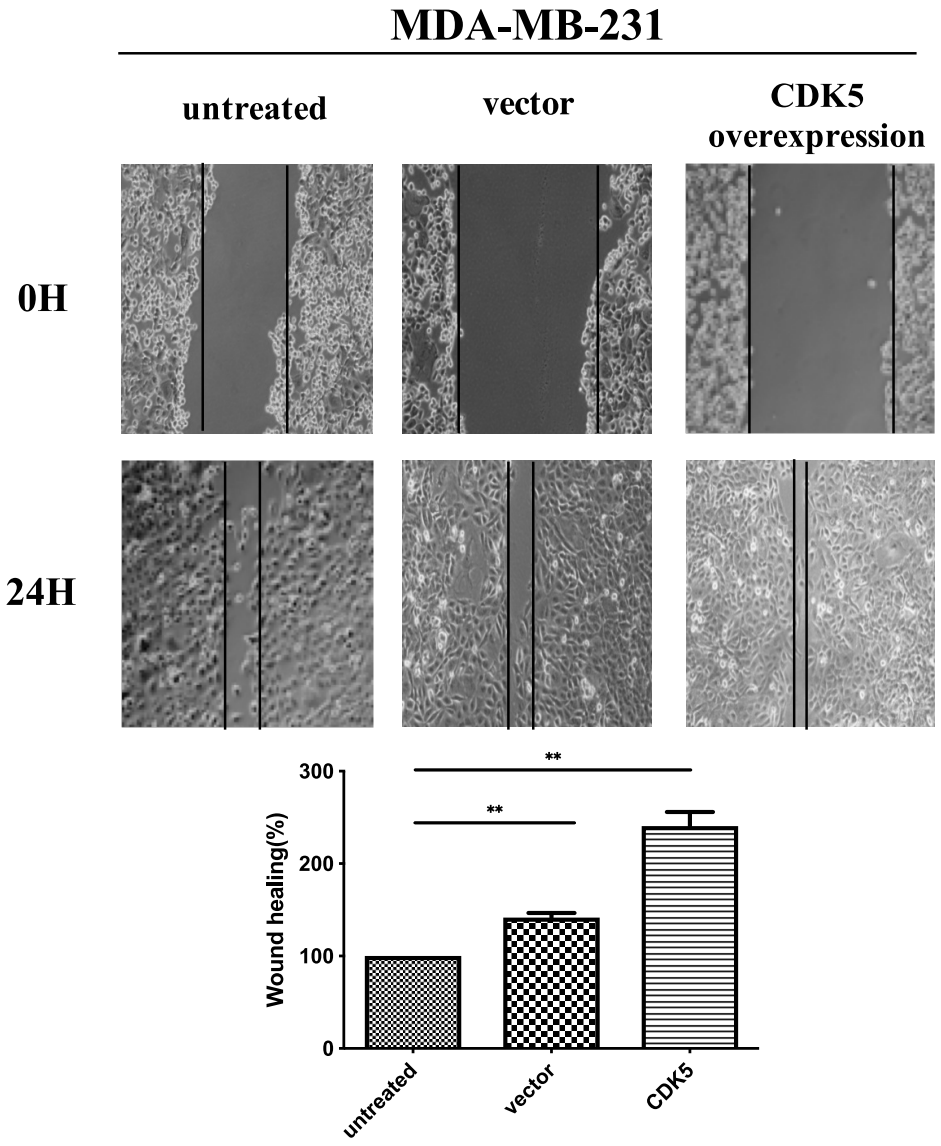

Figure 12: Effect of CDK5 overexpression on cell migration in MDA-MB-231 cells. Cells were transfected with CDK5 DNA, a vector or left untreated for 24 hours. Pictures were taken at the beginning and after 24 h. Representative pictures are shown (upper panel). The wound was analyzed with Image J and normalized to the untreated cells (lower panel). Cells overexpressing CDK5 show an increased wound healing compared to control samples. (** $p < 0.01$)

3.3.3 Wound healing assay with roscovitine treatment

We observed a similar effect of roscovitine on the cells as with the CDK5 specific siRNAs. In BT-474 and MDA-MB-231 cells, roscivitine treated samples displayed a decreased level of wound healing compared to untreated cells. These results suggest that CDK5 is an important protein for cell migration in breast cancer.

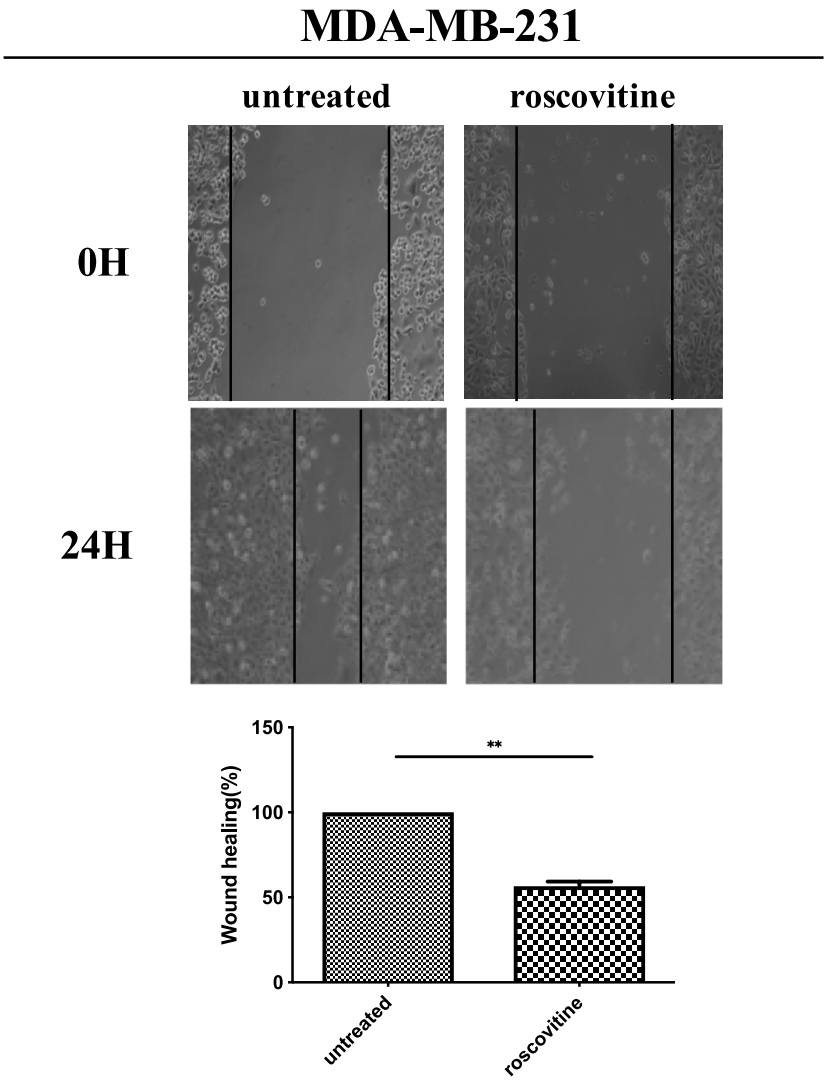

Figure 13: Effect of roscovitine treatment on cell migration in MDA-MB-231 cells. Cells were treated with roscovitine for 24 hours or left untreated. Representative pictures are shown (upper panel). The level of wound closure after 24h was analyzed with Image J and normalized to the untreated cells (lower panel). Roscivitine treatment leads to a significant reduction in wound healing compared to untreated cells. (** $p < 0.01$)

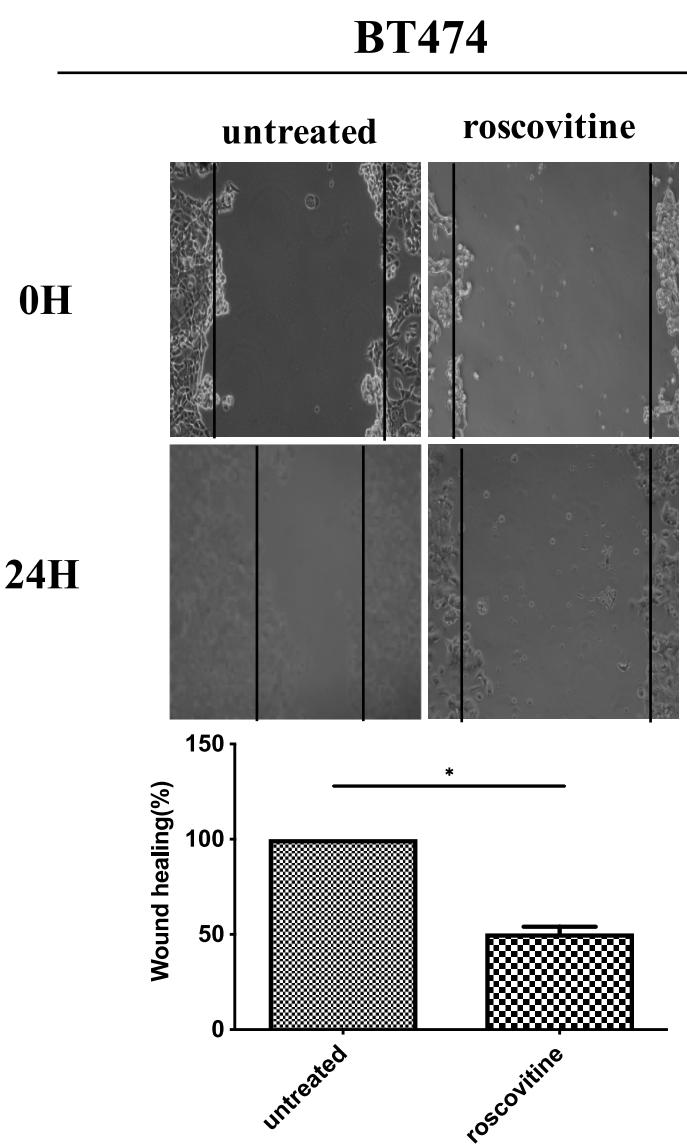

Figure 14: Effect of roscovitine treatment on cell migration in BT-474 cells. Cells were treated with roscovitine for 24 hours or left untreated. Representative pictures are shown (upper panel). Wound closure after 24h was analyzed with Image J and normalized to the untreated cells (lower panel). Also, in BT-474 cells, roscovitine treatment leads to decreased wound healing. (* $p < 0.05$)

3.4 CDK5 is a positive regulator of the mTOR pathway in breast cancer

Western blotting is a method which differentiates specific proteins through their molecular weight using gel electrophoresis (165). The expression levels can then be analyzed and compared to other proteins. Common loading control proteins are β-actin as well as α-tubulin, which are used to compare expression levels between samples with different protein amounts.

We examined phosphorylation levels of the main members of the mTOR pathway, which is known to be of importance in many types of malignancies as expanded on section 1.5.

3.4.1 CDK5 silencing and overexpression

Western blots show decreased phosphorylation levels of mTOR and S6 in both cell lines after siRNA silencing of CDK5. Overexpression of CDK5 in MDA-MB231 cells on the other hand, leads to increased levels of phosphorylated mTOR and S6. The total protein levels of mTOR and S6 remained unchanged in both treatments. Surprisingly, a comparable influence of the different treatments on the integrin β4 phosphorylation levels was observed as well, suggesting that CDK5 may play a role in the regulation of integrin β4.

Western blotting results for the BT-474 cells also show decreased phosphorylation levels of AKT, mTOR and integrin β4 upon siRNA silencing of CDK5.

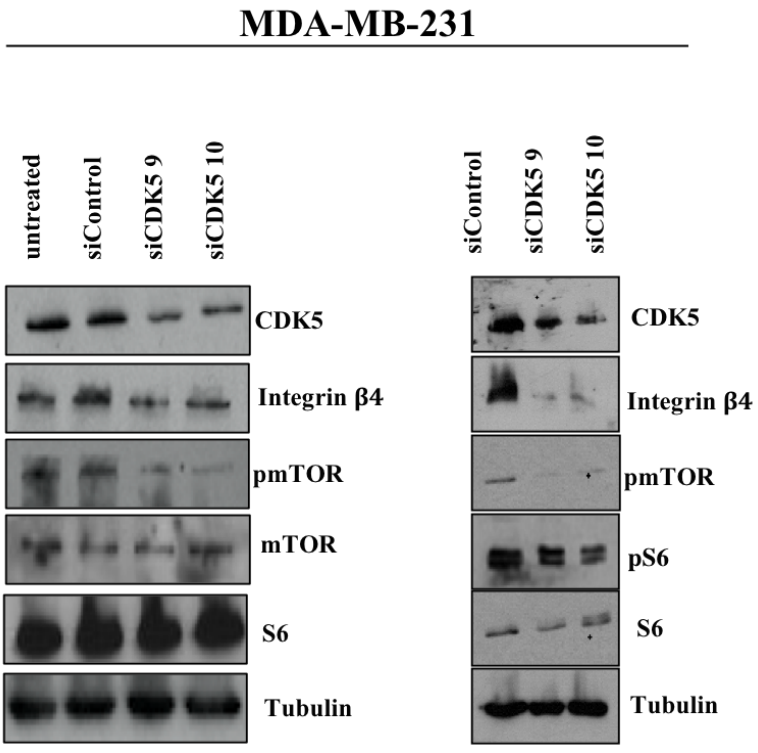

Figure 15: Effect of CDK5 silencing on key proteins of the mTOR pathway in MDA-MB-231 cells. MDA-MB-231 cells were transfected with CDK5 inhibiting siRNAs or left untreated. CDK5 inhibition leads to reduced phosphorylation levels of mTOR, S6 and integrin β4.

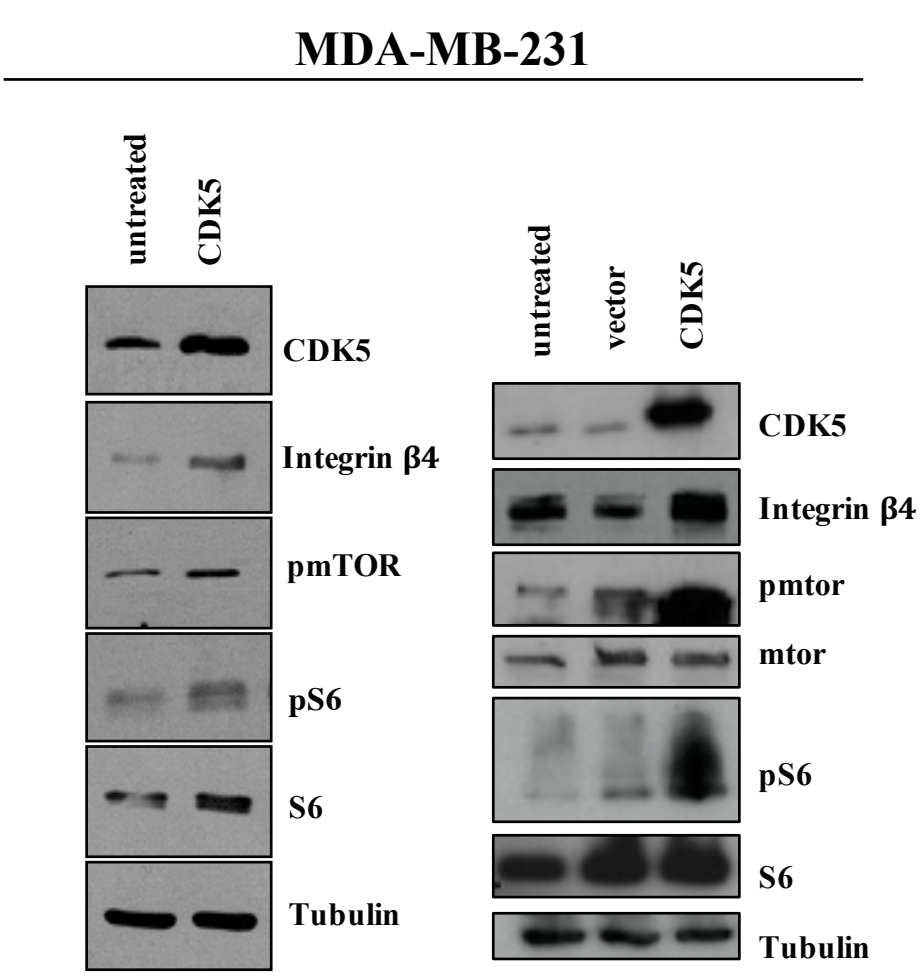

Figure 16: Effect of CDK5 overexpression on key proteins of the mTOR pathway in MDA-MB-231 cells. MDA-MB-231 cells were transfected with CDK5 DNA, an empty vector or left untreated. Increased levels of phosphorylated mTOR, S6 or integrin β 4 are observed in CDK5 overexpressing cells.

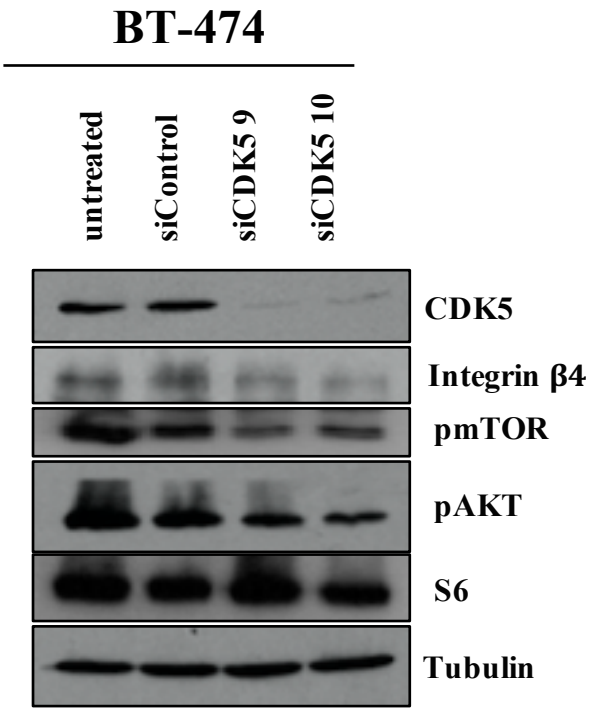

Figure 17: Effect of CDK5 silencing on key proteins of the mTOR pathway in BT-474 cells. BT-474 cells were transfected with CDK5 inhibiting siRNAs or left untreated. Upon CDK5 inhibition, integrin β4, mTOR and AKT display decreased phosphorylation levels.

3.4.2 Roscovitine treatment in MDA-MB-231 cells

Treatment with roscovitine (20 nM) led to reduced phosphorylation levels of CDK5 in agreement with it being used as a CDK inhibitor. Several key proteins of the mTOR pathway are also downregulated, such as AKT, mTOR itself and S6 (see Figure 18). Integrin β4 activity also seems to be affected by addition of roscovitine.

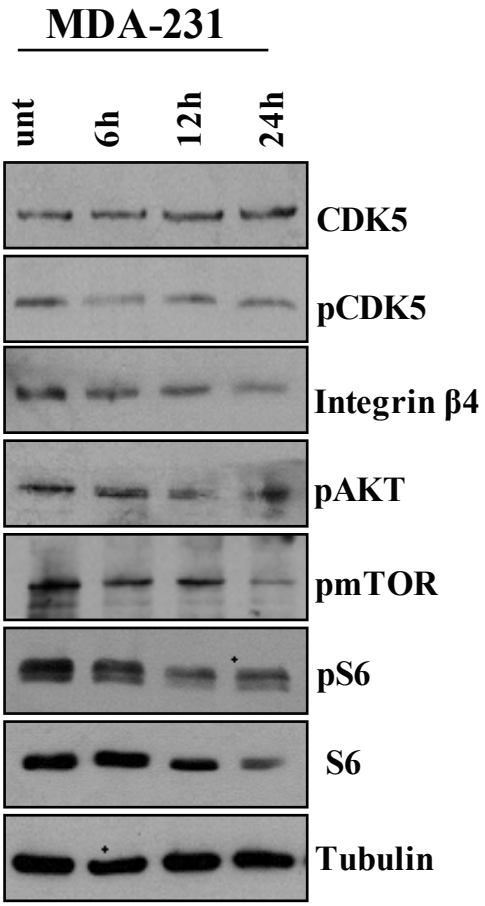

Figure 18: Effect of roscovitine treatment on key proteins of the mTOR pathway in MDA-MB-231 cells. MDA-MB-231 cells were treated with roscovitine (20 nM) for different durations. A steady decrease in the phosphorylation levels of several key proteins of the mTOR pathway such as AKT, mTOR and S6 is observed. Additionally, integrin β4 activity also seems to decrease over time.

3.4.3 Integrin β4 is overexpressed in BT-474 and MDA-MB-231 cells

Western blots revealed increased levels of integrin β4 in the analyzed BT-474 and MDA-MB-231 cell lines compared to another common breast cancer cell line MCF-7 (see Figure 19). As shown before, siRNA silencing of CDK5 leads to decreased levels of integrin β4.

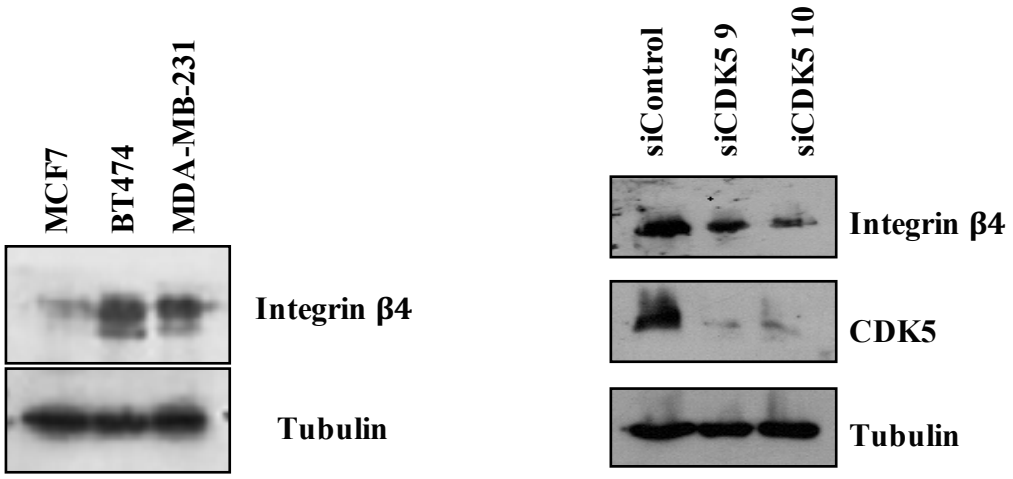

Figure 19: Effect of siRNA CDK5 silencing on integrin β4 and levels of integrin β4 in other breast cancer cell lines. MDA-MB-cells cells were transfected with CDK5 inhibiting siRNA or left untreated (left panel). As seen before, CDK5 inhibition also leads to downregulation of integrin β4. Levels of integrin β4 were measured in MCF-7, BT-474 and MDA-MB-231 cells (right panel). The two analyzed cell lines BT-474 and MDA-MB-231 show higher levels of integrin β4 compared to MCF7 cells.

4 Discussion

In the past few decades the treatment of breast cancer was improved by targeted therapies. Nevertheless, the treatment options for advanced and metastatic breast cancer as well as HER2-positive and especially triple-negative breast cancer are not yet satisfying. Therefore, the identification of proteins like CDK5 as targets for new treatments is indispensable. The role of CDK5 in cancer was described in different studies that showed a correlation between CDK5 and the development of cancer.

We showed that CDK5 might be a possible prognostic marker in breast cancer and could be influencing the clinical outcome. We examined the role of CDK5 using different assays, targeting CDK5 significantly by reducing tumor growth, cell proliferation and cell migration. Furthermore, we observed for the first time that CDK5 might be connected to the AKT/mTOR pathway and integrin β4 in MDA-MB-231 and BT-474 cells.

We additionally investigated the clinical role of CDK5 by using the TCGA data set and the Kaplan-Meier analysis. High expression of CDK5 seems to correlate with poorer overall survival and disease-free survival. Furthermore, we examined the expression of CDK5 in different breast cancer cell lines. We found that CDK5 was overexpressed in BT-474, MDA-MB-231 and ZR-75. MDA-MB-231 and BT-474 are highly invasive breast cancer cell lines and are categorized as basal-like and HER2-positive breast cancer. Other studies confirm this suggested important role of CDK5 for breast cancer progression. Levacque *et al.* performed an analysis using *Oncomine microarray online data mining software* and observed an overexpression of CDK5 in breast tumors and other cancer types (166). Liang *et al.* showed high CDK5 expression in estrogen receptor-negative and HER2-positive breast cancer tissues. They also measured a high expression of it in grade III breast cancer tissues (70).

The three characteristics of estrogen receptor negativity, HER2 positivity and high grade of tumors are all known to determine the prognosis of breast cancer patients and are consistent with the analysis of our Kaplan-Meier plots as well as the TCGA data set. The correlation of CDK5 expression and poor prognosis has also been shown in other cancer types as described in section 1.3.

Furthermore, CDK5 might be a new important tumor marker for different cancer types, since it also correlates with poor clinical outcomes in other cancer types. Zhang *et al.* performed a microarray analysis in 89 colon cancer patients. They showed that higher levels of CDK5 are associated with an advanced stage, poor tumor differentiation, greater tumor size and more nodal metastases. Patients with CDK5 overexpression had a poorer clinical outcome, suggesting CDK5

as a possible biomarker for the detection of patients at higher risk (72). Levacque *et al.* used Kaplan-Meier survival analysis and found that overexpression of CDK5 correlates with lower survival rates in multiple myeloma patients treated with the proteasome inhibitor bortezomib (166). In another study, knockdown of CDK5 led to higher sensitivity to bortezomib and another proteasome inhibitor carfilzomib in multiple myeloma cells (167). Zhang *et al.* suggested CDK5 as a new prognostic marker in multiple myeloma patients undergoing bortezomib treatment. Hsu *et al.* showed that higher levels of mRNA prostate-specific antigen (PSA) were associated with higher CDK5 levels (83). PSA itself is an important prognostic determinant in prostate cancer (168). The blood results of prostate cancer patients with high CDK5 levels revealed higher PSA concentrations. These results could be verified *in vitro* as well. Additionally, higher levels of CDK5 and its activator p35 correlated with higher levels of androgen-receptor positivity in tumor tissues compared to non-tumor tissues, suggesting CDK5 as a regulator of the androgen receptor, which is associated with cancer progression itself (83). Therefore, CDK5 could be also a new marker for prognosis and possible cancer progression in prostate cancer.

Liang *et al.* suggested CDK5 as an important marker in breast cancer. They showed that overexpression of CDK5 leads to an increase in EMT markers suggesting a new role as a regulator of EMT processes. EMT can occur in different types, including physiological processes like embryogenesis, organ development and tissue regeneration, as well as pathological ones like organ fibrosis or cancer progression metastasis (169). It was already shown that higher EMT transcriptions factors are associated with a poor clinical outcome in metastatic breast cancer patients (170). CDK5 might be a possible marker for advanced stages of breast cancer as suggested by Liang *et al*.

ZR75-1 cells show a higher expression of CDK5 as well, which represent an estrogen receptor positive cell line, but is also HER2-positive (171). Higher expression levels of CDK5 were observed by Mandl *et al.* in the estrogen receptor positive MCF7 cell line. They compared the CDK5 expression in cancer cell lines MCF-7, T24 and MDA-MB-231 to non-cancer cell lines MCF10A (71). Upadhyay *et. al* also showed overexpression of CDK5 in MCF-7 and MDA-MB-231 cells as well as active CDK5 by using an *in vitro* assay in both cell lines. Our data suggests that CDK5 expression is associated with expression of HER2, which was also shown by Liang *et al.* (70).

Breast cancer cell lines are as heterogeneous as the disease. Recently, efforts have been made to individualize the breast cancer therapy based on the immunoprofile and molecular characteristics of breast cancer (172,173). The analysis of cancer cells and the correct use of breast cancer cell

lines is the focus of many studies. Every breast cancer cell line represents different groups of breast cancer, which show variable prognosis and treatment response.

MDA-MB-231 cells were originally derived from the metastatic site of an adenocarcinoma of a female human breast. They are described as basal-like, since they are ER-negative, PR-negative and HER2-negative (174). A more detailed molecular classification shows that the MDA-MB-231 cells are claudin-low as they have a low expression of claudin-3, claudinin-4 and claudinin-7. They also show low levels of Ki-67 and E-Cadherin (175). Dias *et al.* showed that claudin-low tumors are associated with high-grade tumors, larger size tumors, more lymphocytic infiltrates and diagnosis at a younger age. They also showed that MDA-MB-231 cells have higher levels of markers for epithelial-mesenchymal transition and breast cancer stem cells (176). All in all this breast cancer cell line is highly aggressive and invasive. Therefore, it is often used in studies for drug treatment option of triple-negative breast cancer, which is the breast cancer subtype with the lowest survival rate.

BT-474 cancer cells were derived from a ductal carcinoma of a female breast. These cells are categorized as luminal B as they are ER-positive, PR-positive and HER2-positive. In their molecular classification, they show high levels of Ki-67 (175). They are sensitive to trastuzumab, a HER2-positive antibody, and are often used to investigate strategies to overcome resistance to anti-HER2 therapies (177).

The breast cancer cell lines MDA-MB-231 and BT-474 represent highly invasive breast cancer types with poor prognosis and loss of therapeutic options in patients with advanced stages of breast cancer. Therefore, all lines were chosen for the investigation of CDK5.

We used different *in vitro* assays which are usually performed when analyzing the aggressiveness of cancer cell lines. We used three different setups. CDK5 silencing and overexpression as well as roscovitine treatment are effective ways to specifically increase or decrease levels of CDK5. It was possible to identify the importance of the CDK5-dependent tumor characteristics. As controls siControl, empty vectors and wildtypes were used.

Inhibition of CDK5 via silencing and roscovitine treatment showed significant reduction of tumor growth compared to the control and wildtype cells. This indicates that CDK5 plays an important role in tumor growth. Similar results were obtained in other breast cancer cell lines in a study by Mandl *et al* (71). The importance of CDK5 for colony growth was investigated in a study of Chiker *et al.* (87). Colony formation assays in the breast cancer cell lines HCC-1954 and BT-549 were performed, which were known to have low levels of CDK5 mRNA. BT-549 cells belong to the claudin-low subtype. The HCC-1955 cells are ER-positive, PR-negative and show high expression

levels of HER2. The results of the experiments in these cell lines performed by Chiker *et al.* can be compared to our results, since they represent similar types of breast cancer, although the expression levels of CDK5 are low. The inhibition of CDK5 in the cell lines resulted in reduced colony numbers compared to the siRNA control and wildtype cells. These results are similar to our results, although we focused on the colony sizes of the cells rather than the number of colonies per well. A significant decrease in the number of colonies was also observed by Xu *et al.* after generation of a MDA-MB-231 cell line with a stable knockdown of CDK5 (178). Furthermore Liang *et al.* verified these results *in vivo* as they used a nude mouse xenograft tumor transplantation model where they injected breast cancer cells (MDA-MB-231 and BT-549) with silenced CDK5 (70). The treated mice showed a significant reduction in tumor weight and tumor size.

Similar results after CDK5 silencing and roscovitine treatment were obtained in other *in vitro* and *in vivo* experiments, but in different cancer types. Merk *et al.* compared CDK5 knockout mice to control mice after injecting them with B16F1 melanoma cells (90). The knockout mice showed a reduction in tumor growth. These results were verified *in vitro* in another study performed by Bisht *et al.*, where knockdown of CDK5 showed a significant effect on colony formation in SKMel melanoma cells (179). Zhuang *et al.* developed stable CDK5 silenced and overexpressed colorectal cancer cell lines. Knockdown of CDK5 showed a reduction in colony size and colony number (72). The additional treatment with roscovitine resulted in a higher decrease in these cell lines. Merk *et al.* performed roscovitine treatment on a glioblastoma xenograft model and observed a significant reduction in tumor growth (90). In CDK5 knockout mice the treatment with roscovitine led to a slightly higher decrease in tumor growth as well.

Roscovitine treatment and CDK5 silencing resulted in a significant reduction of cell proliferation as observed in the performed SRB assay. The same effect of roscovitine on cell proliferation in MDA-MB-231 was shown by Goodyear and Sharma (180). Additionally, Upadhyay *et al.* performed a cell proliferation assay where they showed dose-dependent decreased proliferation in MDA-MB-231 and MCF-7 cell lines after roscovitine treatment, CDK2/5 inhibition and CDK5 silencing (181). Interestingly, roscovitine treatment elicited a stronger response in MCF-7 compared to MDA-MB-231 cells.

Liang *et al.* didn´t observe a change in cell proliferation after knockdown of CDK5, but did so after roscovitine treatment (70). This might be due to knockdown of CDK5 via virus infection, which was performed to inhibit CDK5. All of the mentioned studies performed MTT assays to measure the cell proliferation. We used SRB assays because they are known to have a higher sensitivity and lower variation between cell lines as described by Keepers *et al.* (182).

Liu *et al.* also studied the effect of CDK5 on cell proliferation in A549 cells, which are a non-small cell lung cancer cell line. Knockdown of CDK5 via siRNAs and roscovitine treatment led to decrease in cell proliferation *in vitro* and *in vivo* (183). Similar results were also shown in medullary thyroid cancer cells after inhibition of CDK5 via siRNA and roscovitine treatment (89). Our scratch assay showed a significant reduction of the wound healing capabilities after silencing of CDK5 and roscovitine treatment. To verify these results, we performed CDK5 overexpression as well. The same results were obtained by Liang *et al.*, using a transwell assay where they observed less migration of MDA-MB-231 and B549 cells after CDK5 silencing and roscovitine treatment. They also detected similar effects in invasion assays (70). Additionally, our results are in agreement with the study of Mandl *et al.* (71). Xu *et al.* obtained similar results in MDA-MB-231 cells with stable knockdown of CDK5 (178). They also observed these changes in two MDA-MB-231 cell lines with stable knockdown of CDK5-interacting proteins called KIAA0528 and FIBP, which were identified before using proteomic analysis. They detected a significant reduction in their wound healing assays in all of their knockdown cell lines. They verified these results in soft agar assays as well.

The effect of CDK5 on cell migration was also observed in studies of other cancer cells. Bisht *et al.* performed wound healing assays in cells with knockdown of CDK5, which led to a slower wound closure in melanoma cells. They verified these results with Matrigel-coated Boyden chamber assays (179). Similar results were observed using roscovitine treatment and CDK5 inhibition via siRNA on prostate cancer cells, suggesting a crucial role for CDK5 in motility of prostate cancer cell lines as well (82).

Liu *et al.* investigated the role of CDK5 in motility and migration of lung cancer cells. In scratch assays the repopulation of the gap was significantly reduced after CDK5 silencing and roscovitine treatment (183). Feldmann *et al.* established a pancreatic cancer cell line with dominant-negative CDK5. Similar results were observed after knockdown of CDK5 in another pancreatic cancer cell line. They identified a similar role of CDK5 in the motility of pancreatic cancer performing wound healing assays (80).

A dysregulation of the AKT/ mTOR pathway is known to be involved in cancer growth, survival and migration as well as chemoresistance. Our western blotting results showed decreased levels of activated mTOR and S6 in CDK5 silenced cells. Overexpression of CDK5 in MDA-MB231 showed increased levels of activated mTOR and S6. Interestingly, the levels of the unphosphorylated mTOR and S6 stayed the same. Surprisingly a change in the integrin β4 levels was observed too, suggesting that CDK5 may regulate the mTOR pathway as well as integrin β4.

Therefore, a link to CDK5 could be a possible explanation for our results from the assays we performed.

CDK5 was linked to different pathways in breast cancer. Liang *et al.* focused in their study on the regulation of TGF-β1-induced EMT through CDK5 (70). TGF-β1 itself is able to induce and maintain EMT (184). Interestingly, the MCF10A cell line, which is derived from a mammary gland, showed an elongated fibroblast-like morphology after being cultured in TGF-β1. These cells had significantly lower levels of epithelial marker E-cadherin and higher levels of mesenchymal markers like N-cadherin and α-smooth muscle actin (α-SMA). The same cells showed overexpression and higher kinase activity of CDK5 as well as upregulation of its activator p35. The TGF-β1 inhibitor LY364947 inhibited CDK5 and p35 protein expression. In CDK5 silenced MCF10A cells cultured in TGF-β1, the cells showed an increase in epithelial markers and kept their physiological morphology. Additionally, a decrease in mesenchymal markers was seen. Opposite effects were obtained for the overexpression of CDK5, where the cells retained their morphology. Liang *et al.* studied the relationship between CDK5 and focal adhesion kinase (FAK), which is phosphorylated by CDK5 and known to be involved in breast cancer motility (185,186). Silencing of CDK5 and roscovitine treatment led to less phosphorylation of FAK in MDA-MB-231 and BT549 cells. Furthermore, they studied the effect of CDK5 on the modulation of the cytoskeleton by connecting the presence of CDK5 to the formation of f-actin bundles.

In a study of Navaneetha-Krishnan *et al.* CDK5 was connected to the intrinsic apoptotic pathway in breast cancer cells and therefore its mitochondrial function (69). CDK5 silencing in MDA-MB-231 cells was correlated with higher levels of ROS. They also showed that CDK5 silencing led to higher levels of mitochondrial depolarization and fragmentation, which are associated with apoptosis. In summary, CDK5 loss led to increased apoptosis, which is compatible with studies, where CDK5 inhibition correlated with higher chemotherapy sensitivity in breast cancer (48,187). In breast cancer cells CDK5 has not yet been linked to the mTOR pathway or its downstream targets. However, other cancer types are known where CDK5 is influences the mTOR pathway. In prostate cancer, phosphorylation of AKT is involved in its progression (81). CDK5 silencing was associated with lower levels of activated AKT. For AKT activation, the phosphorylation at position serine 473 is necessary, which might be mediated by CDK5. The total AKT levels remained the same. They also investigated the downstream key proteins like S6 and glycogen synthase kinase 3 β (GSK3β). CDK5 silencing resulted in downregulation of activated S6 and GSK3β. Total protein levels stayed the same. These results are comparable to our results. In a co-immunoprecipitation experiment, an interaction between CDK5 and AKT was observed.

Additionally, the hyper activation of AKT was less efficient in CDK5 silenced prostate cancer cells. Interestingly, it was also found that CDK5 stabilizes the androgen receptor by phosphorylation, showing again the important role of CDK5 for tumor growth. Nevertheless CDK5 is also able to regulate the growth of prostate cancer independently of androgen receptors (81,188).

CDK5 was also linked to the PI3K-AKT signaling pathway in glioblastoma cells. CDK5 phosphorylates the GTP-binding protein phosphatidylinositol 3-kinase enhancer, which is a regulator of the PI3K-AKT pathway. By phosphorylating the phosphatidylinositol 3-kinase enhancer at Ser-279, CDK5 regulates the activity of AKT, leading to growth and migration of glioblastoma cancer cells (189).

We also observed overexpression of integrin β4 in CDK5 overexpressing cell lines MDA-MB-231 and BT-474. Interestingly the silencing of CDK5 also led to less activity of integrin β4. A connection between integrin β4 and CDK5 in cancer progression has not been explained before. Our western blot indicates however, that there might be a connection between these two proteins. Higher levels of integrin β4 were found in basal-like breast cancer cells in a study of Lu *et al.* (131). They analyzed the data of 105 tumor samples from patients with invasive ductal breast carcinomas. They observed significant overexpression of mRNA and protein levels of integrin β4 in basal-like breast cancer, which is consistent with our observed higher levels of integrin β4 in MDA-MB-231 cells. Surprisingly, there was no correlation in HER2-positive breast cancer tumors. They also performed a gene analysis and found that integrin β4 gene expression correlates with a poorer clinical outcome in breast cancer patients. These findings were confirmed by Tagliabue *et al.*, who showed an association between α6β4 expression and poor prognosis (190). Similar findings were observed in a study of Diaz *et al.* (191). They showed that elevated levels of integrin β4 mRNA correlate with increased tumor size and higher tumor grade in early stages of breast cancer, which suggests that integrin β4 is involved in breast cancer progression. In another study, it was shown that higher levels of integrin β4 correlate with a higher probability to relapse during a 5-year-period after chemotherapy in patients with triple-negative breast cancer (133).

Recently, increased levels of integrin β4 were found in mesenchymal-like triple-negative cancer cells suggesting it as a marker for cancer stem cell populations of mesenchymal carcinoma (133). Efforts have been made to identify other markers to identify these tumor-initiating cells or cancer stem cells. Several characteristic proteins such as CD44 and CD24 have been found to be

expressed on the surface of these cells (192,193). Since cancer stem cells are known to lead to relapse and metastasis (194,195), they are important candidates for new treatment (196).
The above-mentioned findings show that CDK5 and integrin β4 might be two new possible targets for the treatment of breast cancer. Both can potentially also be used as markers to identify patients with poorer prognosis. This group of patients for example could then benefit from a more aggressive therapy. More *in vitro and in vivo* experiments are necessary to investigate and understand the mechanism of this possible new target in breast cancer.

5 Conclusion

The treatment of breast cancer has changed significantly over the past decade. The improvements due to novel treatment options not only prolonged survival for patients, but also led to increased quality of life. Nevertheless, treatment of triple-negative and HER2-positive as well as advanced or resistant breast cancer is still challenging. The identification of new treatment options and improved understanding of breast cancer is needed.
Our study suggests CDK5 as a new possible target in triple-negative and HER2-positive breast cancer. Our hypothesis based on our experiments is that CDK5 activates the mTOR pathway and induces a higher activation of integrin β4, which leads to increased cell growth and migration. Our results indicate a new role for CDK5 and its clinical relevance in breast cancer progression. Additionally, we suggest CDK5 as a new biomarker in breast cancer. Further *in vivo* studies should reveal the potential of CDK5 as a new treatment target for patients with triple-negative and HER2-positive breast cancer types and especially advanced as well as metastatic breast cancer. Future experiments should focus on the mechanism of CDK5 regulating tumor migration, proliferation and invasion.

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7 Abbreviations

α-SMA	α-smooth muscle actin
4EBP1	Eukaryotic initiation factor 4E-binding protein 1
AML	Acute myeloid leukemia
APS	Ammonium persulfate
CDK	Cyclin-dependent-kinase
CDKL	Cyclin-dependent-like-kinases
CDK5	Cyclin-dependent-kinase 5
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
DMEM	Dulbecco´s modified Eagle medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemilumescent
EDTA	Ethylenediamine tetraacetic acid
EDTA-T	Ethylenediamine tetraacetic acid with Trypsin
EMT	Epithelial-mesenchymal transition
ER	Estrogene receptor
FAK	Focal adhesion kinase
FCS	Fetal calf serum
GSK3β	Glycogen synthase kinase 3 β
HASH1	Human achaete-scute homologue-1
ITGB4	Integrin β4
mTORC1	Mechanistic target for rapamycin complex 1
mTORC2	Mechanistic target for rapamycin complex 2
OS	Overall survival
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide-3-kinase
PIP2	Posphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PR	Progesterone receptor

PSA	Prostate-specific antigen
PTEN	Phosphatase and tension homolog protein
RHEB	Ras homolog enriched in brain protein
S6	Ribosomal S6
RIPA	Radioimmunoprecipitation assay buffer
RFS	Relapse-free survival
RNA	Ribonucleic acid
RPM	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate polyacrylamide
SIRNA	Small interfering RNA
SRB	Sulforhodamine B
TBST	Tris-buffered saline and Polysorbate 20
T-ALL	T-cell acute lymphoblastic leukemia
TCGA	The Cancer Genome Atlas
TEMED	Tetramethylethylenediamine
TSC1/2	Tuberous sclerosis complex 1 and 2

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„Ich, Negin Karimian, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: **„A novel role of CDK5 in tumor growth, migration and proliferation of breast cancer cell lines MDA-MB-231 and BT-474“** selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Publikationsliste

Jacob J, Favicchio R, Karimian N, Mehrabi M, Harding V, Castellano L, Stebbing J, Giamas G. LMTK3 escapes tumour suppressor miRNAs via sequestration of DDX5. Cancer Letters. 2016;372(1):137–46.

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