Aus dem Experimental and Clinical Research Center, Campus Buch,

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

On the functional relevance of the gene DEP domain containing 1 in breast cancer cells

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 23. Mai 2013

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Abbreviations

ADH	Atypical ductal hyperplasia
AM	Acetoxymethyl ester
Ct	Threshold Cycle
DCIS	Ductal carcinoma in situ
DEPC	Diethyl pyrocarbonate
DEP domain	Disheveled, EGL-10, Pleckstrin-domain
DEPDC1	DEP domain containing 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FACS	Fluorescence activating cell sorter
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HER2	Human epidermal growth factor receptor negative
IDC	Invasive ductal carcinoma
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NEAA	Non essential amino acids
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEM	Positron emission mammography
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
siRNA	Small interfering RNA
TNM	Tumour, node, metastasis
UICC	International Union Against Cancer
VNIP	Van Nuys Prognostic Index
WHO	World Health Organization

1 Introduction

1.1 Invasive breast cancer

Breast cancer is a heterogeneous disease encompassing a wide variety of pathological entities and a wide range of clinical characteristics[1]. This carcinoma originates from the terminal ductal-lobular unit of breast tissue and has two different growth types: invasive or non-invasive. Most breast cancers are invasive at the time point of diagnosis[2]. Invasive breast cancer spreads outside the membrane of the lobule or milk duct into the surrounding breast tissue, and can then migrate into the lymph nodes in the armpit or beyond to other organs. Invasive ductal carcinoma (IDC) is the most common type of invasive breast cancer, which accounts for 70% to 80% of all breast carcinomas[2]; infiltrating (invasive) lobular carcinoma accounts for about 10% to 15%[3]. In addition, there are other rare forms (about 2%) such as medullary, papillary, mucinous and tubular carcinomas. These different tumour types differ in their histologic features, clinical characteristics and their outcome.

1.1.1 Epidemiology

Breast cancer is the most frequent cancer among women. According to the International Agency for Research on Cancer, approximately 1.2 million new cases of invasive breast cancer and 410,000 breast cancer deaths occur every year worldwide[4]. In the United States, breast cancer accounts for nearly 34% of all cancers diagnosed among women[5]. Meanwhile, based on a report from the Association of Population-based Cancer Registries and Robert Koch Institute, 50,000 women are expected to develop breast cancer every year in Germany, with breast cancer being the most frequent diagnosed form of cancer among women. Moreover, the standardized incidence rate in Germany is extremely high compared to the incidence rate in the rest of the world (**Fig. 1**). The risk to develop breast cancer is strongly related to age (**Fig. 2**), with 81% of cases occurring in women aged 50 years and over. The lifetime risk of a woman developing breast cancer is about 10%-12%[6,7]. In addition, 458,400 women died from breast cancer in 2008, accounting for 14% of the total cancer deaths and 6% of total deaths in the female population[8]. The mortality under the estimation of the World Health Organization (WHO) also showed that breast cancer was still the leading cause of female cancer deaths in the European Union, account-

ing for 16% of a total 88,101 deaths[9]. Breast cancer is the primary cause of cancer death in Germany (16.5/100,000 women)[9] and breast cancer is also the most frequent cause of death in women between 40 and 55 years old. However, data from the study on Surveillance Epidemiology and End Results reported in 2011 show that[10] the 5-year breast cancer-specific survival rate in the USA was 89.2%, higher than that of other common cancers. Furthermore, although the incidence and mortality increased steadily for a long time in parallel, mortality in most developed countries during the past 15 years has remained stable or declined, especially among younger women. Both the increased incidence and reduced mortality are attributed to greater use of mammography screening leading to increased detection of breast cancers too small to be felt, and earlier initiation of treatment.
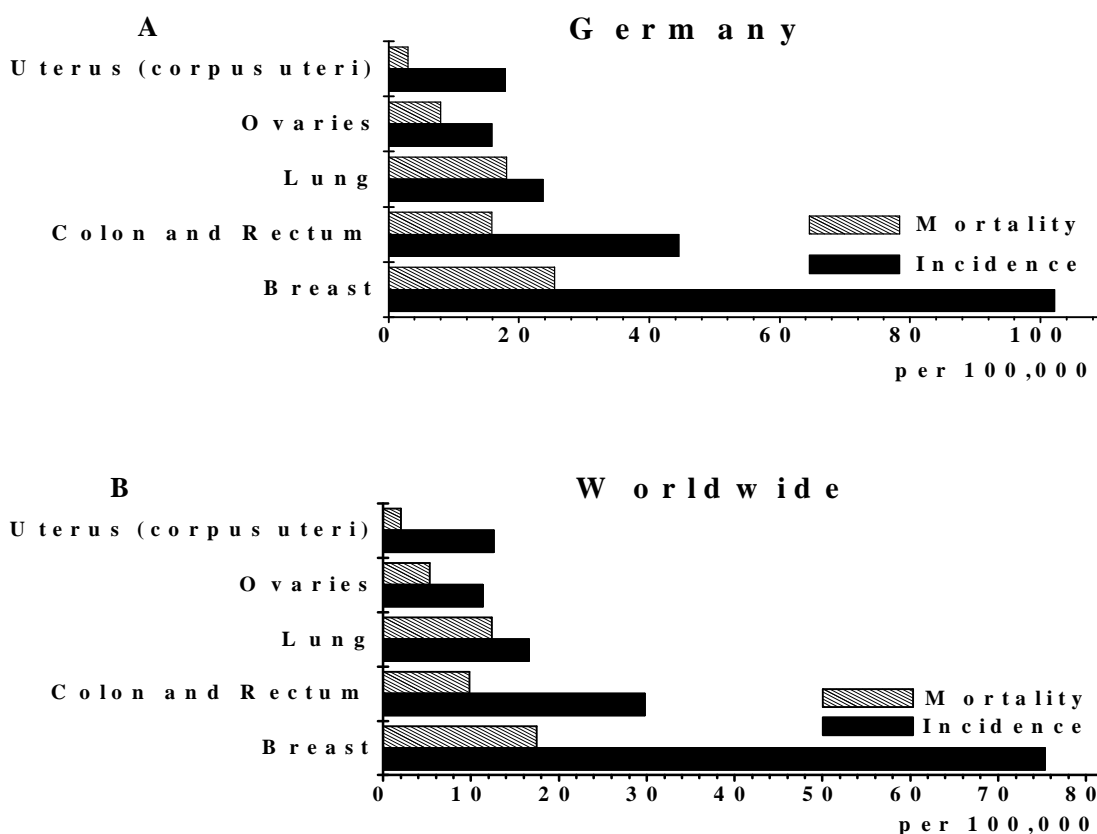
Fig. 1. New cases and mortality in the most common cancers of women in 2006.
(A) In Germany, *(B)* In the world as a whole. Figures adapted from "Cancer in Germany" published jointly by the Association of Population-based Cancer Registries and the Robert Koch Institute, the 7th edition
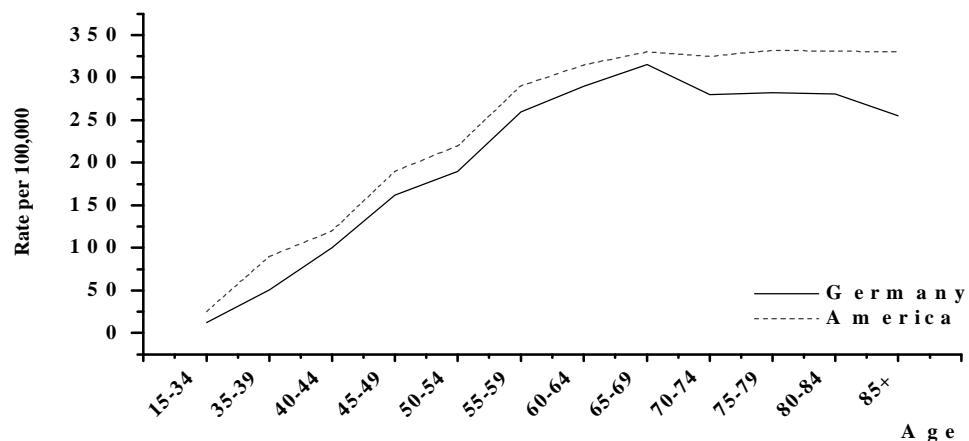
Fig. 2. Age-related female breast cancer incidence rate in Germany and America during 2005-2006.
Figures adapted from North American Association of Central Cancer registries, report 2011.

The vast majority of breast cancers are non-hereditary and occur sporadically, but there are still 27% of cases regulated by hereditary and external risk factors[11]. Only 5%-10% of all breast cancer cases are due to inherited genetic mutations[12]. Breast cancer is not exclusively a disease of women, but about 1% of breast cancer cases affect males[13]. Other factors associated with increased breast cancer risk are sex[14], age, nulliparity and no breastfeeding[15].

1.1.2 Classification

Given the multiple different schemes and complexity of the disease, pathologists used to divide breast cancer into several categories. Current classification is primarily based on descriptively morphological entities whose prognostic significance is demonstrated by retrospective analysis[16]. This is an effective way to determine potential prognostic factors, to select treatment approaches and to estimate the outcome for each individual suffering from breast cancer. The typical classification systems usually include the three aspects: the grade of the tumour, the TNM classification of the tumour and the stage of the tumour.

1.1.2.1 Grading

The grading of breast cancer depends on the microscopic similarity of breast cancer cells to normal breast cells, known as the degree of histological differentiation of tumor cells. The technique of grading for breast cancer was developed by Bloom and Richardson[17] and is now rec-

ommended by the WHO in a modified form. Breast cancer is classified according to the criteria of histology and cytology into three grades, low grade, intermediate grade and high grade. Grading is fundamentally derived by assessing the glandular formation, and abnormalities of the nuclear and the cellular appearance. The more similar the cancer cells are to normal cells, the better the cells are differentiated. Well differentiated is given a grade of 1 (G1), G2 is moderately differentiated, and G3 is low or undifferentiated cells. The less differentiated the cells are, the more immature they are, the higher the tendency to spread is and thus the higher the malignancy of the cancer is, accompanied by a higher probability of metastasis and possibly a poorer survival rate.

1.1.2.2 TNM classification

Developed by the International Union Against Cancer (UICC), the TNM classification is an internationally recognized and widely used system for the description of the anatomic extent of cancer [18]. The TNM classification describes the size of tumor (T), the number of lymph nodes involved (N) and occurrence of distant metastases beyond the regional lymph nodes (M). The extension of the primary tumor (T) is usually described according to its size between zero and four. N and M comprise at least two categories each: absence (N0/M0) and presence (N1-N3/M1) of lymph node or distant metastasis. **Table 1** shows an overview of the TNM classification for breast cancer[19]. Tumors classified prior to treatment are called clinical TNM (cTNM), and pathologic TNM (pTNM) is used to classify tumors after resection.

1.1.2.3 Staging

Staging is the process of grouping patients according to the extent or severity of an individual's cancer[20]. There are five basic stages, zero to IV, according to how large the tumor is and how far it has spread. This division assists in making appropriate treatment choices. Additionally, it allows an improved assessment of prognosis and an optimal comparability of different therapeutic programs. The pathological staging (staging by surgery) consists of the three parts: the clinical staging (information obtained prior to surgery, such as by mammography), the histopathological investigation of primary tumor and the statement of lymph nodes. The current universally accepted and most frequently used staging of breast cancer is based on the UICC[21], which in turn, is based on the TNM classification system (**Table 2**).

Table 1: TNM classification of breast cancer

T	T0	No evidence for primary tumor
	Tis	Carcinoma in situ. Intraepithelial or invasion of lamina propria
	T1	Tumor 2cm or less in greatest dimension
	T2	Tumor more than 2cm but less than 5cm in greatest dimension
	T3	Tumor more than 5cm in greatest dimension
	T4	Tumor of any size directly invades to chest wall (including ribs, intercostal muscles and serratus anterior muscle, but not the pectoral muscles) or skin

N	N0	No regional lymph node metastasis
	N1	Metastasis in 1- 3 regional lymph node
	N2	Metastasis in 4-9 regional lymph nodes
	N3	Metastasis more than 9 regional lymph nodes

M	M0	No distant metastasis
	M1	Distant metastasis (including extension to one or more ipsilateral supraclavicular lymph node)

Table 2: Breast cancer staging grouping

	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0,T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0,T1,T2	N2	M0
	T3	N1,N2	M0
Stage IIIB	T4	N0,N1,N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

1.1.3   Diagnosis and detection

For women at normal risk of breast cancer, several diagnostic procedures could be taken into consideration. Following the clinical breast exams by health care professionals, a number of imaging techniques, such as X-ray mammography, ultrasound, magnetic resonance imaging (MRI) and elastography of paramount importance in the early detection of breast cancer. A biopsy is taken if these imaging tests find a breast change (or abnormality) that raise the suspicion of cancer. Biopsy is the only definitive way to determine whether cancer is really present.

Mammography is a special type of X-ray examination taken to look for abnormal growth or changes in breast tissue before it can be felt. Thus it is the gold standard for breast cancer screening and early detection. There are two types of mammography examinations[22]: screening and diagnostic mammography. The former is done in asymptomatic women, which greatly improves the chance for successful treatment and decreases the risk of late-stage, although its benefit is under discussion. The latter is performed in symptomatic women. Its main purpose is to determine the exact size and location of breast abnormalities and to image the surrounding tissue and lymph nodes, contributing to a reduction in mortality of breast cancer[23]. Although the various studies differed in their design, clinical trials have found a probably relative age-dependent reduction of mortality in breast cancer by 20% - 40% with mammography[24,25]. However, mammography also missed about 10% of the tumors, described as "false negatives"[26]. This is not only due to the dense tissues obscuring the cancer, but also partly due to the fact that the appearance of cancer cells largely overlaps with the normal tissue during the mammography.

1.2 Ductal carcinoma in situ

According to the histopathological classification, breast cancer is subdivided into noninvasive and invasive cancer, mainly originating from the terminal ductal or lobular unit of breast tissue. The former is characterized by not invading the basement membrane and is thus confined within the ducts or lobules and termed carcinoma in situ. There are two types defined as ductal carcinoma in situ (DCIS) and lobular carcinoma in situ. A schematic representation of the structure of the breast localization within lobules and ducts is shown in **Fig. 3**.

DCIS is characterized by neoplastic proliferation of malignant mammary epithelial cells, which are separated from the fat tissue in vessels and connective tissue by an intact basement membrane. DCIS occurs, as already mentioned, within the milk ducts of the mammary gland. It is sort of a "precursor" to the development of invasive breast cancer.

When DCIS is diagnosed, it is strictly a local lesion, which can be cured with adequate local therapy. Consequently, DCIS does not infiltrate into the surrounding tissue and there is no need of chemotherapy. If an invasive carcinoma has been formed, the chance of the development of secondary cancers in other organs is also increased. Furthermore, when metastasis is diagnosed, it becomes a kind of systemic disease that requires deeper and more comprehensive treatment,

coupled with worse progress.

A Construction of Breast

B Cross-section of Ducts

Pectoral Muscle

Ribs

Fat

Lobules

Ducts

Nipple

Connective Tissue

Normal

DCIS

Invasive Carcinoma
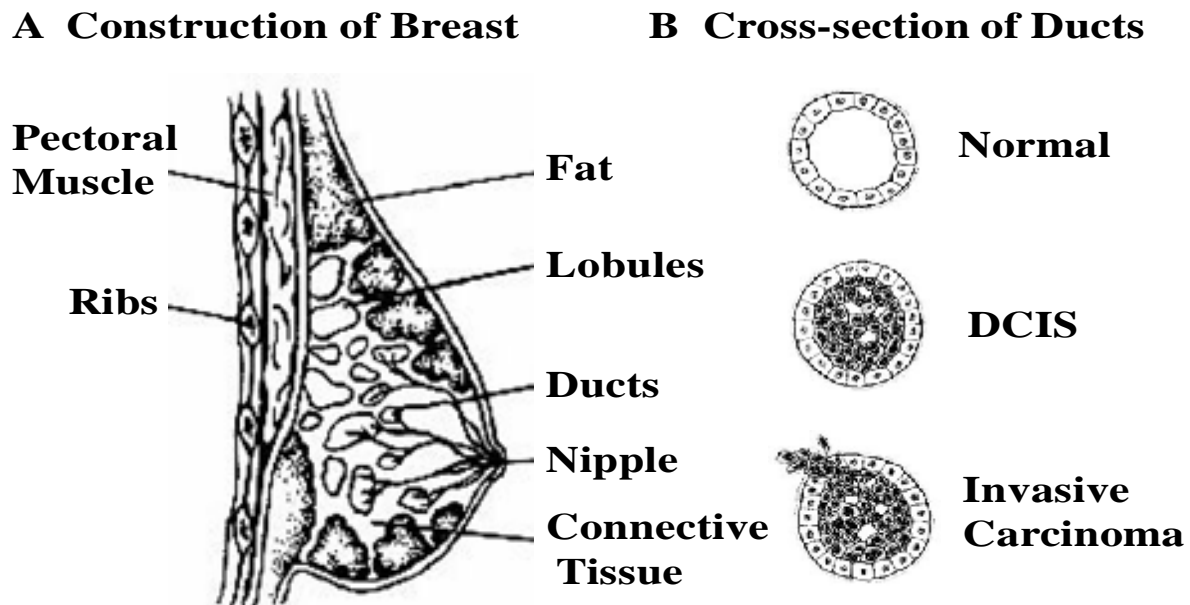
Fig. 3. Schematic representation of the anatomy of breast localization with lobules and ducts. The left image shows the breast anatomy (*A*), right the illustration of a normal milk duct is shown, as well as ducts in DCIS and invasive carcinoma (*B*).

1.2.1   Incidence and detection

Currently, mammography is the gold standard for the early diagnosis of invasive and non-invasive breast tumors. With increased use of mammography screening, which started in the 1980s, detection of DCIS has risen dramatically in parallel especially for women with an age of over 50 years[27]. Now DCIS is discovered in about 20%-45% of newly diagnosed breast cancers and 30% of all breast cancers[28]. However, before the use of mammography screening became widespread, DCIS accounted for less than 5% of breast cancers[29]. If left untreated, nearly 50% of DCIS may progress to invasive ductal carcinoma, which is of a great variability during this period of time[30,31]. Thus, a particular interest in the sensitive detection of DCIS lesion before the transition to an invasive stage emerged and spread. The necessity for early detection is supported by the fact that up to 14% of DCIS are present in microinvasive stages[32].

45% DCIS detected by mammography are characterised by microcalcifications[31]. However, a large proportion of DCIS lesions do not show microcalcification. Nevertheless, the accuracy of mammography for the detection of DCIS is insufficient. The main problem is the underestima-

tion of the extension of DCIS, as well as the underestimation of the number of tumor foci in patients with multifocal disease[27,33].

Kuhl et al.[34] recently published an extensive study on DCIS-screening. They used a method of MRI scan in addition to mammography for diagnostic assessment and screening. A total of 7,000 women were enrolled in this study, in which 167 cases of DCIS were diagnosed. With the aid of MRI examination, 92% of DCIS cases were detected, whereas only 56% of DCIS cases were diagnosed by mammography alone. In high-risk patients with high-grade DCIS, MRI detected 98% of these lesions. By contrast, only 52% were discovered by mammography alone. Disadvantages of MRI investigation are the extremely high cost and lack of qualified personnel for the evaluation of the images. Therefore, MRI examination can probably not replace mammography in the near future.

A further possibility for improved detection of DCIS would be positron emission mammography (PEM). PEM is independent of the presence of microcalcifications and would therefore be a huge advance for the detection of DCIS[35]. Several researches currently suggested that PEM would be of great value in the preoperative identification of malignancy and be a support of conventional breast screening, although some low-grade tumors are not identified with PEM. Therefore, as new tools evolve, a significant and maximum optimization is necessary to enable high detection.

An early detection of DCIS lesions is of paramount importance, since the disease is highly curable at this stage, at which the 10-year cancer-free survival of DCIS is over 97%[36]. Early detection and thus early treatment of DCIS would be the best way to reduce mortality of breast cancer[37].

1.2.2 Classification

So far, the classification of DCIS is based on its characteristic microscopic growth pattern and nuclear features. There are five main types according to the predominant architecture: comedo type, cribriform type, solid type, papillary type and micropapillary type. The comedo type shows a multilayered, neoplastic epithelium, which surrounds a central necrotic area. The cribriform type shows a screen-like proliferation pattern with evenly spaced circular glands or ducts. Tumor cells completely filled with affected breast ducts indicate the solid type. The papillary type is

classified by a fibrovascular framework with epithelial proliferation. The micropapillary type can be discerned by pseudopapillary proliferation without fibrovascular scaffold. However, this classification has various disadvantages. It should be noted that the structure patterns are poorly defined and partially overlap. Several different growth patterns may occur within the same lesion, resulting in a large mixed category[38]. In addition, as a biologically relevant classification, these terms taken into consideration are imperfect, without predictive characters regarding the rate of recurrence or the development of invasive carcinoma. Recent studies have shown that classifications which took the nuclear grading or intraductal proliferation into account obtained meaningful results in terms of local recurrence risk[39]. The Holland classification[40] and the Van Nuys Prognostic Index (VNIP)[41] should be particularly emphasized.

The classification for the malignancy of DCIS lesions due to the histological and cytological criteria of Holland et al.[40] distinguishes well-differentiated, intermediately differentiated and poorly differentiated DCIS. The well-differentiated DCIS is composed of cells with monomorphic, regularly spaced nuclei containing fine chromatin, inconspicuous nucleoli and few mitoses. The intermediately differentiated DCIS shows cells with some pleomorphism but not as strong as in the poorly differentiated group. In poorly differentiated DCIS, one can find atypia, a high mitotic activity and distinct necrosis.

The Van Nuys classification is based on nuclear grading as well as the presence of comedo-type necrosis[42]. Comedo-type necrosis is calcified necrosis within the milk ducts. There are three types, with different probability of relapse. The low-grade DCIS has a low nuclear grade and no necrosis; the intermediate-grade DCIS also has a low nuclear grade but with necrosis; the high-grade DCIS is defined by a high nuclear grade and with (or without) necrosis[42]. The VNIP was derived from the Van Nuys classification[41]. Its significance is calculated from three measurable prognostic factors: the size of the tumor, the width of margin and pathologic classification.

These three factors in the VNIP mentioned above are summarized (**Table 3**). For each parameter, points are assigned from one to three and then totaled to give an overall score (VINP score) ranging from three to nine which is a guideline for further therapy. Patients with VNIP scores of three or four can be considered for treatment with excision only, patients with five to seven require additional radiation therapy, eight and nine points indicate mastectomy[43]. Age as a fourth parameter is sometimes included in the index (USC/VNPI). Patients over 60 years of age

receive one point, patients between 40 and 60 get two points and three points are assigned to patients younger than 40.

Table 3: Van Nuys Prognostic Index (VNPI)

Tumor Size (mm)	Points	Margin Width (mm)	Points	Malignancy	Points
≤15	1	≥10	1	low-grade (non high nuclear grade, no necrosis)	1
16-40	2	1-9	2	intermediate-grade (non high nuclear grade, with necrosis)	2
>40	3	<1	3	high-grade (high nuclear grade, with or no necrosis)	3

Several tumor molecular markers have been examined in DCIS, especially growth factors and hormone receptors. For the estrogen receptor, 50% - 70% of DCIS express this receptor[44]. Besides, it was revealed that the expression of estrogen receptor in well differentiated DCIS lesions is higher than that in poorly differentiated ones[45]. The expression of progesterone receptors appears to be similar to the estrogen receptors. The positive estrogen receptor status in DCIS is also a significant predictive marker for the decision of adjuvant hormonal therapy. Recently, the estrogen receptor was shown to be the primary molecular marker in DCIS detection, with demonstrated relevance to clinical treatment methods.

There are large numbers of studies that have examined the expression of other biomarkers in DCIS. The most frequently analyzed markers are the gene products of the oncogene ERBB2 (HER2) and the tumor suppressor gene p53[46]. There are also analyses of proliferation markers, DNA content, cell cycle regulatory proteins, growth factors and tumor-associated proteases. However, it is impossible to detect a significant correlation between the expression of these markers and the clinical course; neither did we manage to identify a marker that characterizes the transition from DCIS to invasive breast carcinoma.

For the tumor suppressor p53, it has been proved that mutations correlate significantly with tumor grade, presence of necrosis and the mitotic index. Mutations of the p53 gene may be detected in about 25% of all DCIS[47]. HER2 is amplified in approximately 30% of DCIS, but this is mainly in high-grade lesions[48]. The proliferation marker Ki-67 is a valuable marker to distin-

guish between fast and slow proliferating tumors. Meanwhile, the expression of Ki-67 was shown to correlate with the tumor grade[49].

1.2.3 Therapy

Regarding the management of DCIS, there is still disagreement. With increased knowledge about DCIS treatment, the decision becomes more complex. The primary goal is the complete surgical removal of DCIS. In recent decades, breast-conserving surgery was established as an alternative to mastectomy (breast removal). Mastectomy is currently performed only in large, diffusely growing lesions or in case of multicentricity. For large DCIS lesions with a high proportion of comedo necrosis, a careful clinical examination indicated a possible lymph node involvement.

The risk of DCIS patients to form metastasis is low, but the development of local recurrence is high. About 50% of all cases of local recurrence after DCIS therapy developed into invasive cancer and were thus life-threatening. Local recurrence after breast-conserving surgery occurs in high-grade carcinomas within shorter intervals than in well differentiated carcinomas. The rate of local recurrence after breast-conserving surgery for DCIS can be reduced by subsequent exposure to irradiation. According to the 10-year of follow-up reported by the European Organization for Research and Treatment of Cancer study, a reduction of local recurrence by 47% is achieved after irradiation[36]. Mastectomy is associated with the lowest local recurrence risk of 2%[50], there is, however, an overtreatment for a variety of low-grade DCIS. Often, as already mentioned, the VNPI and the statement after breast-conserving surgery are used for the therapeutic decision.

The German Cancer Society recommends carrying out an irradiation after breast-conserving therapy for DCIS. However, in the case of tumors that are smaller than 2 cm or low-grade DCIS with more than 10 mm tumor-free resection margin, adjuvant radiotherapy can be used.

1.2.4 Models of tumor progression

It took a long time to understand the genesis of breast cancer as a multi-step process[51]. This model assumes a gradual progression from normal breast epithelium to atypical hyperplasia occurring, which is regarded as the forerunner of the in situ stage. For invasive breast cancer, there are four stages: normal --- atypical ductal hyperplasia (ADH) --- DCIS--- infiltrating ductal car-

cinoma (IDC). The model was mainly based on pathological and epidemiological data. Recent research results suggest that the linear progression is too simple a model and that the relationship between the levels of progression is far more complex than previously thought. Therefore, the analysis of marker molecules is an important prerequisite for understanding tumor progression and to establishing an exact treatment of various subtypes of DCIS.

DCIS is defined according to the degree of differentiation and genetic changes of the invasive carcinoma, indicating that it is a precursor lesion. Extensive similarities between DCIS and IDC were revealed by comparative genomic hybridization or by analysis of microsatellite instability[52]. Thus, a series of genetic changes in IDC have also been found in the DCIS lesions[53]. Various cytogenetic analyses demonstrated that early lesions such as ADH are later found in advanced non-invasive and invasive carcinomas[54]. This strengthened the hypothesis that the capacity of invasion and biology of a developing carcinoma are already predetermined in the pre-invasive stage. In order to understand the genesis of breast cancer in a better way, a series of cytogenetic studies were conducted[55,56]. Summarizing these results, it was concluded that there were two main groups of DCIS, which could result from gains or losses of chromosomal materials at specific loci[57-59]. The group of well differentiated carcinomas (low nuclear grade) are characterized mainly by the loss of chromosome 16q and gains of 1q. They are predominantly estrogen receptor positive. Poorly differentiated carcinomas, however, show a variety of changes, by gains of chromosome 11q13, and high-grade DCIS and high-grade IDC show amplification of chromosome 17q12. These carcinomas are predominantly estrogen receptor negative[59].

Strong similarity between DCIS and IDC was revealed by several gene expression profiling studies[60,61]. Moreover, distinct expression patterns with different degrees of histological differentiation characteristics were identified[60]. This refuted the linear progression model again. By contrast, low-grade DCIS usually leads to well differentiated IDC and poorly differentiated IDC are formed from high-grade DCIS.

Based on these findings, depicted in **Fig. 4** is a tumor progression model[59], showing both the genetic changes as well as the information obtained by using microarray technology. In this model, the two groups mentioned are strictly separated, and within each group, a vertical progression is shown.
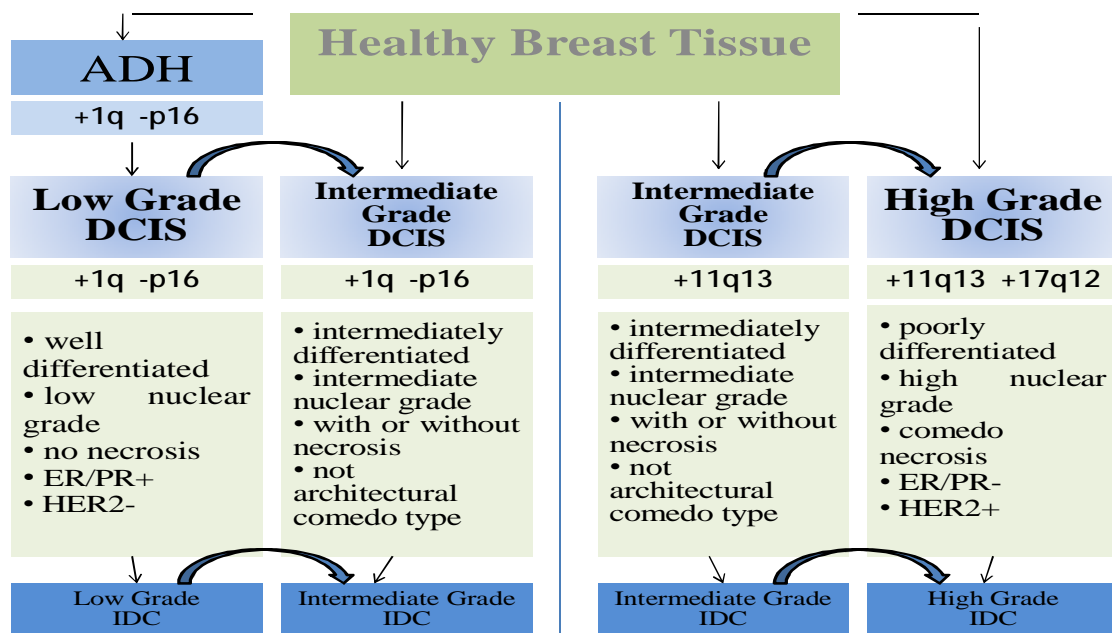
Fig. 4. Schematic representation of a tumor progression model for ductal carcinoma involving the genetic changes and gene expression analyses.

ER = estrogen receptor, PR = progesterone receptor. Based on Moulis and Sgroi. Breast Cancer Res 2008, 10:302.

1.3 DEP domain containing 1 gene

As early diagnosis of DCIS is of utmost importance, finding new biomarkers for disease staging and detection, for monitoring of the disease response to therapy and prediction of patient prognosis seems to be imperative[62]. During our previous research[63] performing whole human microarray analyses of human DCIS samples, we found seven putative tumor markers which are strongly expressed at a very early stage of premalignancy and preneoplasia of breast carcinomas. One of them, DEP domain containing 1 (DEPDC1) is a newly detected gene in this regard.

1.3.1 Structure and Distribution of DEPDC1

Located in 1p31.2, DEPDC1 has two transcriptional variants, DEP (Dishevelled, EGL-10, Pleckstrin) domain-containing protein 1A isform 1 (NCBI Reference Sequence: NM_001114120.1) and DEP domain-containing protein 1A isoform 2 (NCBI Reference Sequence: NP_060249.2). Isoform 1 (also known as DEPDC1-V1: GeneBank Accession *AB281187*) is chosen as the canonical sequence and isoform 2 (also known as DEPDC1-V2: GeneBank Accession *AB281274*) differs from the canonical sequence in one exon. Both contain a highly con-

served DEP domain, and interact with zinc finger transcription factor ZNF224. While DEPDC1-V1 also contains a RhoGAP-like domain, part of it is missing in DEPDC-V2.

DEPDC1 is a newly described gene that is highly overexpressed in bladder cancer cases, but hardly detectable in any human normal tissue apart from the testis[64,65], so it can be regarded as a cancer/testis antigen. Meanwhile, overexpression of DEPDC1 was found in lung adenocarcinoma associated with a worse prognosis[66]. Furthermore, our findings demonstrated that DEPDC1 was significantly up-regulated in DCIS and IDC[63].

1.3.2 Biological functions

Proteins containing DEP domain regulate a broad range of cellular functions including a large number of signaling proteins. For instance, the "D" of DEP domain originates from the drosophila melanogaster disheveled gene, which is known to be an adaptor in the Wnt signaling pathway [67]; the "E" of DEP domain originates from EGL-10, which is considered as a regulator of G-protein signaling protein and negatively regulates signaling by G-protein coupled receptors in Caenorhabditis elegans[68]; the "P" of DEP domain originates from pleckstrin, which modulates signaling in platelets and neutrophils[69]. Although DEPDC1 contains a highly conserved DEP domain, there are still many open questions with regard to its pathophysiologic roles for the growth of human cancer cells.

Coimmunoprecipitation and immunocytochemistry experiments revealed that the DEP domain in DEPDC1 interacts and is colocalized with ZNF224, a Kruppel-like zinc-finger protein being a transcriptional repressor that represses aldolase A gene transcription[70]. Thus, DEPDC1 is recognized to be probably involved in transcriptional regulation of many genes as a transcriptional corepressor.

1.3.3 Gene expression of DEPDC1 in cancer and clinical aspects

Strong staining of endogenous DEPDC1 protein in the nucleus of bladder cancer cells was detected by immunocytochemical staining analysis[65]. The growth of bladder cancer cells was significantly inhibited through the suppression of DEPDC1 expression with small-interfering RNA (siRNA)[65]. Consequently, it can be suggested that DEPDC1 might play an essential role in the growth of bladder cancer cells and would be a promising molecular-target for novel therapeutic

drugs or cancer peptide-vaccine to bladder cancers. Recently, similar results were found by Harada et al. who indicated that DEPDC1 contributes to bladder cancer oncogenesis[64]. According to this study, the DEPDC1-ZNF224 complex could probably play a critical part in bladder carcinogenesis. Apoptosis of bladder cancer cells in vitro and in vivo could be induced by inhibiting the ZNF224-interacting domain in DEPDC1[64]. Besides, a DEPDC1-derived peptide vaccine has been shown to effectively induce peptide-specific cytotoxic T lymphocytes in 66.7% (4/6) of advanced bladder cancer[71], leading to a stable disease and prolongation effect of overall survival.

In addition, an involvement of upregulation of DEPDC1 in lung adenocarcinoma was determined recently[66], and a high expression of DEPDC1 was associated with poor prognosis.

In the PrognoScan database, DEPDC1 is shown to be positively expressed in bladder cancer, breast cancer, multiple myeloma, lung adenocarcinoma and melanoma. A high expression is coupled with a poor outcome of diseases. Therefore, DEPDC1 could be a novel target for diagnosis and therapy in various cancers. Furthermore, our previous publication has shown that DEPDC1 is significantly up-regulated in breast cancer (**Fig. 5**)[63]. Possibly, our findings of an enhanced expression of DEPDC1 in human DCIS samples will allow an early detection of DCIS in the future, thus leading to early treatment and longer survival of patients who are at high risk of developing invasive breast carcinomas[63]. In summary, these results suggest that DEPDC1 is a key molecule for the development of cancer in various entities. However, the detailed mechanisms which get activated by DEPDC1 or which pathways are involved have not been elucidated yet.
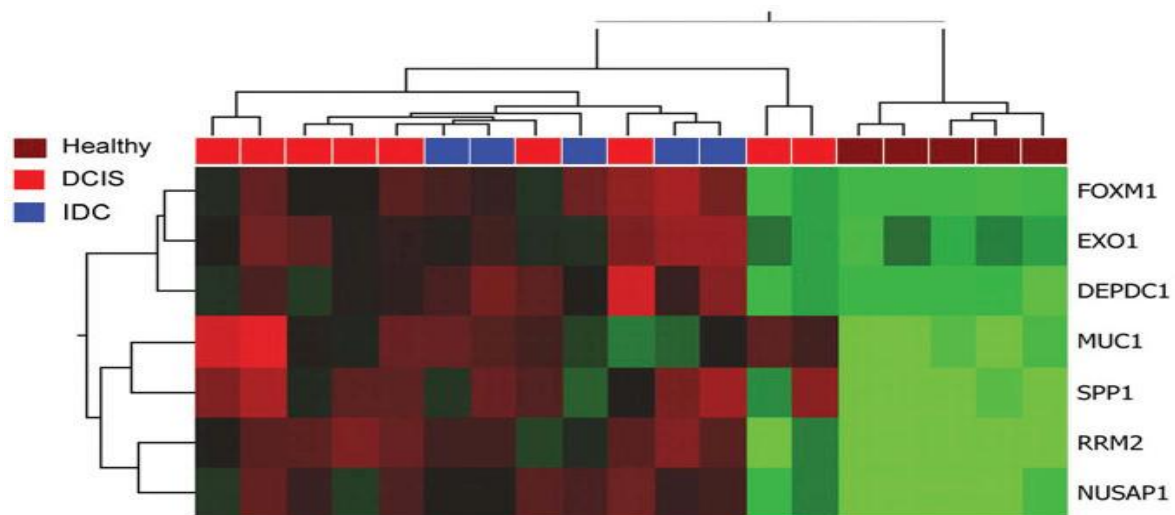
Fig. 5. Microarray analysis of seven putative tumor marker genes including DEPDC1 in breast cancer.
Each row represents a probe set and each column a human sample. Red indicates upregulation, green is downregulation, and black means no change. A strong upregulation of DEPDC1 gene in malignant DCIS and IDC compared to the healthy samples is found.

Although the clinical significance of DEPDC1 is still under active investigation, encouraging results associated with DEPDC1 detection and metastatic progression in patients have provided a reliable estimate of disease survival at an earlier stage. Thus, DEPDC1 might become a prognostic weapon against earlier death of patients with various cancer diseases.

2. Aims of the study

As mentioned above, DEPDC1 is a novel, recently detected gene highly overexpressed in various carcinomas. Up to now, only five references with regard to DEPDC1 could be found in MEDLINE and PubMed databases. The functional regulation of DEPDC1 for the development and invasion of cancer cells has not been clarified yet. Therefore, the aim of this thesis is to characterize the functional relevance of DEPDC1 in breast cancer based on experiments promoting DEPDC1 gene overexpression or inhibiting its expression by RNA-interference in an in vitro model. In order to investigate thoroughly the effect of DEPDC1 in breast cancer cell lines, two different cell lines were chosen: MDA-MB-231 (an aggressive fibroblast-like cell showing a high expression of DEPDC1) and MCF-7 (a less aggressive cell showing a low level of DEPDC1 expression). Both cell lines were utilized to assess various functional parameters, such as cell proliferation, apoptosis and migration.

3. Materials and Methods

3.1 Material

This section describes the materials used in this work sorted in alphabetical order.

3.1.1 Biologic substances

Plasmid for DNA transfection:

DEPDC1: was transfected using pcDNA^{TM} 3.1/V5-His TOPO$^{®}$ TA Expression Kit

(Invitrogen^{TM})

Probes for Taqman gene expression assay:

DEPDC1 (Hs00854841_g1)   (Assay on Demand^{TM})

Hu GAPDH (Probe dye: VIC-MGB)   (Applied Biosystems)

The gene ID number is 4325792, and the RefSeq is NM_002046.3.

Reagents:

TransFectin^{TM} Lipid Reagent: (Bio-Rad)

DharmaFECT$^{®}$ transfection reagent: (Thermo Scientific)

Mouse monoclonal antibody towards DEPDC1 (#H00055635-   (abnova)
M05)

Goat anti-mouse antibody conjugated to horseradish peroxid-   (Abcam)
dase (#97245)

Antibody diluent with background reducing components   (Dako)

Mayer's Hematoxylin (#008001)   (Invitrogen)

VectaMount^{TM} Mounting Medium (#H5507)   (Vector)

ImmPACT^{TM} DAB peroxidise substrate   (Vector)

Add 1 drop approximately 30 µl of ImmPACT™ DAB Chromogen concentrate to 1 ml ImmPACT™ DAB Diluent to make the substrate solution.

siRNA:

FlexiTube siRNA Hs DEPDC1 (20 nmol):   (QIAGEN)

The powder was added to 1ml siRNA buffer, making the stock concentration 20 µM, and filter sterilized through filters, make aliquots to 100µl, stored at -20°C.

Allstars Neg. Control siRNA (20 nmol)   (QIAGEN)

The powder was added to 1ml siRNA buffer, making the stock concentration 20 µM, and filter sterilized through filters, make aliquots to 100µl, stored at -20°C.

3.1.2 Cell lines

3. Materials and Methods

Two human breast cancer cell lines derived from pleural effusion, MCF-7 (estrogen receptor positive, ER$^+$) and MDA-MB-231 (ER$^-$; progesterone receptor negative, PR$^-$; human epidermal growth factor receptor negative, HER2$^-$), obtained from the American Type Culture Collection, were used in this study. Details of these cell lines are given in **Table 4**.

Table 4: Cell lines

Name	Growth	Receptor Expression	Origin
MCF-7	adherent	estrogen receptor positive (ER$^+$)	ATCC$^®$, HTB-22^{TM}
MDA-MB-231	adherent	triple-negative (ER$^-$, PR$^-$, HER2$^-$)	ATCC$^®$, HTB-26^{TM}

3.1.3 Media, buffers and solutions

Accutase (100ml):   (PAA)

Make aliquots to 5ml, store at 4°C for preservation.

Amphotericin B: (PAA)

Make aliquots to 5ml, store at 4°C for preservation.

Diethylpyrocarbonate (DEPC)- treated water (Pryogen-free):   (Invitrogen)

Foetal Bovine Serum (FBS):   (PAA)

Filter sterilized through filter and store at -20°C.

G418 disulfate salt solution (50 mg/ml):   (Sigma)

Filter-sterilized H$_2$O to 50 mg/ml and make aliquots to 10ml, stored at 4°C.

Glutamine (2mM):

L-Glutamine (200mM, 100x)   (PAA)

Produce a 1:100 dilution, filter-sterilized H$_2$O to 50 ml and make aliquots to 5ml, stored at -20°C.

MCF-7 Culture Medium (100ml):

    10% FBS Gold (10ml)   (PAA)

    1% 2mM Glutamin (1ml)   (PAA)

    1% 100mM Na-Pyruvate (1ml)   (PAA)

    1% Non Essential Amino Acids (NEAA) (1ml)   (PAA)

    1% P/S (1ml)   (PAA)

    Insulin, human 10mg/ml ready to use (246ul)   (Sigma)

    RPMI 1640 (85.6ml)   (PAA)

Add 1ml Glutamin (2mM) before using, stored sterile at 4°C for preservation.

3. Materials and Methods

MDA-MB 231 Culture Medium (100ml):

10% FBS Gold (10ml)	(PAA)
1% Amphotericin B (1ml)	(PAA)
1% 2mM Glutamin (1ml)	(PAA)
RPMI 1640 (88ml)	(PAA)

Add 1ml Glutamin (2mM) before using, stored sterile at 4°C for preservation.

MEM Non Essential Amino Acids (NEAA, 100x Concentrate):   (PAA)

Make aliquots to 5ml, stored at 4°C for preservation.

P/ S (100Units/0.1mg/ml):

Penicillin (10,000Units)/ Streptomycin (10mg/ml)   (PAA)

Produce a 1:100 dilution, make aliquots to 5ml and stored at -20°C..

1x PBS (to wash the cells) (100ml):

P/S (1ml)	(PAA)
Gentamicin(10mg/ml, 200mM) (0.5ml)	(PAA)
Phosphate buffered saline(1x PBS) (98.5ml)	(PAA)

Store sterile at 4°C for preservation.

Trypsin (0.05%)/ EDTA (0.02%):   (PAA)

Filter sterilized through a 0.2 μm filter unit and make aliquots to 10ml, stored at -20°C

3.1.4 Kits

Cell apoptosis assay kit (FITC Annexin V Apoptosis Detection):   (BD Pharmingen[TM])

Purified Recombinant Annexin V (0.5mg/ml)

FITC Annexin V

R-phycoerythrin Staining Solution

10x Annexin V Binding Buffer

Cell invasion assay kit (Matrigel[TM] Invasion Chamber):   (BD BioCoat[TM])

24-Well BD Falcon[TM] TC Companion Plate

Falcon Cell Culture inserts, containing 8 micron pore size PET membrane with a thin layer of Matrigel Basement Membrane Matrix

RNA isolation kit (RNeasy Micro Kit):   (QIAGEN)

RNeasy MinElute[®] Spin Columns

QIAshredder Spin Columns

Buffer RLT. 1% β-mercaptoethanol must be added to RLT before use

Buffer RW1

Buffer RDD

Buffer RPE (4x, concentrate). The working solution is diluted in four time-volume 100% ethanol

Rnase-free water

Collection Tubes (1.5ml and 2ml)

Carrier RNA, poly-A

RNase-Free DNase Set: DNase I stock solution: DNase (1500 Kunitz units). Dissolve the lyophilized DNase in 550µl of the RNase-free water provided. (Inject RNase-free water into the vial using an RNase-free needle and 1ml syringe); mix gently by inverting the vial (never vortex). And divide it into single-use aliquots. Stored at -20°C for six months.

<u>TaqMan kit (RNA UltraSense^{TM} One-Step qRT-PCR System):</u>　　　(Invitrogen)

RNA UltraSense^{TM} Enzyme Mix (250µl)

RNA UltraSense^{TM} 5X Reaction Mix (2 x 1ml)

50x ROX Reference Dye (100µl). This must be stored in the dark

Sufficient reagents are provided for 100 reactions

3.1.5 Technical equipment and instruments

Centrifuges:

Microlitercentrifuge CT15RE	(VWR)
Varifuge 3.0R	(Heraeus)
Megafuge 2.0R	(Heraeus)
Galaxy Mini Centrifuge Kinetic Energy 26 Joules	(VWR)

Flow cytomery:

FACSCantoTM Π Glow Cytometer	(Becton Dickinson)
CELLQuest Pro® software	(Becton Dickinson)

Freezers:

4°C and -20°C	(Liebherr)
-40°C	(GFL)
-80°C (Ultra Low temperature Freezer C660)	(Labotect)

Incubators:

Shaking incubator	(SHELLAB)
Certomat® RM Shaking incubator	(B.Braun)

3. Materials and Methods

Automatic CO_2 incubator	(Heraeus)

Microscopes:

Inverse VisiCam 5.0	(VWR)
Olympus BX50	(Olympus, Japan)

Real-time PCR:

MicroAmp^{TM} Optical 384-well Reaction plate	(Applied Biosystems)
SDS 2.4 software	(Applied Biosystems)
Micro Amp^{TM} Optical Adhesive Film	(Applied Biosystems)
7900HT Fast Real-Time PCR System	(Applied Biosystems)

RNA quality control:

Agilent 2100 Bioanalyzer	(Agilent)
2100 expert software B.02.05.SI360	(Agilent)

RNA quantity control:

ND-1000 spectrophotometer	(NanoDrop®)
ND-1000 software V3.1.0	(NanoDrop®)

Other equipment for cell culture:

C-Chip (DHC-N01)	(Digital Bio)
Cell culture flasks (25cm^2/75cm^2)	(Falcon®)
Cell culture hood	(BDK)
Combitips® (5ml)	(Eppendorf)
Culture dishes	(TPP®)
Culture Insert	(ibidi)
Culture plates (6-well/24-well), polypropylene	(BD Falcon^{TM})
Cytospin 3	(Shandon)
Heating bath B15	(Thermo Scientific)
Magnetic-stirrer Waterbath	(Thermo Electron)
Multipette® Plus	(Eppendorf)
Pipetus	(Hirschmann)
Rotator SB3	(Stuart)
Serological pipette, sterile (1ml, 5ml, 10ml, 25ml)	(BD Falcon^{TM})
Sterile single use filter unit (0.2μm)	(Sartorius)
Tips (yellow, blue)	(Eppendorf)
Tubes (15ml/50ml), polypropylene conical	(BD Falcon^{TM})

5ml Polystyrene Round-Bottom Tube	(BD Falcon[TM])
Vacusafe-Safety Vacuum System	(IBS)
Vortex Genie 2TM	(Bender & Hobein)

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Cell maintenance

MCF-7 cells were cultured in RPMI 1640 medium supplemented with 1% (v/v) NEAA and 10% (v/v) FBS, and containing 100 units penicillin / 0.1 mg/ml streptomycin, 1% (v/v) Na-pyruvate and 2mM glutamine. The cells were maintained in a 37°C incubator with 5% CO_2. The first medium changing occurred 24h later, and the complete medium was changed every three days.

MDA-MB-231 cells were cultured in RPMI 1640 medium containing 10% (v/v) FBS, 1% amphotericin B, and 1% 2mM glutamine. The cells were maintained in a 37°C incubator with 5% CO2. The first medium changing occurred 24h later, and then the complete medium was changed every three days

3.2.1.2. Cell passaging

Both cell lines were subcultured at 72 hour intervals (almost growing 80%) by removing the old culture medium, washing with an appropriate amount of PBS buffer (3ml per T25cm^2) gently. Trypsin/EDTA (e.g. 1.0 ml of 0.25% (w/v) per T25cm^2 flask) was added to neutralise the buffer used before and then discarded. More trypsin/EDTA (0.5ml per T25cm^2 flask) was added.

When the cell cloned ball began to sufficiently disperse (working 5 to 6 minutes, in 37°C incubator), additional fresh culture medium was added at a ratio of 1:10.

3.2.2 siDEPDC1-mediated knockdown in MDA-MB-231 cells

3.2.2.1 Transfection of MDA-MB 231 using siDEPDC1

Preparation

Cell seeding number: 1 x 10^5 cells/well in 6-well plate; transfection concentrations: 5nM; final concentration of DharmaFect reagent: 2μl/well

Procedures

Prepare 5 μM siRNA solution in serum-free medium RPMI 1640. In separate tubes, dilute the siRNA and the appropriate DharmaFect transfection reagent with RPMI 1640. Gently mix the contents of each tube by pipetting carefully up and down. Incubate for 5 minutes at room temperature. Mix each new tube by pipetting carefully up and down. Incubate for 20 minutes at room temperature. Add 3200 μL of antibiotic-free complete medium to the mix in last step, for a final volume of 4000μL transfection medium (2ml/well) and a final siRNA concentration of 5nM. Remove culture medium and wash by 2ml PBS once. Add 2ml transfection medium to each well. Incubate cells at 37°C in 5 % CO_2 for 48–72 hours (over the weekend) for mRNA analysis later.

3.2.2.2 RNA isolation and quality/quantity control

Preparation

Cells: remove the medium and wash with PBS gently. Extract the PBS and disrupt the cells by adding 350μl buffer RLT; DNase I stock solution incubation mix.

Procedures

Pipet the lysate in every collection tube directly into a QIAshredder spin column placed in a 2ml collection tube, and centrifuge 2min at 15000 revolutions per minute (rpm).

Add 350μl 70% ethanol to the lysate and mix. Transfer the sample to an RNeasy MinElute spin column placed in a 2ml collection tube and centrifuge for 15s at 10000 rpm. Discard the flow-through. Add 350μl buffer RW1 to the RNeasy Min Elute spin column and centrifuge for 15s at 10000 rpm. Discard the flow-through. Add 80μl DNase I stock solution incubation mix directly to each RNeasy MinElute spin column membrane, and place at room temperature for 15min. Add 350μl buffer RW1 to the RNeasy MinElute spin column and centrifuge for 15s at 10000 rpm. Discard the flow-through and collection tube. Place the RNeasy MinElute spin column in a new 2ml collection tube. Add 500μl buffer RPE to the spin column and centrifuge for 15s at 10000 rpm. Discard the flow-through. Add 500μl 80% ethanol to the RNeasy MinElute spin column and centrifuge for 2min at 10000 rpm. Discard the flow-through and collection tube. Place the RNeasy MinElute spin column in a new 2ml collection tube. Open the lid of the spin column, and centrifuge at 15000 rpm for 5min. Discard the flow-through and collection tube. Place the RNeasy MinElute spin column in a new collection tube (1.5ml), add 16μl RNase-free water directly to the

centre of the spin column membrane. Then centrifuge at 4°C for 1min at 15000 rmp to elute the RNA.

The RNA concentration was measured by UV spectrophotometry with software ND-1000 V3.1.0. The samples whose quantity beyond 100ng/μl, and A260/A280 ratio greater than 2.0, A260/A230 ratio greater than 1.8 was used in a further quality control and experiment. Quality control was performed with Agilent 2100 Bioanalyzer to check for genomic DNA contamination and RNA decay. And only samples with a RIN (RNA integrity number) of more than seven were taken.

3.2.2.3 Taqman Real Time PCR

Dilute the isolated RNA with DEPC to 100ng/μl, total volume is 10μl, vortex and centrifuge. Prepare a master mix on ice of all components according to DEPC---Master Mix---housekeeper---Assay on Demand (DEPDC1)---ROX---RNA Ultrasense Enzyme, in one 0.5ml epitube, as specified below:

Make four aliquots of 33.25μl in each 0.5ml-epitube, and add 1.75μl diluted RNA separately to every aliquot (without DEPC epitube). Gently mix. Add 10μl/well in 384-well plate (three well for every sample), keep the RNA 50ng/well

Use optical adhesive film, close the plate, centrifuge at 1200 rpm for 30 second. The program of the real time instrument to perform cDNA synthesis immediately followed by polymerase chain reaction (PCR) amplification was carried out according to the protocol shown below:

Temperatures (°C)	Time
50	15min
95	2min
40 cycles of:	
95	15sec
60	30sec

The relative quantitation of gene expression was performed with comparative CT Method (ΔΔCt). In this experiment the expression levels of a target (DEPDC1) and an endogenous control (GAPDH) were evaluated. The levels of these amplicons in a series of siRNA-treated samples are

compared to an untreated calibrator sample.

3.2.2.4 Functional determination of cell proliferation

Perform the siRNA in 6-well plates first. After the 48h of incubation with the siRNA, wash cells with PBS twice gently, add 100µl of trypsin / EDTA, and collect the wells of the same treatment together in a 15ml-Falcon-tube.

Seed 10^4 cells/well for all the treatments in the five plates of 24-well (after 6 hours, cells must be attached to the plate. This point is considered time point 0) with the time interval 24 h. Perform the Calcein protocol as follows: warm up PBS and Calcein; remove medium from wells (it is very important to completely remove the medium because phenol red can interfere); wash with PBS twice; prepare the Calcein staining solution: add 1µl Calcein into 1ml PBS; add 350µl staining solution/well; incubate for 60 min at 37°C; measure plate with the OMEGA plate reader: bottom optic, 480nmexcit/520emiss, gain 1000; 90% required value, shake for 5sec before reading.

3.2.2.5 Functional determination of apoptosis

Remove the cell-culture medium of MDA-MB-231, add 500µl of accutase and incubate 20min. Prepare 4 x 15ml Falcon polypropylene tubes: Control; 2µl DharmaFect; Allstars Neg. 25nM; siRNA DEPDC1 25nM.

After incubation with accutase, add 500µl PBS, and collect the cells with the PBS. Wash each well with 1000µL of PBS twice, collect all the volume. Centrifuge 1200rpm/5min, discard the flow-through, and resuspend cells in 4ml PBS, vortex for mixture.

Aliquot samples into 4 tubes (1ml/tube), centrifuge 1100rpm/4min, remove PBS, and resuspend cells in: controls (100µl of FACS Buffer); FITC tubes (10µl FITC Annexin V + 100µl of FACS buffer); propidium iodide (PI) tubes (5µl PI + 100µl of FACS buffer); Mix tubes (10µl FITC Annexin V + 5µl PI + 100µl of FACS buffer). Vortex for mixture, incubate at 37°C for 30 min in the dark, and add 500µl of FACS Buffer to the samples before measuring.

3.2.2.6 Functional determination of cell invasion

Remove the medium from 6-well plate after siDEPDC1 transfection (72 hours), wash twice with 1ml PBS and exhaust this wash solution by vacuboy. Add serum-free medium RPMI 1640

2ml/well, incubate for 24h at 37°C in a 5% CO_2 incubator. Thaw Matrigel (12 inserts, four transwells per treatment: Allstars Neg./ siDEPDC1/ DharmaFECT) from freezer to room temperature, dilute Matrigel (500μl /insert and 700μl /well) in serum-free medium, at 37°C in a 5% CO_2 incubator for 2h.

Harvest cells from the 6-well plate with 500μl accutase per well, incubate for 20min at 37°C in a 5% CO_2 incubator. Wash cells twice with serum-free medium, collect the wash solution and centrifuge to 15ml Falcon^{TM} tube with 1ml serum-free medium. Mix the cells gently with 1ml syringe, count the cells with c-chip, and dilute the cells to $1x10^5$ / ml with serum-free media in 5ml Polystyrene Round-Bottom tube. After the incubation of 2 hours, remove the media from Matrigel, add into media with 20% FBS 750μl per well, fill the lower chamber of transwells with 500μl cellsolution ($1x10^5$/ml), at 37°C in a 5% CO_2 incubator for 22h. The next day, remove the media from transwells, collect the media, wash Matrigel with PBS once. Harvest cells from transwell and well with 750μl accutase, wash with serum-free media once and collect the wash solution in the same epitube. Count the cells.

3.2.3 DEPDC1 overexpression in MCF-7 cells

3.2.3.1 Cloning of DEPDC1 gene into pcDNA3.1 vector

Isolation of RNA from MDA-MB-231was performed as described previously. Run PCR with the following reaction components:

	Per reaction	Four tubes (backup DNA)
RNase-free water	33 μl	138,6
5x QIAGEN OneStep RT-PCR Buffer	10 μl	42
dNTP Mix (containing 10 mM of each dNTP)	2 μl	8,4
Primer sense	0.5 μl	2,1
Primer antisense	0.5 μl	2,1
QIAGEN OneStep RT-PCR Enzyme Mix	2 μl	8,4
Template RNA	2 μl	8,4
	50 μl	210

The thermal cycler conditions are described in the table below:

	Time	$T^{ao}C$
Reverse transcription:	30 min	45
Initial PCR activation step	15 min	95
3-step cycling: 40 cycles		
Denaturation:	10sec	94
Annealing:	1min	58
Extension:	3min	68
Final extension:	10min	68
End	∞	4

PCR products were checked with 1% agarose gel electrophoresis. We obtained a single band of approximately 3000pb and then purified the product with the MinElute PCR purification Kit. The following reaction was performed using theAmpliTaq DNA Polymerase (Applied Biosystems):

Reagent	µl per reaction
10x AmpliTaq DNA Buffer	**2**
dNTPs (from the topo kit)	**1**
AmpliTaq DNA Polymerase	**1**
PCR product	**10**
H$_2$0	**6**

This mixture was incubated at 72°C for 10 minutes. The table below describes the settings of the TOPO® Cloning reaction for depdc1transformation into chemically competent TOP10 E. Coli.

Reagent	µl per reaction
Fresh PCR product	4
Salt solution	1
Topo® Vector	1
Final volume	6

The mixture of the TOPO® Cloning reaction and the chemically competent TOP10 E. Coli were incubated together on ice for 30 minutes. After this incubation time we let them stay 2 min at 42°C and another 2 minutes at 4°C. The bacteria were incubated 2 hours with medium SOC shaking horizontally at 37°C. The recombinants were selected on solid Luria-Bertani (LB) medium containing ampicillin (100µg/ml). Plasmid DNA was extracted with the QIAprep® Miniprep Kit.

The structure of the construct (insertion and orientation) was analyzed by restriction analysis (FastDigest® EcoRV) and PCR.

3.2.3.2 Transfection of MCF-7 using DEPDC1 plasmid

The day before transfection, inoculate 24-well plates with 5×10^4 of MCF-7 cells/well in 0.5ml complete medium, at 37°C in a 5% CO_2 incubator overnight.

The following day, transect the MCF-7 cells in the 24-well plate: six wells only MCF-7 cells as black control; six wells with 1.0 µl TransFectin reagent. As for the other twelve wells, choose six wells: for each well, prepare 0.5µg plasmid DEPDC1 in 50 µl of serum-free medium and 1.0 µl TransFectin reagent in 50 µl of serum-free medium, gently mix the DNA and TransFectin solutions together by tapping or pipetting, incubate 20 minutes at room temperature. Add 100 µl of the DNA–TransFectin complexes directly to cells in complete medium; choose the rest six wells as MOCK control: for each well, prepare 0.5µg empty plasmid pcDNA3.1 in 50 µl of serum-free medium and 1.0 µl transfectin reagent in 50 µl of serum-free medium, gently mix the empty plasmid and TransFectin solutions together by tapping or pipetting, incubate 20 minutes at room temperature. Add 100 µl of the DNA–TransFectin complexes directly to cells in complete medium. Incubate the cells at 37°C in a 5% CO2 incubator. Replace transfection medium with fresh complete medium after 24 hours.

3.2.3.3 G418 selection of DEPDC1 expression MCF-7 cells

After the 24hours' incubation with fresh culture medium for MCF-7 DEPDC1 transfection cells, refresh the medium with G418 medium (complete culture medium containing 800 µg/ml of G418) for selection of stable clones.

At a ratio of 800 µg G418 / ml, refresh G418 medium the first time after three days' incubation. While the concentration of G418 is reduced to 700µg/ml during the next medium change, this whole selection period can take 10 to 14days. There will be a massive cell death and most of the cells will wash off the bottom of the plate, leaving colonies of stable cells behind.

To pick clones, prepare 6-well plate with 1ml medium containing 700µg G418. Rinse the 24-well plate with PBS and then add 200µl 0.25% (w/v) trypsin/EDTA per well, incubating for 5min.

Then for every well add warm 800μl G418 medium (700μg/ml), colonies can be transferred into the new 6-well plate at a 1:1 ratio.

3.2.3.4 RNA isolation and quantity/quality control

RNA was extracted using the RNeasy Micro Kit according to the manufacturer's instructions, the method is the same at that described in "3.2.2.2", and only the samples whose quantity beyond 100ng/μl, A260/A280 ratio greater than 2.0, A260/A230 ratio greater than 1.8, and with the RIN of more than seven were taken for further testing.

3.2.3.5 Taqman gene expression assay

Using the primer DEPDC1 and housekeeper GAPDH, set up two sample groups: DEPDC1 0.5μg as test group, empty plasmid 0.5μg as MOCK group, and use DEPC-treated water for NTC, with the same method as that described in "3.2.2.3".

3.2.3.6 Immunocytochemistry

Remove the culture media and rinse once with PBS, centrifuge cells at 1200rpm for 5min; remove the PBS and immediately add 100% alcohol with a temperature of -20°C, and put the tube in -20°C freezer for 10 min. Centrifuge again, remove the absolute alcohol and add PBS. Fixed cells are kept at 4°C for subsequent immunostaining.

To this end, cells were incubated for 30 min with 0.3% H_2O_2 +0.3% BSA in PBS at room temperature, followed by incubation for 20 min with ready-to-use (2.5%) normal horse blocking serum. Then cells were incubated with mouse monoclonal antibody to DEPDC1 diluted 1:500 for 1 hour, antibody dilute solution was taken as substitute for the self negative control in each group. After washing with PBS for 5 min, the cells were stained with goat anti-mouse antibody conjugated to horseradish peroxidase at a dilution of 1:500 for 45 min. After washing with PBS for 5 min, cells were incubated with peroxidase substrate solution for 20 min. The reaction was stopped with ddH_2O and then counterstained with hematoxylin solution for 1 min. The slides were washed twice for 2 min each, and mounted with VectaMount. Immunostaining images were obtained under light microscope.

3.2.3.7 Functional determination of cell proliferation

According to the protocol in Section 3.2.2.4.1, just use non-treated MCF-7 breast cancer cell line as control group, use pcDNA3.1 empty vector transfected-MCF-7 cells as negative group, use DEPDC1 plasmid transfected-MCF-7 cells as the test groups which included three clones (test 6, test 7 and test 10).

3.2.3.8 Functional determination of apoptosis

According to the protocol in Section 3.2.2.4.2, but use non-treated MCF-7 breast cancer cell line as control group, use pcDNA3.1 empty vector transfected-MCF-7 cells as negative group, use DEPDC1 plasmid transfected-MCF-7 cells as the test groups which included three clones (test 6, test 7 and test 10). Meanwhile, before the FITC Annexin V test, two different methods were used: one was the normal way without any change; the other one incubated in 37°C for three hours with rolling and then test with Annexin.

3.2.3.9 Functional determination of cell migration

Prepare cell suspension with $1x\ 10^5$ cells/ml. Make non-treated MCF-7 breast cancer cell line as control group, pcDNA3.1 empty vector transfected-MCF-7 cells as negative group, DEPDC1 plasmid transfected-MCF-7 cells as the test groups which included three (test 6, test 7 and test 10).

Apply 70 µl into each well of culture insert. And fill the outer area with 400 µl cell medium. Incubate at 37°C and 5% CO_2.

After 24 hours (appropriate cell attachment), gently remove the culture insert by using sterile tweezers. Watch the reaction of cells migrating into the cell-free gap by microscope every 24 hours.

4. Results

4.1 Expression of DEPDC1 gene in a panel of breast cancer cell lines:

Previous results from the work of our laboratory showed that DEPDC1 is a putative tumor marker for early breast cancer. DEPDC1 is strongly expressed at very early stages of premalignancy, as well as in invasive breast carcinomas. In order to find a possible relation between the expression of DEPDC1 gene and the malignancy of different breast cancer cell lines, we examined the endogenous expression of this gene by quantitative RT-PCR (**Fig. 6**). The MDA-MB-231 breast cancer cell line showed the highest expression of DPEDC1 among all the cell lines studied. In addition, Hs578Bst breast cancer cells barely expressed DEPDC1 under normal conditions. Similarly, the MCF-7 breast cancer cell line expressed DEPDC1 only at very low levels. Among the three breast cancer cell lines, MDA-MB 231 cells have the highest metastatic ability. In contrast, Hs578Bst cells which have been cultivated originate from normal breast tissue, and MCF-7 cells show only minimal invasiveness. Therefore, these results may indicate a relationship between DEPDC1 and aggressiveness of breast cancer cells.

As such, these data showed the direction for future studies: MDA-MB-231 cells were chosen to clone the full-length cDNA of the DEPDC1. Similarly, this cell line was chosen for experiments which were designed to examine the effect of DEPDC1 on cellular functions by gene silencing using siRNA. Alternatively, for overexpression of the DEPDC1 protein, MCF-7 cells were chosen in order to examine if these cells become more invasive after upregulation of the expression of DEPDC1.
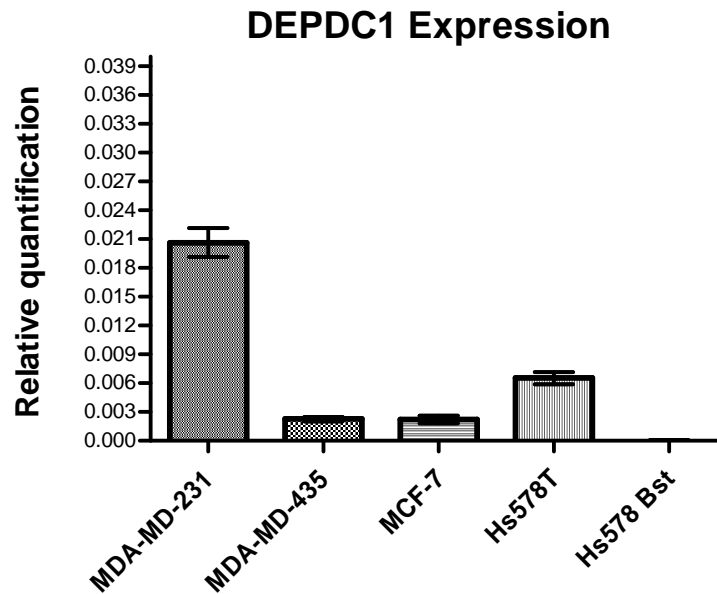
Fig. 6. Different DEPDC1 expression in different breast cancer cell lines.

RNA was extracted using the RNeasy Mini Kit, according to the protocol described in the "Materials and Methods" section. RNA quality was checked with BioAnalyzer. RNA with a RIN (integrity number) value below 9 was discarded. TaqMan® Gene Expression Master Mix was used to perform real-time PCR (RT-PCR). The mRNA relative levels were normalized to the mean value used $\Delta\Delta Ct$ method in comparison to GAPDH expression. All the experiments were performed at least in triplicate and data were shown as mean ±standard error.

4.2 Inhibition of DEPDC1 expression in MDA-MB-231 cells by the siRNA-treatment

In order to examine the effects of DEPDC1 on cell functions, the mRNA-expression of DEPDC1 was inhibited by siRNA-treatment. We chose the breast cancer cell line MDA-MB-231, which displayed the highest amount of DEPDC1 on mRNA. DEPDC1 expression was checked with GAPDH-controlled RT-PCR in cells treated with DEPDC1 siRNA, cells treated with a negative control siRNA (Allstar) and cells treated only with the transfection reagent DharmaFect. Cells treated with siDEPDC1 showed a strong DEPDC1 down-regulation. DEPDC1 expression of siDEPDC1 treated cells was about 90% lower than that of the group treated with DharmaFect alone (transfection reagent) which was considered as control group (**Fig.7**). The Allstar group (Negative siRNA treated cells) showed nearly 15% inhibition of DEPDC1 expression in comparison to the control group.

These results indicated that treatment with DEPDC1 siRNA leads to an effective knockdown of

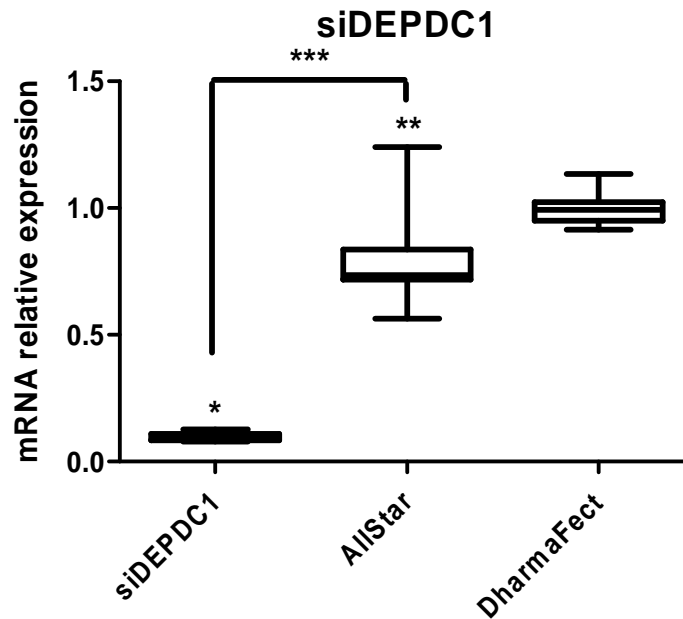the expression of endogenous DEPDC1 in breast cancer cell line MDA-MB-231.

Fig. 7. Inhibition of DEPDC1 expression after the siRNA-treatment in MDA-MB-231 breast cancer cells.

The siRNA-sequence directed towards DEPDC1 sequence included both possible transcripts (gene bank accession NM_001114120 and NM_017779). 100000 cells/well were seeded, incubated overnight to ascertain attachment to the plate, and then cells were incubated with siRNA at a concentration of 5nM. The DharmaFect group (co-transfected with an equal concentration of the transfection reagent) and Allstar group (transfected with an equal amount of corresponding negative siRNA) were treated in the same way. After 48 h of treatment, cells were harvested and RNA was isolated. DEPDC1 mRNA expression was determined by quantitative RT-PCR. The mRNA relative levels were normalized to the mean value by using the $\Delta\Delta$Ct method in comparison to GAPDH expression. Graph shows the mean normalized DEPDC1 expression of three independent experiments performed in quadruplicates; error bars represented SEM. Statistical significance was assessed by t-test (*$p < 0.001$, **$p < 0.005$ compared with control group). Normalized DEPDC1 expression of the siDEPDC1 group was compared to the Allstar negative control by t test for unpaired samples (***$p < 0.001$).

4.2.1 Effect of DEPDC1 expression on proliferation

In order to examine the effect of DEPDC1 on cell proliferation, we counted viable cells using the Calcein-AM assay which detects the capacity of the intracellular esterase to convert non-fluorescent Calcein-AM into green-fluorescent calcein. The fluorescent calcein is well retained in the cytoplasm of living cells. This property is useful to carry out fluorescence-based microplate assays for the determination of cell viability. Cell density was determined by replacing the medium with 0.35 ml of 1µM calcein Solution. For measuring the plates at distinct incubation times,

an OMEGA plate reader (480 nm excit/520 nm emission) was used.

First, a standard curve of the ratio of Calcein-AM staining versus cell number was prepared (**Fig. 8A**): cell numbers were measured after seeding cells for 6 h. This standard curve gives the relationship between the cell number and the fluorescence of calcein. Linear data are shown and the values represent means ± standard error.

Next, we measured the effect of siRNA-treatment at different times after seeding. Cells were treated with DEPDC1 siRNA or with a negative control siRNA (Allstar) and compared to cells treated only with the transfection reagent DharmaFect. Cell numbers were counted every 24h after siRNA treatment (5nM) using the metabolism of Calcein-AM dye. Each point is the average value of three wells. As depicted in **Fig. 8B**, cells treated with DEPDC1 siRNA grew much slower than the cells of the control groups.

Taken together, these observations indicate that the expression of DEPDC1 gene has a strong stimulating effect on the proliferation of MDA-MB-231 breast cancer cells.
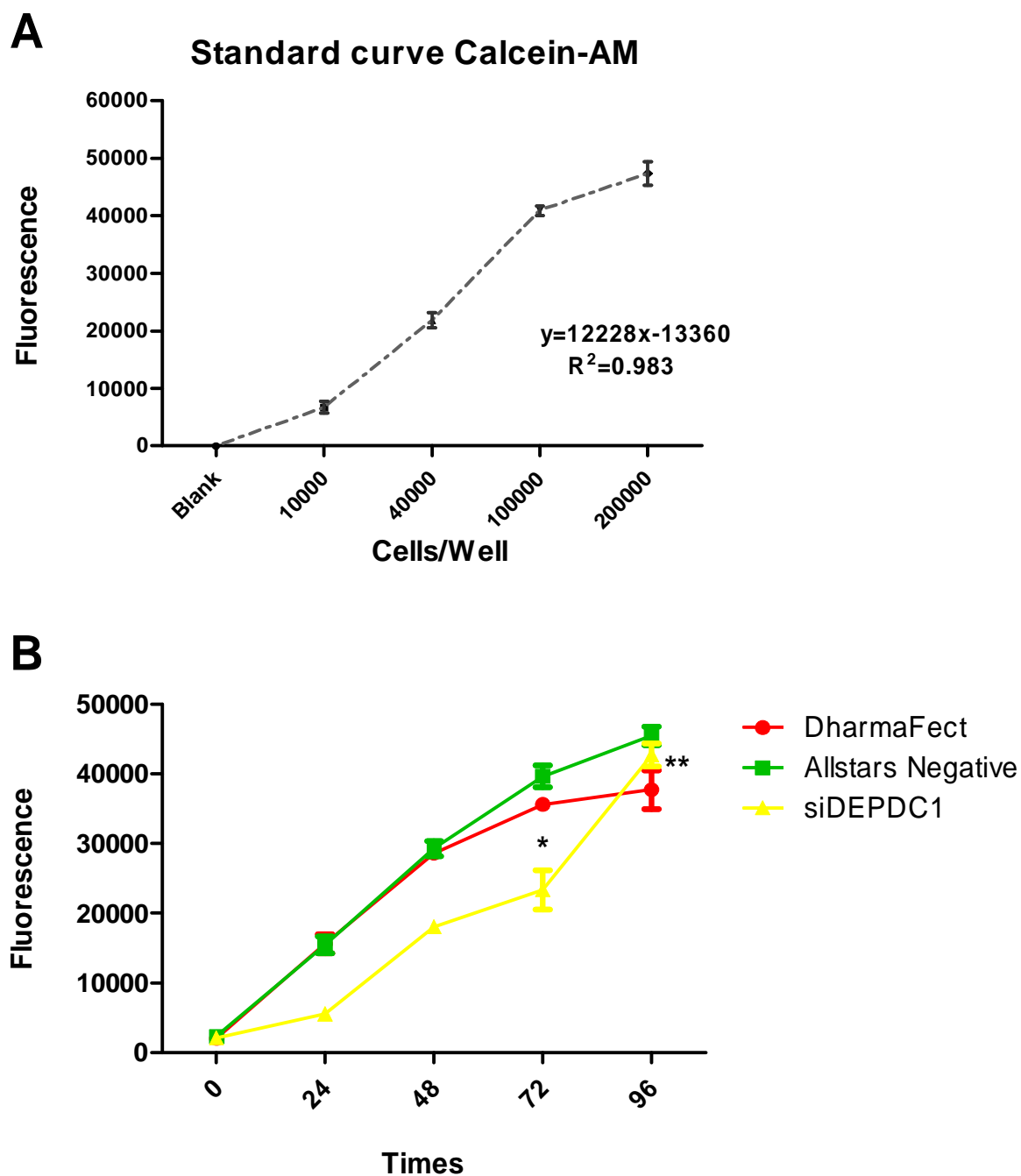
A

B

Fig. 8. Silencing of DEPDC1 inhibits proliferation of MDA-MB-231 cells.

(A) The standard curve of cell number-dependent response with the fluorescence of Calcein-AM, statistical analysis used linear regression. *(B)* Cell numbers were counted at different time points after seeding. The strongest suppression of proliferation was obtained at 72 h (*$p < 0.0009$, compared to the DharmaFect control group), reaching an inhibition of 62%. There was no statistical significance at 96 h (**$p > 0.05$, compared to DharmaFect control group), indicating that the siRNA-effect is lost after 96h due to degradation of the siRNA.

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4.2.2 Effect of DEPDC1 expression on apoptosis

In order to determine whether the siDEPDC1-treatment affects the apoptosis of MDA-MB-231 breast cancer cells, 10^5 cells per well were seeded in a 6 well plate and transfected with siDEPDC1, Allstar negative control or only DharmaFect. Apoptosis was examined using the FITC Annexin V Apoptosis Detection Kit. Cell pellets were resuspended in FACS buffer containing 10µl Annexin or/and 5µl PE to distinguish between apoptotic and necrotic cells. The percentage of cells undergoing early-stage apoptosis (Annexin V-FITC positive) or late-stage apoptosis/necrosis (Annexin V-FITC and PE positive) was measured with a BD FACSCanto II flow cytometer. The results clearly showed an increase in the number of apoptotic cells after treatment with siDEPDC1 compared to DharmaFect and Allstar negative control cells.

As shown in **Fig. 9A**, the early stages of apoptosis which are characterized by changes in mitochondrial membrane potential and cell membrane asymmetry, were significantly increased in the siDEPDC1 group. No such increase was observed in the apoptotic level for the Allstar negative control compared to the control cells. Besides, the late apoptosis characterized by DNA fragmentation and loss of cell membrane permeability was analyzed (**Fig. 9B**). The siDEPDC1 played the role in maintaining pro-apoptotic effect. However, there was no statistical difference between the Allstar negative control group and the DharmaFect control.

These findings demonstrate that the DEPDC1 gene expression might block apoptosis of MDA-MB-231 breast cancer cells.
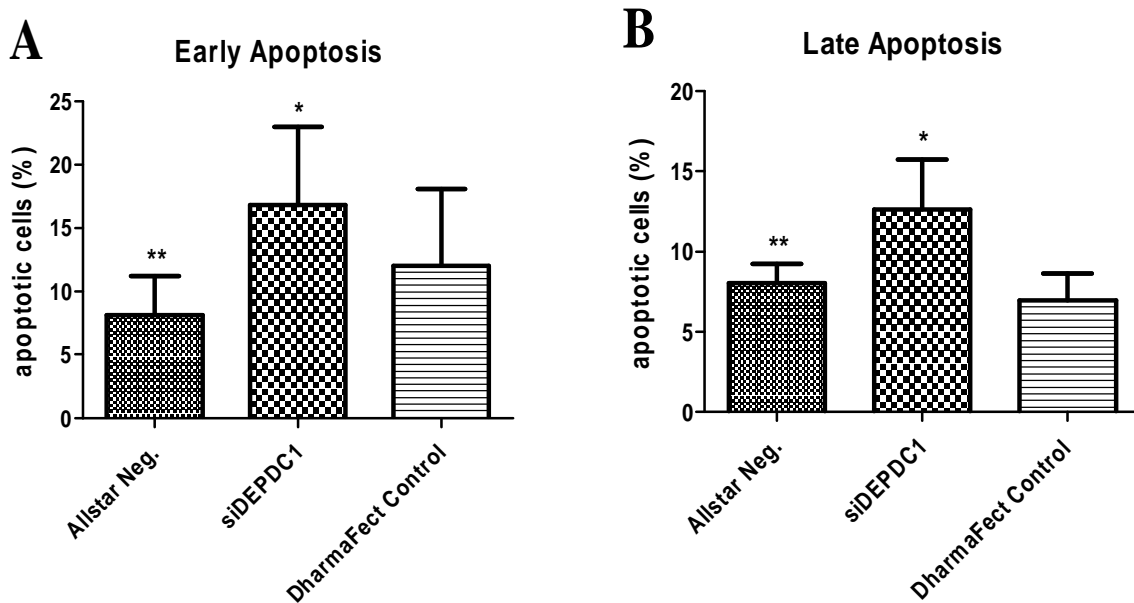
Fig. 9. Effect of DEPDC1 on apoptosis in MDA-MB-231 cells.
Early *(A)* and late *(B)* apoptosis were analyzed among three groups of treatments. The graph showed the mean percentage of apoptotic cells (+ SE) performed in triplicates. Apoptosis values of cells were compared to their corresponding controls by t-test for unpaired samples. In both stages of early apoptosis and late apoptosis, differences between DharmaFect control and Allstar negative control samples were not significant (**$p > 0.05$), while the pro-apoptotic effect of siDEPDC1 was significantly higher (*$p < 0.05$, compared to DharmaFect control group).

4.2.3 Effect of DEPDC1 expression on cell cycle distribution

In order to confirm the results obtained in the apoptosis assay and clearly depict possible changes in cell division, cell cycle analysis was performed. Numbers of cells in the different cycles are depicted in **Fig. 10.** For comparison we used cells treated with DharmaFect alone, Allstar negative control and siDEPDC1. The distribution of cells in the G1 (enables cells to grow and primes to DNA synthesis), S (DNA replication), and G2/M (mitosis) phases of the cell cycle can be determined by measuring the DNA content. Mostly, cells remain in the G1 phase, which is thus numerically the most predominant phase and shows up as the largest peak. In S phase, DNA synthesis commences and chromosomes replicate; entering into G2/M phase, each chromosome has sister chromatids. Therefore, G2 phase cells have twice the cellular DNA content compared to the G1 cells. S-phase cells are distributed between the G1 and G2/M populations. The DNA content of the cells was quantitatively measured by flow cytometry analyses. The cell cycle profiles are shown in **Fig. 10B, 10D & 10F** with gates P3, P4 and P5, separately gating cells in G1, S phase

and G2/M.

Of the control cells treated only with DharmaFect, as shown in **Fig. 10B**, 72.2% were in G1 stage, 11.9% in S phase and 12.6% in G2 phase. No statistical difference was observed in the DNA content of cells for the Allstar negative control compared to the control cells (**Fig. 10D**). For the MDA-MB-231 cells treated with siDEPDC1 in **Fig. 10F**, 79.1% of the cells were at the G1 phase, 10.0% were at the S phase and 8.0% were at the G2 phase. In comparison to the control cells in **Fig. 10B**, it is evident that the treatment of siDEPDC1 transfection induces G1/S arrest. Furthermore, as the majority of cells are at G1, cells probably accumulate at G1 due to siDEPDC1 transfection; by contrast, DNA synthesis (S phase), growth (G2 phase) and mitosis (M phase) are reduced.

DEPDC1 therefore appears to induce both DNA synthesis and mitosis. This result confirms our data displayed in the previous section on apoptosis showing that DEPDC1 plays a significant role in the increased growth of MDA-MB-231 breast cancer cells.
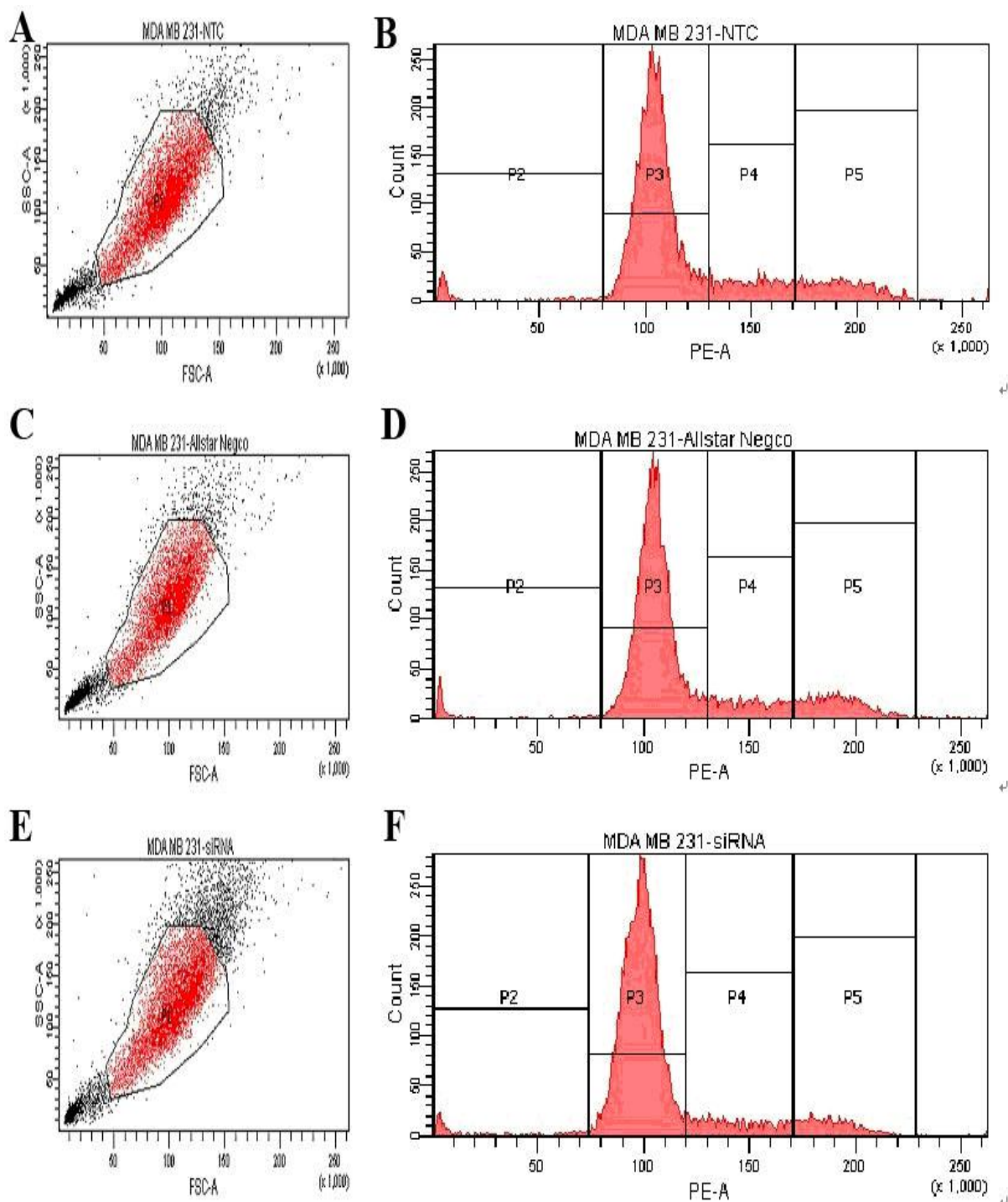
Fig. 10. PE 575/26nm Channel Cell Cycle Analysis in MDA-MB-231 cells.
Left side: side scatter versus forward scatter for MDA-MB-231 cells separately treated with DharmaFect *(A)*, with Allstar negative control *(C)* and with siDEPDC1 *(E)*, these DNA content gatings are placed on FSC x SSC (cell size x granularity). Right side: DNA content frequency histograms representing the number of cells in G1 stage, S stage and G2/M stage, MDA-MB-231 cells treated with DharmaFect *(B)*, with Allstar negative control *(D)* and with siDEPDC1 *(F)* are displayed using PE 575/26nm-A parameter.

4.2.4 Effect of the DEPDC1 expression on invasion

In order to assess the effect of DEPDC1 on the invasive ability of MDA-MB-231 breast cancer cell line, Boyden Chamber invasion assays were performed. Cells were seeded into separate inserts and the invasion rate was quantified by counting the cells which migrate through the pores of the membrane at the bottom of the insert. MDA-MB-231 cells treated with Allstar Negative control were set as a mock sample (**Fig. 11**). Cells treated with DharmaFect were considered as the control group, and the invasion levels of these cells were normalized to the mean value of the control group which was regarded as 100% (not shown).

The cells treated with siDEPDC1 showed a loss of approximately 70% in migration capacity in comparison to DharmaFect-treated cells. However, cells treated with Allstar negative control showed only a 30% decrease compared to the control group. This finding demonstrates a pro-invasive effect of DEPDC1 on MDA-MB-231 breast cancer cell migration.
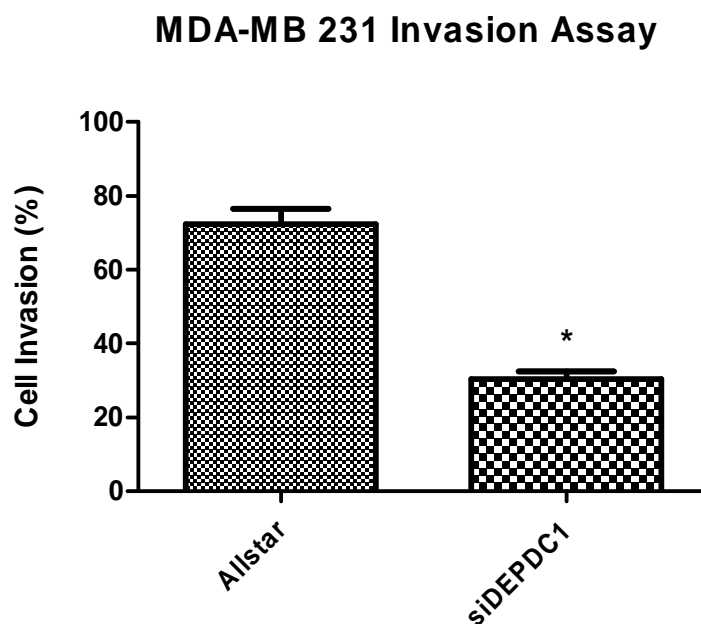
Fig. 11. Effect of DEPDC1 on invasion in Boyden Chambers with Matrigel.
The anti-invasive effect of siDEPDC1 was determined using Boyden Chambers filled with Matrigel. On the horizontal axis the different treatment groups are indicated. Cells treated with siDEPDC1 showed a 70% loss of invasive capacity, cells treated with Allstar Negative lost 30% of the invasive capacity. Statistical significance was assessed using the t test (*p < 0.0001, compared to the Allstar negative mock group)

4.3 Overexpression of DEPDC1 in MCF-7 cells by plasmid transfection

In order to further analyze the functional significance of DEPDC1 on tumour cell aggressiveness in an alternative way, the effects of DEPDC1 overexpression of cells normally having a low DEPDC1 expression was examined. To this end, we used the MCF-7 breast cancer cell line which expresses the DEPDC1 gene only lowly. The DEPDC1 gene obtained with PCR from MDA-MB-231 cells was subcloned into pcDNA3.1 vector (see "Materials and Methods"), followed by the knock-in of MCF-7 cells and cultivation of the clones with G418 for selection of stable DEPDC1 overexpression clones

To ensure successful DNA plasmid transfection to the MCF-7 cell line, GAPDH-controlled RT-PCR was performed. Results showed that the mRNA-expression of DEPDC1 was indeed stimulated drastically after cloning DEPDC1 into the MCF-7 breast cancer cell line. Non-treated MCF-7 cells were used as a control group and pcDNA3.1 empty vector transfected-MCF-7 cells were used as the negative group. Three clones were grown which had been transfected independently with the DEPDC1-containing plasmid (called test 6, test 7 and test 10). There was an effective DEPDC1 up-regulation in all three test groups compared to the control group (**Fig. 12**), especially in test 7 which showed an approximately four fold the DEPDC1 expression in comparison to the negative clone. There was no significant difference in DEPDC1 expression between the negative group and the control group, which indicates that there was no cytotoxicity caused by the transfection reagent or process or other nonspecific effects.

Overall, these data demonstrated that the DEPDC1 expression is upregulated in the MCF-7 test clones 6, 7 and 10.
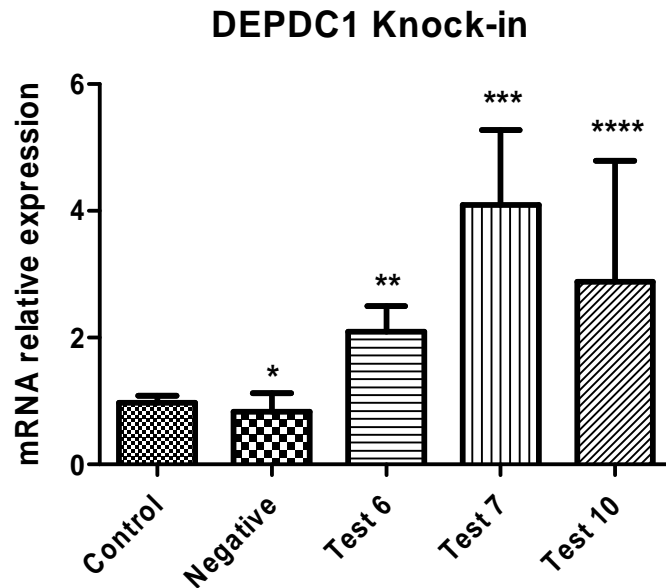
DEPDC1 Knock-in

Fig. 12. Enhanced DEPDC1 expression after cloning DEPDC1 into MCF-7 breast cancer cells.

We used pcDNA™3.1/V5-His-TOPO® as empty vector. The plasmid DNA (DEPDC1 RNA was isolated from MDA-MB-231 cells) was extracted with the QIAprep® Miniprep according to the protocol described in the "Materials and Methods" section. 0.5 ug plasmid (test group) and the same amount of empty vector (negative group) were transfected into MCF-7 cells set as 5×10^5 cells per well. The control group (non-treated MCF-7 cells) was kept without any transfection. After 24 h of treatment, G418 was added to the medium for selection of stable clones containing a DEPDC1 insert. The mRNA relative levels were normalized to the mean value using the ΔΔCt method in comparison to GAPDH expression. Graph shows the mean normalized DEPDC1 expression of five independent experiments performed in quadruplicates and the error bars represent the SEM. Statistical significance was assessed using the t test. DEPDC1 expressed significant upregulation in test groups compared to the control group (**$p < 0.01$; ***$p < 0.02$; ****$p < 0.05$ compared with control group). No statistical significance was assessed for the normalized DEPDC1 expression between the negative group and the control group (*$p > 0.05$).

4.3.1 Immunocytochemical evaluation of DEPDC1 overexpression

In order to analyze the overexpression of DEPDC1 on MCF-7 test 7 cells transfected with DEPDC1 on the protein level and to confirm the results of Taqman PCR in advance, an immuno-cytochemistry assay was performed. MDA-MB-231 cells which normally have a high DEPDC1 expression were used as a positive control and non-treated MCF-7 cells, which express the DEPDC1 gene only at a low level, as a negative control. For each group, there was one staining without primary antibody (mouse monoclonal antibody towards DEPDC1, see "Materials and

Methods"), taken as control in this group. We used goat anti-mouse antibody conjugated to horse-radish peroxidase as the secondary antibody. This immunocytochemistry reaction refers to the process of detecting DEPDC1 protein in breast cancer cells by exploiting the principle of antibodies (human IgG2A here) binding specifically to antigens (DEPDC1 protein here) in tumor cells. The horseradish peroxidase is an enzyme which produces a brown staining that can be analyzed microscopically.

As expected, MDA-MB-231 cells showed positive immunostaining (ranging from + to +++) in 90% of cells investigated and thus have a high DEPDC1 expression also on the protein level (**Fig. 13**). In contrast, non-treated MCF-7 cells showed nearly no immunostaining compared to the secondary antibody-only control. However, MCF-7 test 7 cells are quite similar to MDA-MB-231 cells in this regard, showing a strongly positive immunoreactivity for DEPDC1 is hereby demonstrated.

Our observations by immunocytochemistry prove that there is a significant difference in DEPDC1 protein expression between non-treated MCF-7 control and test 7 clones, implying an upregulation of DEPDC1 in MCF-7 test 7 clones. Therefore, the treatment with DEPDC1 plasmid transfection leads to an effective overexpression of the DEPDC1 gene in breast cancer cells MCF-7 also on the protein level.
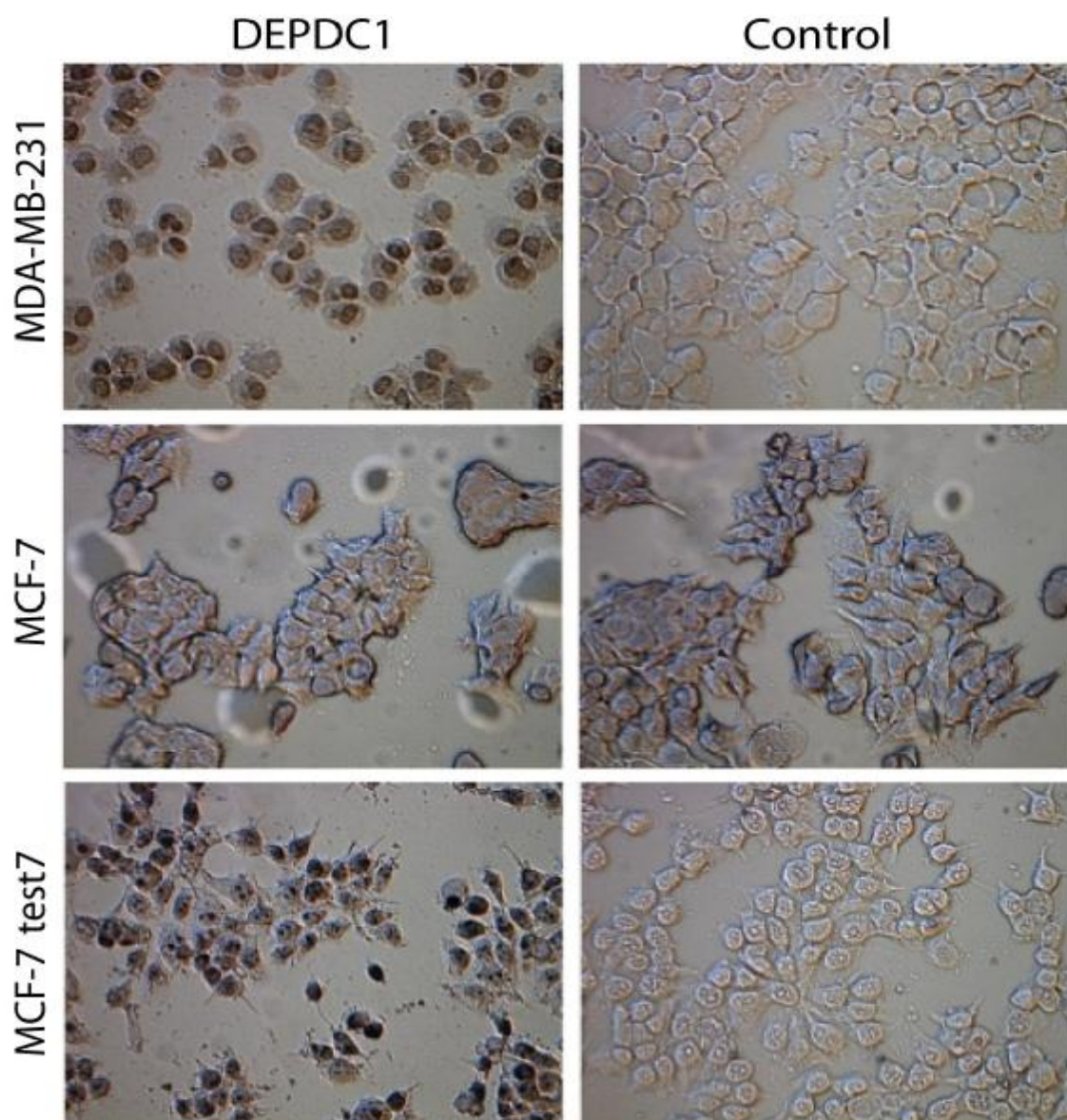
Fig. 13. Immunocytochemical identification of DEPDC1 overexpression in MCF-7 cells.
Breast cancer cells were stained for DEPDC1 according to the protocol described in the "Materials and Methods" section. The DEPDC1-positive cells are stained in brown color. Pictures on the left show cells stained with a monoclonal antibody to DEPDC1; pictures on the right show cells incubated only with the secondary antibody. MCF-7 test 7 clones were similar to MDA-MB-231 cells, showing a strong DEPDC1 immunostaining compared to the MCF-7 group. Magnification: 200x.

4.3.2 Effect of DEPDC1 overexpression on proliferation

In order to examine the effect of DEPDC1 on proliferation in MCF-7 cells, we counted viable cells using the Calcein-AM assay which quantified the capacity of intracellular esterase to convert

non-fluorescent Calcein-AM into green-fluorescent calcein (described in Section 4.2.1).

First, a standard curve of the ratio of Calcein-AM-staining versus cell number was prepared (**Fig. 14A**). Cells were counted after seeding non-treated cells for 6 hours. The standard curve gives the relationship between cell number and the fluorescence of calcein. Linear data of living cells were shown and the values represented means ± standard error.

Next we measured the effect of DEPDC1 overexpression at different times after seeding. Cells transfected with DEPDC1 plasmid or with pcDNA3.1 empty vector were seeded and compared to cells without any treatment. Cell numbers were counted every 24 h after cell seeding using the metabolism of Calcein-AM dye. Each point represents the average value of three wells. As depicted in **Fig. 14B**, all cells transfected with DEPDC1 plasmid in three test groups grow much faster than the cells of control group.

In summary, these observations indicate that the DEPDC1 gene has a strong stimulating effect on the proliferation of MCF-7 breast cancer cells.
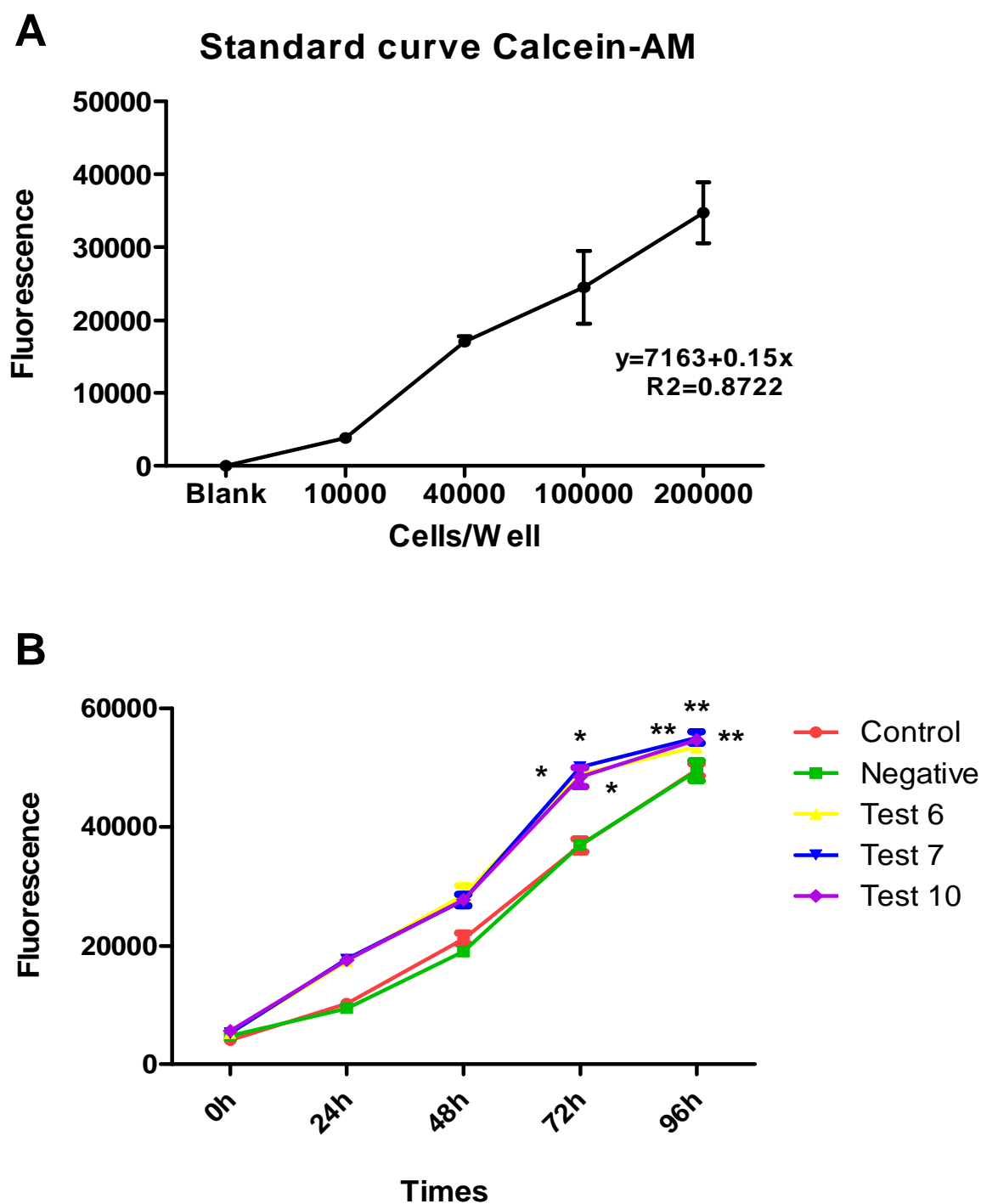
Fig. 14. Effect of DEPDC1 overexpression on proliferation of MCF-7 cells.

(A) The standard curve of cell numbers in relation to the fluorescence of Calcein-AM. Linear regression was used in statistical analysis. *(B)* Cells were counted at different time points after seeding. The maximal activation of proliferation was obtained at 72 h (*p < 0.001, compared to the control group), reaching an activation of 40%. There was no statistical significance at 96 h (**p > 0.05, compared to the control group).

4.3.2 Effect of DEPDC1 overexpression on apoptosis

In order to determine whether DEPDC1 gene affects apoptosis of MCF-7 breast cancer cells, 10^5 cells/well of three different groups (non-treated MCF-7 control, empty vector transfected negative mock and DEPDC1 plasmid transfected test samples) were seeded in a 6 well plate. Apoptosis was examined using the FITC Annexin V Apoptosis Detection Kit by Flow Cytometry. For this method, cells need to be dissociated, since individual cell have to be counted. If the cells cluster together, no measurement is possible. However, MCF-7 cells tend to form clusters. Therefore, we compared cells dissociated from the culture plates in the conventional way (mentioned in Section "Materials and Methods") and cells dissociated in a second manner that includes incubation of cells in 37℃ for three hours under agitation or rolling before starting the Annexin assay.

The results without rolling of the cells (**Fig. 15A & 15B**) clearly showed a reduction in the number of apoptotic cells for DEPDC1 transfected cells compared to control cells. As shown in **Fig. 15A**, the DEPDC1 expressing test groups showed an insignificant reduction in the early stages of apoptosis. With regard to late apoptosis (**Fig. 15B**), DEPDC1 had a dramatic anti-apoptotic effect in the test groups, especially in the test 7 group which showed a decrease by 19.7% in the number of apoptotic cells compared to the control group. Although the negative group clearly expressed lower apoptotic ability than the control group, this was of no statistic significance. These findings demonstrate that the DEPDC1 gene expression might be responsible for an anti-apoptotic effect in MCF-7 breast cancer cells.

In contrast, no significant differences were found between the cell clones if rolling was applied (**Fig. 15C & 15D**). For early apoptosis (**Fig. 15C**), test 7 showed no anti-apoptotic ability. Moreover, all test groups and the negative group showed a comparable apoptotic level compared the control group. In late apoptosis (**Fig. 15D**), the most apoptotic cells were still in test 7 and the least apoptotic cells in the negative group, without any statistical significance compared to the control group. These data demonstrated that the DEPDC1 gene probably did not play a part in the anti-apoptotic effect in MCF-7 breast cancer cell line.

Although with the same samples and the same FTTC Annexin V apoptosis detection kit, conflicting findings were obtained. The problem might be that the MCF-7 breast cancer cells are not appropriate for this experiment. Therefore, no conclusion can be drawn about the role of DEPDC1 in the apoptosis of MCF-7 cells.
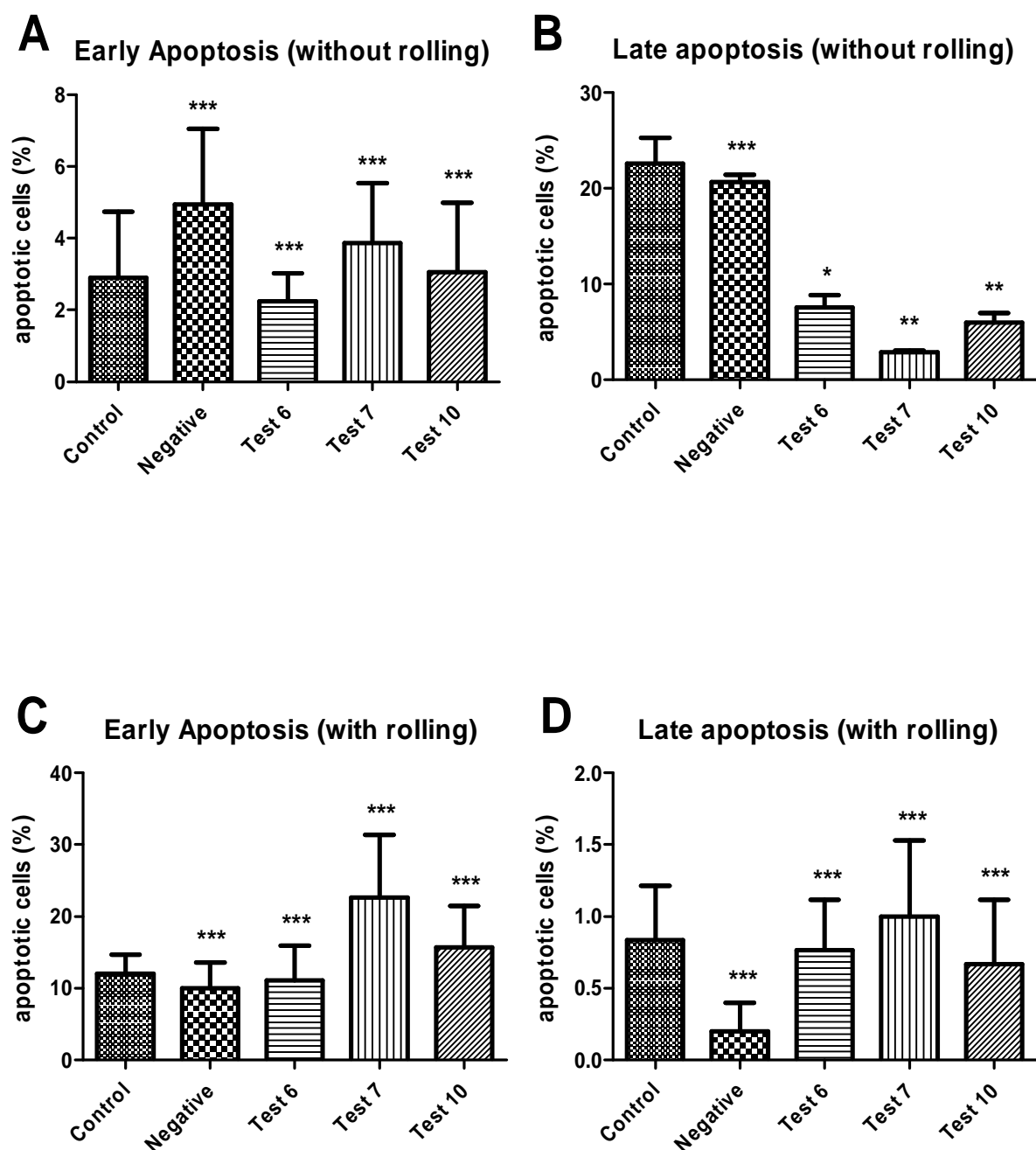
Fig. 15. Effect of DEPDC1 overexpression on apoptosis in MCF-7 cells.

Top lane: early *(A)* and late *(B)* apoptosis without rolling incubating before Annexin test. Bottom lane: early *(C)* and late *(D)* apoptosis with rolling incubating before Annexin test. Graph shows the mean percentage of apoptotic cells (+ SE) performed in triplicates. Apoptosis values of cells were compared to their corresponding controls using the t test for unpaired samples (*$p < 0.05$; **$p < 0.01$; ***$p > 0.05$ compared to the control group).

4.3.3 Effect of DEPDC1 overexpression on migration of MCF-7 cells

In order to determine whether DEPDC1 also affects the migration rate of MCF-7 breast cancer cells, a wound healing or scratch assay was performed. For comparison we added MDA-MB-231 cells, which express DEPDC1 strongly as a positive control.

Instead of simple scratching, culture-inserts were put into a 24-well plate after seeding of 10^5 cells/ml to create a "wound" in the cell monolayer. After 24 hours, the culture-inserts were gently removed and cell migration to the cell-free gap was investigated with a microscope. Images were captured at regular intervals (24, 48, 72 and 96 hours) during cell migration, and compared to quantify the migration rate of cells. Representative examples are shown in **Fig. 16**. As expected, MDA-MB-231 cells have a high migration capability and were able to quickly close the gap. In contrast, non-treated MCF-7 cells and tests 6 and 10 showed no closing of the gap. Interestingly, cells in test 7, which expressed DEPDC1 strongly, showed a strong capability to close the gap and were quite similar to MDA-MB-231 cells in this regard.

Our observations of cell migration prove that there is a significant difference in wound closure between non-treated MCF-7 control and test clones, implying a strong effect of DEPDC1 on migration. Therefore, DEPDC1 seems to have a strong stimulating effect on invasion in breast cancer cells.

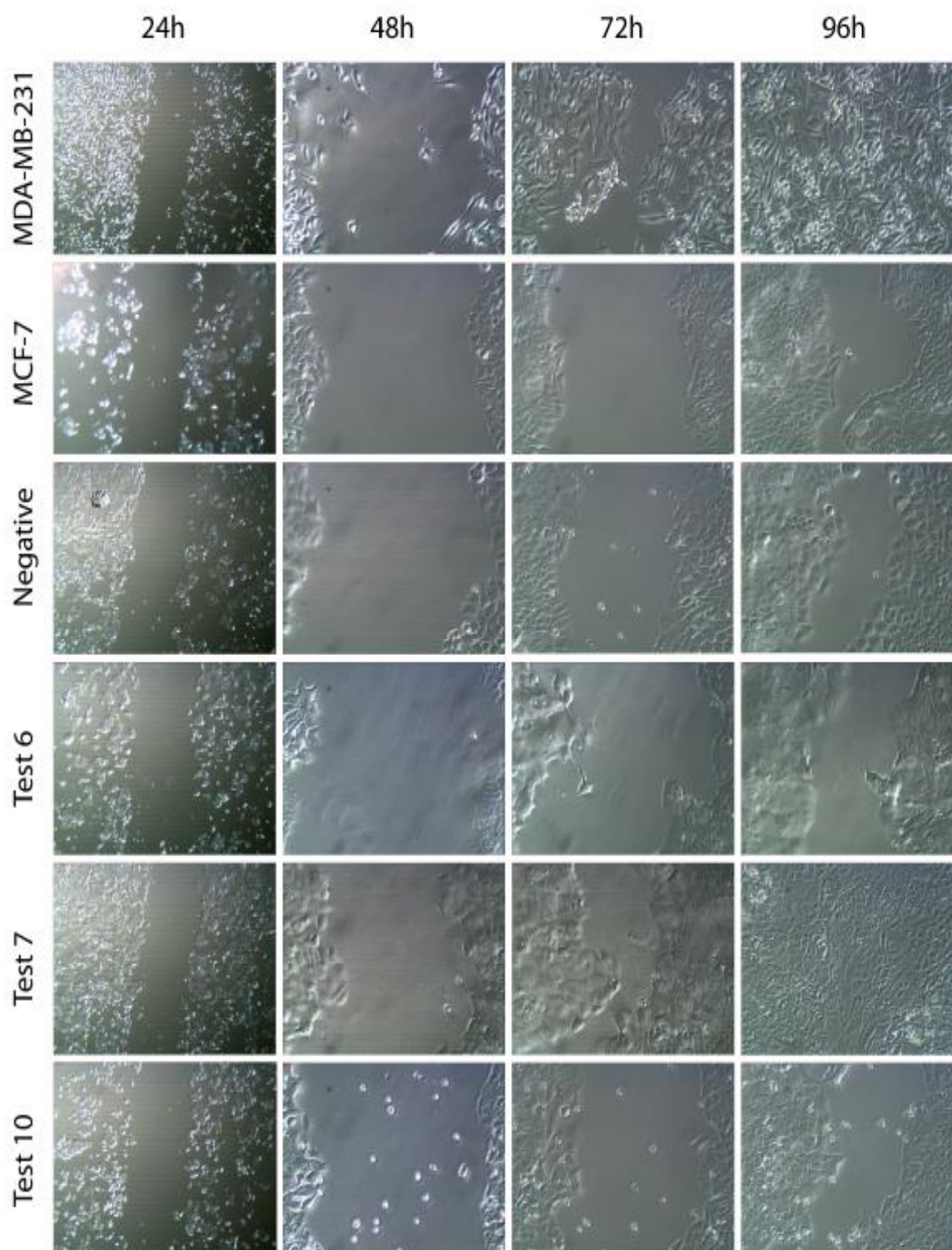**Fig. 16. Effect of DEPDC1 on migration in wound healing.**

Migration capability is shown at four time intervals of wound healing in MCF-7 cells with DEPDC1 overexpression and MDA-MB-231 cells. MCF-7 test 7 clones were similar to MDA-MB-231 cells in which they showed a strong invasive capability compared to the control group. Magnification is 40x at 24h and 100x at 48h, 72h and 96h, respectively.

5. Discussion

In this thesis, the hypothesis that DEPDC1 up-regulation is associated with an increased growth and invasive ability of human breast carcinoma cells, as well as with a reduced apoptosis level, was addressed and evaluated in an in-vitro model using different breast cancer cell lines.

5.1 Significance of investigating the relevance of DEPDC1 gene for breast cancer development

As outlined, DEPDC1 has been implicated recently to play a critical role in the carcinogenesis of different carcinomas. DEPDC1 was shown to be highly overexpressed in the majority of clinical bladder cancer samples, but did not show an expression in surrounding normal bladder cells[65]. In addition, the DEPDC1 gene was also demonstrated to be overexpressed in lung adenocarcinomas and was found to be were positively correlated with a poor outcome[66]. Hence, DEPDC1 might be a candidate diagnostic and therapeutic target for various carcinomas.

Given the contribution of DEPDC1 gene to cancer progression in general, we were interested to model the role of this gene in breast cancer. Breast cancer is not only a serious physical disease, it also can have a serious emotional effect[72]. Therefore, a method of sensitive detection of early lesions before the transition to an invasive stage has become a truly international endeavor, demanding an increased awareness. In a previous study, we found seven putative tumor markers that were strongly expressed at a very early stage of premalignancy and preneoplasia of breast carcinomas, DEPDC1 being one of them[63]. To better understand the possible relation of DEPDC1 gene and breast cancer, in this study the expression of this gene was examined in several breast cancer cell lines. The present study provides, for the first time, a detailed systemic analysis of DEPDC1 expression in different breast cancer cell lines. These data suggest a relationship between DEPDC1 and breast cancer aggressiveness.

MDA-MB-231, a human breast cancer cell line originally obtained from a patient at M.D. Anderson Cancer Center, Houston, is an estrogen receptor (ER)-negative cell line and thus not responsive to estrogen[73]. In addition, MDA-MB-231 is considered as a triple-negative breast cancer cell line, not only because of the absence of estrogen, but also because it lacks the expression of progesterone and HER2 receptors[74]. Higher histological grade and early metastatic spread tend

to be associated with these triple-negative breast cancers, leading to poor prognosis and outcome[75]. MDA-MB-231 cells are able to grow on agarose which is an indicator of transformation and tumorigenicity[76]. Besides, these cells display a relatively high colony forming efficiency and are considered to be highly invasive and metastatic[77-79] with typical fibroblast structures. Here the MDA-MB-231 breast cancer cell line showed the highest expression of DPEDC1 gene among all the cell lines studied.

MCF-7 (Michigan Cancer Foundation-7, referring to the institute where it was established[80]), is a human breast adenocarcinoma ER-positive control cell line, and unreceptive to vimentin[81] which promotes tumor cell invasiveness. MCF-7 cells are luminal epithelial like cells, growing in monolayers. They have minimal ability to invade through an artificial basement membrane in vitro[82,83]. Here the MCF-7 breast cancer cell line has a significantly lower level of DEPDC1 gene expression.

Hs578Bst was derived by A.J. Hackett, et al.[84] from normal breast tissue peripheral to an infiltrating ductal carcinoma which was the source of another cell line, Hs578T. It is an immortalized non-tumorigenic and non-metastatic breast epithelial cell line[84]. In fact, Hs578Bst cells were normally used to represent normal breast epithelial cells. Here the Hs578Bst breast cancer cell line barely expressed DEPDC1 in normal conditions.

These findings are in accordance with recently published data by Okayama H et al.[66] demonstrating that overexpression of DEDPC1 in lung adenocarcinoma patients corresponds to a worse prognosis. Furthermore, our results suggest that the MDA-MB-231 breast cancer cell line might be the most suitable cell line for examination of DEPDC1. Besides, the MCF-7 breast cancer cell line was identified to be more suitable than the Hs578Bst breast cancer cell line for knock-in expression of DEPDC1 to investigate the function of DEPDC1 in an alternative way.

Returning to what has been published regarding DEPDC1 in bladder cancer cells, overexpression of DEDPC1 was examined in six bladder cancer cell lines[65]. In that study, the anti-DEPDC1 antibodies performed by immunocytochemical staining and western blotting analysis were both detected in bladder cancer cells. It is an interesting possibility that DEPDC1 interacts and is colocalized with zinc finger transcription factor ZNF224, to play a critical role in bladder carcinogenesis[64]. In fact, approximately 30%-40% of breast cancers are estimated not to possess ER[85]. ER-negative breast cancer cells are known to be more aggressive than those that exhibit

ER-positive status[86]. It is suggested that ER-positive breast cancer cells, such as MCF-7 rely on ERs to enhance growth, while ER-negative breast cancer cells, like MDA-MB-231 probably need a separate mechanism to support their motility and invasion. There has been evidence suggesting that ER-negative breast cancer cells may depend on epidermal growth factor receptor (EGFR) for their growth and viability[85]. However, not only hormones and EGFR are linked with breast cancer, but zinc finger too, has probably been linked with breast cancer. Zinc concentrations were found to be higher in biopsies removed from mastectomies or lumpectomies than in normal breast tissues[87]. The effects of hormones and growth factors may play an important role in inducing zinc transporters that mediate breast tumor growth. Therefore, future work analyzing the contributions of DEPDC1 to breast cancer can focus on the zinc finger transcription factor.

5.2 Functional relevance of DEPDC1 expression in breast cancer cells

Tumorigenesis, characterized by uncontrolled cell growth, tumor formation and migration, is associated with various alterations in genes related to regulation of proliferation, apoptosis and invasion of tumor cells[88]. Thus, when the genes involved in molecular events leading to tumorigenesis have been investigated, the identification of functional relevance of gene expression in tumor cells becomes critical in developing effective therapeutic strategies. As reported, a number of proteins containing the DEP domain have been identified in various organisms ranging from yeast to humans. The DEP domain is identified to mediate intracellular signaling through regulation of protein stability[89,90]. Mammalian regulators of G-protein signaling also contain this domain and regulate signal transduction by increasing the GTPase activity of G-protein alpha subunits, thereby driving them into their inactive guanosine diphosphate-bound form. Recently, it was reported that DEP domain appeared to be involved in membrane association[91,92]. Therefore, the notion that the location of DEPDC1 is in the nucleus of bladder cancer cells is controversial[65]. Hence, although DPEDC1 contains the DEP domain, it may have unique cellular functions different from other DEP-domain molecules reported previously[90].

The transcriptional cofactor DEPDC1 gene has been implicated in cellular functions including the control of cell growth and the regulation of cell apoptosis in bladder cancer cells[65]. However, as previously alluded to, very little is known about the expression of DEPDC1 and its potential role in cancer. Kanehira M et al. initially published a paper in 2007 stating that knockdown of

DEPDC1 significantly suppressed the growth and remarkably increased the apoptosis in bladder cancer cells, thus suggesting that DEPDC1 is a positive regulator of bladder cancer progression[65]. This was confirmed by Okayama H et al. who showed that by gene expression profiling of 226 primary lung adenocarcinomas, high DEPDC1 expression correlated with worse prognosis, reduced 5-year recurrence-free survival and a higher tumor grade[66].

Since no publication about the functional relevance of DEPDC1 in breast cancer has been published yet, the potential role of DEPDC1 in breast cancer progression is still completely unknown. Having established DEPDC1 expression in different breast cancer cell lines, we observed significant differences between the nonaggressive MCF-7 cells and aggressive MDA-MB-231 cells, showing that high expression of DEPDC1 was associated with a more malignant behavior in breast cancer. Based on this finding, we investigated further the functional effects of DEPDC1 expression in two different breast cancer cell lines. We sought to determine if knockdown of DEPDC1 would alter the tumorigenic capacity in MDA-MB-231 cells, or if knock-in of DEPDC1 into MCF-7 cells would make them more invasive, by measuring their proliferative, apoptotic and invasive characteristics.

Proliferation is one of the cellular mechanisms involved in cancer progression. Our results demonstrated that the DEPDC1 gene has a strong effect on the proliferation of MDA-MB-231 breast cancer cells. We noted a decrease in the number of MDA-MB-231 cells treated with DEPDC1 siRNA compared to controls. A strikingly similar enhanced proliferation was observed in DEPDC1 plasmid transfected-MCF-7 cells, which grow much faster than the control cells. Although to date, no report has been published on the effects of DEPDC1 overexpression on cancer aggressiveness, there was one related to the effects of DEPDC1 knockdown in bladder cancer cells[65]. This study showed that the proliferation of bladder cancer cells got remarkably suppressed due to the down-regulation of DEPDC1 which supports our findings. Thus, silencing of DEPDC1 in cancer cells has an anti-proliferative effect. Interestingly, the strongest suppression by siDEPDC1 and the maximal activation by DEPDC1 overexpression on proliferation were both obtained at 72 h after cell seeding. In addition, there was no significant decrease (or increase) in the proliferation of MDA-MB-231 cells treated with siDEPDC1 (or DEPDC1 transfected MCF-7 cells) at 96 h. There are three possible explanations for these changes of the effects of DEPDC1 overexpression or silencing on cell proliferation:

1. DEPDC1 alone does not have the transforming activity. DEPDC1 has been proved to be a transcription factor. In bladder cancer cells, it interacted with ZNF224 to a complex, leading to significant growth activation[64]. In breast cancer cells here, DEPDC1 gene may perhaps also act as a transcriptional cofactor, it achieved the maximal interaction with another protein within this 72 h. While, the other unknown protein might be only expressed in small quantities in MDA-MB-231 cells and MCF-7 cells. Therefore, at 96 h the growth suppression or activation lost in the both cell lines due to the depletion of another protein, resulting in the absence of the complex which played the critical role in proliferation.

2. DEPDC1 has GTPase activator activity. A number of proteins containing the DEP domain are believed to be able to increase the GTPase activity. RhoC (Ras homolog gene family, member C), is a GTPase[93]. The RhoC GTPase has been reported to be overexpressed in inflammatory breast cancer[94]. Thus, DEPDC1 has a significant effect on proliferation in breast cancer cells by increasing the GTPase. Furthermore, the activity tends to peak within the first 72 h after seeding due to the effect of GTPase in controlling cytoskeletal reorganization[95]. Furthermore, the unidirectional change of the GTPase from the GTP-bound form to the GDP-bound form by hydrolysis of the GTP through GTPase-activity, effectively switches the GTPase off. In turn, the effects of DEPDC1 on proliferation owing to increased activity of GTPase disappear at 96 h.

3. With regard to MDA-MB-231 cells, there might be another possibility for the loss of an inhibitory effect on proliferation by siDEPDC1 at 96h. The knockdown of DEPDC1 expression in MDA-MB-231 cells was obtained by means of DEPDC1 siRNA transfection. While the transduction of siRNA into cells leads to only a transient knockdown of DEPDC1 expression. The siDEPDC1 is eventually lost over about 5 rounds of cell replication, just 96 h after transfection.

There is little quantitative data about the apoptosis frequency in tumors compared to our knowledge about cell proliferation[96]. However, Annexin V assay offers the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity[97]. Our results assay demonstrated that DEPDC1 gene has a strong effect on the apoptosis of MDA-MB-231 breast cancer cells. By Annexin V assay we noted significantly more apoptotic cells in siDEPDC1 treated MDA-MB-231 cells, both in early and late stage of apoptosis. This finding was consistent with

the research by Kanehira, M et al.[65]. Curiously however, these anti-apoptotic changes were not literally observed in DEPDC1 plasmid transfected MCF-7 cells. According to the very nature of the process of apoptosis, it involves scattered single cells in which the early stages can not be recognized and the end-stage apoptotic bodies undergo rapid phagocytosis[98]. However, MCF-7 cells usually form clusters[99]. Consequently, two independent Annexin V assays were employed to detect the effects of DEPDC1 overexpression in MCF-7 cells via cell apoptosis. One way was to include a three-hour 37 ℃ rolling incubation of the cell preparations and before the Annexin assays to obtain more single cells; the other way was conventional treatment without rolling incubation. Our results demonstrated that by the conventional method without rolling incubation, less viable single cells were obtained during the Annexin V assay, but DEPDC1 gene was contributing to the anti-apoptosis of breast cancer cells. On the contrary, there was no significant decrease in the apoptosis of DEPDC1 plasmid treated MCF-7 cells after rolling incubation, although more viable single cells were present. This discrepancy between these results highlights that future studies will have to address more specifically the possible mechanisms whereby DEPDC1 might influence apoptosis. It also highlights the limitations of in vitro assays. The rolling incubation undoubtedly assured more single cells, but the involvement of multiple step preparation might change the distribution of live, apoptotic and necrotic cells in the cell population. It has been asserted that any undesired artifact in an in vitro assay might cause forced cell damage[100]. Hence, no conclusion here can be drawn regarding the role of DEPDC1 in the anti-apoptotic effect of MCF-7 cells. These results suggest that the breast cancer cell line MCF-7 is not well suited for analyzing the anti-apoptotic effect of DEPDC1.

However, the proliferation and Annexin V assay are only two of several functional characteristics that are relevant to cancer progression. For example, cell cycle is a key contributor to describe the process of replicating DNA and dividing a cell. Thus we proceeded to investigate the nuclear DNA content in each MDA-MB-231 cell of a population with different treatments using flow cytometry. In addition to determining the relative cellular DNA content, we were also able to identify the MDA-MB-231 distribution during the various phases of the cell cycle. The treatment of DEPDC1 siRNA prevented MDA-MB-231 growth and induced a significant S phase (DNA synthesis) arrest. These studies indicated that DEPDC1 gene controls the G1-S transition cell cycle phase in MDA-MB-231 breast cancer cells. Since no result about the involvement of DEPDC1

gene in the regulation of the cell cycle has been published yet, it is not known how DEPDC1 regulates the cell cycle. However, since DEPDC1 knockdown MDA-MB-231 cells showed an increase in the number of cells in G1 phase as well as an increase in the number of apoptotic cells, this probably suggests an effect of DEPDC1 on the same pathway that controls breast cancer growth and death. By contrast, DEPDC1 seems not to affect the cell cycle function of MCF-7 cells. There is little variation in cell cycle distribution in all three test groups overexpressing DEPDC1 compared to the control. The cell cycle progression was unchanged by DEPDC1 over-expression treatment in MCF-7 cells possibly confirming the hypothesis that DEPDC1 is regu-lated differently in MDA-MB-231 cells and MCF-7 cells. DEPDC1 might exert a proliferative effect but not an apoptotic effect in MCF-7 cells. Therefore, with regard to MCF-7 cells, DEPDC1 may stimulate cell proliferation rather than decreasing programmed cell death. This probably could account for the present findings that cell viability and cell cycle distribution are impaired by the DEPDC1 overexpression effect in MCF-7 cells.

Having demonstrated an effect of DEPDC1 expression on proliferation and apoptosis in breast cancer cells, we further investigated whether DEPDC1 might affect migration. For MDA-MB-231 cells treated with siDEPDC1, we proceeded to examine cell invasion capacity of these cells, which is related to and encompasses cell migration, except that cells do more than migrate. We observed a significant decrease in the cells which migrate through the pores of the membrane at the bottom of the insert. The Boyden chamber assay suggested that DEPDC1 plays a role in the invasion of MDA-MB-231 cells. Interestingly, this contrasted with work showing that overexpres-sion of DEPDC1 does not enhance cell migration or invasion potential in bladder cancer cells[64]. It is probably due to different mechanisms that DEPDC1 participates in the progression of several human cancers. Regarding the MCF-7 cells transfected with DEPDC1 plasmid, it is not possible to evaluate the functional effect of DEPDC1 on invasion using the Boyden chamber, owing to their weak invasive capability. Hence, cell migration monitored in real time was examined using a microscope. We carried out wound healing assays with MCF-7 cells transfected with DEPDC1 plasmid. These studies showed that cells in test 7 are indeed similar to MDA-MB-231 cells with significantly increased wound closure and increased cell motility. The other test groups (test 6 and test 10) that were also transfected with DEPDC1 plasmid, however, showed the same wound clo-sure as the control and clearly compromised cell motility. What may account for the differences in

migration of test groups with the same treatment is not clear yet.

Firstly, the wound healing assay examines the ability of a cell culture to recolonize the wound, which is dependent on both migration and cellular growth. Although this method is well suited for studying tissue injury, it has limitations when assessing tumor cell motility[101]. On the one hand, tumor cells are caused to migrate across a rigid and planar substrate, which hardly resembles an in vivo situation, where cancer cells migrate into an extracellular matrix[102]. On the other hand, scratching the cell monolayer induces morphological changes and physically stresses the cells at the edge of the wound, which changes intracellular signaling events[103,104].

Moreover, the three test groups derived from three independent clones, even though they were all transfected with DEPDC1 plasmid. On one hand, individual differences are inevitable, which determines the possibility of discordance between each group. On the other hand, although the overexpression of DEPDC1 in MCF-7 cells was obtained by the supplementing the medium with G418 for obtaining stable transfectants, these cells should ideally express DEPDC1 in all clones and at constant levels over prolonged periods of time. However, the DEPDC1 plasmid in reality can get lost gradually due to the passaging of cells. The effect of DEPDC1 on migration partly depends on the percentage of plasmid lost. According to previous observation from qRT-PCR that test 7 cells show a higher DEPDC1 expression than test 6 and test 10 and that an antibody towards DEPDC1 could detect strong signals in test 7 clones during in immunocytochemistry, quicker migration in test 7 clones should be expected.

In this thesis, the use of two breast cancer cell lines of different aggressive capability has allowed us a unique opportunity to begin studying the fundamental effect of DEPDC1 in the regulation of breast cancer progression. The MDA-MB-231 cells provided an excellent model to investigate the role DEPDC1 plays in proliferation, apoptosis, cell cycle and finally cell invasion. The MCF-7 cells showed marked differences in proliferation and migration between cells with overexpressed DEPDC1 and non-treated cells, supporting the obtained data with a higher expression of DEPDC1 in more aggressive cells. However, it must be noted that the MCF-7 cells have not proved to be a good model for assessing DEPDC1 function in apoptosis and cell cycle.

The identification of functional effects of DEPDC1 in breast cancer cells poses several open questions with regard to tumor genesis. For example, the present study provides, for the first time, a detailed functional analysis of DEPDC1 expression in breast cancer cells. However, do these

effects arise from DEPDC1 and how does DEPDC1 act in breast cancer? To answer this question it would be necessary to compare the respective functional domains based on the structure of the DEPDC1 gene. Furthermore, these findings, although promising, must be contextualized with the fact that the role of DEPDC1 in a clinical setting of breast cancer is still to be discovered. Therefore, how is DEPDC1 expressed in breast cancer tissues of patients? To answer this question it would be necessary to collect patient samples of breast cancer and analyze the relevance between DEPDC1 expression and patient history. And last, but not the least, to date, there are no cancer therapies on the market which specifically target DEPDC1. Can the novel DEPDC1 gene be suggested as valid and successful drug delivery target? To answer this question it would be necessary to explore in vivo studies, to investigate the functional role of DEPDC1, and to analyze the effect of drug suppressing DEPDC1 expression. As an extension to the results obtained from this study, these experiments are suggested to be the direction of our future work. Undoubtedly these studies will help obtain a clearer picture of DEPDC1 function in breast cancer biology.

Overall, we have identified a possible relation between the expression of DEPDC1 gene and the malignancy of different breast cancer cell lines. Subsequently we showed that DEPDC1 significantly influences proliferation, apoptosis, cell cycle and migration of breast cancer cells, leading us to hypothesize that DEPDC1 plays an important role in breast cancer progression. This wealth of evidence indicates the potential involvement of DEPDC1 in breast cancer initiation and progression. The ultimate aim of any research in labs is to benefit the world, to help the people. What we expect most in this study on DEPDC1 in breast cancer is to provide a valuable theoretical basis for clinical treatment. To date, our study, for the first time, has suggested much potential for future consideration of DEPDC1 as a potential therapeutic target, a biomarker or even as a drug for systemic delivery in breast cancer. However, since only little is known in the field of DEPDC1, as discussed above, the revolution has not prevailed yet, comrades we still need to struggle!

6. Summary

Background / Aims: Breast cancer is the most frequent cancer among women. In Germany, 50,000 women are expected to be diagnosed with breast cancer every year, ranking first over all the other diagnosed forms of cancer among women. DEP domain containing 1 (DEPDC1) has been implicated recently to play a critical role in carcinogenesis. In a previous study, it was found to be highly expressed already during early stages of breast cancer. Therefore, in order to better understand the possible relation of the DEPDC1 gene and breast cancer, appropriate breast cancer cell lines were selected to examine the expression of this gene. In addition, the functional role of DEPDC1 in proliferation, apoptosis, cell cycle and migration was analyzed.

Materials and Methods: Expression of the DEPDC1 gene was analyzed in five breast cancer cell lines by GAPDH-controlled real-time quantitative polymerase chain reaction (RT-PCR). By DEPDC1 siRNA transfection, DEPDC1 was silenced in the MDA-MB-231 breast cancer cell line, which displayed the highest amount of mRNA DEPDC1 expression. The functional effects of DEPDC1 knockdown in MDA-MB-231 cells were analyzed using the Calcein-AM assay for proliferation, the FITC Annexin V detection for apoptosis, Flow cytometry assay for nuclear DNA content distribution in cell cycle, and Boyden Chamber assays for invasion. Moreover, DEPDC1 was overexpressed stably by transfection into the MCF-7 breast cancer cell line, which normally expressed the DEPDC1 gene only at a low level. The DEPDC1 gene was obtained by PCR from MDA-MB-231 cells and then subcloned into pcDNA3.1 vector. Transfection of the DEPDC1 plasmid was followed by G418 selection to assure stable cloning of DEPDC1. Immunocytochemistry confirms DEPDC1 overexpression on the protein level. The functional effects of DEPDC1 overexpression in MCF-7 cells were analyzed by using Calcein-AM assay for proliferation, FITC Annexin V Apoptosis Detection with and without 3-hour 37°C rolling incubation for apoptosis, and wound healing assays for migration.

Results: Among all breast cancer cell lines studied, MDA-MB-231 cell showed the highest DEPDC1 mRNA-expression, while MCF-7 cells expressed DEPDC1 at a very low level. MDA-MB-231 cells treated with DEPDC1 siRNA grew much slower than control cells with a maximal inhibition of 62% at 72 h. This treatment resulted also in more apoptotic cells in both early and late stage of apoptosis, led to cell arrest in S phase and a significant loss of invasion capacity. Re-

garding the MCF-7 cells, clones could be established which have been transfected with DEPDC1 plasmid (including test 6, test 7 and test 10). Compared to the non-treated cells, the cells in test groups grew much faster with a maximal activation of 40% in test 7 at 72 h. However, conflicting findings were obtained in apoptosis assay. Most interestingly, MCF-7 clone test 7 cells bearing high DEPDC1 expression were similar to MDA-MB-231 cells, showing a significantly increased wound closure.

Conclusion: This study, for the first time, indicates a relationship between DEPDC1 and aggressiveness of breast cancer cells and demonstrates that DEPDC1 could be a novel therapeutic target for inhibiting cell growth in breast cancer. The inhibition of DEPDC1 is strongly associated with proliferation, apoptosis, cell cycle, invasive ability and therefore related to breast cancer progression. However, although DEPDC1 overexpression in MCF-7 cells has significant effects in proliferation and migration, we have to note that the overexpression of DEPDC1 here is not a perfect model to analyse the functional role DEPDC1 plays within the organism. Further analysis of DEPDC1 at protein level and in vivo study may add to a better understanding of the relevance of this gene for breast cancer. Moreover, studies on the mechanisms of action of DEPDC1 in breast cancer can probably help to develop new therapeutic approaches.

7. Zusammenfassung

Hintergrund / Ziele: Brustkrebs ist die häufigste Krebsart bei Frauen. In Deutschland erkranken jedes Jahr etwa ca. 50.000 Frauen an Brustkrebs. Dem Gen DEP doamin containing 1 (DEPDC1) wird seit kurzem einer kritischen Rolle für die Karzinogenese verschiedener Tumorarten zugeschrieben. In einer früheren Studie unserer Arbeitsgruppe wurde eine erhöhte Expression von DEPDC1 bereits in frühen Stadien von Brustkrebs gefunden. Das Ziel dieser Arbeit war es die Rolle von DEPDC1 für die Proliferation, Apoptose, Zellzyklus und Migration von Brustkrebszellen besser zu verstehen.

Materialien und Methoden: Die Expression des DEPDC1-Gens wurden in fünf Brustkrebs-Zelllinien über quantitative real-time Polymerase-Kettenreaktion (RT-PCR) analysiert. Durch DEPDC1 siRNA Transfektion wurde die Expression von DEPDC1 in der MDA-MB-231 Brustkrebs-Zellenlinie inhibiert, die die höchste mRNA Expression von DEPDC1 zeigte. Die funktionellen Auswirkungen einer DEPDC1 Inhibition in MDA-MB-231-Zellen wurden unter Verwendung der Calcein-AM-Probe für die Proliferation, von FITC Annexin V Apoptosis Detection zur Bestimmung der Apoptose und Flow Cytometry mit Propidium Jodid für die Zellzyklus-Analyseuntersucht. Für Messungen der Invasion wurde ein Boyden-Chamber Assay eingesetzt. In einem zweiten Ansatz wurde DEPDC1 durch Transfektion in MCF-7 Brustkrebs-Zellenlinie überexprimiert, die das DEPDC1-Gen normalerweise nur sehr gering exprimiert. Das DEPDC1-Gen wurde durch PCR von MDA-MB-231 Zellen isoliert und in den pcDNA3.1 Vektor eingebaut. Nach der Transfektion des DEPDC1 Plasmids wurde unter G418 Gabe eine Selektion der Zellklone durchgeführt. Die Überexpression von DEPDC1 konnte in immunocytochemischen Untersuchungen bestätigt werden. Anschließend wurden die funktionellen Auswirkungen der DEPDC1 Überaktivität in MCF-7 Zellen untersucht. Dies wurde wie oben dargestellt durchgeführt. Zusätzlich wurde ein Wundheilungsassay für die Untersuchung der Migration der Zellen eingesetzt.

Ergebnisse: Unter allen untersuchten Brustkrebs-Linienzellen zeigten die MDA-MB-231 Zelle die höchste DEPDC1 mRNA-Expression, während MCF-7 Zellen DEPDC1 nur auf sehr niedrigem Niveau aktiv waren. MDA-MB-231-Zellen, welche mit DEPDC1 siRNA behandelt wurden wuchsen signifikant langsamer. Diese Behandlung verstärkte auch sowohl das Früh- und

Spätstadium der Apoptose. Weiterhin führte die Behandlung zu einem Arrest der Zellen in der S-Phase und zu einem signifikanten Verlust der Invasionskapazität. Die MCF-7 Zellen, die DEPDC1 überexprimierten wuchsen schneller und zeigten eine erhöhte Migrationsfähigkeit, die der von MDA-MB-231 Zellen entsprach. Widersprüchliche Ergebnisse wurden im Apoptose Assay für MCF-7 Zellen erzielt.

Schlussfolgerung: Diese Studie zeigt zum ersten Mal eine Verbindung zwischen einer erhöhten DEPDC1-Expression und der Aggressivität von Brustkrebs-Zellen. Die Resultate legen nahe, dass DEPDC1 ein neuartiges therapeutisches Ziel für die Hemmung des Zellwachstums bei Brustkrebs sein könnte. Die Hemmung der DEPDC1-Expression verringert signifikant Proliferation, Zellteilung, Apoptose, und Invasionsfähigkeit von MDA-MB-231 Zellen. Entsprechende Ergebnisse wurden nach Überexpression von DEPDC1 in MCF-7 Zellen gefunden. Weitere Analysen des DEPDC1-Gens auf der Proteinebene und durch in vivo-Studien sind zum besseren Verständnis der Rolle dieses Gens für Brustkrebs erforderlich. Darüber hinaus könnten solche Studien möglicherweise helfen neue therapeutische Ansätze zu entwickeln, die auf der Inhibition des DEPDC1 fußen.

8. References

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Acknowledgments

First of all, I have to say that this thesis would not have been possible without the support and thoughtfulness of an amazing Postdoc, Dr. Qing Wang from whom I have learnt so much more than just science. Her support and encouragement has been the driving force in my whole work in Germany. She is an inspiring mentor and I am truly indebted to her.

To Dr. habil. Wolfgang Kemmner, I am extremely grateful for the opportunity that you provide to me in your laboratory. I would like to extend my sincere thanks to your continual teachings and assistance whenever I was having trouble with understanding scientific studies or principles.

I would also like to acknowledge the technicians in our lab, Sabine, Gudrun, Claudia. They are full of joy to be with and wonderful advisors to provide me with great advice when I study in the lab as a foreigner. I truly appreciate their intelligence and expertise on academic and personal issues.

Another member that I would like to thank is Chenming Ma, he allowed me to participate in parts of his work and taught me the thesis skills. I thank him for being a good friend in both study and life.

Besides, I would like to take this opportunity to acknowledge and show my appreciation for all Chinese members who have contributed to the success and achievement of my education in Charité - Universitätsmedizin Berlin. Especially Yu Shi, Qiu Jiang, Wukun Liu, it was you that taught me how to troubleshoot when things were not working, how to think more like a scientist; it was you that provided me with moral support during both a challenging and happy time.

Finally, special thanks to my parents, it is your encouragement, optimism and unceasing support that led to the harvest of my project. I acknowledge you for everything that you have done for me.

Thesis is thesis, but it is not only thesis. It is more like a process exploring and finding truth. My experience throughout this process turned out to be more amazing than I expected owing to you, to all of these individuals mentioned above. Thanks!

Erklärung

„Ich, Yicun Man, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: On the functional relevance of the gene DEP domain containing 1 in breast cancer cells selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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