Technische Universität München
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Regulation of the tetraspanin and metastasis suppressor KAI1 and its splice variant by the estrogen system and their interaction with the epidermal growth factor receptor in hormone receptor-positive breast cancer cells

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Abstract

The tetraspanin and tumor suppressor KAI1 was found to regulate important processes in tumor biology by suppressing cell migration, invasion, and proliferation. It was found to be downregulated or lost in many cancer entities, correlating with poor prognosis of cancer patients. Thus, possibilities to restore KAI1 expression in cancer would be of great interest, also for clinical translation. However, so far, the mechanisms underlying the regulation of KAI1 expression are far from being completely understood. Moreover, a splice variant of KAI1 wild type (KAI1-wt), KAI1-sp was shown to promote tumor progression and metastasis. Based on earlier data showing an inverse correlation between KAI1 and the expression of the estrogen receptor (ERα) in ER-positive breast cancer, indicating an estrogen (E2)-mediated repressing effect on KAI1 expression, we aimed to investigate possible differential regulation of KAI1-wt and KAI1-sp by the E2/ER system by use of stable breast cancer cell transfectants overexpressing either KAI1-wt or KAI1-sp. We confirmed an inverse correlation between ERα and KAI1 expression in MCF7 WT cells, however, in our study, KAI1 expression was found to be induced by E2. In KAI1-wt transfectants ERα expression was reduced and further lowered upon E2 stimulation. ERα levels were below detection in KAI1-sp transfectants. Still, here, E2 stimulation decreased KAI1 expression. This was also noted in ER-negative MDA-MB-231 WT cells and KAI1-sp transfectants. The inverse correlation between KAI1 and the ER could not be upheld. Thus, we explored possible interdependent effects between the E2/ER system, the two KAI1 proteins and the known interaction partner of KAI1-wt and the ER, the epidermal growth factor receptor (EGFR), crucially involved in cancer progression. KAI1-wt has been shown before to downregulate the EGFR, whereas ER activation was observed via its phosphorylation and/or its association with e.g. the EGFR. Moreover, E2 was found to activate intracellular signaling events similar to those activated by the epidermal growth factor (EGF) suggesting a cross-communication via divergent signaling cascades. We found that EGFR expression and activation (p-EGFR) was drastically increased in MCF7 KAI1-sp transfectants, whereas it was below detection in WT and KAI1-wt cells independent of E2 stimulation. MDA-MB-231 WT exhibited low endogenous (p-)EGFR levels which were further lowered by E2. EGFR levels were similar in KAI1-wt transfectants with increased activation. As seen in MCF7 KAI1-sp transfectants, also in MDA-MB-231 KAI1-sp-transfected cells, (p-)EGFR levels were drastically increased, pointing to pro-oncogenic effects.

Since all of these factors studied are involved in tumor growth, we next investigated the net outcome of this functional protein crosstalk on cell proliferation. In all three cell lines, KAI1-wt

expression attenuated cell proliferation, which is in accordance with its tumor suppressor function. On the opposite, cell proliferation was enhanced upon the expression of KAI1-sp, however there was no effect of E2 stimulation. This supports the idea of the differential functional role of the two KAI1 protein variants in tumor cell biology, on one hand, the metastasis suppressor KAI1-wt and, on the other, KAI1-sp, which does not only abrogate the functions of KAI1-wt, but even more, promotes tumor biological effects in favor of tumor progression. Furthermore, the mutual crosstalk between KAI1, the ERα, and the EGFR underlines the important role of KAI1 in the complex molecular cross-communication within biological events. These observations support the strategy that KAI1-wt and its wide range of interaction partners could be a potential therapeutic target in order to improve disease outcome.

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List of Abbreviations

Ab	Antibody
appr.	Approximately
APS	Ammonium persulfate
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLSM	Confocal laser scanning microscope
conc.	Concentration
dest.	Distilled
DIC	Differential interference contrast
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
E2	Estrogen
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen-responsive element
FCS	Fetal calf serum
FN	Fibronectin

GAM	Growth arrested cell culture medium
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hours
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid
HER2	Human epidermal growth factor receptor 2, CD340
HR	Hormone receptor
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IgG	Immunoglobulin G
KAI1	Kangai-1, CD82, 4F9, C33, GR15, IA4, SAR2, ST6, TSPAN27
kDa	Kilo Dalton
LEL	Large extracellular loop
min	Minutes
miRNA	MicroRNA
mM	Milli Molar
NaCl	Sodium chloride
NFκB	Nuclear factor κB
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKC	Protein kinase C
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT	Room temperature
Sd	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

sec	Seconds
SEL	Small extracellular loop
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
sp	Splice
TBS	Tris-buffered saline
TEM	Tetraspanin enriched microdomain
TEMED	Tetramethylethylenediamine
TMD	Transmembrane domain
Tris	Tris-(hydroxymethyl-)aminomethane
V	Volt
$^{v}/_{v}$	Volume/volume
Vec	Vector
$^{w}/_{v}$	Weight/volume
WT/wt	Wild type

1 Introduction

Since the very beginning of research on human breast cancer, it had been attempted to define mechanisms how cells malignantly degenerate, attain the ability to grow tumor nests, and metastasize by invasion into the extracellular matrix (ECM) and vasculature. Also, the regulation of tumor biological processes on a cellular and molecular level is being focused on. Two types of genes have been found affecting these processes in case of distinct mutation. On the one hand, oncogenes encourage tumorigeneses, on the other, tumor suppressors protect tissue from degeneration to cancer cells. In this context, also a member of the tetraspanin family, KAI1, had been identified as a tumor and metastasis suppressor in prostate cancer (Dong et al., 1995) which will be in the focus of the present work with respect to its effects on cell proliferation and the protein expression of some selected interaction partners of KAI1 in human breast cancer.

1.1 Clinical aspects of breast cancer

Worldwide 19.3 million new cancer diagnoses were documented in 2020. Among these were 2.3 million breast cancer cases, which is equivalent to 11.9%. (Ferlay et al., 2021) About 490,000 men and women get diagnosed with cancer in Germany every year. Taking demographic changes into account, the number of overall cancer cases will rise by at least 20% until 2030. Based on current statistics of the *Robert Koch Institute* (RKI), one out of eight women will be affected by breast cancer in her life. In 2017, out of 230,050 first diagnosed cancer cases in women 67,300 patients were afflicted with breast cancer, which equals 29% and makes breast cancer by far the most common cancer entity in women. (Barnes, 2016; Gurung-Schönfeld I, 2021)

Many risk factors for developing breast cancer have been identified. Especially hormonal influences play an important role. Hormonal exposure over many years due to an early first and a late last menstrual bleeding, childlessness, giving birth at high age for the first time, and hormonal medication primarily after the menopause raise the risk for breast cancer. Also, obesity, low physical activity, as well as high density of breast tissue and so-called risk lesions count to risk factors. (Merino Bonilla et al., 2017)

Moreover, in about five to ten percent of breast cancer cases, a positive family history is found. In this context, genetic mutations causing breast cancer have been identified. A quarter of hereditary cases is linked to germline mutations. The best characterized genes responsible

due to their mutations are *BRCA1* and *BRCA2*, which also lead to a higher risk for patients to get afflicted with ovarian as well as pancreas, colon, prostate, and gastric cancer. (Baretta et al., 2016)

The histological classification of breast cancer follows the *World Health Organization* (WHO) classification. Most breast cancer subtypes belong to the histological group of "invasive carcinoma of no special type" (NST) (Sinn and Kreipe, 2013). Additionally, tumor tissue is classified into three groups characterizing cell differentiation after *Elston and Ellis*. According to this classification, tumor cells are categorized as well, moderately, or poorly differentiated. (Elston and Ellis, 1991) Furthermore, the expression of hormone receptors (HR), the estrogen receptor (ER), the progesterone receptor (PR), as well as the human epidermal growth factor receptor 2 (HER2) is evaluated. In immunohistochemical staining the proliferation index Ki-67 is determined. This leads to a classification into different groups (see Table 1) which affects the therapeutic approach and the prognosis. (Merino Bonilla et al., 2017)

subtype	*ER/PR-status*	*HER2-status*	*Ki-67*
Luminal A	positive	negative	low
Luminal B	positive	negative	high
		positive	any level
HER2-positive	negative	positive	any level
Triple-negative	negative	negative	any level

Table 1. Histopathological classification of breast cancer (Merino Bonilla et al., 2017)

Clinical staging is defined according to the *Tumor-Node-Metastasis* (TNM)-based and the *Union for International Cancer Control* (UICC)-classification. These classifications consider tumor size, lymph node status, and metastasis. (Giuliano et al., 2017) The appropriate choice of individualized therapy regimen is decided based on pathology, histology, and TNM/UICC-stage. Surgery, chemotherapy, radiotherapy, and endocrine therapy in case of HR-positive breast cancer, are some of the options. (Leitlinienprogramm Onkologie, 2018; Crees et al., 2020)

Through constant improvement of therapeutic options and treatment strategies, overall survival rates have improved to 88% after five years and 82% after ten years. Still, breast cancer is the leading cause of death by cancer in women accounting for 17.4% in Germany. (Kaatsch et al., 2017) Screening programs have contributed to the improvement of survival rates (Merino Bonilla et al., 2017). The German legal prevention program, for example, allows a palpation of

the breast for women over the age of 30 once a year. Women between the age of 50 and 69 are encouraged to get a mammography screening every two years (World Health Organization, 2014). However, the five-year survival rate for metastatic breast cancer is only 22% (Sledge, 2016).

In addition to tumor size, involvement of lymph nodes, postoperative residual tumor, histological grading, HR- and HER2-status, and metastasis throughout the body is a prognostic key factor (Sledge, 2016).

1.2 Main tumor biological characteristics of breast cancer metastasis

Like many other solid tumors, breast cancer metastasizes by dissemination through the blood and the lymphatic vascular system as well as metastasis *per continuitatem*. Metastasis *per continuitatem* describes tumor growth in adjacent tissues like the chest wall, the contralateral mammary gland, or the skin. Lymphatic metastases occurs especially in axillary lymph nodes. However, 70% of patients suffer from hematogenous spread of tumor cells forming bone metastases in the spine, pelvis, and long bones. Furthermore, lung, liver, and pleura are often involved. The main challenge is a frequent and early metastasis, as most deaths are caused by metastasis and not the primary tumor. (Scully et al., 2012; Merino Bonilla et al., 2017)

The ability of solid tumors to form metastases requires changes in numerous physiological functions (see Figure 1). Tumor cells are attached to the extracellular matrix (ECM) and neighbor cells by adhesion receptors of the integrin superfamily as well as cadherins. The ECM is a three-dimensional net containing a variety of peptides, like collagens and glycosaminoglycans. In order to detach from the primary tumor, tumor cells loosen these adhesions and invade local tissue towards lymphatic or blood vessels where they travel to distant tissue and organs. There, they re-adhere to the endothelium within vessels and extravasate. Tumor cells stimulate the development of new blood vessels, called angiogenesis in order to proliferate and form new tumor clusters. (Guo and Giancotti, 2004; Hunter et al., 2008; Okegawa et al., 2004; Scully et al., 2012; Theocharis et al., 2016)

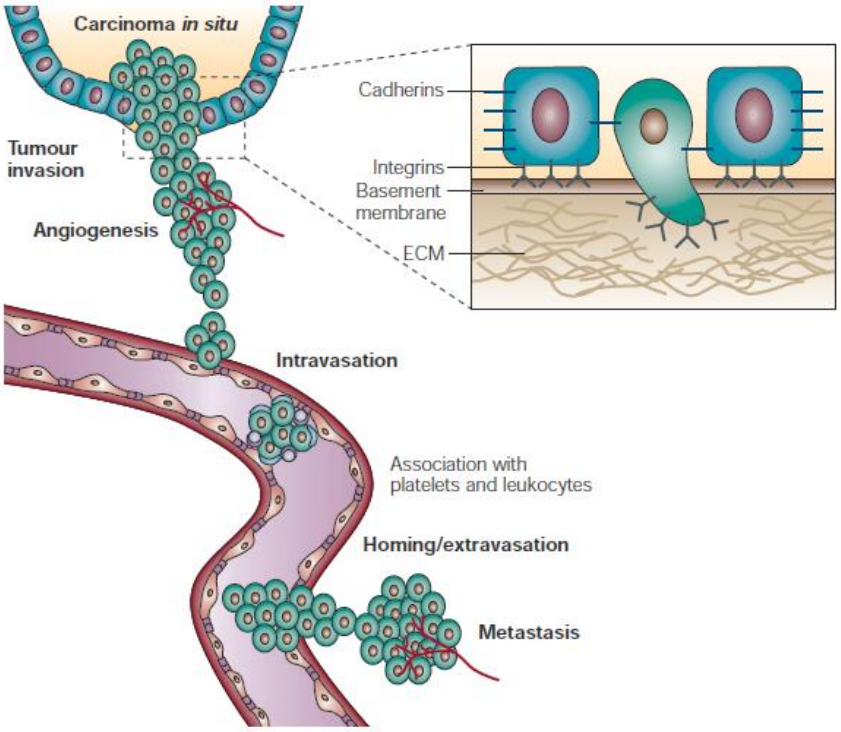


Figure 1. Schematic illustration of the process of metastasis (Guo and Giancotti, 2004)

During the process of metastasis tumor cells detach from the primary tumor by loosening their E-cadherin-dependent cell-cell adhesion followed by the invasion through the basal membrane towards lymphatic or blood vessels. After intravasation, tumor cells circulate, form aggregates with platelets and leukocytes, until they re-adhere to the endothelium of the vessels in distant target organs in order to extravasate. Through angiogenesis, tumor cells attain the ability to proliferate and expand to new tumor clusters which may grow to form metastases. Tumor cells are depicted in green with light brown nuclei. (Guo and Giancotti, 2004)

How these molecular mechanisms are altered and how tumor cells attain the ability to detach, disseminate, and form metastases is still being studied in order to precisely understand tumor development and progression. Two types of genes have been identified, oncogenes and tumor suppressors, which affect cellular functions and degeneration to tumor cells in case of distinct mutations. Oncogenes are dominantly inherited genes and encourage tumorigenesis if one allele is mutated. In contrast, tumor suppressor genes, which are subject to recessive inheritance, protect tissue from developing cancer and, if both alleles are mutated, malignant degeneration is favored. For human breast cancer, oncogenes, like *ErB-B2* coding for HER2, and tumor suppressor genes like *BRCA1/2* are important. (Lee and Muller, 2010)

Tumor suppressors are proteins that reduce the ability of tumor dissemination by modulating cell adhesion, motility, proliferation, and invasion. Several tumor suppressors have been detected, such as mitogen-activated protein kinase 4 (MKK4) in ovarian cancer (Spillman et

al., 2007) or the *RB1*-gene in retinoblastoma (Mendoza and Grossniklaus, 2015). Scientific focus of the present study was laid on the role of a putative tumor suppressor of the tetraspanin protein family, KAI1 and its role in cell growth and protein expression.

1.3 The tetraspanin KAI1

KAI1 belongs to the family of tetraspanins, also called *transmembrane 4 superfamily* (TM4SF). So far, about 33 tetraspanins have been identified in men, most of which are ubiquitously expressed and some display tissue-specific expression. Tetraspanins show a characteristic architecture (see Figure 2) encompassing four highly conserved transmembrane domains (TMD) forming one large extracellular loop (LEL) and one small loop (SEL). The amino-terminus and the carboxy-terminus are both located on the intracellular side of the plasma membrane. Additionally, there is one small intracellular loop. (Termini and Gillette, 2017)

The tetraspanin KAI1 was first isolated in 1995 in prostate cancer. The coding gene consists of 267 amino acids and is located on chromosome 11p11.2 (Dong et al., 1995). KAI1 is also known as the leucocyte surface molecule CD (cluster of differentiation) 82 or C33 (Bienstock and Barrett, 2001).

The TMD 1, 3, and 4 contain the polar amino acids asparagine, glutamine, and glutamate which contribute to the assembly of the protein itself and the interaction with other tetraspanins and surface molecules (Bari et al., 2009). Moreover, the transmembrane domains play a central role in protein maturation (Cannon and Cresswell, 2001). The LEL consists of a conserved complex of three helices and a variable domain made up by two helices. The constant region holds one conserved amino acid motif of *cysteine – cysteine – glycine* (CCG) and two single cysteine residues. These cysteines form disulfide bonds ensuring protein stability and correct protein architecture. The conserved domain contributes to the interactions with other tetraspanins. (Kitadokoro et al., 2001) The variable domain is unique and differs between tetraspanins. Its main functions relate to the mediation and modulation of interactions with other (membrane) proteins. (Levy and Shoham, 2005)

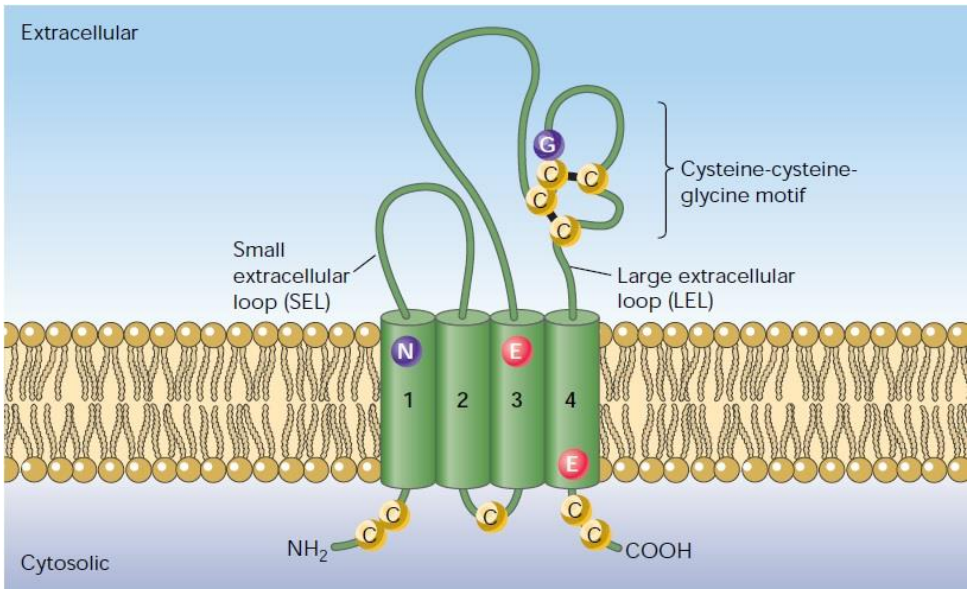


Figure 2. Scheme of the structure of tetraspanins (Levy and Shoham, 2005)

The characteristic architecture of a tetraspanin is based on four transmembrane domains (green cylinders, 1-4), a small (SEL) and a large extracellular loop (LEL). Furthermore, there is a short intracellular loop. The amino-terminus (NH_2) and the carboxy-terminus (COOH) lie intracellularly. In the LEL, the typical cysteine-cysteine-glycine motif forms disulfide bridges with further cysteine residues. The number of disulfide bonds varies between the different tetraspanins. The highly conserved transmembrane domains contain the polar amino acids asparagine, glutamine, and glutamate. (Levy and Shoham, 2005)

The LEL of the tetraspanin KAI1 contains six cysteines forming three cysteine bridges. Besides the cysteine residues, it harbors three asparagine residues for posttranslational N-linked glycosylation. (Ono et al., 2000) Glycosylation of tetraspanins is described to contribute to correct protein folding, transit through the endoplasmic reticulum, and cellular tetraspanin functions (Ono et al., 1999; Scholz et al., 2009).

The SEL contains 17 amino acids (Tsai and Weissman, 2011). Little is known about its function. The SEL of the tetraspanin CD81 contains a β-strand which interacts with the LEL for its proper folding and therefore affects its function. (Seigneuret, 2006) The intracellular loop of KAI1 contains two cysteines close to the interface of the membrane. These cysteine residues are post-translationally palmitoylated, which is implicated in the association with other membrane molecules and tetraspanins. (Zhou et al., 2004)

The amino-terminus of KAI1 consists of ten and the carboxy-terminus of 14 amino acids. Both termini are located intracellularly and contain three further cysteine residues (C5, C251, C253). Like in the inner loop, these cysteines are palmitoylated. (Zhou et al., 2004) Furthermore, the

carboxy-terminus contains a special tyrosine-based sorting motif. This region is instrumental for internalization processes from the cell surface and interaction with lysosomes and endosomes. The basic structure of this sorting sequence holds the code *Y-X-X-Φ* with tyrosine (Y), two variable amino acids (X), and one amino acid with a bulky hydrophobic residue (*Φ*). (Bonifacino and Dell'Angelica, 1999; Berditchevski and Odintsova, 2007)

Through this special molecular structure, tetraspanins obtain the ability to interact with one another and associate with a variety of other proteins and membrane receptors. As tetraspanins have no intrinsic activity of their own, they function by recruiting these interaction partners. Tetraspanins connect and organize their specific binding partners into a multimolecular network called *tetraspanin web* or *tetraspanin enriched microdomain* (TEM). (Charrin et al., 2009) The interaction of tetraspanins with other molecules occur on different levels. A direct, so-called primary interaction exists between a specific tetraspanin and other proteins, whereas secondary interactions occur between different members of the tetraspanin family. Primary interactions may not be easily chemically disrupted. Secondary, indirect associations are sensitive to mild detergents. Third level-, or tertiary interactions describe a link between a tetraspanin and other proteins. Depending on the cell type, the TEM contains one or different members of the tetraspanin family, as well as other proteins in order to execute cell-specific functions. One specific tetraspanin is therefore capable of associating with different proteins in different cell types. Another remarkable characteristic of the TEM is a lateral dynamic organization of the cell membrane and the interaction with intracellular signaling pathways enabled by tertiary crosstalk. (Hemler, 2001; Boucheix and Rubinstein, 2001; Levy and Shoham, 2005)

Tetraspanins therefore influence a variety of physiological cellular processes by associating with a plethora of proteins in the TEM concerning especially cell proliferation, adhesion, differentiation, motility, and migration (Ruseva et al., 2009; Miller et al., 2018; Deissler et al., 2007). Moreover, the modulation of a variety of other processes is documented, such as immune responses, growth of nerve tissue, sperm-egg fusion, vascular functions, and homeostasis (Zhu et al., 2002; Mela and Goldman, 2009; Zhang et al., 2009; Jankovičová et al., 2015; Greenberg et al., 2018; Reyes et al., 2018; Zou et al., 2018). However, the precise involvement and interplay of different tetraspanins in pathophysiological processes like tumor development, progression, and metastasis are still not fully understood (Hemler, 2001).

1.4 The functions of the tumor metastasis suppressor KAI1

The tetraspanin KAI1 was already defined as a tumor metastasis suppressor. Thus, the loss of KAI1 is associated with poor patient prognosis through development of metastases and tumor growth. This negative correlation between the expression of KAI1 and tumor progression was determined for several tumor entities. These are esophageal, gastric, colorectal, pancreas, lung, and thyroid carcinomas, as well as the gynecological cancers of the ovary, cervix, endometrium, and breast. (Liu et al., 2001; Liu et al., 2003a; Liu et al., 2003b; Chen et al., 2004; Guo et al., 2015; Upheber et al., 2015; Zhou et al., 2015; Miller et al., 2018; Zeng et al., 2018; Liu et al., 2019)

As described in the previous chapter, KAI1 – like all tetraspanins – associates with other proteins and receptors within the TEM. By this, tumor biological processes of KAI1 are modulated, as there is no intrinsic function of KAI1. Various interaction partners of KAI1 have been identified, including the tetraspanins CD9 and CD81, which allows a coordination within the TEM. KAI1 is also associated with adhesion proteins, like cadherins, and integrins of the β_1-, β_2-, and β_3-subfamilies. Moreover, the immunoreceptors CD4, CD8, EWI2/PFRL, and molecules of the *Major Histocompatibility Complex* (MHC) Type I and II count to interaction partners of KAI1, which is important for the activation of B- and T-cells. KAI1 interacts with the membrane protein KAI1-COOH-terminal interacting tetraspanin (KITENIN) and is able to weaken its promoting effect concerning cell adhesion, invasion, and motility. Furthermore, a correlation between KAI1 and intracellular signaling proteins, such as the protein kinase C (PKC), was found. Other very important interaction partners of KAI1 are growth factors and growth factor receptors, such as the epidermal growth factor receptor EGFR (see chapter 1.7). (Lee et al., 2004; Liu and Zhang, 2006; Upheber et al., 2015)

The interaction of KAI1 with its partners may occur by different ways (see Figure 3). Direct interactions depend on physical contact between the proteins and cause a *touch and down* or a *physical hindrance* mechanism. Thus, KAI1 is able to accelerate the endocytosis rate of a receptor such as the EGFR (see chapter 1.7) by the *touch and down* mechanism or attenuate adhesion activity of integrins by *physical hindrance*. Furthermore, KAI1 can exert its function after its phosphorylation by indirectly affecting proteins of intracellular signaling pathways. (Tonoli and Barrett, 2005)

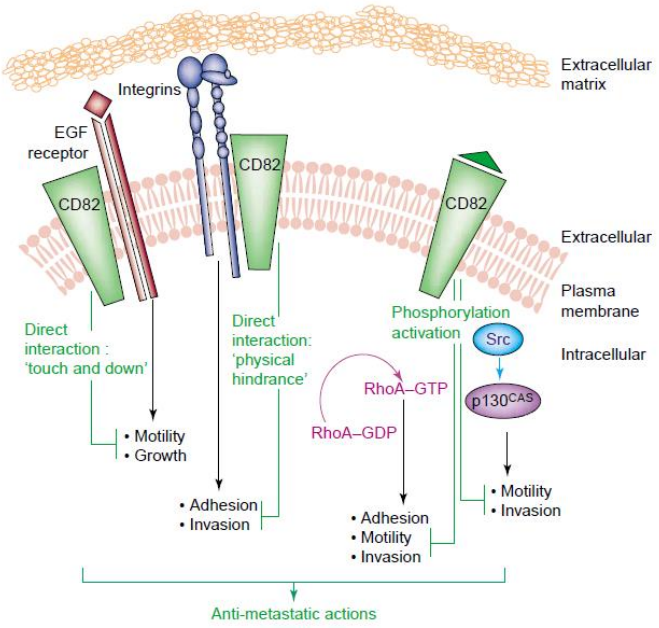


Figure 3. Functional mechanisms of the metastasis suppressor KAI1 (Modified after Tonoli and Barrett, 2005)

Different mechanisms of how KAI1/CD82 exerts its functions were suggested. KAI1 directly interacts with, for example, the EGFR and integrins. Internalization of the EGFR is accelerated by KAI1 by the touch and down mechanisms, whereas KAI1 interferes with the physiological activity of integrins by the physical hindrance mechanism. Proteins involved in intracellular signal transduction pathways, such as the RhoA and the Src pathway, are affected by KAI1 after its phosphorylation activation. KAI1 thereby suppresses cell motility, growth, adhesion, and invasion, as the hallmarks of metastasis. Green lines indicate the inhibitory actions of KAI1, black arrows show processes mediated by the interaction partners EGFR, integrins, and intracellular proteins. (Tonoli and Barrett, 2005)

Together with its interaction partners, KAI1 suppresses cell migration, invasion, motility, and proliferation (Ono et al., 1999; Malik et al., 2009; Ruseva et al., 2009; Tsai and Weissman, 2011; Upheber et al., 2015; Miller et al., 2018). Therefore, KAI1 takes over a central role in tumor biology by preventing tumor progression and metastasis. Thus, it is of great scientific interest to understand the regulation of its expression. However, the control over KAI1 expression and how it is downregulated or even lost is so far only incompletely understood.

1.5 The splice variant of KAI1

One mechanism for transcriptional modification may be executed by alternative splicing. Hereby, coding sequences, called coding exons, are assembled, and linked in a different order,

or single exons are not transcribed. By this, different mRNAs are formed from one single gene, creating structurally and/or functionally different proteins. Depending on the localization of a removed exon, the function of the protein may be attenuated or even completely lost. (Bush et al., 2017)

In 2003, such a splice variant of KAI1 (KAI1-sp) was discovered in a gene study on important genes for metastasis in gastric cancer. The amplification of the gene of the KAI1 protein in reverse transcription polymerase chain reaction (RT-PCR) produced two proteins of different length. Besides the wild type KAI1 protein (KAI1-wt), a shorter gene product lacking Exon 7 was identified in sequencing analysis. Exon 7 contains 84 base pairs, respectively 28 amino acids (position 215-242) and encodes the carboxy-terminal part of the LEL as well as the proximal part of the fourth TMD. The selective loss of exon 7 was found to cause structural changes of the KAI1 protein (see Figure 4). Possible different effects of KAI1-wt and KAI1-sp were studied in tumor tissue compared to healthy tissue. (Lee et al., 2003)

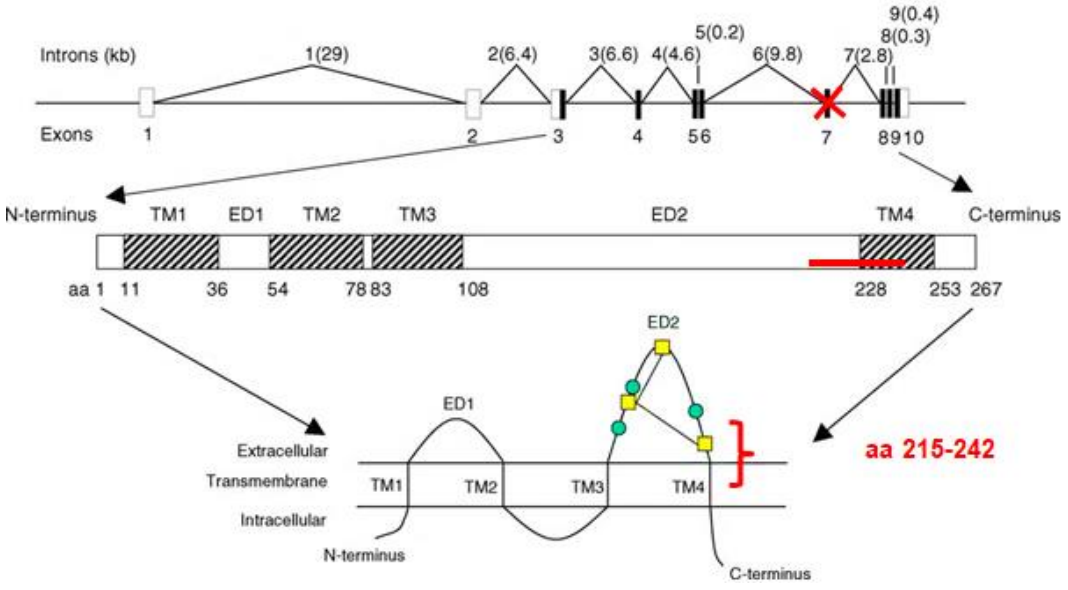


Figure 4. Scheme of the KAI1 gene and the site of alternative splicing (Modified after Jackson et al., 2005)

The KAI1 gene contains 10 introns and 9 exons. Non-coding introns are depicted by white boxes and coding exons by black boxes. In the process of alternative splicing exon 7 is lost (crossed out in red) which codes for the distal part of the extracellular domain 2 (ED2) and the proximal part of the fourth transmembrane domain (TM4). Hatched areas in the bar structure indicate the four transmembrane domains (TM1-4), ED1 and ED2 represent the extracellular domains of the KAI1 protein. In the secondary protein structure, green circles depict potential sites for glycosylation, and yellow squares cysteine residues for disulfide bridges. The splice variant of KAI1 lacks the amino acids 215-242 (marked in red). (Modified after Jackson et al., 2005)

The expression of KAI1-sp, compared to KAI1-wt, had a favorable effect on tumor growth and metastasis in gastric cancer and, consequently, was associated with poor patient prognosis. Combined with clinical data, on one hand, it was discovered that patients with a high expression level of KAI1-sp in the primary tumor tissue as well as in regional or distant metastases in lymph nodes and peritoneum had a significantly reduced survival rate. On the other, in patients with good prognosis, KAI1-sp was expressed on extremely low levels in primary tumor and metastatic tissue. (Lee et al., 2003)

In *in vitro* experiments in a colon carcinoma cell line, functional alterations caused by the structural difference between KAI1-sp and KAI1-wt were investigated. Here, the expression of KAI1-sp led to an increased cell-ECM adhesion, an attenuated association with the integrin $\alpha_3\beta_1$, and a reduced colocalization with E-cadherin. Furthermore, KAI1-sp provoked a significantly increased motility and invasive potential, whereas KAI1-wt was associated with reduced invasiveness. In an *in vivo* mouse model for colon carcinoma, mice injected with KAI1-sp cell transfectants developed metastases in the lungs, liver, and peritoneum, whereas mice injected with KAI1-wt cell transfectants suffered from lung metastases only. The outcome of further experiments in this mouse model also showed that the expression of KAI1-sp is accompanied by greater tumor growth, cell migration, invasiveness, as well as cell adhesion. Thus, it was stated, that the absence of exon 7 and its encoded region appears to alter the association of KAI1-wt with its interaction partners. This seems to be involved in the loss of the protective functions of KAI1-wt in tumorigenesis and metastasis. (Lee et al., 2003)

The differential role of KAI1-wt and its splice variant was already explored in human ovarian and triple-negative breast cancer in the research group of Prof. Dr. Reuning. In an KAI1-transfected ovarian cancer cell model, it was shown that KAI1-sp altered its crosstalk with integrin $\alpha_v\beta_3$ thereby reducing cell adhesion and inducing cell migration. Moreover, the expression of KAI1-sp provoked enhanced cell proliferation compared to KAI1-wt. In HR-negative human breast cancer cells expressing KAI1-sp an accelerated cell proliferative activity was noted, corresponding to an upregulation of the expression of the EGFR, while KAI1-wt reduced cell proliferation. Furthermore, it was shown that KAI1-sp induced an accelerated cell motility compared to KAI1-wt going along with the activation of the focal adhesion and Src kinase. Taken together, these results support the idea that KAI1-sp alters tumor biological effects in favor of tumor progression and metastasis in triple-negative human breast cancer. (Miller et al., 2018; Upheber et al., 2015)

Not only transcriptional modifications like alternative splicing have been identified to cause structural changes of the molecule leading to the loss of KAI1 and its tumor suppressive

functions (Lee et al., 2003). Transcription factors seem to modulate cellular expression of KAI1 by increased repression or decreased activation of transcription (Liu and Zhang, 2006). For example, NFκB, β-catenin, and p53 were described in this context (Dong et al., 1997; Shinohara et al., 2001; Kim et al., 2005). Loss of heterozygosity did not turn out as a cause of KAI1 downregulation (Dong et al., 1996). It is also possible, that post-translational modifications induce alterations of the KAI1 molecule and its functional capacity (Tonoli and Barrett, 2005). Furthermore, it was found that KAI1 is upregulated upon treatment with *Fulvestrant*, a selective estrogen receptor (ER) degrader (SERD), in ER-positive breast cancer cells suggesting an ER-mediated effect on the KAI1 expression (Christgen et al., 2008).

1.6 Functions of the estrogen receptor

A plethora of physiological processes in human cells are influenced by estrogen (E2) and the two estrogen receptors (ER), ERα and ERβ. ERα is physiologically expressed especially in the tissue of the uterus, ovary, testes, and breast, but is also found in bone, liver, and adipose tissue. The ERβ is present in non-gonadal tissue, such as colon, bone marrow, or lung. Both receptors are expressed in the brain where ERα plays an important role in neuroendocrinology. (Cui et al., 2013) As the ER is found in so many different tissues, it does not come as a surprise that its malfunction is implicated in various diseases (Jia et al., 2015).

The ER belongs to the super family of nuclear receptors. The two isoforms ERα and ERβ, are encoded by the respective gene on different chromosomes. The encoding genes for ERα and ERβ are located on chromosome 6q25.1 and 14q23.2, respectively. ERα contains 595 and ERβ 530 amino acids. (Rondón-Lagos et al., 2016; Arnal et al., 2017)

ERα is expressed in about 50-80% of human breast cancer cases (Huang et al., 2014). It was found to be one of the most important prognostic factors for breast cancer as an increased patient survival rate is observed in ERα-positive breast cancer cases (Knight et al., 1977; Burns and Korach, 2012; Matthews and Sartorius, 2017). The role of ERβ in human breast cancer is not yet fully understood, although its involvement in ovarian, endometrial carcinoma, as well as in endometriosis, fibroids, *Alzheimer's* disease, cardiovascular disease, *Parkinson's* disease, and osteoporosis has been documented. Therefore, the understanding of functions and interacting mechanism of the ER is crucial for developing new therapeutic strategies. (Burns and Korach, 2012; Cui et al., 2013)

Estrogen pathways are initiated by E2 binding to plasma membrane bound or cytoplasmic ERs. By binding E2 to an ER in the cytoplasm, a conformational change within ERs is triggered and ER-dimers are formed. The thereby activated ER internalizes into the nucleus and binds to the estrogen-responsive element (ERE) on the DNA, where it regulates transcription either directly or indirectly. Indirect modulation of transcription happens by the communication between the activation factors 1 and 2 of the ER with other co-factors, such as steroid receptor coactivator 1 (SRC-1). Membrane bound ERs are able to activate cytoplasmic signaling pathways after binding E2. On one hand, the ER initiates a variety of intracellular signaling pathways after E2-binding, on the other, data show a crosstalk of the ER with other receptors on the cell membrane, suggesting that ER-pathways are also mediated by other protein kinases, such as the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), or PKC. (Hall et al., 2001; Cui et al., 2013)

Taken together, the ER is involved in the regulation of many physiological processes like cell proliferation, adhesion, invasion, migration, anti-apoptosis, and cell differentiation. Abnormal function of the ER leads to various diseases such as cancer or inflammation. It therefore takes a central role in tumor biology. (Jia et al., 2015)

1.7 The epidermal growth factor receptor and its cell biological role

Besides the central position of the ER in breast cancer, also the EGFR was identified as a key cellular player in tumor biology. The EGFR, encoded by gene *ErbB-1*, belongs to the group of receptor tyrosine kinases which mediate many signaling pathways affecting cell growth, differentiation, migration, and cell death. (Sasaki et al., 2013)

The group of *ErbB* receptors has been studied in different tumor entities like ovarian cancer, breast cancer, and non-small cell lung cancer. Mutations and/or overexpression of the EGFR and its activated and thus phosphorylated form (p-EGFR) lead to poor patient prognosis, metastasis, increased invasiveness, and drug resistance. Furthermore, new therapeutic approaches targeting these receptors, especially the EGFR, are under close clinical investigation. (Wang, 2017)

The EGFR consists of a single polypeptide chain encompassing 1186 amino acids. It occurs as an inactive monomer and is activated upon ligand binding, such as epidermal growth factor (EGF), the transforming growth factor-α (TGF-α), and the heparin-binding EGF-like growth

factor, respectively. Ligand binding induced EGFR dimerization and the intracellular tyrosine kinase is activated leading to the initiation of various intracellular signaling pathways, which affect cell survival and apoptosis, cell proliferation, growth, motility, and invasiveness. (Dutta and Maity, 2007; Magkou et al., 2008; Wang, 2017)

Most interestingly, the EGFR has also been identified as a direct interaction partner of KAI1-wt on the cell surface. KAI1-wt does not influence EGFR ligand binding but causes a desensitization of the ligand-dependent activation, which leads to an attenuation of EGFR dimerization. It was shown that KAI1-wt is associated with the compartmentalization of the EGFR. Moreover, upon the loss of KAI1-wt, an increased EGFR dimerization as well as a decreased EGFR diffusion within the plasma membrane is provoked. Consequently, the amount of cell-surface-expressed EGFR varies as a function of the cellular KAI1-wt expression. (Odintsova et al., 2000; Odintsova et al., 2003; Danglot et al., 2010) Besides the cell surface distribution of the EGFR, KAI1-wt was demonstrated to affect the recruitment of intracellular proteins crucial for the initiation of the intracellular signaling pathways of the EGFR (Berditchevski and Odintsova, 2016). This is supported by the findings of *in vitro* studies. In human ovarian cancer cells, the overexpression of KAI1-wt was associated with a lower total amount of EGFR, whereas the overexpression of KAI1-sp led to an upregulation of cellular EGFR expression and an increase of its amount on the cell surface (Upheber et al., 2015). In human breast cancer cells the loss of KAI1-wt and the overexpression of KAI1-sp was correlated with elevated EGFR levels and associated with an enhanced proliferative activity and cell motility (Miller et al., 2018). Summarizing, KAI1 is described as an essential trafficking molecule involved in the organization of the architecture of multiprotein complexes within the cell surface.

1.8 Aims of the study

The differential role of the tumor suppressor KAI1-wt and its splice variant KAI1-sp displaying pro-oncogenic functions on tumor progression and metastasis in various tumor entities is a central issue in cancer research. Since KAI1-wt is lost in many different tumor types, going along with poor patient prognosis, the regulation of its expression is of major scientific interest in view of promoting its tumor suppressive functions in cancer cells. However, the control over KAI1 expression is so far only incompletely understood.

Thus, the present study aimed at

- gaining insight into the regulatory role of E2 in concert of ERα for KAI1 expression levels in HR-positive human breast cancer cells.
- investigating the breast cancer cell proliferative activity as a function of
    - KAI1-wt and KAI1-sp expression in ERα-positive breast cancer cells by use of stably transfected cell models overexpressing KAI1-wt or KAI1-sp. As controls served ERα-negative human breast cancer and melanoma cells.
    - cell stimulation by E2.
    - the expression and activation of the EGFR as one of the important functional cooperation partners of KAI1-wt.

2 Materials

2.1 Cell lines

Three different cell lines were used in this study.

MCF7

The main cell line of interest in this study was the ER-positive human breast cancer cell line MCF7, which was derived from a malignant pleural effusion of a 69-year-old female patient diagnosed with a metastasized adenocarcinoma of the breast. Soule et al. (1973) established this cell line at the Michigan Cancer Foundation and first characterized morphology and genetics. Today the MCF7 cell line is one of the most important ER-positive breast cancer cell lines for experimental research (Comşa et al., 2015). In this study, MCF7 cells were stably transfected with expression plasmids encoding either KAI1-wt or KAI1-sp.

MDA-MB-231

The breast cancer cell line MDA-MB-231 is classified as a triple-negative adenocarcinoma cell line, meaning that it does not display the ER, PR, and HER2, respectively, in considerable amount or not at all. It had originally been established from a 51-year-old female patient's malignant pleural effusion in 1973. (Brinkley et al., 1980)

In preliminary work of the research group of Prof. Dr. Reuning, MDA-MB-231 cells were stably transfected with expression plasmid encoding KAI1-wt or KAI1-sp. In order to achieve cell transfectants harboring comparable amounts of the two KAI1 variants, the batch of transfected cells were subcloned by limited dilution. Stably transfected cell clones were kindly provided for the projects of the present study.

MDA-MB-435

In 1976, MDA-MB-435 cells were first isolated from a malignant pleural effusion of a 31-year-old female patient suffering from an adenocarcinoma of the breast. It was described as a triple-negative breast cancer cell line. (Brinkley et al., 1980)

However, Ross et al. (2000) performed cDNA microarrays to analyze gene expression in various cancer cell lines and the data showed a similarity of MDA-MB-435 cells to melanoma cells. Further research using polymerase chain reaction (PCR) and immunohistochemistry to

compare genes of MDA-MB-435 and melanoma cells substantiated the hypothesis of their melanoma origin (Ellison et al., 2002). Moreover, the evaluation of gene expression discovered an identical gene expression to melanoma cells (Rae et al., 2007). After many years of debate and research involving fluorescence *in situ* hybridization (FISH), genotyping, immunostaining, and molecular analysis, the MDA-MB-435 cell line is today considered a melanoma, and not a breast cancer cell line, as all results were consistent (Korch et al., 2018).

Like given for MBA-MB-231 cells, MDA-MB-435 cells were also stably transfected with either KAI1-wt or KAI1-sp-encoding expression vectors in preliminary work of the research group of Prof. Dr. Reuning. Transfections with the empty expression plasmid served as control. All stably transfected cell clones were kindly provided for the present study.

Expression plasmid

Plasmid pcDNA3.1/Hygro	Thermo Fisher Scientific, Waltham, Massachusetts, USA

Human KAI1-wt and KAI1-sp cDNA, respectively, were generated by PCR reactions in preliminary work of the research group of Prof. Dr. Reuning and subcloned into the expression vector pcDNA3.1/Hygro (Miller et al., 2018; Ruseva et al., 2009; Upheber et al., 2015).

2.2 Antibodies

Primary and secondary antibodies were used in Western blot analysis and immunocytochemical staining procedures.

Antigen	*Name*	*Company*	*Stock conc. [µg/µl]*
EGFR	purified mouse anti-EGFR clone 13	Becton-Dickinson Biosciences, Franklin Lakes, New Jersey, USA	0.25
ERα	sc-8002	Santa Cruz Biotechnology, Dallas, Texas, USA	0.2

Antigen	*Name*	*Company*	*Stock conc. [µg/µl]*
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	MAB374	Merck Millipore, Darmstadt, Germany	1
KAI1	TS82b	Diaclone, Besançon, France	1
p-EGFR	Y845	R&D Systems, Minneapolis, Minnesota, USA	1

Table 2. Primary antibodies

Name and Conjugation	*Company*
Alexa-488-conjugated goat-anti-mouse Immunoglobulin (IgG) (A32723)	Thermo Fisher Scientific
Alexa-488-conjugated goat-anti-rabbit IgG (A-11034)	Molecular Probes by Thermo Fisher Scientific, Eugene, Oregon, USA
horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (G-21040)	Thermo Fisher Scientific
HRP-conjugated goat-anti-rabbit IgG (G-21234)	Life Technologies by Thermo Fisher Scientific, Carlsbad, California, USA

Table 3. Secondary antibodies

2.3 Chemicals

Name/Description	*Company*
Acetic acid 96%	Carl Roth, Karlsruhe, Germany
APS (Ammonium persulfate)	Sigma-Aldrich, St. Louis, Missouri, USA

Name/Description	*Company*
Biotinylated protein ladder #7727	Cell Signaling Technology, Danvers, Massachusetts, USA
BSA (Bovine serum albumin)	Sigma-Aldrich
DMEM (*Dulbecco's modified eagle medium*)	Thermo Fisher Scientific
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich
DPBS (*Dulbecco's phosphate buffered saline*)	Thermo Fisher Scientific
E2 (Estrogen)	Sigma-Aldrich
EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich
EDTA 1% $^{w}/_{v}$ (cell culture)	Merck Millipore
Ethanol 80% $^{v}/_{v}$	Otto Fischar, Saarbruecken, Germany
FCS (Fetal calf serum)	Thermo Fisher Scientific
Fibronectin (FN)	Corning, Corning, New York, USA
Glycerol	Carl Roth
Glycine	Carl Roth
HEPES (4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid)	Carl Roth
HEPES (cell culture)	Thermo Fisher Scientific
Hygromycin B	Thermo Fisher Scientific
Immobilon Western HRP Substrate	Merck Millipore
L-Arginine	Sigma-Aldrich
L-Asparagin	Sigma-Aldrich
Lipofectin	Thermo Fisher Scientific
Methanol ≥ 99%	Carl Roth
Milk powder	Carl Roth
NaCl	Carl Roth
Paraformaldehyde (PFA)	SERVA Electrophoresis, Heidelberg, Germany

Name/Description	*Company*
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Pierce ECL Westernblot substrate	Thermo Fisher Scientific
Prestained Protein Ladder PageRuler™ 26619	Thermo Fisher Scientific
Protease inhibitor cocktail (Complete, EDTA-free)	Roche Diagnostics, Mannheim, Germany
Rotiphorese® Gel 40 (acrylamide)	Carl Roth
SDS (Sodium dodecyl sulfate)	Carl Roth
TEMED (Tetramethylethylendiamin)	AppliChem, Darmstadt, Germany
Tris-(hydroxymethyl-)aminomethane	Carl Roth
Triton X-100	Carl Roth
Trypan Blue 0.4% $^{w}/_{v}$	Sigma-Aldrich
Trypsin 0.5%	Thermo Fisher Scientific
Tween® 20	Sigma-Aldrich

Table 4. Applied Chemicals

2.4 Technical equipment

Name/Description	*Company*
Axiovert 25 microscope	Carl Zeiss Microscopy, Jena, Germany
Centrifuge 5424R	Eppendorf, Hamburg, Germany
Centrifuge Labofuge 400R	Thermo Fisher Scientific
ChemiDoc™ XRS+	Bio-Rad Laboratories, Munich, Germany
Confocal laser-scanning microscope (CLSM) Axio Observer.Z1 LSM 700	Carl Zeiss Microscopy
Electrophoresis Power Supply PowerPac™ 3000	Bio-Rad Laboratories
Eppendorf Reference ® pipettes	Eppendorf

Name/Description	*Company*
Finnpipettes	Thermo Fisher Scientific
Heracell 150i incubator-CO_2	Heraeus
Herasafe safety cabinet	Heraeus, Hanau, Germany
Mini Centrifuge myFuge™	Benchmark Scientific, Sayreville, New Jersey, USA
Multipette® plus	Eppendorf
Neubauer counting chamber and cover glass	LO – Laboroptik, Friedrichsdorf, Germany
Pipetus®	Hirschmann Laborgeräte, Eberstadt, Germany
Polymax 2040	Heidolph, Schwabach, Germany
Precision scale 434	Kern & Sohn, Balingen, Germany
Scale BP1200	Sartorius, Goettingen, Germany
Thermomixer 5436	Eppendorf
Vortex mixer Reamix 2789	Karl Hecht, Sondheim, Germany
Vortex mixer REAX 2000	Heidolph
Western Blot combs, buffer tanks, gel holder cassettes, electrode assembly	Bio-Rad Laboratories

Table 5. Technical equipment

2.5 Software

For performing and evaluating confocal laser scanning microscopy (CLSM) the software *ZEN black edition 2011* (Carl Zeiss Microscopy) was used. As the visualization software for Western blot analysis served *Image Lab™ 6.0.1 2017* (Bio-Rad Laboratories).

2.6 Laboratory consumables

Name/Description	*company*
Cell scraper	Greiner Bio-One, Kremsmuenster, Austria
CELLSTAR® filter cap cell culture flasks	Greiner Bio-One
CELLSTAR® centrifuge tubes	Greiner Bio-One
CELLSTAR® serological pipettes	Greiner Bio-One
Cryogenic tubes	Thermo Fisher Scientific
Eppendorf tubes	Sarstedt, Nuembrecht, Germany
Falcon® 6-/24-/96-well multiwell plates	Corning
Microchamber slides	Sarstedt
Pasteur glass pipettes	Hirschmann Laborgeräte
Pipette tips	Sarstedt
Roti®-PVDF membrane	Carl Roth
Sterile filters Minisart®	Sartorius, Goettingen, Germany

Table 6. Utilized laboratory consumables

3 Methods

3.1 Cell culture

3.1.1 Cell cultivation and subculture

All three cancer cell lines used for the present study were cultivated under the same conditions at 37 °C and 5% (v/v) CO_2 in cell culture flasks. Cell growth medium (composition see Table 7) was changed every 2-3 days. At a confluence of 80%, cells were passed into new cell culture flasks every 3-4 days. For that, the growth medium was aspirated, cells washed from the growth area with PBS, and afterwards incubated with the cell splitting solution containing 0.05% (v/v) EDTA in PBS at 37 °C for 5-10 min. Then, cells were resuspended in PBS, collected into 10 ml falcon tubes, and centrifuged at 12,000 x g at 20 °C for 3 min. After removal of the supernatant, cells were resuspended in fresh growth medium, and transferred into a new flask.

Stable KAI1-wt and KAI1-sp cell transfectants carrying the selection marker Hygromycin on the expression plasmids were grown in cell culture medium containing additional 200 µg/ml Hygromycin B.

Cell growth medium	
DMEM GlutaMAX™	500ml
HEPES	1 mM
FCS	10% (v/v)
L-Arginine	550 mM
L-Asparagine	272 mM

Table 7. Ingredients of the cell growth medium

3.1.2 Cell counting

Cell numbers were counted by using a *Neubauer* hemocytometer. It contains nine large squares of 1 mm edge length. The depth of the counting chamber measures 0.1 mm. The cell suspension was mixed, and therefore diluted, with 50 µl trypan blue in order to stain dead cells which were not counted. With capillary forces the suspension was drawn into the chamber.

Using a microscope, the cell numbers in the four corner squares were counted. Hence, the number of cells was calculated as follows:

(Counted cell number/4) x dilution factor x 10,000 = cell number/ml

3.1.3 Cryoconservation and thawing of cells

In order to establish cell stocks, cells were cryoconserved in liquid nitrogen. For that, cells were detached from the growth area of the cell culture flask, washed in PBS, and centrifuged as described in chapter 3.1.1. Afterwards, the cell pellet was resuspended in freezing medium (90% ($^{v}/_{v}$) FCS, 10% ($^{v}/_{v}$) DMSO) and transferred into cryogenic tubes. These were quickly placed on ice and stored at -80 °C. After 3-4 days, cell stocks were transferred into liquid nitrogen at -196 °C for long-term storage.

For the thawing of cells, cold cell growth medium was added, the cell suspensions passed into falcon tubes, and centrifuged at 12,000 x g for 3 min. After resuspension in cell growth medium, cells were transferred into small cell culture flasks. Cells were at least splitted twice prior to their use in experiments.

3.1.4 Stable transfection of MCF7 cells with KAI1-wt or KAI1-sp

For stable transfection, MCF7 cells were seeded on a multiwell plate (growth area 9.6 cm^2) and cultured for 24 h until they reached a confluency of 60-70%. Cells were washed once in PBS and covered with FCS-free cell culture medium (GAM). Lipofectin was used as transfection vehicle and preincubated in GAM for 45 min at room temperature (RT). The expression plasmid containing the KAI1-wt- or KAI1-sp-cDNA was dissolved in GAM and mixed with the lipofectin/GAM solution and incubated for 15 min at RT. Transfections with the empty expression vector served as control. Then, the medium was aspirated and cells were recovered in fresh GAM. The DNA/lipofectin solution was carefully dripped onto the cell monolayers and incubated at 37 °C. After 6 h of incubation, the transfection medium was removed, and the cell layer covered with complete cell culture medium. After further cultivation for 3 days, the medium was aspirated and cell culture medium containing 200 µg/ml Hygromycin B added. As the expression plasmids carry the selection marker Hygromycin, transfected and thus Hygromycin-resistant clones were selected from untransfected cells. At a confluence of 80%, cells were detached from the cell growth area of the flasks and passed on for further cultivation (see chapter 3.1.1). The success of transfection was determined by

measuring the cellular KAI1-wt or KAI1-sp protein content by immunocytochemical staining and Western blot analysis as described below (see chapters 3.3 and 3.4).

3.1.5 Generation of cellular lysates

Cells were seeded on 6-well cell culture plates at a density of 500,000 cells/well. After 3 h, when cells established firm adhesion, E2 was added and the cell culture plates were incubated for another 48 h. After removing the cell culture medium, cells were washed in ice cold PBS and incubated with a special lysis buffer (composition see Table 8) for 15 min. Then cells were removed from the growth area with a cell scraper and transferred into an *Eppendorf* tube. After centrifugation at 12,000 x g for 4 min, the supernatant was passed into a new *Eppendorf* tube and used for determination of protein concentration prior to Western blot analysis. Cell lysates were stored for further use at -20 °C in aliquots to avoid repeated thawing.

Cell lysis buffer	
HEPES pH 7.5	50 mM
NaCl	150 mM
EDTA	1 mM
Glycerole	10% ($^{v}/_{v}$)
Triton X-100	1% ($^{v}/_{v}$)
Complete™ protease inhibitor cocktail	0.1% ($^{v}/_{v}$)

Table 8. Ingredients of the cell lysis buffer

3.2 Cell proliferation assay

For measuring the cell proliferative activity, cells were seeded at a density of 20,000 cells per well in triplicates on 24-well multiwell cell culture plates (1.9 cm^2) in 1 ml cell growth medium per well and cultivated.

After established cell adhesion within 3 h, as starting point, cell numbers were determined by cell counting, which was repeated after incubation of 24, 48, 96, and 120 h. Growth medium was removed and cells carefully washed in PBS. Cells were detached in 10% ($^{v}/_{v}$) trypsin in PBS at 37 °C for 8 min. The cell suspensions were collected in *Eppendorf* tubes and 50 µl per tube of trypan blue was added in order to exclude blue-stained dead cells form counting. The

determination of the cell number was conducted as described in chapter 3.1.2. The cell number of each well was counted three times.

3.3 Immunocytochemical staining

In order to determine the extent of the expression of KAI1, the EGFR, and p-EGFR, immunocytochemical staining was performed.

Detection of KAI1

Cells were seeded at a density of 40,000 cells per well on microchamber cell culture slides which were coated with 5 µg/ml of the ECM protein fibronectin (FN) for 30 min at RT and cultivated overnight at 37 °C. In order to compare unstimulated and E2-stimulated cells, cells were first cultivated for 3 h on the microchamber cell culture slides to allow firm cell adhesion before E2 stimulation and then incubated for 24 h overnight. Cell growth medium was removed, and the cell monolayers washed in PBS. Then, cells were fixed in PBS, 4% ($^{w}/_{v}$) PFA (pH 7.4) for 15-20 min at RT and thereafter washed thrice in PBS. Unspecific binding of antibodies to cell surfaces was minimized by incubating the cells with PBS, 2% ($^{w}/_{v}$) BSA for 30 min at RT. Cells were again washed in PBS and incubated with the primary antibody (Ab) targeting KAI1 at a concentration of 10 µg/ml PBS, 1% ($^{w}/_{v}$) BSA for 2.5 h at RT. The supernatant was removed and after six washes in PBS, cells were incubated with the secondary goat-anti-mouse-IgG conjugated by the fluorophore Alexa-488 at a concentration of 2 ng/ml PBS, 1% ($^{w}/_{v}$) BSA at RT in the dark. After 45 min, cells were repeatedly washed six times in PBS, the chamber frame removed, and the slides mounted by a cover slip. The measurement of fluorescence intensity was determined by CLSM. As control for cell autofluorescence, cells were incubated in the presence of the secondary Alexa-488 labeled antibody alone.

Detection of the EGFR and the p-EGFR

In order to determine the expression levels of the EGFR and the p-EGFR, respectively, cells were seeded, cultivated overnight at 37°C, and fixed as described above. All PBS washing steps in between were also performed as outlined above. Unspecific binding sites were blocked in PBS, 2% ($^{w}/_{v}$) BSA for 30 min at RT. The cells were incubated with the primary antibody directed to the EGFR or the p-EGFR for 2.5 h at RT at a concentration of 2.5 µg/ml and 10 µg/ml PBS, 1% ($^{w}/_{v}$) BSA. Alexa-488-conjugated secondary antibodies were incubated at a concentration of 2 ng/ml PBS, 1% ($^{w}/_{v}$) BSA for 45 min at RT in the dark. For detecting the EGFR a goat-anti-mouse-IgG was used, for measuring p-EGFR cells were incubated with a

goat-anti-rabbit antibody. Fluorescence intensity was determined by CLSM. As control for cell autofluorescence, cells were incubated in the presence of the secondary Alexa-488 labeled antibody alone.

3.4 Western blot analysis

For Western blot analysis, extracted cellular proteins were separated by gel electrophoresis and subsequently transferred onto PVDF membranes.

Quantification of protein concentrations in cell lysates

Protein concentrations in cell lysates were determined following the *bicinchoninic acid assay*, short BCA assay. The Pierce BCA Protein Assay Kit by *Thermo Fisher Scientific* was used according to the manufacturers specifications. This method is based on the *Biuret* reaction, which describes the reduction of bivalent copper to monovalent copper. Together with BCA, it forms a color complex and leads to a color change to violet. This enables the photometric measurement of protein concentrations by means of an established protein concentration calibration curve using a dilution series of the protein standard BSA.

First, Tris-buffered saline (TBS) stock solution (composition see Table 9) was diluted 1:10 in distilled water (H_2O_{dest}) to generate the working solution for the dilution of the cell lysates. 50 µl of the protein standards and the cell lysate were applied to a 96-well multiwell plate and mixed with 200 µl of BCA reagent per well. The plate was incubated for 2 h at RT. Subsequently, the absorption was measured by use of an ELISA reader at a wavelength of 570 nm and the protein concentrations calculated by means of the protein calibration curve.

TBS stock solution	
NaCl	1.5 M
1 M Tris-HCl pH 8.0	0.5 M

Table 9. Ingredients of TBS stock solution

SDS gel electrophoresis

At least 25 µg of total protein of each protein lysate were used for the separation by *sodium dodecyl sulfate polyacrylamide gel electrophoresis* (SDS-PAGE). Protein samples were resuspended in electrophoresis buffer (ingredients see Table 10) and then incubated at 95 °C

for 4 min after reducing or non-reducing buffer was added (ingredients see Table 11), depending on the manufacturers specifications of the antibody used for protein detection.

The 10% PAGE-separating gel (ingredients see Table 12) for the gel electrophoresis was poured between two sealed glass plates and covered with ethanol to prevent contact to oxygen and to obtain an even gel line for precise sample application. After complete gel polymerization, the ethanol was drained off. The 4% ($^{w}/_{v}$) PAGE-stacking gel (ingredients see Table 13) was added on top of the separating gel containing a comb to form pockets for sample application. Afterwards, the polyacrylamide gel was fixed in the gel holder cassette and put into a buffer tank containing electrophoresis buffer. The comb was removed, and the protein samples pipetted into the pockets of the stacking gel. A prestained protein ladder was also added to determine the molecular weight of the protein of interest. The buffer tank was closed with the electrode assembly and the SDS-PAGE was run at a voltage of 100 V until the probes reached the bottom of the separating gel.

Electrophoresis buffer	
Glycine	1.6 mM
Tris	250 mM
SDS	1% ($^{w}/_{v}$)

Table 10. Ingredients of the electrophoresis buffer

Non-reducing buffer	
Tris/HCl (pH 6.8)	90 mM
Glycerole	30% ($^{v}/_{v}$)
SDS	7% ($^{w}/_{v}$)
Bromophenol blue	0.01% ($^{w}/_{v}$)
For reducing buffer add:	
2-mercaptoethanol	20% ($^{v}/_{v}$)

Table 11. Ingredients of the non-reducing and reducing buffer

10% PAGE-separating gel	
1.5 M Tris-HCl pH 8.8	2.5 ml
10% ($^{w}/_{v}$) SDS	100 µl
Acrylamide 40%	2.5 ml
10% ($^{w}/_{v}$) ammonium persulfate (APS)	50 µl
TEMED	5 µl
H_2O_{dest}	4.8 ml

Table 12. Composition of the 10% PAGE-separating gel

4% PAGE-stacking gel	
0.5 M Tris-HCl pH 8.8	2.5 ml
10% ($^{w}/_{v}$) SDS	100 µl
Acrylamide 40% ($^{w}/_{v}$)	1 ml
10% ($^{w}/_{v}$) APS	50 µl
TEMED	10 µl
H_2O_{dest}	6.3 ml

Table 13. Composition of the 4% PAGE-stacking gel

Western blotting

Cellular proteins were separated by SDS-PAGE in an electrical field. In the process of Western blotting these proteins were transferred from the gel onto a *polyvinylidene difluoride* (PVDF) membrane, followed by protein detection using the respective specific primary antibodies. First, a PVDF membrane and six filter papers were cut to the size of the electrophoresis gel. The membrane was swiveled in methanol and the filter papers soaked in wet-blotting buffer (composition see Table 14) before use. Then a sponge and three filter papers were positioned on the anode part of the assembly cassette. The electrophoresis gel and the membrane were put thereon minding a bubble-free buildup. After three more layers of filter paper and an additional sponge on the side of the cathode, the cassette was closed. The transfer was performed in a filled buffer tank on ice with the power of 350 mA for 90 min. Subsequently, protein bands were detected by specific primary antibodies. The different incubation conditions for the respective antibody are displayed in Table 15. Unspecific binding of antibodies to cell surfaces was minimized by incubating the membrane in TBST wash (composition see Table 16) containing 5% ($^{w}/_{v}$) milk powder for 90 min. The membrane was washed three times in

TBST prior to the application of the primary antibody. The incubation with the primary and later the secondary antibody was performed on a shaker for even distribution over the membrane. The membrane was then washed again in TBST thrice and thereupon incubated with the HRP-conjugated secondary antibody for 1 h at RT. The enhanced chemiluminescence (ECL) substrate was prepared according to the manufacturers information and the membrane panned therein for 3 min at RT after three more TBST washes. Protein bands were then visualized by chemiluminescence detection.

Differences in protein loading and blotting efficiency were normalized by reprobing the membranes with a mAb directed to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Band signal intensities were evaluated by use of the Bio-Rad Imager *Gel Doc™ XR +* and *ChemiDoc™ XRS +* Systems with the software *Image Lab™* and normalized to those recorded for GAPDH.

Wet-blotting buffer	
Tris	25 mM
Glycine	192 mM
Methanol	20% ($^{v}/_{v}$)

Table 14. Ingredients of the wet-blotting buffer

Targeted antigen	*Stock conc. µg/µl*	*Dilution*	*Incubation time*	*Temperature*
EGFR	0.25	1:2,500	overnight	4 °C
ERα	0.2	1:500	overnight	4 °C
GAPDH	1	1:10,000	1 h	RT
KAI1	1	1:300	overnight	4 °C
p-EGFR	1	1:2,000	overnight	4 °C

Table 15. Incubation conditions for respective primary antibodies

TBST wash	
NaCl	1.5 M
1M Tris-HCl pH 8.0	0.5 M
Tween® 20	0.1% ($^{v}/_{v}$)

Table 16. Ingredients of the TBST wash

4 Results

Previous data from the research group of Prof. Dr. Reuning had documented that tumor biological processes, like cell adhesion, proliferation, and migration, are influenced as a function of the expression of KAI1-wt in human breast cancer cells. Furthermore, an enhanced cellular level of the splice variant KAI1-sp plays a role in increased tumor growth and invasiveness. (Miller et al., 2018) In the present study, we aimed at exploring the tumor biological effects of the two KAI1 proteins, KAI1-wt and KAI1-sp, and the effects of E2 stimulation on cell proliferation and the expression of ERα and EGFR in HR-positive human breast cancer cells. For this, we established an *in vitro* cell model of MCF7 WT cells and respective stable cell transfectants expressing either KAI1-wt or KAI1-sp. Vec-transfected cells served as controls as well as HR-negative MDA-MB-231 and MDA-MB-435 cells.

4.1 Establishment of a human breast cancer cell model expressing either KAI1-wt or KAI1-sp upon stable cell transfection

In order to investigate the tumor biological effects of KAI1-wt and KAI1-sp in human MCF7 breast cancer cells which harbor a low content of KAI1 protein, we generated stable cell transfectants with expression vectors coding for KAI1-wt or KAI1-sp. As controls served mock transfectants.

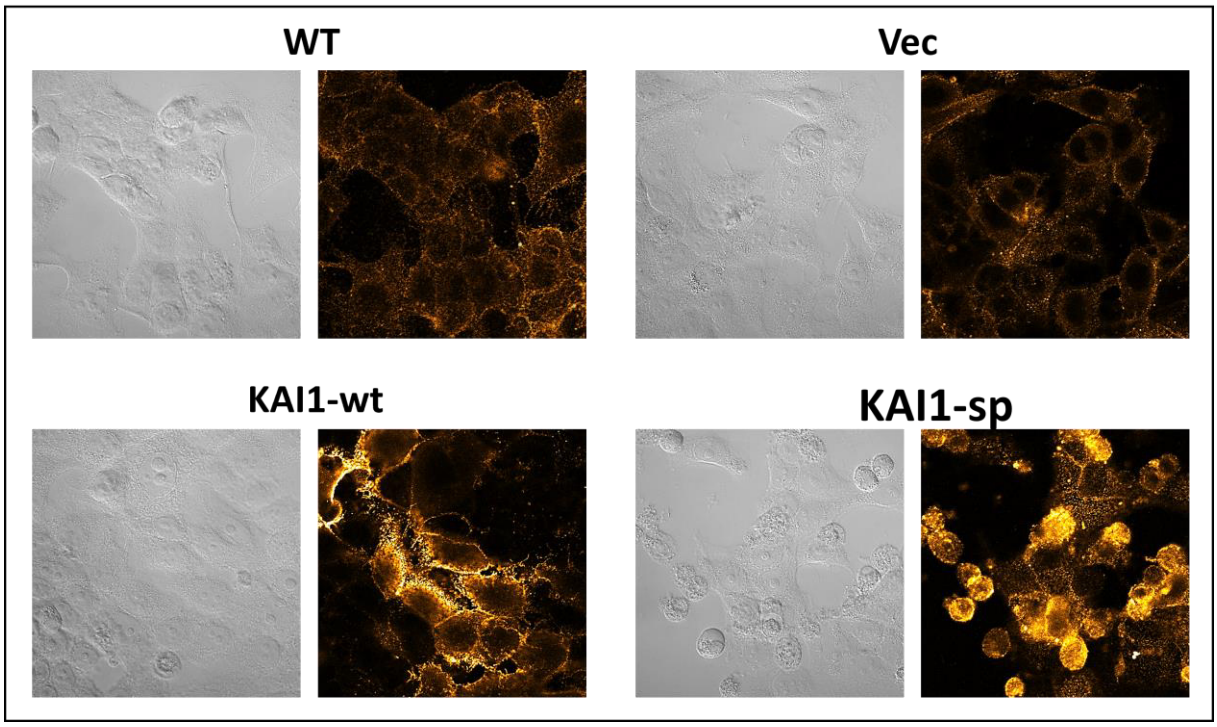
KAI1 transfectants of the two cell lines MDA-MB-231 (breast cancer) and MDA-MB-435 (melanoma) had been previously established by the research group of Prof. Dr. Reuning and were kindly provided for the present study.

Cells which were successfully transfected display antibiotic resistance to Hygromycin B which was therefore used as a selection marker. After isolation of stably transfected cells, the expression levels of KAI1 protein were evaluated by immunocytochemical staining and CLSM analysis as described. The staining of the KAI1 proteins on MCF7 cells proved successful cell transfection and KAI1 expression on the cell membrane.

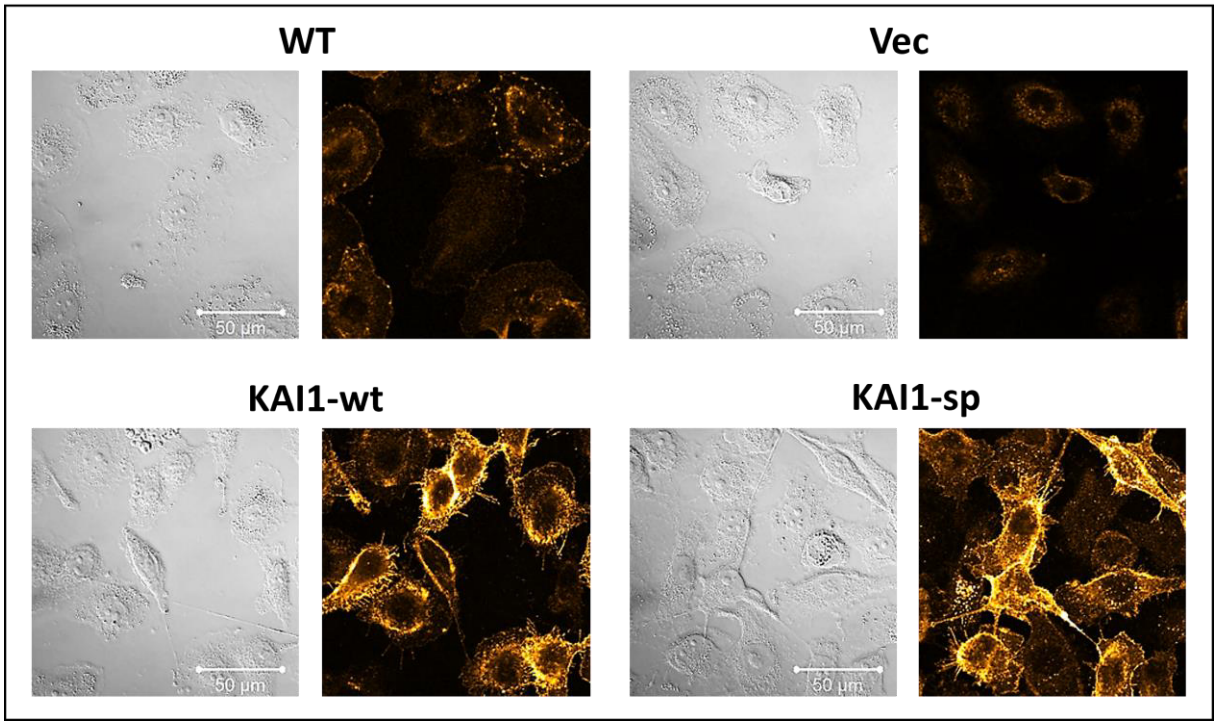
The WT cells of all three cell lines, MCF7, MDA-MB-231, and MBA-MB-435, respectively, and the corresponding Vec transfectants exhibited low and comparable KAI1 expression levels.

Immunocytochemical staining revealed comparably high fluorescence signal intensities for KAI1-wt and KAI1-sp transfectants (see Figure 5).

A) MCF7



B) MDA-MB-231



C) MDA-MB-435

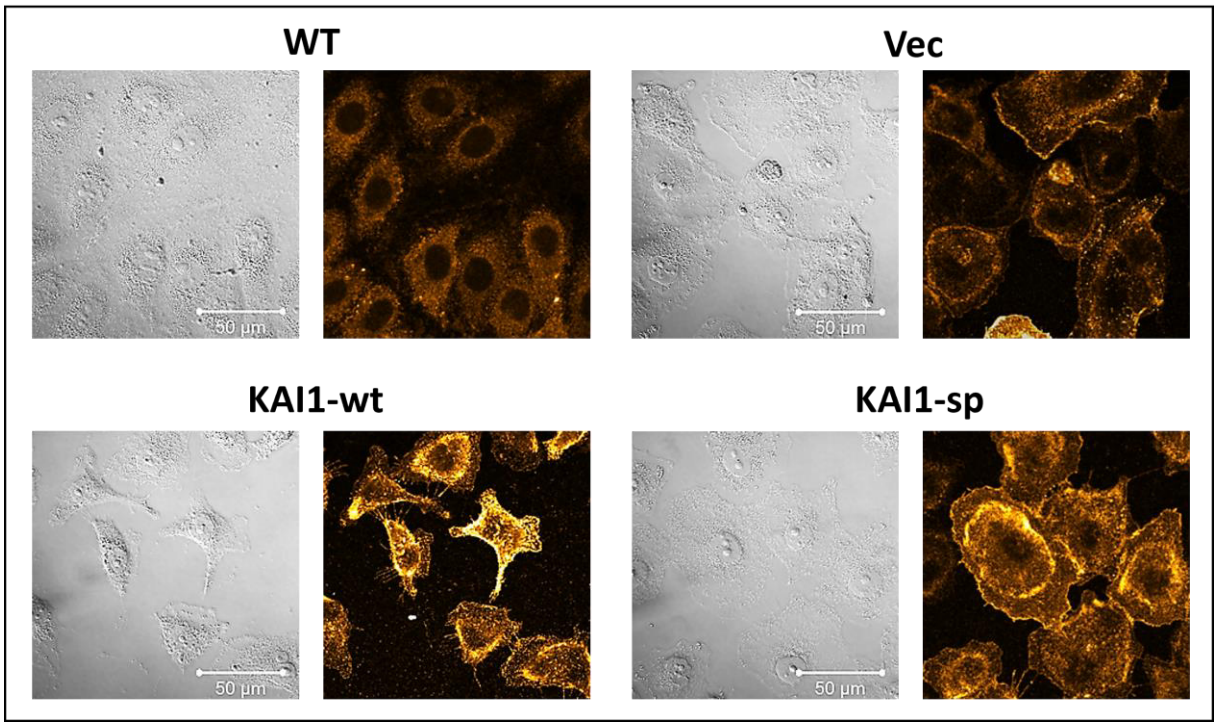


Figure 5. Immunocytochemical staining of KAI1 proteins in MCF7, MDA-MB-231, and MDA-MB-435 cells and their respective transfectants

KAI1 protein expression was detected by immunocytochemistry in MCF7, MDA-MB-231, or MDA-MB-435 cells and the respective transfectants thereof. As described, all cell transfectants were grown on microchamber cell culture slides and stained. Representative fluorescence images are depicted with the corresponding differential interference contrast (DIC) images. Fluorescence signal intensity was evaluated by CLSM and converted into a pseudo glow scale: low intensity (red), medium intensity (yellow), and high intensity (white). In all three cell lines the respective WT cells and Vec transfectants showed comparably low KAI1 expression levels. KAI1-wt and KAI1-sp transfectants presented with similarly elevated KAI1-expression levels.

By microscopical inspection of MCF7 WT cells, it was noted that cells obtained a characteristic spread shape upon adhesion to the underlying cell growth area with elongated filo- and lamellipodia. KAI1-wt-transfected MCF7 cells maintained their characteristic appearance by developing a spread shape upon adhesion. This cell morphology was also noticed for Vec transfectants.

Following stable cell transfection with KAI1-sp cDNA, a different cell morphological phenotype was observed with a more rounded cell shape and thus a smaller spreading area when compared to MCF7 WT cells or KAI1-wt transfectants.

Representative images from immunocytochemical staining are shown for MCF7 WT cells and KAI1-wt transfectants, as well as KAI1-sp transfectants (see Figure 6).

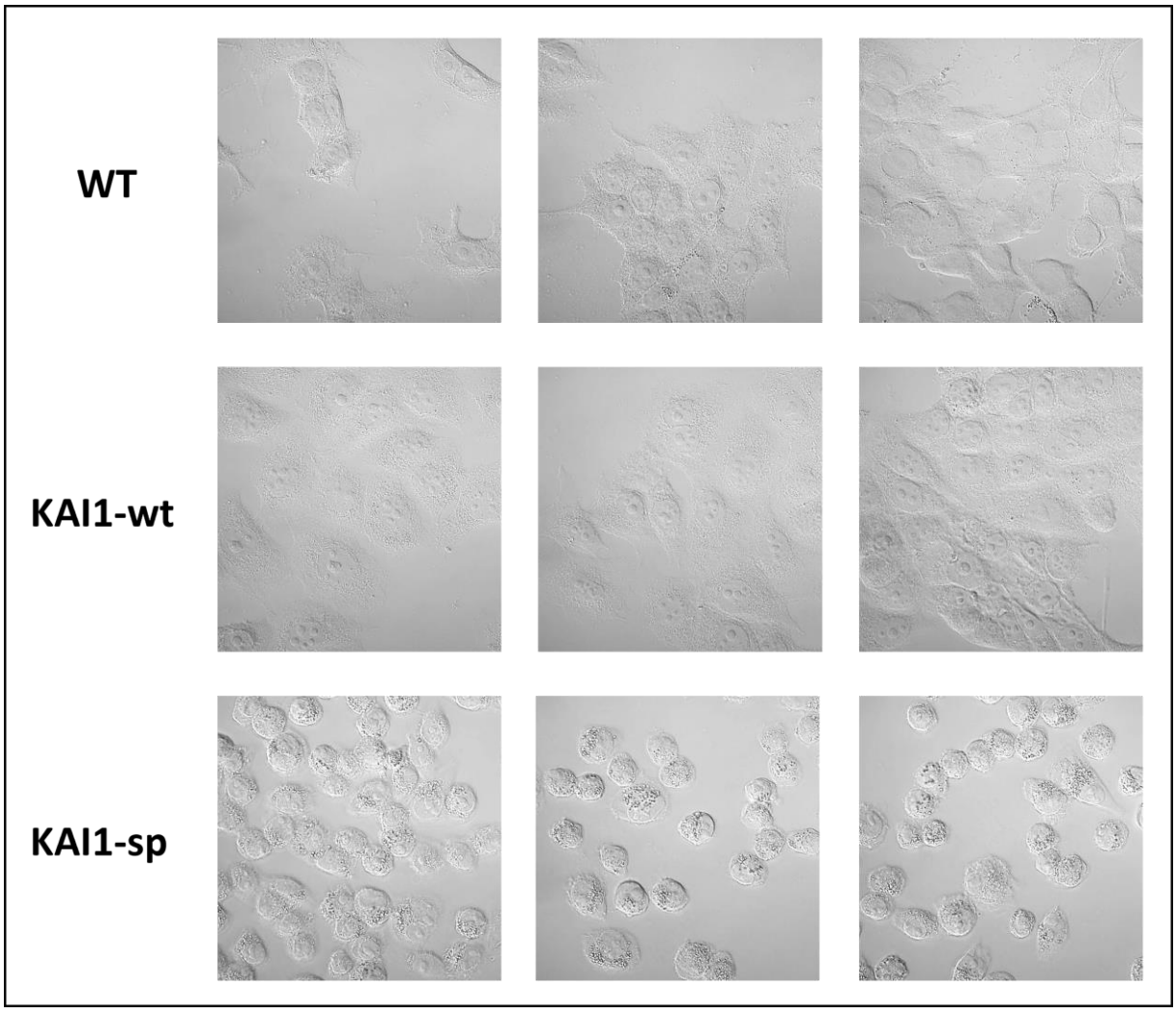


Figure 6. Characteristic cell morphology of MCF7 WT cells, KAI1-wt, and KAI1-sp transfectants

MCF7 WT cells obtained a spread shape upon adhesion to the cell growth area. KAI1-wt transfectants did not change this phenotype. However, KAI1-sp-transfected MCF7 cells displayed a more rounded cell shape and therefore a smaller spreading area upon adhesion. Representative images are shown for WT cells, KAI1-wt, and KAI1-sp transfectants, respectively.

4.2 Effect of KAI1-wt and KAI1-sp and cell exposure to estrogen on cellular expression of the estrogen receptor-α

It is well known that the ER plays an important role in tumor biology (Jia et al., 2015). Therefore, possible effects of KAI1-wt and KAI1-sp on the expression of ERα were investigated by Western blot analysis. In parallel, we studied ERα expression as a function of cell exposure to E2 (0.6 ng/ml). For this, WT cells, Vec-, KAI1-wt-, and KAI1-sp-transfected MCF7, MDA-MB-231, and MDA-MB-435 cells, respectively, were grown on 6-well cell culture plates in the absence or presence of E2 (0.6 ng/ml). After 48 h, cells were lysed, followed by Western blot analysis of ERα expression as described. Identical membranes were reprobed with an antibody directed to the house keeping protein GAPDH in order to control for equal protein loading on the SDS gels and blotting efficiency of each lysate applied. ERα appeared in the SDS-gel at a molecular weight of appr. 66 kDa.

MCF7 WT cells showed a strong band for ERα, indicative of a high receptor expression. Values for signal intensity obtained for ERα protein bands in Vec, KAI1-wt, and KAI1-sp transfectants, respectively, in the absence or presence of E2 are given as n-fold over endogenous expression in unstimulated WT cells, which was set to “1.0” (see Figure 7).

The observed high endogenous amount of cellular ERα in MCF7 WT cells was reduced upon stimulation by E2 (0.6-fold). Vec transfectants obtained an expression level of ERα, corresponding to around one third of the expression level in WT cells. Like in WT cells, cellular ERα expression was lowered after exposure to E2 (0.1-fold). In KAI1-wt transfectants, the expression levels for ERα were half as high as in WT cells. Upon E2 stimulation, the expression was downregulated to 0.2-fold. In KAI1-sp cell transfectants, the expression of ERα was even further diminished not reaching the detection limit of Western blot analysis, independent of the presence of E2.

Since MDA-MB-231 as well as MDA-MB-435 cells are classified as ERα-negative, as expected, no signals for ERα expression were detectable in any of the cell variants which did not change upon E2 stimulation.

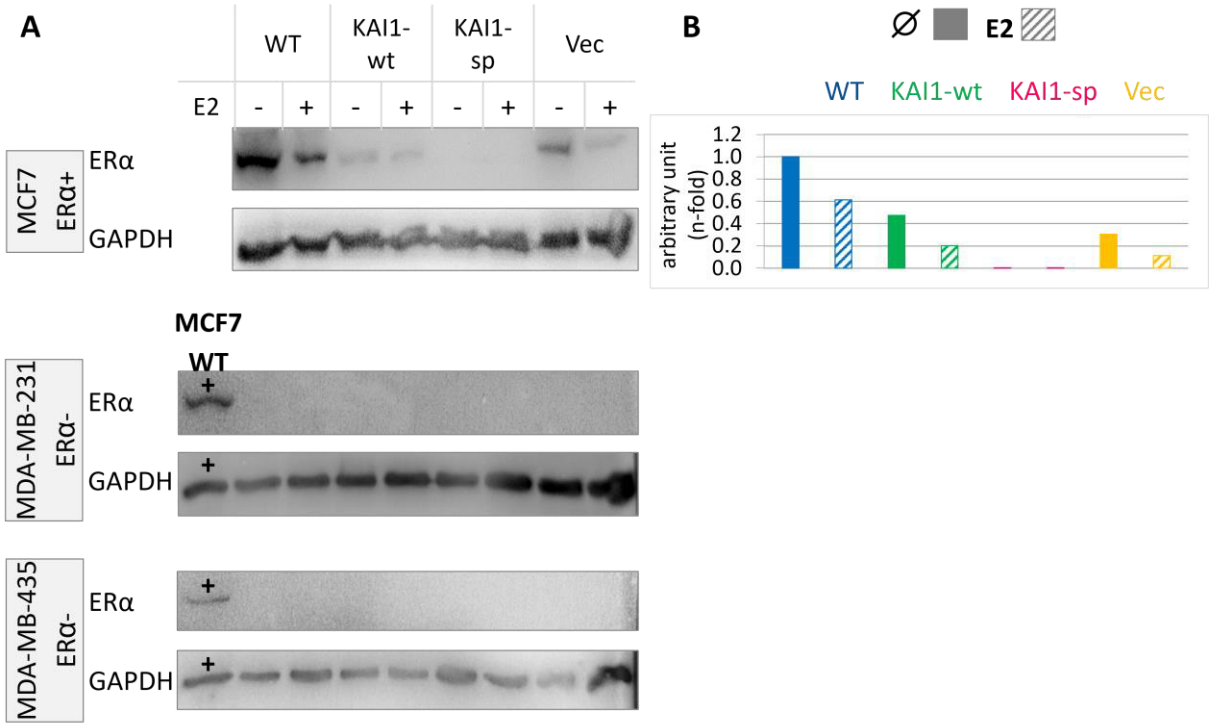


Figure 7. Western blot analysis of the cellular expression of ERα in MCF7, MDA-MB-231, or MDA-MB-435 WT cells and their respective KAI1 transfectants as a function of cell stimulation by E2

ERα-positive MCF7 cells were compared to ERα-negative MDA-MB-231 and MDA-MB-435 cells, respectively. MCF7, MDA-MB-231, and MDA-MB-435 cells, respectively, as well as their corresponding transfectants were cultivated unstimulated (Ø/-) or stimulated by 0.6 ng/ml E2 (E2/+). A) Cellular ERα levels were detected by Western blot analysis. In order to normalize the signal intensity of the obtained ERα bands, Western blot membranes were reprobed with an antibody directed to GAPDH. Typical and representative images are shown. In the Western blots for ERα-negative cell lines, lysates of MCF7 WT cells were applied into lane 1 as positive control for ERα staining. B) Signal intensity for ERα protein bands was densitometrically analyzed by the software Image Lab™ and normalized to the signals obtained for GAPDH. Data are given as n-fold by setting the normalized signal intensity of unstimulated MCF7 WT cells to "1.0".
MCF7 WT cells presented with a high endogenous level of cellular ERα, which was reduced upon E2 stimulation. In Vec transfectants ERα levels accounted for a third compared to WT cells and were also reduced upon E2 stimulation of cells. In KAI1-wt transfectants, half of the amount of ERα was detected compared to WT cells which further decreased upon E2 stimulation. In KAI1-sp transfectants, in the absence or presence of E2, ERα-levels were below the detection limit. As expected, in unstimulated as well as stimulated MDA-MB-231 and MDA-MB-435 cell transfectants ERα levels were below the detection limit.

4.3 KAI1 expression in stable cell transfectants as a function of estrogen exposure

The regulation of cellular KAI1 expression is still a matter of debate in the current literature. To this end, we were interested whether E2 might exert a possible regulatory effect on the expression of the KAI1 proteins in ERα-positive MCF7 WT cells and their respective cell transfectants. We investigated the expression of the KAI1 proteins as a function of E2 stimulation by Western blot analysis and immunocytochemical staining. ERα-negative MDA-MB-231 and MDA-MB-435 cells and their respective KAI1 transfectants served as controls.

KAI1 detection by Western blot analysis

WT cells, Vec-, KAI1-wt-, and KAI1-sp-transfected MCF7, MDA-MB-231, and MDA-MB-435 cells, respectively, were grown on 6-well cell culture plates in the absence and presence of E2 (0.6 ng/ml). After 48 h, cells were lysed and subjected to Western blot analysis for KAI1 detection as described. Identical membranes were reprobed with an antibody directed to the house keeping protein GAPDH in order to control for equal protein loading on the SDS-gels and blotting efficiency of each sample applied. KAI1 is highly glycosylated and thus appears on a 10% SDS-PAGE as a broad protein band ranging in the molecular weight between 34 and 60 kDa. The signal intensity of protein bands representing KAI1 proteins was determined by using the software *Image Lab*™ and normalized to the signals of GAPDH as described under “Methods”. Signals obtained for unstimulated WT cells were set to “1.0” (see Figure 8).

MCF7 WT cells presented with a low endogenous amount of KAI1 protein. Successful stable KAI1 transfection of MCF7 cells was proven in KAI1-wt transfectants (11.9-fold higher expression levels than WT cells), whereas KAI1-sp cell transfectants displayed a 6.0-fold higher KAI1 expression level than WT cells. E2 stimulation of MCF7 WT cells led to a 2.6-fold increased KAI1 expression compared to unstimulated cells. In Vec transfectants, an only minor effect of E2 was noted on KAI1 expression levels. Most interestingly, E2-stimulated KAI1-wt transfectants exhibited a small increase of KAI1 expression levels compared to this respective transfectant in the absence of E2 (11.9-fold to 12.8-fold). On the opposite, in KAI1-sp transfectants, E2 stimulation led to obviously lower KAI1 expression levels compared to that in the absence of E2 (6.0-fold to 1.1-fold).

ERα-negative MDA-MB-231 WT cells presented with a higher endogenous KAI1 protein level than MCF7 WT cells which was comparable to the respective Vec transfectant. MDA-MB-231 KAI1-wt and KAI1-sp transfectants, respectively, increased KAI1 protein expression by stable

cell transfection over WT cells (KAI1-wt transfectants: 3.2-fold, KAI1-sp transfectants: 1.9-fold). Unexpectedly, although being ERα-negative, E2-stimulated cells presented with a slight increase of KAI1 expression levels: WT cells 1.6-fold, KAI1-wt transfectants 3.5-fold, and KAI1-sp transfectants 2.6-fold. Vec transfectants showed slightly lower KAI1 expression upon E2 stimulation (1.5-fold to 1.0-fold).

In ERα-negative MDA-MB-435 WT cells endogenous levels of KAI1 were below detection. KAI1-wt and KAI1-sp transfectants presented with increased KAI1 expression with no effect of E2 stimulation (data not shown).

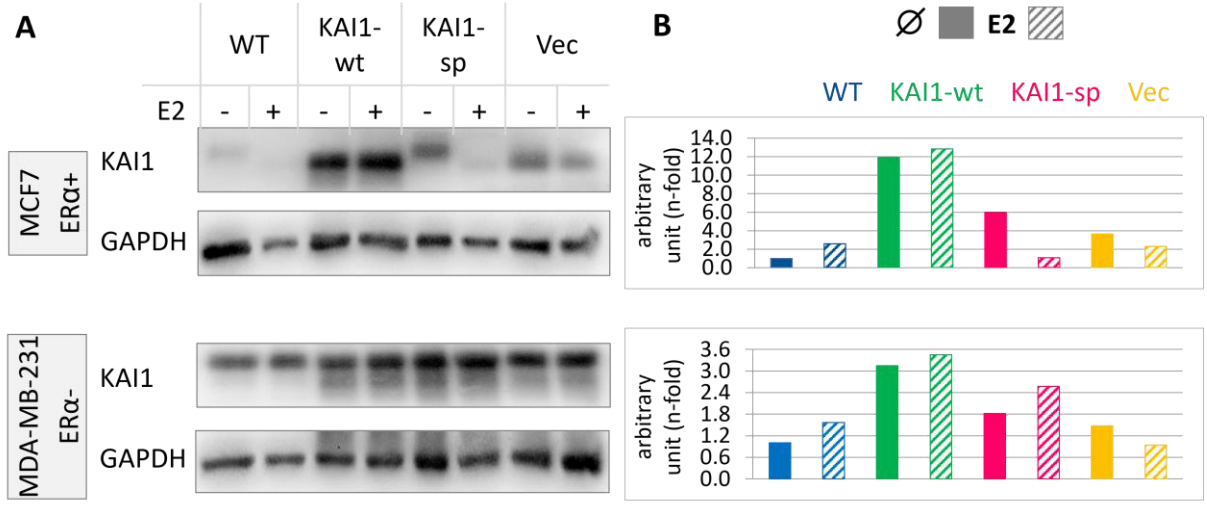


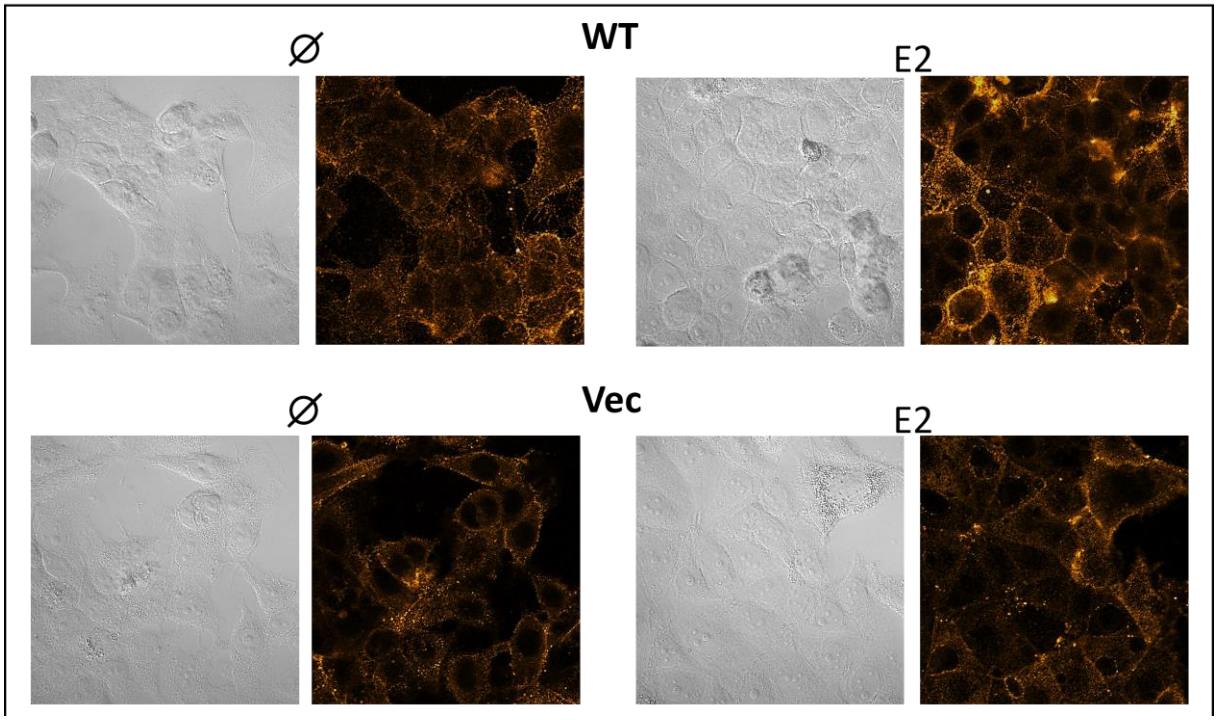
Figure 8. Western blot analysis of the cellular KAI1 expression in MCF7 or MDA-MB-231 WT cells, and their respective KAI1 transfectants as a function of E2 stimulation

MCF7 or MDA-MB-231 WT cells and their respective KAI1 cell transfectants were cultivated in the absence (Ø/-) or presence of 0.6 ng/ml E2 (E2/+) for 48 h. A) Cellular KAI1 expression was detected by Western blot analysis. In order to normalize the signal intensity of the obtained KAI1 bands, Western blot membranes were reprobed with an antibody directed to GAPDH. Typical and representative images of Western blots are shown. B) KAI1 signal intensity on Western blots was densitometrically analyzed by using the software Image Lab™ and normalized to the signals obtained for GAPDH. Data are given as n-fold by setting the normalized signal intensity of the respective unstimulated WT cells to "1.0".
In both cell lines, successful KAI1 transfection was confirmed by the detection of higher KAI1 protein levels compared to WT cells and Vec transfectants. KAI1 protein expression was increased in MCF7 WT cells and KAI1-wt transfectants upon E2 stimulation. However, in KAI1-sp and Vec transfectants, respectively, decreased KAI1 expression levels were observed upon cell exposure to E2. In ERα-negative MDA-MB-231 WT cells, higher endogenous KAI1 levels were detected compared to MCF7 WT cells. In KAI1-wt and KAI1-sp transfectants higher KAI1 levels were noted compared to WT cells. E2 stimulation led to further increased KAI1 levels in WT cells, KAI1-wt, and KAI1-sp transfectants, whereas in Vec transfectants KAI1 levels were slightly lower upon E2 exposure.

Immunocytochemical staining of KAI1 proteins

KAI1 protein expression as a function of E2 stimulation was further analyzed by immunocytochemical staining. For this, MCF7 WT cells and the respective transfectants were seeded on microchamber cell culture slides in the presence or absence of 0.6 ng/ml E2 and cultivated overnight. After cell fixation and incubation with the corresponding antibody, KAI1 proteins were stained (see Figure 9). Cell staining in the absence of the primary antibody served as control, which were negative (data not shown).

We detected an elevation of KAI1-wt and KAI1-sp due to successful stable cell transfection when compared to the low endogenous KAI1 levels in MCF7 WT cells. The expected cell surface localization of KAI1 was confirmed. Upon cell exposure to E2, MCF7 WT cells displayed an increased fluorescence signal intensity for KAI1. In E2-stimulated KAI1-wt transfectants signal intensity was similar to unstimulated cells. In KAI1-sp transfectants signal intensity for KAI1 appeared less intense upon E2 stimulation when compared to the respective unstimulated cells. These findings by immunostaining corresponded well with the results obtained by Western blot analysis.



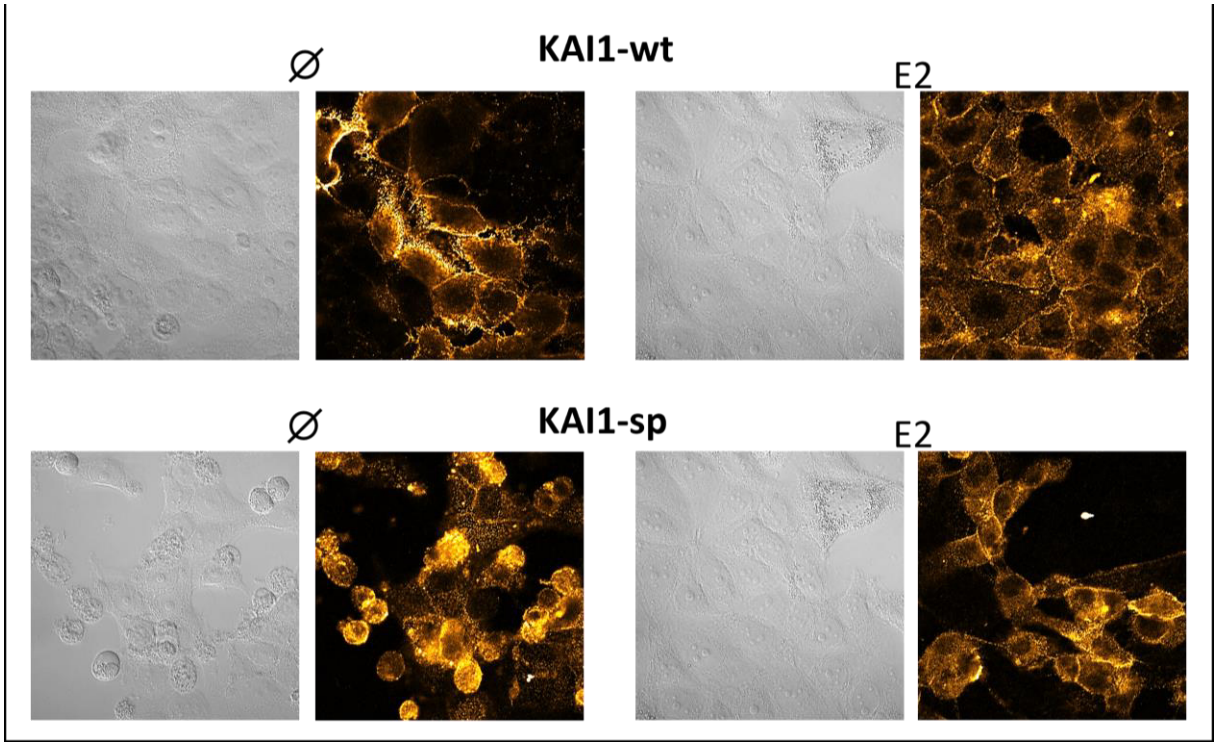


Figure 9. Immunocytochemical staining of the two KAI1 protein variants in MCF7 WT cells and the respective stable KAI1-wt and KAI1-sp transfectants in the presence or absence of E2

By immunocytochemical staining, in MCF7 cells, KAI1 expression levels were evaluated as a function of E2 stimulation. Cells were grown on microchamber cell culture slides in absence (Ø) or presence of E2 (0.6 ng/ml) and stained for KAI1 as described. In MCF7 WT cells, E2 exposure led to a slightly stronger fluorescence signal intensity for KAI1. In KAI1-wt transfectants signal intensity was similar to unstimulated cells. In KAI1-sp transfectants a downregulation of KAI1 expression was noticeable upon cell exposure to E2.

4.4 Effects of KAI1-wt, KAI1-sp, and estrogen on the cellular expression of the epidermal growth factor receptor

The EGFR has been identified to hold a central position regarding breast cancer biology. It plays an important role in proliferation of cancer cells, tumorigenesis, and metastasis. (Sasaki et al., 2013) Here, we studied possible regulatory effects of the two KAI1 variants and E2 on the expression of the EGFR by Western blot analysis and immunocytochemistry.

EGFR detection by Western blot analysis

Cells were cultivated in the absence or presence of E2 (0.6 ng/ml) for 48 h, thereafter lysed and Western blot analysis performed as described. The EGFR protein bands appeared at a molecular weight of appr. 175-180 kDa. The endogenous EGFR expression level in unstimulated WT cells of each cell line was set to “1.0” and set in relation to the results achieved for the respective Vec, KAI1-wt, and KAI1-sp transfectants and stimulated samples (see Figure 10).

In ERα-positive MCF7 WT cells, in the presence or absence of E2, EGFR expression levels were below the detection limit of Western blot analysis, which was also true for KAI1-wt and Vec transfectants. In contrast, KAI1-sp expression led to a markedly high upregulation of the EGFR expression with no further changes upon cell exposure to E2.

In ERα-negative MDA-MB-231 WT cells, low endogenous EGFR expression was observed, which was even more lowered upon E2 stimulation (0.8-fold). Lower EGFR expression was shown in Vec transfectants (0.3-fold), which was increased upon cell exposure to E2, reaching the level of that in unstimulated WT cells. KAI1-wt-transfected cells showed similar EGFR levels like WT cells (0.9-fold), which was slightly elevated upon E2 stimulation (1.2-fold). Stable transfection with KAI1-sp led to a further elevation of EGFR-expression (1.9-fold over WT cells), which was halved upon cell exposure to E2.

ERα-negative MDA-MB-435 WT cells showed considerable endogenous EGFR expression, which was lower in Vec and KAI1-wt transfectants (0.7-fold compared to WT cells). E2 had only slight effects on EGFR expression levels in MDA-MB-435 WT cells, KAI1-wt, or Vec transfectants (WT cells: 1.0-fold to 0.9-fold, KAI1-wt transfectants: 0.6-fold to 0.5-fold, Vec transfectants: 0.7-fold to 0.9-fold). However, in KAI1-sp-transfected MDA-MB-435 cells, the amount of EGFR was 1.6-fold higher than in WT cells and was further increased upon cell exposure to E2 (2.0-fold).

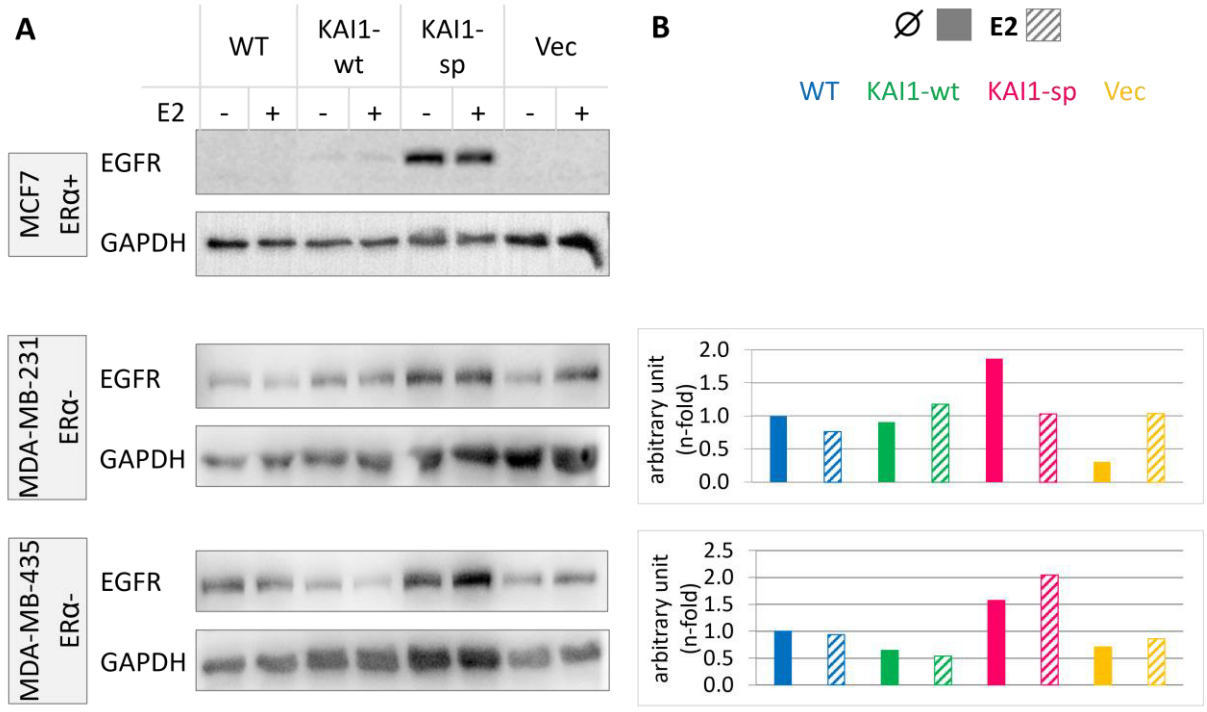


Figure 10. Western blot analysis of the cellular expression of the EGFR in MCF7, MDA-MB-231, or MDA-MB-435 cell transfectants and as a function of the expression of the two KAI1 proteins and E2 stimulation

MCF7, MDA-MB-231, and MDA-MB-435 WT cells and their respective KAI1 cell transfectants were cultivated in the absence (Ø/-) or presence of 0.6 ng/ml E2 (E2/+) over 84 h. A) Cellular EGFR levels were detected by Western blot analysis. In order to normalize the signal intensity of the obtained EGFR bands, Western blot membranes were reprobed with an antibody directed to GAPDH. Typical and representative images of Western blots are depicted. B) Signal intensity for EGFR protein bands was densitometrically analyzed by using the software Image Lab™ and normalized to the signals obtained for GAPDH. Data are given as n-fold by setting the normalized signal intensity of the respective unstimulated WT cells to "1.0".
In MCF7 WT cells, KAI1-wt, and Vec transfectants, EGFR expression levels were below the detection limit, independent of E2 stimulation. In KAI1-sp transfectants, in absence and presence of E2, comparable, markedly increased EGFR expression levels were noted. In MDA-MB-231 WT cells and KAI1-wt transfectants low EGFR expression was observed, which was only slightly affected upon E2 stimulation. In Vec transfectants lower EGFR levels were noted, which were increased after E2 stimulation. KAI1-sp transfectants obtained higher EGFR levels, which were downregulated upon E2 stimulation. MDA-MB-435 WT cells showed higher endogenous EGFR expression, which was lower in Vec and KAI1-wt transfectants, and only slightly affected by E2 stimulation. In KAI1-sp-transfected cells, the amount of EGFR was higher than in WT cells and further increased upon exposure to E2.

Immunocytochemical staining of the EGFR

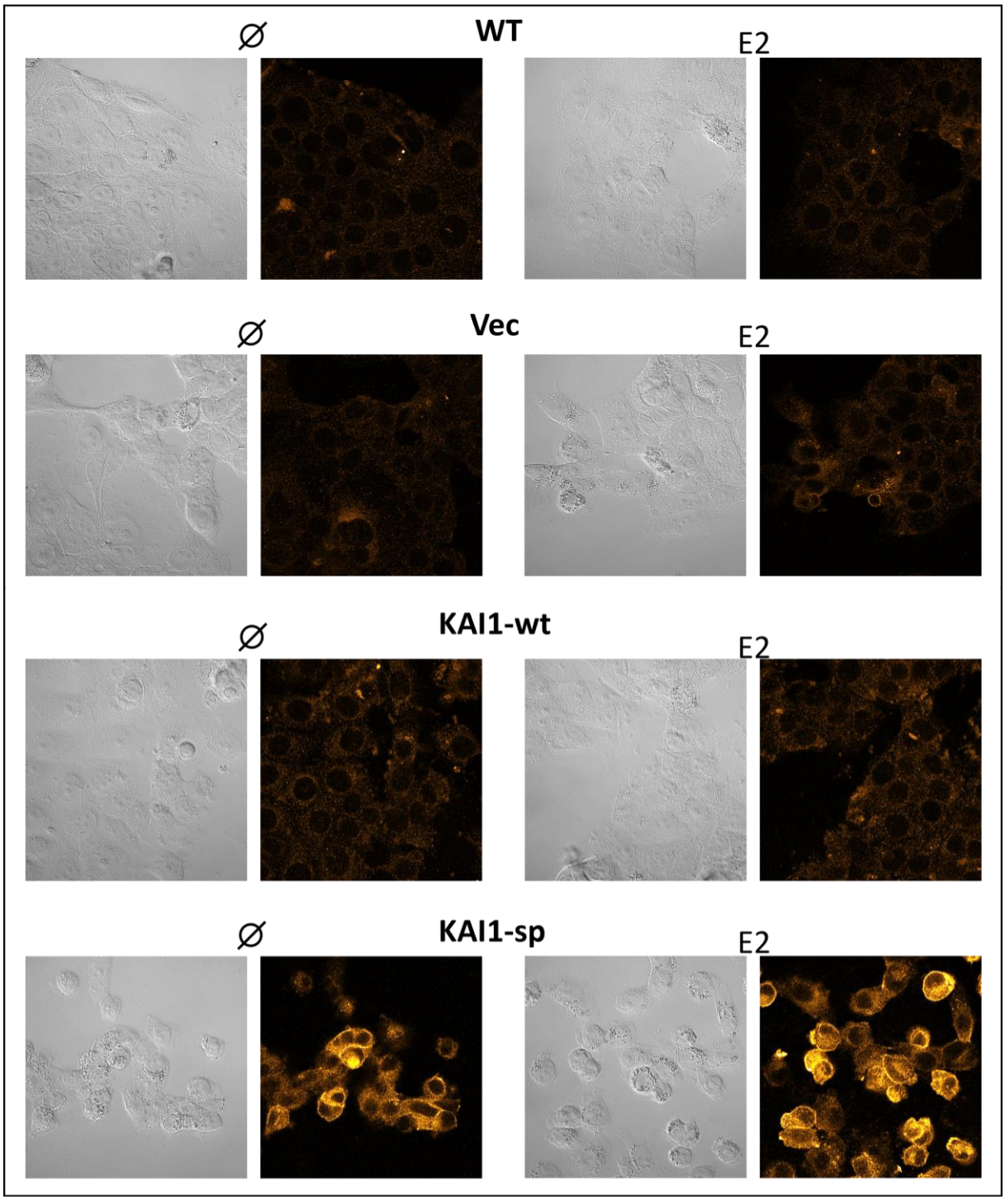
In parallel, EGFR expression was detected by immunocytochemistry. MCF7, MDA-MB-231, or MDA-MB-435 WT cells and their respective Vec, KAI1-wt, and KAI1-sp transfectants were seeded on microchamber cell culture slides in the presence or absence of 0.6 ng/ml E2 for 24 h and EGFR stained as described. Cell staining in the absence of the primary antibody served as control, which were negative (data not shown).

MCF7 WT cells presented with low endogenous EGFR expression levels. Vec and KAI1-wt transfectants showed a similar low fluorescence signal intensity for the EGFR with no effect of E2. However, after stable cell transfection with KAI1-sp, a stronger fluorescence signal intensity for the EGFR was observed, which was even slightly stronger upon cell exposure to E2 (see Figure 11 A).

MDA-MB-231 WT cells and Vec transfectants showed low endogenous levels of EGFR expression. KAI1-wt transfectants displayed a stronger fluorescence signal intensity for EGFR and even brighter signals in KAI1-sp transfectants (see Figure 11 B).

MDA-MB-435 WT cells contained higher endogenous EGFR expression levels than MCF7 WT and MDA-MB-231 WT cells. Vec transfectants presented with a slightly brighter fluorescence signal intensity compared to WT cells. In KAI1-wt transfectants, cellular EGFR expression levels were found to be increased. Strongest fluorescence signal intensity for EGFR was observed in MDA-MB-435 KAI1-sp transfectants (see Figure 11 C).

A) MCF7



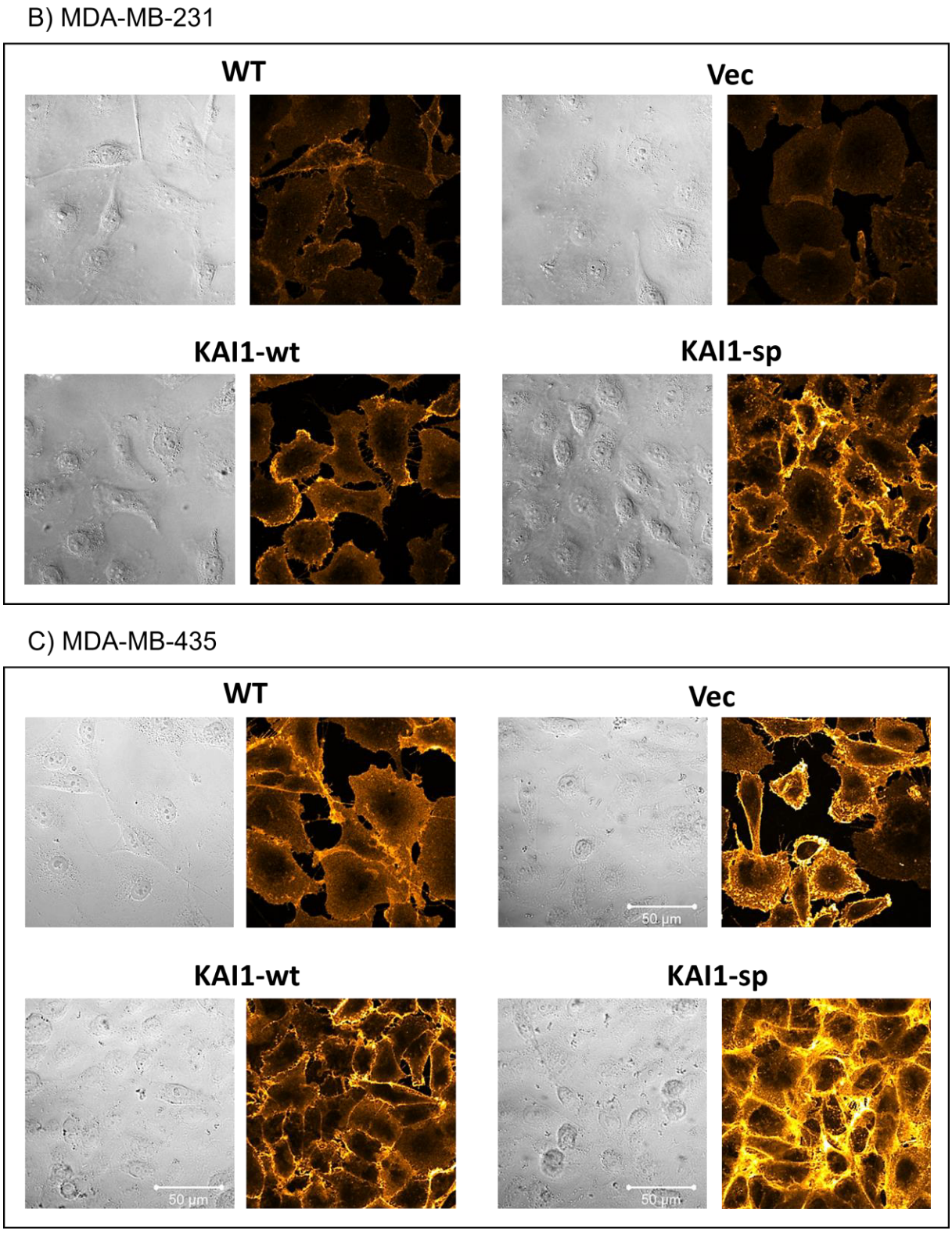


Figure 11. Immunocytochemical detection of EGFR expression levels in MCF7, MDA-MB-231, or MDA-MB-435 cells and their corresponding KAI1 transfectants

MCF7, MDA-MB-231, or MDA-MB-435 cell transfectants were grown on microchamber cell culture slides and stained for EGFR expression as described. MCF7 cell variants were grown in absence (Ø) or presence of E2 (0.6 ng/ml) for 24 h, whereas ERα-negative MDA-MB-231 and MDA-MB-435 cells were only grown in absence of E2. Representative fluorescence images are depicted together with the corresponding DIC images.
A) In unstimulated MCF7 KAI1-sp transfectants highest EGFR levels were observed, whereas the low endogenous levels in MCF7 WT cells were not altered in Vec and KAI1-wt transfectants nor by E2 stimulation. However, in MCF7 KAI1-sp transfectants, E2 stimulation led to a stronger fluorescence signal intensity for the EGFR.
B) MDA-MB-231 WT cells and their Vec transfectants presented a low endogenous expression level of the EGFR. After stable transfection with KAI1-wt, the EGFR expression increased. In KAI1-sp transfectants highest EGFR levels were observed.
C) MDA-MB-435 WT cells contained a notable endogenous amount of EGFR. A similar fluorescence signal intensity for EGFR was observed for KAI1-wt transfectants, whereas Vec transfectants showed a slightly stronger fluorescence intensity signal. KAI1-sp transfectants presented with the highest cellular EGFR expression.

4.5 Effects of KAI1-wt and KAI1-sp and estrogen on the activation status of the EGFR

EGFR activation via phosphorylation was shown to play a central role in tumor biology. It was found that mutations and/or overexpression of the EGFR and its activated form are associated with poor prognosis, metastasis, increased invasiveness, and drug resistance. (Wang, 2017) We here studied whether the activation and thus the phosphorylation status of the EGFR (p-EGFR) was altered as a function of the expression of the two KAI1 variants or upon E2 stimulation by Western blot analysis and immunocytochemistry.

p-EGFR detection by Western blot analysis

Phosphorylated EGFR was detected by a specific antibody directed to the phosphorylation site Y845 within the EGFR protein. Cells were grown in the absence or presence of E2 (0.6 ng/ml) for 48 h, and Western blot analysis performed as described. Protein bands for the p-EGFR appeared on the gels at a molecular weight of appr. 175-180 kDa. The endogenous level of p-EGFR in unstimulated WT cells was set to “1.0”. The results for EGFR activation in the respective Vec, KAI1-wt, or KAI1-sp transfectants, are given as n-fold relative to WT (see Figure 12).

In MCF7 WT cells, Vec, and KAI1-wt transfectants, p-EGFR expression was below the detection limit of the Western blot analysis, independent of the presence of E2. On the contrary,

in KAI1-sp transfectants a marked p-EGFR expression was noticeable in the presence as well as absence of E2.

MDA-MB-231 WT cells presented with moderate endogenous EGFR activation, whereas Vec transfectants disclosed a higher p-EGFR level (3.0-fold compared to WT cells). In KAI1-wt transfectants, a 4.9-fold higher activation status of EGFR than WT cells was noted. E2 stimulation led to a downregulated of EGFR activation in WT cells, KAI1-wt, and Vec transfectants (WT: 0.5-fold, KAI1-wt transfectants: 3.4-fold, Vec transfectants: 1.9-fold). KAI1-sp transfectants displayed p-EGFR expression levels similar to KAI1-wt transfectants (4.6-fold over WT cells) which was further increased as a response to E2 (5.7-fold).

MDA-MB-435 WT cells also displayed moderate endogenous p-EGFR levels which were reduced upon E2 stimulation (0.3-fold). In Vec transfectants, lower p-EGFR levels were found (0.3-fold compared to WT cells) which were elevated in response to E2 (0.5-fold). KAI1-wt transfectants presented with decreased p-EGFR expression (0.1-fold compared to WT cells) which were raised to 0.2-fold by E2 stimulation. KAI1-sp transfectants disclosed comparable p-EGFR levels to WT cells which was slightly reduced by E2 (0.7-fold).

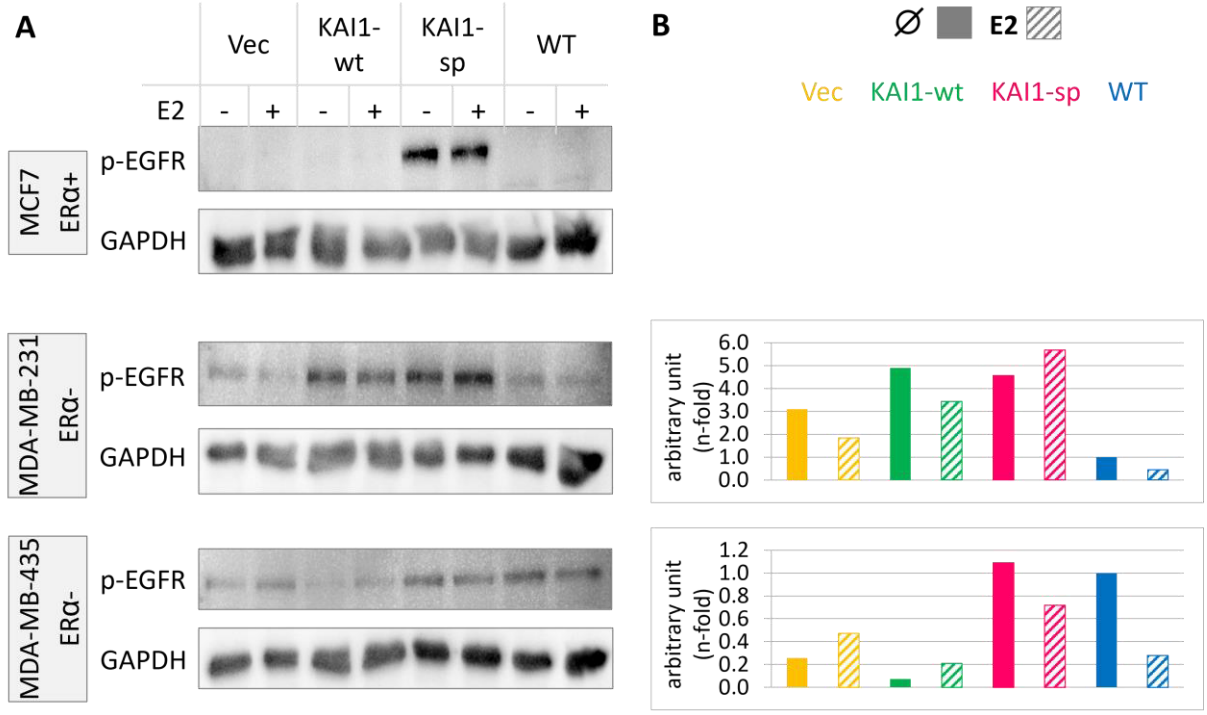


Figure 12. Western blot analysis of the cellular expression of p-EGFR in MCF7, MDA-MB-231, or MDA-MB-435 and their respective KAI1 transfectants as a function of cell stimulation with E2

MCF7, MDA-MB-231, and MDA-MB-435 WT cells and their respective KAI1 cell transfectants were cultivated in the absence (Ø/-) or presence of 0.6 ng/ml E2 (E2/+). A) Cellular p-EGFR expression was detected by Western blot analysis. In order to normalize the signal intensity of the obtained p-EGFR bands, Western blot membranes were reprobed with an antibody directed to GAPDH. Representative images of Western blots are shown. B) Signal intensity of p-EGFR was densitometrically analyzed by the software Image Lab™ and normalized to the signals obtained for GAPDH. Data are given as n-fold by setting the normalized signal intensity of the respective unstimulated WT cells to "1.0".
In MCF7 WT cells, Vec, and KAI1-wt transfectants, respectively, p-EGFR expression was below the detection limit, independent of E2 stimulation. In KAI1-sp transfectants p-EGFR levels were highly increased in the presence and absence of E2. MDA-MB-231 WT cells presented with moderate endogenous EGFR activation, which was higher in Vec and KAI1-wt transfectants. Cell exposure to E2 decreased p-EGFR levels in WT cells, Vec, and KAI1-wt transfectants. In KAI1-sp transfectants, p-EGFR levels were similar to KAI1-wt transfectants, which were further increased upon E2 stimulation. MDA-MB-435 WT cells also displayed moderate endogenous p-EGFR levels, comparable to those in KAI1-sp transfectants, which were reduced upon E2 exposure. KAI1-wt and Vec transfectants showed decreased EGFR activation, with only slight alteration upon E2 stimulation.

Immunocytochemical staining of the p-EGFR

The activation status of the EGFR in MCF7 cells as a function of KAI1 transfection and cell exposure to E2 was further analyzed by immunocytochemistry. For this, cells were seeded on microchamber cell culture slides in presence or absence of E2 overnight and p-EGFR staining performed as described. Cell staining in the absence of the primary antibody served as control, which were negative (data not shown).

MCF7 WT cells and KAI1-wt transfectants, respectively, displayed a similarly low fluorescence signal intensity for the p-EGFR, which was slightly stronger in Vec transfectants. In MCF7 WT cells, KAI1-wt, and Vec transfectants, respectively, exposure to E2 did not alter p-EGFR-levels. The highest activation status of the EGFR was observed in KAI1-sp transfectants also independent of E2 stimulation (see Figure 13). These data are in good accordance with the results obtained by Western blot analysis.

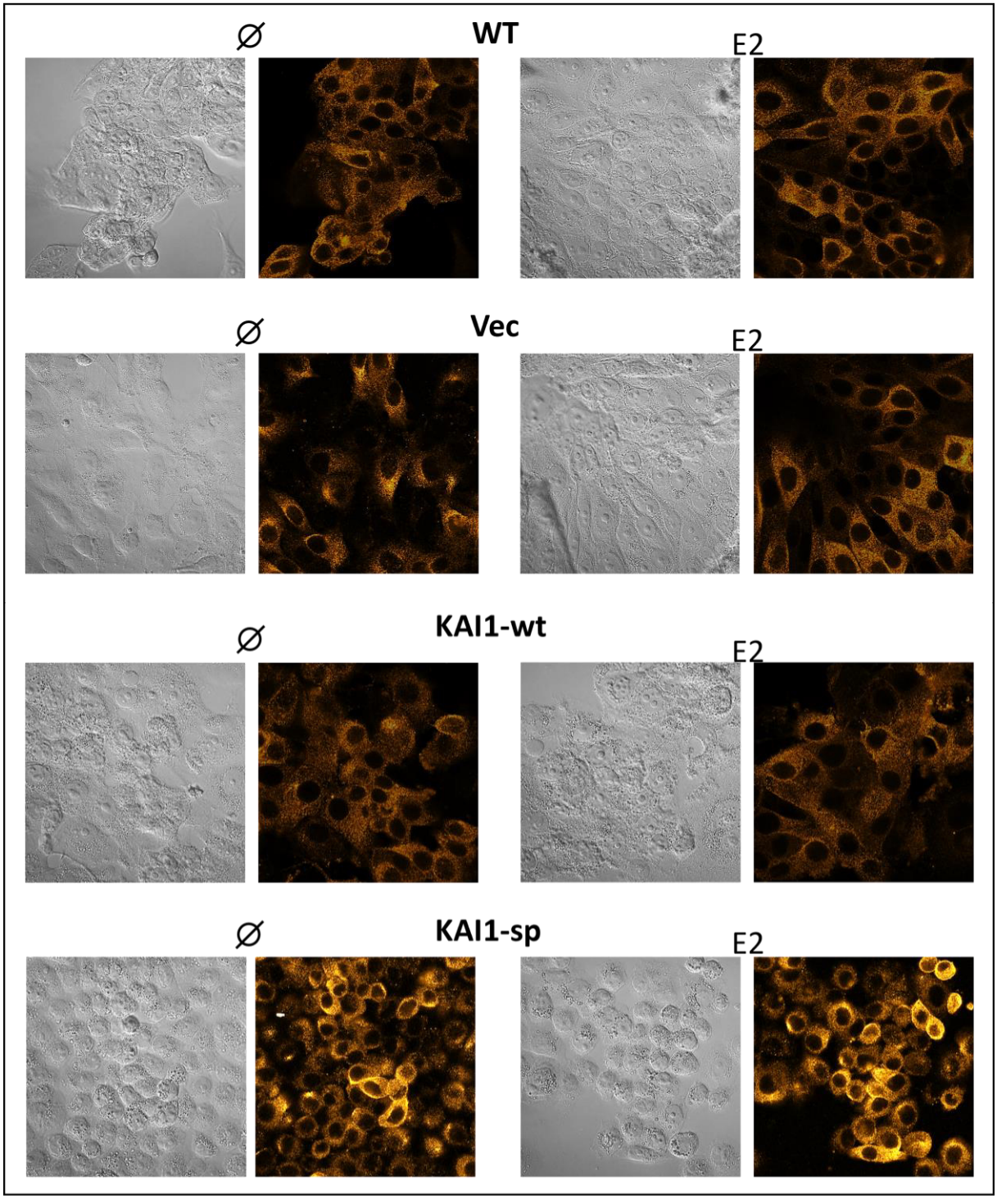


Figure 13. Immunocytochemical detection of p-EGFR levels in MCF7 cells and the respective KAI1 transfectants as a function of E2 stimulation

MCF7 WT cells, Vec, KAI1-wt, and KAI1-sp transfectants, respectively, were grown on microchamber cell culture slides in absence (Ø) or presence of 0.6 ng/ml E2 overnight and stained for p-EGFR expression. Representative fluorescence images are depicted together with the corresponding DIC images. In MCF7 WT cells and KAI1-wt transfectants, respectively, weak

fluorescence signal intensities for p-EGFR were observed, which was slightly stronger in Vec transfectants. KAI1-sp transfectants presented with highly upregulated p-EGFR levels. An alteration of p-EGFR levels upon E2 exposure was not observed.

4.6 Impact of the two KAI1 proteins and exposure to estrogen on cell proliferation

4.6.1 Dose-dependent experiments

The analysis of possible effects of E2 stimulation on breast cancer cell proliferation as well as on protein expression levels of the KAI1 proteins was defined as one aim of the study. To this end, we first performed dose-response assays to define an effective E2 concentration. The proliferation rates of MCF7 WT, KAI1-wt, and KAI1-sp cells, respectively, were measured by proliferation assays as described over a time period of 3 and 84 h upon stimulation with 0.6 ng/ml, 1.5 ng/ml, 3.0 ng/ml, or 5.0 ng/ml of E2. As controls served unstimulated cells. Cells were first cultivated for 3 h to allow firm cell adhesion before E2 stimulation. Thereafter, cell numbers were counted at this starting point and set to “1.0”. After 84 h of further cell incubation in the presence and absence of E2, cell numbers were again counted. Data are depicted as n-fold enhanced cell number over that at the starting point (3 h) of the untreated cells of the respective cell transfectant (see Figure 14).

Unstimulated MCF7 WT cells increased their numbers by appr. 2.7-fold. Upon stimulation with 0.6 and 1.5 ng/ml of E2, respectively, the proliferation rate increased by appr. 3.2-fold, upon cell exposure to 3.0 ng/ml E2 by 3.0-fold. Surprisingly, cells stimulated with 5.0 ng/ml E2 showed a lower proliferative activity comparable to untreated WT cells.

KAI1-wt transfectants proliferated significantly slower compared to WT cells (appr. 2.2-fold decreased cell number). MCF KAI1-wt cell transfectants did slightly alter their proliferative activity upon E2 stimulation compared to unstimulated KAI1-wt transfectants. Upon stimulation with 0.6 ng/ml E2, KAI1-wt transfectants proliferated 2.1-fold, upon E2 stimulation with 1.5 and 3.0 ng/ml E2 to 2.3-fold, and upon E2 exposure to 5.0 ng/ml E2 to 2.1-fold compared to unstimulated KAI1-wt transfectants.

Unstimulated MCF7 KAI1-sp cell transfectants showed significantly increased cell numbers when compared to WT cells (up to 3.3-fold), disclosing a further slight increase upon stimulation with 0.6 or 1.5 ng/ml E2 (up to 3.6-fold), and with 3.0 ng/ml of E2 (up to 3.5-fold).

On the opposite, at 5.0 ng/ml E2, the cell proliferation rate decreased by appr. 2.2-fold compared to untreated KAI1-sp transfectants.

Taken together, a significantly decreased cell proliferation rate was observed in untreated KAI1-wt transfectants compared to WT cells, whereas KAI1-sp-transfected presented with significantly enhanced cell proliferation. The strongest effect of E2 stimulation was noted in MCF7 WT cells after stimulation with 0.6 and 1.5 ng/ml of E2. In the two KAI1 cell transfectants 0.6 and 1.5 ng/ml E2, respectively, only slightly increased cell proliferation rates. Cell exposure to 5.0 ng/ml of E2 did neither affect the proliferation rates of WT cells nor that of KAI1-wt transfectants. However, it drastically reduced proliferation of KAI1-sp transfectants. Based on these results, for all E2 stimulation experiments, we chose an E2 concentration of 0.6 ng/ml.

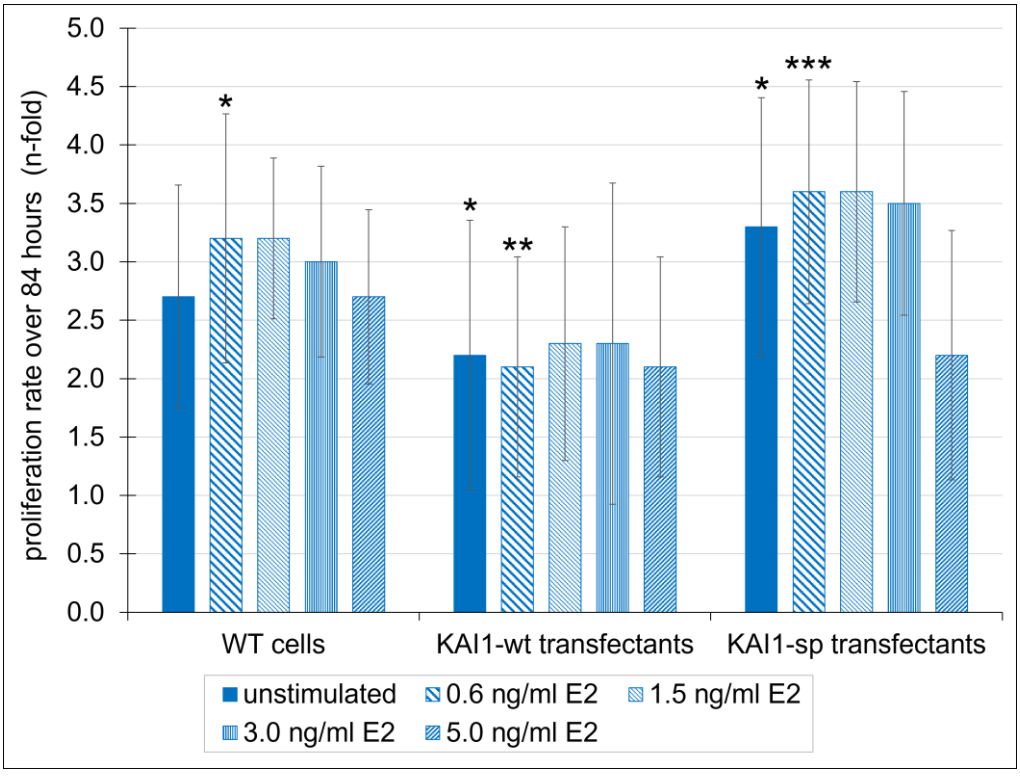


Figure 14. Cell proliferation of MCF7 WT cells, KAI1-wt, and KAI1-sp cell transfectants, respectively, as a function of dose-dependent E2 stimulation (84 h)

In order to define an effective E2 concentration, dose response assays with increasing concentrations of E2 were performed. Cells were seeded in duplicates onto 24-well cell culture plates at a density of 20,000 cells/well. Starting after 3 h of incubation – after cells had established a firm adhesion – cell numbers were counted and the number of the respective unstimulated cell transfectants set to "1.0". E2 was added at the concentration of 0.6 ng/ml, 1.5 ng/ml, 3.0 ng/ml, or 5.0 ng/ml. Cell numbers, determined after 84 h are given as n-fold ± Sd over cell numbers of the respective untreated cell transfectant at 3 h. Statistically significant differences ($p<0.05$) to unstimulated WT cells are indicated by an asterisk, to unstimulated KAI1-wt transfectants by a double asterisk, and to unstimulated KAI1-sp transfectants by a triple asterisk.

Unstimulated MCF7 WT cell numbers increased to appr. 2.7-fold. Upon stimulation with 0.6 and 1.5 ng/ml E2 WT cells increased by appr. 3.2-fold, the effect of 3.0 ng/ml E2 was slightly weaker Exposure to 5.0 ng/ml E2 did not lead to an enhanced cell proliferation in WT cells. Unstimulated KAI1-wt transfectants grew slower (2.2-fold) compared to unstimulated WT cells, whereas KAI1-sp transfectants presented a higher proliferation rate (3.3-fold). KAI1-wt transfectants did slightly respond to E2 stimulation. Upon stimulation with 0.6, 1.5, or 3.0 ng/ml E2, KAI1-sp transfected cells increased by appr. 3.5-fold, 5.0 ng/ml E2 decreased cell numbers (up to 2.2-fold).

4.6.2 Time-dependent measurements of cell proliferation

In order to evaluate possible effects of KAI1-mediated changes on cell proliferation in addition to those due to exposure to E2 in ERα-positive MCF7 cells, we also tested ERα-negative MDA-MB-231 breast cancer cells and ERα-negative MDA-MB-453 melanoma cells in proliferation assays.

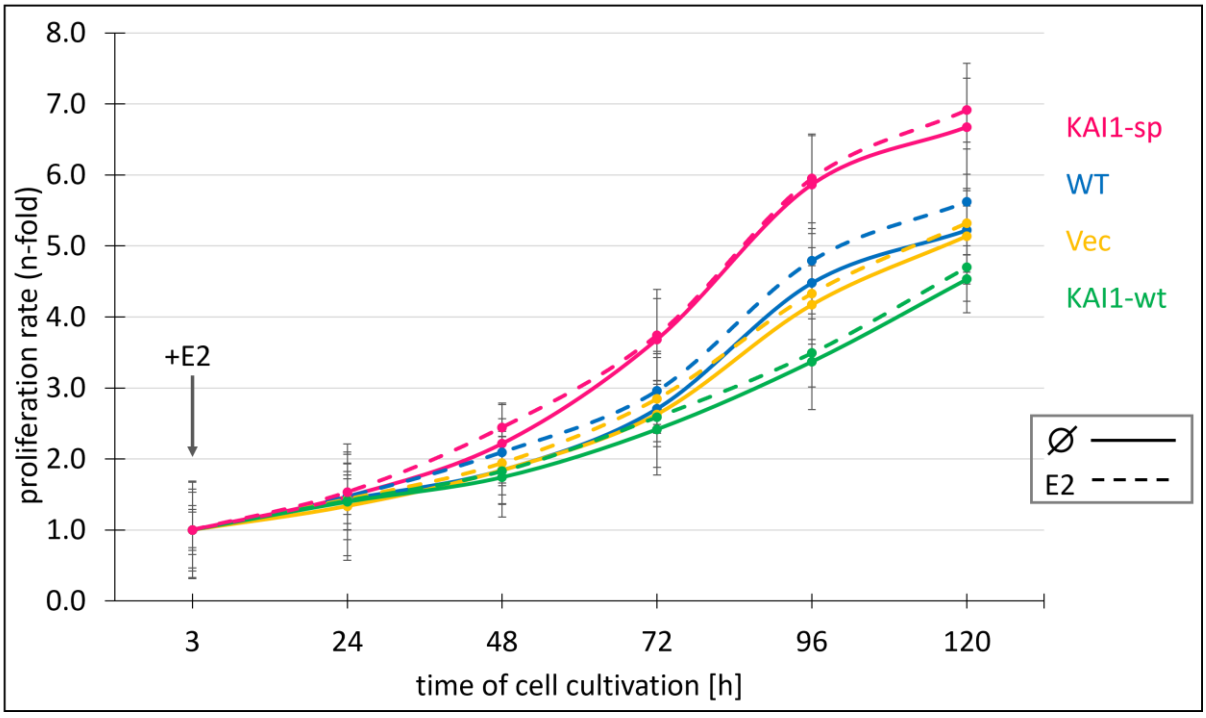
WT cells, Vec, KAI1-wt, and KAI1-sp transfectants, respectively, were seeded in triplicates on uncoated 24-well cell culture plates. Following 3 h after cells had established a firm adhesive phenotype, cells were counted and the cell numbers of the different WT cells and their respective transfectants each set to “1.0”. Cells were then further cultivated in the absence or presence of E2 (0.6 ng/ml) and counted after 24, 48, 72, 96, and 120 h, respectively. Data for all time points are given as n-fold increases over cell numbers determined for each cell variant at the starting point of 3 h.

Within 120 h, MCF7 WT cells and Vec transfectants increased the cell numbers by 5.2-fold and 5.1-fold, respectively. Upon E2 stimulation, a further slight increase was noticed (WT cells: 5.6-fold, Vec transfectants: 5.3-fold). KAI1-wt transfectants presented with a 4.5-fold downregulated proliferative activity compared to WT cells which was almost comparable in the presence of E2 (4.7-fold). The highest proliferation rate was noticeable for KAI1-sp transfectants (appr. 6.7-fold) with a slight further rise upon cell exposure to E2 (6.9-fold) (see Figure 15 A).

In ERα-negative MDA-MB-231 cells, similar effects were noticed. WT cells and Vec transfectants showed 5.5-fold and 5.1-fold increased cell numbers, respectively, over 120 h. Stable KAI1-wt-transfected cells showed lower proliferation rates with only 4.7-fold enhanced cell numbers within 120 h, whereas the cell proliferation of KAI1-sp transfectants was strongly increased (up to appr. 7.0-fold). As expected, in ERα-negative MDA-MB-231 breast cancer cells, E2 exposure did not alter the cell proliferative activity of either cell variant (see Figure 15 B).

Unstimulated ERα-negative MDA-MB-435 melanoma WT cells and Vec transfectants increased the cell number 5.2-fold and 5.1-fold, respectively. KAI1-wt cell transfectants showed a slower proliferation rate up to 4.4-fold within 120 h. However, KAI1-sp transfectants displayed an enhanced proliferative activity, reaching 6.6-fold elevated cell numbers over that at the starting point at 3 h. Like seen in ERα-negative MDA-MB-231 cells, cell exposure to E2 did not affect cell proliferation rates in MDA-MB-435 WT melanoma cells, Vec, and KAI1-sp transfectants, respectively. Unexpectedly, exclusively the proliferation of KAI1-wt transfectants increased to 5.1-fold upon E2 cell stimulation (see Figure 15 C).

A) MCF7



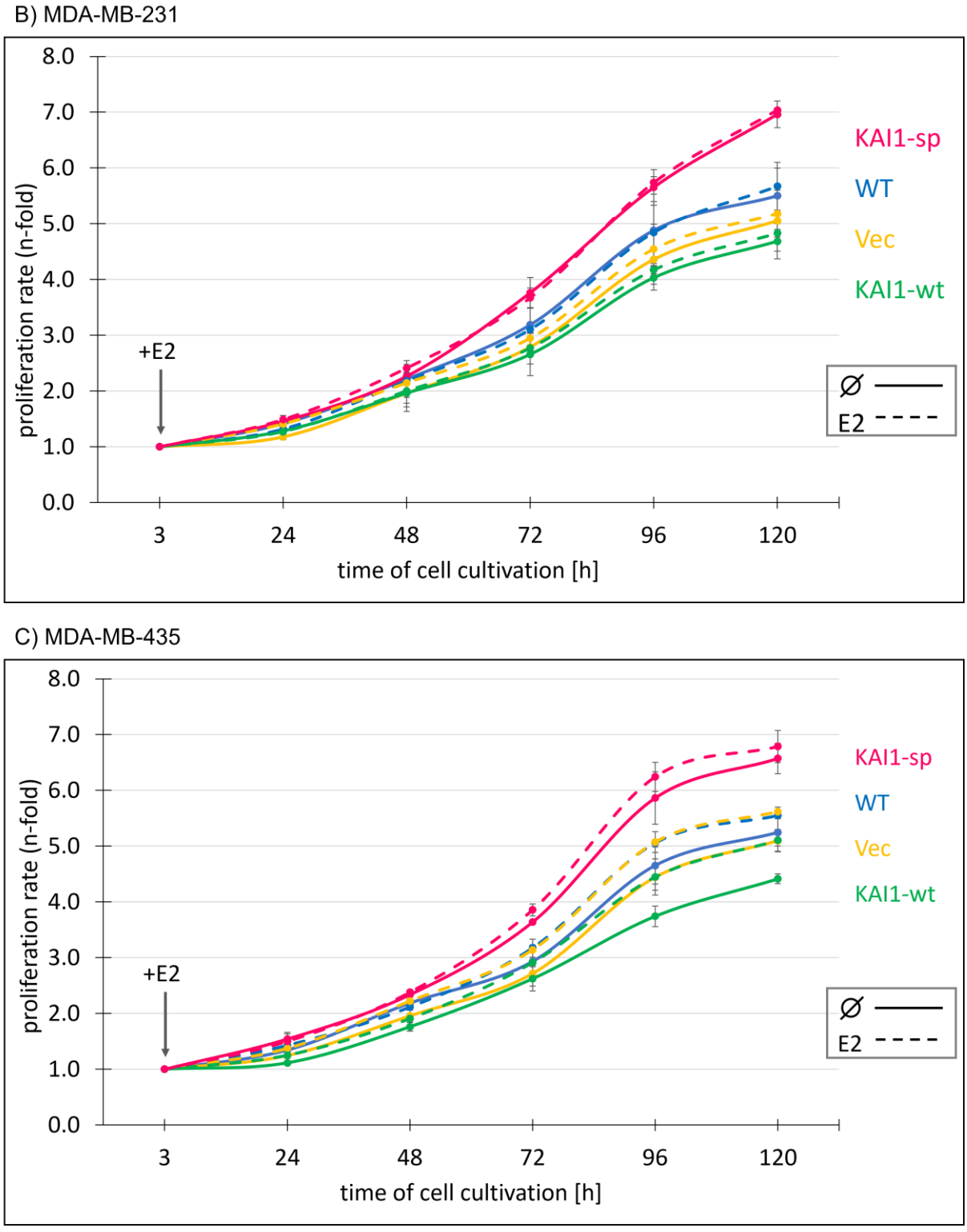


Figure 15. Time course of the impact of the cellular expression of either KAI1 protein variant and E2 exposure on the proliferation rate of MCF7, MDA-MB-231, and MDA-MB-435 cells, respectively, within 120 h of cell cultivation

MCF7, MDA-MB-231, and MDA-MB-435 WT cells, respectively, as well as the corresponding Vec, KAI1-wt, or KAI1-sp transfectants were seeded on 24-multiwell plates in triplicates at a density of 20,000 cells/well. After cells had established a firm adhesive phenotype within 3 h, cell numbers were determined by counting and the cell numbers of the WT cells and their respective transfectants each set to "1.0". Then 0.6 ng/ml E2 was added or cells left untreated (Ø). Cells were counted after 24, 48, 72, 96, and 120 h, respectively. Mean values of triplicates are given as n-fold elevated cell numbers over the starting point of each cell transfectant at 3 h ± Sd.
A) MCF7 cells: Unstimulated MCF7 WT cells increased their cell numbers by 5.2-fold over 120 h, comparable to unstimulated Vec transfectants (5.1-fold), with slight effects of E2 stimulation (WT cells: 5.6-fold, Vec transfectants: 5.3-fold). Unstimulated KAI1-wt-transfected cells presented a notably decreased proliferation rate by 4.5-fold, almost comparable to the proliferation in presence of E2 (4.7-fold). Unstimulated KAI1-sp transfectants remarkably increased their cell numbers by 6.7-fold over 120 h compared to WT cells, with a slight further rise upon E2 stimulation (6.9-fold).
B) MDA-MB-231 cells: Untreated MDA-MB-231 WT cells increased their cell number to 5.5-fold and Vec transfectants to 5.1-fold. KAI1-wt-transfected cells proliferated slower with a 4.7-fold enhanced cell number within 120 h, whereas KAI1-sp transfectants strongly increased the proliferation rate up to 7.0-fold compared to WT cells. As expected E2 exposure had no effect on cell proliferation in ERα-negative MDA-MB-231 cells.
C) MDA-MB-435 cells: Untreated MDA-MB-435 WT cells proliferated by 5.2-fold over 120 h, which was comparable to Vec transfectants. KAI1-wt transfectants showed a weakened proliferative activity (4.4-fold over 120 h), whereas KAI1-sp transfectants obtained a strongly enhanced cell proliferative activity (6.6-fold over 120 h) compared to WT cells. E2 stimulation had no effect on cell proliferation in WT cells, Vec, and KAI1-sp transfectants, but provoked increased proliferation of KAI1-wt transfectants (5.1-fold).

Taken together, in all three investigated cell lines, MCF7, MDA-MB-231, and MDA-MB-435 cells, respectively, KAI1-wt transfection led to a decreased and KAI1-sp transfection to an increased proliferative behavior compared to the respective WT cells. Upon E2 stimulation, MCF7 WT, KAI1-wt, and KAI1-sp transfectants increased their proliferative activity. As expected, E2 stimulation did not affect cell proliferation in ERα-negative MDA-MB-231 and MDA-MB-435 WT cells and the respective transfectants, except for MDA-MB-435 KAI1-wt transfectants.

5 Discussion

In the present study, we investigated whether the regulation of the expression of the tumor suppressor KAI1 is under the control of the E2/ER system in human breast cancer cells. In breast cancer, E2 and EGF activate similar intracellular signaling pathways triggered by the EGFR, which is also an important interaction partner of KAI1 (Lippman et al., 1986; Dickson and Lippman, 1987; Odintsova et al., 2000; Filardo, 2002; Odintsova et al., 2003; Sasaki et al., 2013; Lee et al., 2004; Liu and Zhang, 2006; Upheber et al., 2015; Miller et al., 2018; Sigismund et al., 2018). We characterized the mutual impact of these factors on their respective expression and activation levels and the cellular proliferative response. For this, we established a breast cancer cell model using human HR-positive MCF7 breast cancer cells, which were stably transfected to overexpress either KAI1-wt or its splice variant KAI1-sp. As controls served human HR-negative MDA-MB-231 breast cancer cells as well as human HR-negative MDA-MB-435 melanoma cells.

5.1 The regulation of KAI1 in breast cancer cells by estrogen

Breast cancer is by far the most common cancer type in women accounting for 11.7% of worldwide new cancer cases in 2020 (Ferlay et al., 2021). Furthermore, breast cancer is the leading cause of death in female cancer patients with an overall survival rate of 88% at five and 82% at ten years. For metastatic breast cancer, the survival rate at five years is only 22%. (Barnes, 2016; Kaatsch et al., 2017) In search of new therapeutic targets, cancer research focuses on the identification of factors involved in cancer progression and metastasis, with special interest on putative tumor suppressors. One of them is the tetraspanin KAI1, which is lost or drastically downregulated in a series of different cancer types, including breast cancer. The regulatory mechanisms responsible for this are still not completely understood. So far, some transcription factors were found to putatively modulate KAI1 expression, such as NFκB, β-catenin, or p53 (Dong et al., 1997; Shinohara et al., 2001; Kim et al., 2005; Liu and Zhang, 2006). Also, micro-RNAs seem to modulate KAI1 expression (Christgen et al., 2008; Christgen et al., 2009; Dai et al., 2014; Wang and Liu, 2021). Furthermore, post-translational modifications of the KAI1 molecule by N-glycosylation and palmitoylation were found to alter its functional capacity (Ono et al., 2000; Zhou et al., 2004). Most interestingly, in most ER-positive human breast cancer tissue, a loss of KAI1 mRNA and protein was found, suggesting

a possible impact of the E2/ER system on KAI1 expression (Christgen et al., 2008; Christgen et al., 2009). Thus, in the present work, we focused on the impact of E2 in concert with the ER on the expression levels of KAI1 and modulation of their interaction partner EGFR.

As a prerequisite for E2 effects, we first detected expression levels of the ERα in the different cell lines. As expected, MCF7 WT cells presented with high endogenous ERα expression, which was markedly decreased upon cell stimulation with E2. ERα levels drastically declined in KAI1-wt transfected cells compared to MCF7 WT cells and even more in the presence of E2. Most interestingly, upon KAI1-sp transfection, ERα levels were even further diminished below the detection limit of Western blot analysis, independent of E2 stimulation, comparable to ER-negative MDA-MB-231 breast cancer and MDA-MB-435 melanoma cells. The decreased ERα expression upon cell stimulation with E2 is in line with earlier studies, where a downregulation of ERα mRNA and protein levels was shown in the presence of its own ligand in ER-positive breast cancer cell lines, such as MCF7 cells (Saceda et al., 1988; Kaneko et al., 1993; Pink and Jordan, 1996). Our results, that the expression of both KAI1 proteins provoked a decreased or even a loss of ERα expression by yet unclear regulatory mechanisms, support the idea of an inverse correlation between ER and KAI1 expression. It had already been stated in earlier studies that in a high percentage of ER-positive breast cancer tissue, KAI1 mRNA and protein was lost, suggesting a regulatory effect of the E2/ER system on KAI1 expression. Furthermore, the treatment of breast cancer cells with the ER antagonist and selective estrogen receptor degrader (SERD) *Fulvestrant* provoked a significant increase of KAI1 expression and subsequently inhibited cell proliferation. (Christgen et al., 2008; Christgen et al., 2009) The interaction between KAI1, the ER, the EGFR, and HER2 was earlier evaluated in various human breast cancer cell lines by immunohistochemistry. Results showed a downregulation of KAI1 in 76 % of tumor tissue and a loss of KAI1 expression in 4 of 9 breast cancer cell lines, among these MDA-MB-435 and MCF7. Although an inverse correlation of ER and KAI1 was suggested, there was no significant association of KAI1 expression with other immunophenotypic markers of tumor aggressiveness, such as grade, lymph node status, and EGFR or HER2 expression. (Huang et al., 2005) Taken together, one may assume that patients afflicted with ER-positive breast cancer might benefit from endocrine therapy by increasing KAI1 expression. In fact, multiple clinical studies confirmed the benefit of endocrine therapy by reducing the risk of distant metastases and prolonged patient survival (Rabbani and Mazar, 2007). However, some *in vivo* studies showed, that in ER-negative breast cancer, KAI1 expression is maintained in distant metastases accompanied with poor patient prognosis, proposing that the function of KAI1 is altered (James et al., 2003; Weigelt et al., 2005; Gaedcke et al., 2007). Therefore, a possible

explanation is that although KAI1 is detectable, its tumor suppressive functions may be inactivated as it happens by alternative splicing. Indeed, Lee et al. (2003) first isolated a splice variant of KAI1 in tumor cells of gastric cancer patients. KAI1-sp was shown to not only lose the tumor suppressive functions of KAI1-wt but, even more, exert pro-oncogenic effects in human ovarian and ER-negative breast cancer (Ruseva et al., 2009; Upheber et al., 2015; Miller et al., 2018).

5.1.1 Effect of estrogen on the cellular expression of KAI1

We next investigated whether the cellular expression of KAI1 is regulated by the E2/ER system in human ER-positive breast cancer cells.

In MCF7 WT cells, we found low endogenous KAI1-wt levels, which is in line with the above-mentioned findings of an inverse correlation between the ER and KAI1 (Christgen et al., 2008; Christgen et al., 2009). However, KAI1-wt levels were slightly increased upon cell stimulation with E2. KAI1-wt and KAI1-sp cell transfectants disclosed elevated KAI1 expression levels as proof of successful stable cell transfection. KAI1 expression in KAI1-wt transfectants did not obviously change upon E2 stimulation, whereas, in contrast, in KAI1-sp transfectants, it was drastically reduced in the presence of E2. Compared to MCF7 WT cells, ERα-negative MDA-MB-231 WT cells presented with a higher endogenous KAI1-wt expression, which was only slightly increased upon E2 stimulation. The enhanced levels of KAI1 in KAI1-wt transfectants were further elevated. The drastic downregulation of KAI1 expression in KAI1-sp transfectants upon E2 stimulation was unexpected, since we had noticed a loss of ERα expression in those cell transfectants. This indicated that the inverse correlation between KAI1 and the ER was here not upheld. Moreover, the E2 effect appears rather independent of the ERα. This could also explain why in ERα-negative MDA-MB-231 cells, KAI1 expression was affected by E2 stimulation. It was already reported, that in MDA-MB-231 cells as well as in other breast cancer cells this inverse correlation was not found (Huang et al., 2005).

Since many different physiological processes are provoked by the E2/ER system, it is important to contemplate the complex mechanisms which influence this system. The pathway is usually initiated by E2 binding to membrane-bound or cytoplasmic ERs which trigger the formation of ER-dimers. After internalizing the complex into the nucleus, it binds to the ERE-motif on promotor regions of ER-target genes thereby regulating their transcription. However, membrane-bound ERs are also able to activate intracellular pathways after E2 binding for which ERE binding is not required. (Hall et al., 2001; Cui et al., 2013) By this, membrane-

bound ER activates other intracellular pathways leading to the phosphorylation of various transcription factors, such as the activator protein (AP)-1, STATs, Elk-1, CREB, NFκB, or the stimulating protein (Sp)-1 which again regulate gene transcription by binding to their respective DNA binding motifs (Björnström and Sjöberg, 2005; O'Malley, 2005; Marino et al., 2006; Barton et al., 2018). This suggests that an ERE on the respective gene is not necessarily required to be regulated by stimulation of cells with E2 (O'Lone et al., 2004). In fact, by a search for ERE-motifs in the KAI1 gene promoter using the software *PROMO*, no ERE sites were detectable, which is relevant for endogenous KAI1 expression (Messeguer et al., 2002; Farré et al., 2003).

5.1.2 The expression of the EGFR and its activation status as a function of the two KAI1 proteins and cell exposure to estrogen

The EGFR was already identified as an important interaction partner of KAI1 (Odintsova et al., 2000; Odintsova et al., 2003; Lee et al., 2004; Liu and Zhang, 2006; Sasaki et al., 2013; Upheber et al., 2015; Miller et al., 2018). KAI1-wt does not influence ligand binding to the EGFR but causes a desensitization of the ligand-induced EGFR activation and thus decreases EGFR dimerization as a prerequisite for EGFR signaling. Furthermore, KAI1 suppresses ligand-dependent ubiquitylation of the EGFR which leads to a decreased activation of intracellular signaling pathways. It was also shown that KAI1-wt leads to an increased EGFR compartmentalization and degradation by proteosomes decreasing the cellular amount of EGFR. Consequently, the cell surface distribution of the EGFR varies as a function of KAI1-wt expression. Furthermore, KAI1-wt controls the recruitment of intracellular proteins crucial for the initiation of intracellular signaling pathways. (Odintsova et al., 2000; Odintsova et al., 2003; Danglot et al., 2010; Odintsova et al., 2013; Berditchevski and Odintsova, 2016)

In addition, an interaction between the EGFR and the E2/ER system was suggested as E2 was found to induce a transactivation of the EGFR and, vice versa, the EGFR is thought to modulate ER signaling (Razandi et al., 2004; Song et al., 2005; Song et al., 2007; Song et al., 2009). Based on this complex interplay between the different proteins, we wondered, how the ER, E2, and the two KAI1 proteins influence EGFR expression and its activation.

ERα-positive MCF WT cells presented with low endogenous EGFR expression and activation, which was not altered upon E2 stimulation. This was not surprising as the expression of ERα was diminished to a minimum in MCF7 KAI1-sp transfectants. An inverse correlation of EGFR and ER was already found in *in vitro* studies on multiple ER-positive cell lines, such as MCF7

cells, as well as in tumor tissue (Fitzpatrick et al., 1984; Koenders et al., 1991; Yarden et al., 1996). KAI1-wt-transfected cells presented with similar (p-)EGFR levels like WT cells, independent of the presence of E2. On the opposite, in KAI1-sp transfectants, we found highly increased EGFR expression and activation compared to WT cells with no further E2 effect.

ER-negative MDA-MB-231 WT cells displayed moderate endogenous expression and activation levels of the EGFR. Like in MCF7 cells, KAI1-sp expression induced an upregulation of the EGFR, which was almost halved in the presence of E2. However, the amount of p-EGFR, which also strongly increased in KAI1-sp transfectants, was slightly further increased upon E2 stimulation. Upon KAI1-wt transfection, we measured increased p-EGFR levels, whereas the amount of EGFR was comparable to WT cells. These differential effects of KAI1-wt and KAI1-sp on (p-)EGFR expression are in accordance with earlier research on ER-negative MDA-MB-231 breast cancer cells (Miller et al., 2018). Also, in human ovarian cancer cells, overexpression of KAI1-sp markedly increased the amount of (p-)EGFR and its localization on the cell surface compared to KAI1-wt. Moreover, in KAI1-sp transfectants of human ovarian cancer cells, the observed changes in EGFR protein were shown to be under transcriptional control since a significantly enlarged EGFR promoter activity was noticed in cells overexpressing KAI1-sp. (Upheber et al., 2015)

In MDA-MB-231 KAI1-wt transfectants, while being ER-negative, p-EGFR levels were reduced upon E2 stimulation. Also, in MDA-MB-231 KAI1-sp transfectants, EGFR expression was halved and p-EGFR levels were slightly increased by the addition of E2. These findings also suggest effects which are mediated through ERα-independent signaling pathways as described above.

In fact, in previous studies, it was shown that E2 and EGF cross-communicate by initiating similar converging intracellular pathways and activating similar networks associated with the EGFR, implicating protein kinases, such as the mitogen-activated protein kinase (MAPK). In an *in vitro* study on ER-positive human breast cancer, it had been found that in multiple cell lines, including MCF7 cells, E2 stimulation led to a relevant upregulation of EGFR mRNA, whereas the amount of EGFR protein was suppressed upon the exposure to anti-estrogens. Our results of low (p-)EGFR expression in MCF7 cells with high ERα expression levels are in line with this finding of a relevant interaction between the EGFR and the E2/ER system and an inverse correlation of the EGFR and the ERα in breast cancer cells and tumor tissue. (Yarden et al., 1996)

The findings that the EGFR acts as an interaction partner of the ER and KAI1, as well as the interaction between the ER and KAI1 further support the idea of KAI1 as a key player in organizing multiprotein complexes within the cell surface and being involved in membrane trafficking. KAI1 was found to have many interaction partners including other tetraspanins, integrins, cadherin adhesion proteins, intracellular signaling proteins, growth factors, and growth factor receptors, such as the EGFR. (Hemler et al., 1996; Hemler, 2001; Yang et al., 2001; Berditchevski and Odintsova, 2007; Charrin et al., 2009; Yan et al., 2021) The EGFR plays a central role in tumor biology by modulating cell proliferation, differentiation, apoptosis, and migration in favor of tumor growth (Lee et al., 2004; Liu and Zhang, 2006; Sasaki et al., 2013; Upheber et al., 2015). KAI1-wt was shown to influence EGFR ligand binding, its activation, dimerization, and recruitment of intracellular signaling proteins (Berditchevski and Odintsova, 2016). Thus, an alteration of the expression and function of KAI1-wt might have negative effects on the function and expression of the EGFR and its functions. Indeed, it has been well documented that in many different tumor entities, the downregulation, complete loss, or splicing of KAI1 contributes to enhanced tumor growth and progression, as well as increased metastasis potential. (Yang et al., 2001; Lee et al., 2003; Lee et al., 2004; Guo et al., 2005; Lu et al., 2007; Takeda et al., 2007; Xu et al., 2008; Yang et al., 2008; Ruseva et al., 2009; Upheber et al., 2015; Chai et al., 2017; Guo et al., 2017; Miller et al., 2018; Wang et al., 2018; Zeng et al., 2018)

5.1.3 Effect of KAI1-wt and KAI1-sp on breast cancer cell proliferation

One of the main hallmarks of tumor growth and progression is a dysregulated cell proliferation. Not only KAI1, but also the ER and the EGFR had already been shown to be involved in cancer cell growth (Ono et al., 1999; Lee et al., 2004; Liu and Zhang, 2006; Malik et al., 2009; Sasaki et al., 2013; Upheber et al., 2015; Jia et al., 2015; Miller et al., 2018). Consequently, in the present study, we aimed at investigating how ERα and (p-)EGFR expression, E2 stimulation, and the expression of the two KAI1 protein variants affect the proliferative activity of ERα-positive and ERα-negative breast cancer cells. In both cell lines, MCF7 and MDA-MB-231, a high cellular expression of KAI1-wt led to a downregulation of cell proliferation compared to WT cells, whereas elevated KAI1-sp expression provoked a markedly higher proliferative activity. These results are in line with previous studies, where KAI1-wt reduced and KAI1-sp promoted the proliferation of human ovarian and triple-negative breast cancer cells in favor of

tumor progression and metastasis (Ruseva et al., 2009; Upheber et al., 2015; Miller et al., 2018).

Corresponding results were also reported from other *in vitro* studies. In pancreatic and colon cancer cells, the expression of KAI1-wt was associated with a decreased cell proliferation (Liu et al., 2003a; Guo et al., 2005). In hepatocellular carcinoma cells, high KAI1-wt expression was associated with decreased cell proliferation, moreover, with cell apoptosis and an attenuated invasive ability (Yang et al., 2008). In cancer tissue of patients afflicted with esophageal cancer, a high expression of KAI1-wt was accompanied by decreased cell proliferation, invasion, and metastatic potential. Consequently, lower KAI1-wt expression was associated with a larger number of metastases and poor patient prognosis. (Zeng et al., 2018) Furthermore, in *in vivo* models, the effects of KAI1-wt and KAI1-sp were studied with respect to their impact on tumor growth. Mice injected with KAI1-sp-transfected colon carcinoma cells suffered from a greater tumor size and metastases in the lungs, liver, and peritoneum, whereas mice injected with KAI1-wt-transfected cells developed lung metastases only. (Lee et al., 2003) Also, upon the injection of KAI1-wt-transfected hepatocellular carcinoma cells into mice, a decline of the number of lung metastases was observed. On the opposite, the inoculation of KAI1-sp-transfected hepatocellular carcinoma in mice provoked more aggressive tumors with larger tumor size and a higher number of metastases. (Yang et al., 2008) Intravenous introduction of KAI1-wt-transfected progenitor cells into mice reduced migration and lung metastases in nasopharyngeal carcinoma and pancreatic cancer, respectively (Wang et al., 2018). Moreover, also in *in vivo* studies in mice on breast, lung, or pancreatic cancer, a significantly reduced number of metastatic loci was observed upon the overexpression of KAI1-wt (Yang et al., 2001; Takeda et al., 2007; Xu et al., 2008).

All these findings support the suggestion that KAI1-wt acts as a tumor suppressor and prevents metastasis by suppressing cell proliferation. On the contrary, its splice variant not only loses these tumor protective functions, but even more, turns them into pro-oncogenic effects. One might speculate that this is due to the loss of exon 7 in KAI1-sp, which encodes the C-terminal part of the LEL and the proximal part of the fourth transmembrane domain. As the tetraspanin KAI1 has no intrinsic activity, it mediates its effects by its interaction with other membrane molecules and receptors within the TEMs via distinct domains, located on the LEL. In *in vitro* studies with human breast, lung, colorectal, and prostate cancer cells, it was found that also a mimic molecule of the small extracellular domain of KAI1 suppressed cell adhesion, migration, invasion, and aggregation. Furthermore, *in vivo*, this mimic peptide reduced the number of metastases in the lung. (He et al., 2021)

However, upon E2 stimulation we could not observe relevant changes of the cell proliferative activity in time-dependent cell counting experiments in MCF7 WT cells and the respective transfectants except a slight upregulation of cell proliferation in MCF7 WT cells. Even though, MCF7 WT cells presented with low endogenous EGFR levels, the high ERα expression seems to suffice for increased cell proliferation. This is in line with previous findings, showing that the ERα plays a role in MCF7 cell proliferation by ERα-mediated upregulation of proliferation markers, such as Ki-67, and inhibition of the cell cycle regulators p21 and p53 (Liao et al., 2014). Also, high levels of ERα expression were associated with a higher proliferation rate in MCF7 human breast cancer cells in long-term growth trails over up to 6 months (Katzenellenbogen et al., 1987). Furthermore, in *in vitro* experiments by other researchers, MCF7 cell proliferation was inhibited upon treatment with the SERM *Tamoxifen* or the SERD *Fulvestrant* suggesting that cell proliferation was ERα-dependent (Coezy et al., 1982; Christgen et al., 2008; Gier et al., 2017). As KAI1-wt transfectants displayed a decline of cellular ERα and (p-)EGFR expression, it was not surprising that KAI1-wt transfectants presented with a lower cell proliferation rate, which was not affected by E2 stimulation. In KAI1-sp transfectants, we observed diminished ERα and highly upregulated (p-)EGFR levels. Their expression was independent of E2 stimulation. The drastically upregulated (p-)EGFR expression in KAI1-sp transfected MCF7 and MDA-MB-231 cells, respectively, corresponded to the increased proliferative activity of both cell types. Although MCF7 KAI1-sp transfectants presented with diminished ERα expression, they displayed an enhanced proliferative activity compared to WT cells. This suggests that the increased cell proliferation of MCF7 KAI1-sp transfectants might not depend on the expression of the ERα, but on other signaling pathways probably also triggered by the drastically increased EGFR expression and activation. This inverse correlation between the ER and the EGFR was already described in *in vitro* studies on multiple ER-positive cell lines, such as MCF7 cells, as well as in tumor tissue (Fitzpatrick et al., 1984; Koenders et al., 1991; Yarden et al., 1996). Furthermore, a link between the ER and the EGFR was already shown in human breast cancer cells, where cell stimulation with tetradecanoylphorphol-13-acetate (TPA) triggered an increase of EGFR mRNA, whereas the ER was reduced (Lee et al., 1989). Considering these results, it might be possible, that the drastic upregulation of (p-)EGFR in MCF7 KAI1-sp transfectants might promote hormone independence (Yarden et al., 1996). Also, in cell lines expressing either the ER or the EGFR it was suggested that the expression and regulation of the EGFR may depend on ER expression (deFazio et al., 1992). This close link between the EGFR and the ER expression, and, in addition, the expression of KAI1 support the idea of the ER and the EGFR as independent prognostic factors for tumor progression and patient prognosis. Various studies

investigating tumor size, tumor grade, age at diagnosis, and nodal status, as well as analysis on transcriptional and protein levels confirmed the ER and the EGFR status as suitable prognostic factors in human breast cancer, as which they are used today. (Phung et al., 2019; Hou et al., 2022)

As expected, in ER-negative MDA-MB-231 WT cells and the respective KAI1 transfectants cell proliferation was not affected by E2 stimulation. As mentioned, ER-negative MDA-MB-231 WT cells and KAI1-wt transfectants displayed moderate endogenous (p-)EGFR expression. KAI1-sp expression induced an upregulation of the (p-)EGFR. Even though EGFR levels were almost halved and p-EGFR levels slightly increased upon E2 stimulation in KAI1-sp transfectants, we could not observe an effect of E2 stimulation in cell proliferation. Thus, cell proliferation depends on many more other factors, which were not in the focus of the present investigation.

5.2 Effects of KAI1-wt and KAI1-sp in the human melanoma cell line MDA-MB-435

The MDA-MB-435 cell line was originally isolated from a malignant pleural effusion of a female breast cancer patient and first described as a triple-negative human breast cancer cell line (Brinkley et al., 1980). However, studies comparing gene expression profiles of melanoma and breast cancer cells showed a similarity of MDA-MB-435 cells to melanoma cells (Ross et al., 2000; Ellison et al., 2002). Various further studies performing fluorescence *in situ* hybridization (FISH), genotyping, immunostaining, and molecular analyses discovered that genetic characteristics of MDA-MB-435 cells match with those of melanoma cells. Finally, MDA-MB-435 cells were confirmed to originate from melanoma and not from breast cancer. (Rae et al., 2007; Korch et al., 2018) Thus, in this study, MDA-MB-435 cells serve as a further control for ER-negative cancer cells to study the differential effects of the tumor suppressor KAI1-wt and its splice variant as well as the impact of (p-)EGFR on their respective expression and the effect on cell proliferation. As expected, these cells did not express ERα, which was affected neither by the expression of either of the two KAI1 protein variants nor upon cell stimulation with E2. In these cells, the expression of KAI1-wt and KAI1-sp also affected their cell proliferative activity. Compared to WT cells and Vec-transfected cells, the expression of KAI1-wt led to a decreased proliferation rate. On the contrary, cells overexpressing KAI1-sp showed an enhanced proliferative activity. The determination of EGFR levels and its activation status revealed notable endogenous levels of the (p-)EGFR in WT cells. KAI1-wt transfectants

presented with a lower level of (p-)EGFR expression. On the contrary, cells expressing KAI1-sp disclosed an upregulated expression of the (p-)EGFR. All these results were in good correspondence to earlier research on MDA-MB-435 cells (Miller et al., 2018). Upon cell stimulation with E2, EGFR expression in KAI1-sp transfectants was increased, whereas no effect was noted in WT cells or KAI1-wt-transfected cells. Most interestingly, upon E2 stimulation, p-EGFR levels were increased in WT cells and KAI1-wt transfectants and downregulated in KAI1-sp transfectants. Thus, also in the ERα-negative MDA-MB-435 cells, an ERα-independent signaling pathway initiated by cell stimulation with E2 might be conceivable.

With respect to malignant melanoma, it was already shown that KAI1-wt suppressed cell migration and invasion (Tang et al., 2014). Moreover, KAI1-wt had an inhibitory effect on angiogenesis and metastasis, whereas low KAI1-wt expression levels were associated with poor prognosis of patients suffering from a malignant melanoma (Tang et al., 2015; Zhang et al., 2016; Du et al., 2022; Wang et al., 2023). In summary, also for malignant melanoma, KAI1-wt as well as KAI1-sp could represent valuable markers for predicting the progressive, invasive, and metastatic potential, and, consequently, patient outcome.

6 Outlook

The tumor suppressor KAI1-wt is lost in many cancer types, leading to poor patient outcome. Furthermore, lacking KAI1-wt is associated with drug resistance, low cell differentiation, high cancer recurrence, and reduced disease free and overall survival. (Yan et al., 2021) Thus, it is important to understand the regulation of the expression of KAI1-wt and the wide range of intracellular signaling pathways for the development of strategies to restore its expression in order to improve disease outcome. This is even more important because a splice variant of KAI1 has been identified in several cancer entities, which was shown to not only abrogate the tumor suppressive actions of KAI1-wt but instead enhances pro-oncogenic events leading to greater tumor growth and metastasis. (Lee et al., 2003; Lee et al., 2004; Takeda et al., 2007; Xu et al., 2008; Yang et al., 2008; Ruseva et al., 2009; Upheber et al., 2015; Wang et al., 2018; Zeng et al., 2018) In ER-positive breast cancer, an inverse correlation between KAI1 and the ER has been suggested, as KAI1 mRNA and protein were lost in most ER-positive tumor tissues. Furthermore, treatment with *Fulvestrant*, an ER antagonist and selective estrogen receptor degrader (SERD) increased KAI1 expression in ER-positive breast cancer tissue. (Christgen et al., 2008; Christgen et al., 2009) However, in the present study on ER-positive MCF7 breast cancer cells, we found increased KAI1 expression upon cell stimulation by E2.

Thus, future studies are still needed to better understand the regulation of KAI1 expression in different cancer cells. Indeed, in recent years, several attempts had already been made in various (pre-) clinical trials to directly apply KAI1-wt protein, targeting its regulatory factors or, alternatively, via its adenoviral restoration (Mashimo et al., 2000; Yang et al., 2001; Lee et al., 2003; Lee et al., 2004; Takeda et al., 2007; Xu et al., 2008; Yang et al., 2008; Wang et al., 2018; Viera et al., 2021). One of the more recent approaches seek to mimic the properties of KAI1-wt. Treating cancer cells with a peptide imitating the small extracellular domain of KAI1 resulted in reduced cell adhesion, invasion, migration, and cell-cell aggregation of human colorectal, breast, prostate, and lung cancer cells *in vitro* and reduced pulmonary metastases *in vivo* in a mouse model (He et al., 2021). Another promising novel option to inhibit tumor progression and metastasis was explored by microRNA (miRNA) therapeutics. In triple-negative breast cancer, transfection of cancer cells with the miRNA miR-124 resulted in reduced cell proliferation, invasion, and metastasis. (Ji et al., 2019; Shi et al., 2019) Also, in ER-positive MCF7 cells, cell transfection with the miRNA miR-125a-5p led to decreased cell proliferation and migration, as well as increased apoptosis (Liang et al., 2019). There is no data yet on miRNAs precisely inducing KAI1-wt expression, but a search on known miRNAs found that about 500 miRNAs, of which many are found in breast cancer, are predicted to

target KAI1 expression due to their RNA sequence (Wong and Cheah, 2020; Viera et al., 2021).

In summary, a future challenge will be the elucidation of the molecular mechanisms underlying the control of KAI1-wt expression in order to develop specific therapeutic concepts for the restoration of the tumor suppressive functions of KAI1-wt in cancer cells and to block the pro-oncogenic effects of KAI1-sp. This will pave the way into clinical trials regarding further cancer treatment options, also for patients afflicted with ER-positive breast cancer. Moreover, the detection of KAI1-wt and KAI1-sp will have the potential to serve as a valuable biomarker for predicting the prognosis for cancer patients.

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