

A Placenta-Specific Gene Ectopically Activated in Many Human Cancers Is Essentially Involved in Malignant Cell Processes

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Abstract

The identification and functional characterization of tumor-specific genes is a prerequisite for the development of targeted cancer therapies. Using an integrated data mining and experimental validation approach for the discovery of new targets for antibody therapy of cancer, we identified PLAC1. PLAC1 is a placenta-specific gene with no detectable expression in any other normal human tissue. However, it is frequently aberrantly activated and highly expressed in a variety of tumor types, in particular breast cancer. RNAi-mediated silencing of PLAC1 in MCF-7 and BT-549 breast cancer cells profoundly impairs motility, migration, and invasion and induces a G₁-S cell cycle block with nearly complete abrogation of proliferation. Knockdown of PLAC1 is associated with decreased expression of cyclin D1 and reduced phosphorylation of AKT kinase. Moreover, PLAC1 is localized on the surface of cancer cells and is accessible for antibodies which antagonize biological functions of this molecule. These features, in summary, make PLAC1 an attractive candidate for targeted immunotherapeutic approaches. [Cancer Res 2007;67(19):9528–34]

Introduction

Antibody-based cancer therapies have been successfully introduced into the clinic and have emerged as the most promising therapeutics in oncology over the last decade. Eight antibodies have been approved for treatment of neoplastic diseases, most of them, however, in lymphoma and leukemia (1). Only three monoclonal antibodies (mAb), namely Herceptin, Avastin, and Erbitux, address solid cancer types, which account for >90% of cancer-evoked mortality. The substantial remaining medical need, the significant clinical benefit of approved mAbs, and their considerable commercial success altogether motivated a wave of innovative approaches, standing poised not only to develop antibody-based therapies for extended groups of patients but also to improve their efficacy (2, 3).

One of the challenges to be mastered for the advent of the next generation of upgraded antibody-based cancer therapeutics is the selection of appropriate target molecules, which is the key for a favorable toxicity/efficacy profile.

In addition to lack of expression in toxicity relevant normal tissues, robust and high expression on the surface of tumor cells and exhibition of a tumor-promoting function are desirable characteristics for an ideal antibody target (4).

The search for genes tightly silenced in the vast majority of healthy tissues moves into the focus of attention the intriguing observation that genes of the gametogenic and/or trophoblastic lineage are frequently ectopically activated and robustly expressed in human cancer. Based on phenotypical similarities between germ cells, pregnancy trophoblast, and cancer cells, John Beard proposed as much as 100 years ago a “trophoblastic theory of cancer” (5, 6). The discovery of the sporadic production of chorionic gonadotropin, α -fetoprotein, carcinoembryonic antigen, and other trophoblastic hormones by cancer cells provided the first molecules shared between neoplastic and trophoblastic cells (6–10). The concept was reignited by the inauguration of the steadily growing cancer/germline class of genes, which represents >100 members, each expressed in a variety of tumor types. The observation that entire trophoblastic and gametogenic programs escape transcriptional silencing and are ectopically activated in cancer cells (11, 12) holds the promise that within this class of genes with exquisitely selective tissue distribution, appropriate targets for mAb therapy may be discovered.

Applying an integrated data mining and validation approach for the identification of trophoblastic genes with aberrant induction in cancer, we discovered PLAC1, which has been originally described as placenta-specific gene. Our investigations unraveled features, which qualify PLAC1 as a highly attractive target for antibody-based therapy of breast cancer and other tumor types.

Materials and Methods

Tissues and cell lines. Recombinant DNA work was done with the official permission and according to the rules of the state government of Rheinland-Pfalz. Tissues were obtained as human surplus materials during routine diagnostic or therapeutic procedures and were stored at -80°C until use. Breast cancer cell lines MCF-7 and BT549 were cultured in DMEM/10% FCS.

RNA isolation, reverse transcription-PCR, and real-time reverse transcription-PCR. RNA extraction, first-strand cDNA synthesis, reverse transcription-PCR (RT-PCR), and real-time RT-PCR were done as previously described (13). For end-point analysis, PLAC1-specific oligonucleotides (sense 5'-AAA TTT GGC AGC TGC CTT CAC-3'; antisense 5'-TGA TGC CAC ATT CAG TAA CAC-3'; 60°C annealing) were used in a 35-cycle RT-PCR. Real-time quantitative expression analysis was done in triplicates in a 40-cycle RT-PCR. After normalization to hypoxanthine phosphoribosyltransferase (sense 5'-TGA CAC TGG CAA AAC AAT GCA-3'; antisense 5'-GGT CCT TTT CAC CAG CAA GCT-3'; 62°C annealing) PLAC1 transcripts in tumor samples were quantified relative to normal tissues using $\Delta\Delta\text{CT}$ calculation. Specificity of PCR reactions was confirmed by cloning and sequencing of amplification products from arbitrarily selected samples.

Bioinformatics. For in silico cloning of trophoblast-specific molecules, we modified and adapted a data mining strategy described in detail

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Cell proliferation analysis. At 24 h after transfection with siRNA duplexes, 1×10^4 cells were cultured for 48 h in medium supplemented with 10% FCS. Proliferation was analyzed by measuring the incorporation of BrdUrd into newly synthesized DNA strands using the DELFIA cell proliferation kit (Perkin-Elmer), according to the manufacturer's instructions, on a Wallac Victor² multilabel counter (Perkin-Elmer).

Cell cycle analysis. Cells were cultured in medium supplemented with 10% FCS in varying concentrations. At 72 h after transfection with siRNA duplexes, cells were harvested, ethanol-fixed, and stained with propidium iodide before flowcytometric DNA content analysis. Cells in the different phases of the cell cycle were quantified using CellQuest Pro (BD Biosciences) and FlowJo (Tree Star) flowcytometric analysis software. Apoptotic cells were quantified by Annexin V staining 48 and 72 h after siRNA transfection.

Cell migration and *in vitro* invasion assay. Cell migration assays were conducted in transwell chambers with 8.0- μ m pore membranes (BD Biosciences) with cells cultured in serum-free medium for 12 h before experiments. For siRNA experiments, cells were transferred to serum-free conditions 24 h after transfection with siRNA duplexes as described above. Cells (4×10^4) in 400 μ L serum-free culture medium were added to the upper chamber. The bottom chambers contained 800 μ L culture medium supplemented with 5% FCS as chemoattractant. At 24 h later, cells that had migrated to the bottom side of the membrane were fixed in ice-cold methanol; membranes were excised, placed on microscope slides, and mounted with Hoechst (Dako) for fluorescence microscopy. Cells in five random visual fields (100 \times magnification) were counted for each membrane. All experiments were done in triplicates. Effects on chemokinesis of cells were analyzed using the same experimental setup with chemoattractant added to both the upper and lower chambers. For *in vitro* invasion assays, the upper chambers were prepared with 100 μ L Matrigel (BD Biosciences) diluted to 1 mg/mL in serum-free medium. Chambers were incubated at 37°C for 5 h for gelling.

Results and Discussion

To identify placenta-specific trophoblastic genes, we adapted a genome-wide data mining strategy, which we had originally developed for *in silico* identification of germ cell-specific molecules (11, 13, 14). In principle, hierarchical keyword search

of Genbank was combined with digital cDNA library subtraction for prediction of authentically placenta-specific genes.

One of the candidates we identified by this approach was PLAC1. Previous reports have described this gene as restricted to cells of the trophoblast, in which it is detected throughout gestation (20). Moreover, PLAC1 has been hypothesized to be involved in placental growth regulation (21).

We investigated *PLAC1* mRNA in a comprehensive set of normal and neoplastic tissue specimens by end-point RT-PCR and quantitative real-time RT-PCR. We confirmed that *PLAC1* expression is confined to placenta. In all other normal tissue specimens, transcript amounts are below or just at the detection limit of highly sensitive RT-PCR (Fig. 1A). The only exception is testis, albeit with transcript levels 3 to 4 logs lower than those observed in placenta.

In 38% (86 of 225) of primary tumor specimens across different cancer types and 55% (22 of 40) of tumor cell lines, however, we found aberrant activation of this gene with otherwise tightly controlled transcription (Fig. 1A; Supplementary Table S1). Fifty-one of sixty-two (82%) primary breast cancer samples scored positive for *PLAC1* expression (defined as at least 100-fold above background in nontrophoblastic normal tissues), with 24% (15 of 62) showing low (100-fold to 1,000-fold), 40% (25 of 62) showing intermediate (1,000-fold to 10,000-fold), and 17% (11 of 62) showing high (>10,000-fold) expression (Fig. 1B). Moreover, we found *PLAC1* transcription in 21 of 50 (42%) lung cancer samples (Supplementary Fig. S1), as well as in gastric and ovarian cancer (Supplementary Table S1). Induction of *PLAC1* did not correlate with histologic subtype, tumor stage, or tumor grade.

For several germ cell and trophoblast-specific genes, it has been reported that their expression in cancer cells correlates with demethylation of CpG islands in the respective promoter regions (11, 12, 22–24). Consequently, such genes are easily activated in nonexpressing cells by experimental genomic demethylation using compounds, such as 5-aza-2'-deoxycytidine. *PLAC1*, however, was not inducible by 5-aza-2'-deoxycytidine treatment (Supplementary Fig. S2), suggesting that its expression is regulated by factors other

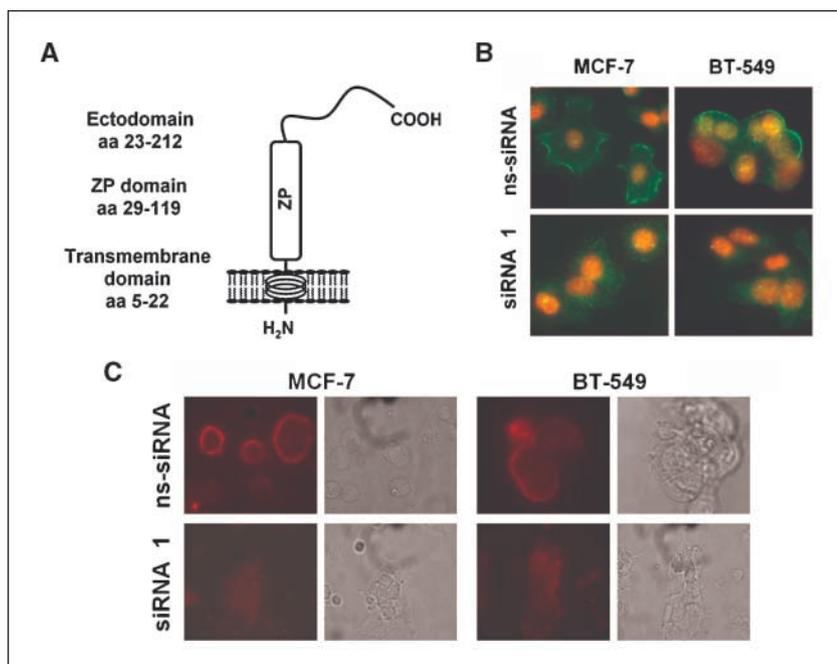


Figure 2. *PLAC1* is a cell surface protein. A, predicted topology and structure of *PLAC1* protein. Staining of methanol-fixed (B) and nonfixed (C) MCF-7 and BT-549 breast cancer cells with anti-*PLAC1*/C-term antibody after transfection with *PLAC1*-specific siRNA (*siRNA1*) or non-silencing siRNA.

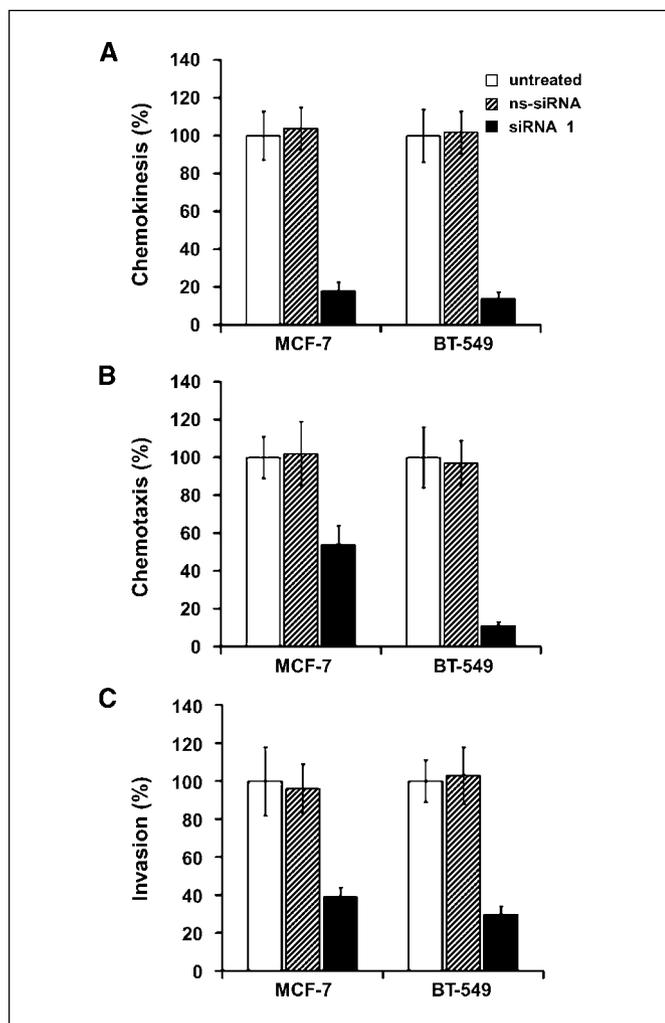


Figure 3. PLAC1 expression promotes motility, migration, and invasion of breast cancer cells. *A*, chemokinesis (motility) analysis in transwell migration assays with 5% FCS added to the upper, as well as the lower, chamber was analyzed after 12 h. *B*, chemotaxis analysis of MCF-7 and BT-549 cells in transwell migration assays 12 h after 5% FCS has been added to the lower chamber only to obtain a gradient. *C*, analysis of chemotactic invasion into Matrigel 24 h after 5% FCS as chemoattractant has been added to the lower chamber.

than DNA methylation. This is in line with the low CpG content of the PLAC1 promoter (data not shown).

For analysis of protein expression, we raised a polyclonal rabbit antibody (rabbit anti-PLAC1/C-term) against a PLAC1-specific peptide epitope (NP_068568, amino acids 117–127). Specificity of the antibody was verified by gene silencing of PLAC1 using siRNA. To exclude siRNA off-target activity experiments were conducted with two sets of PLAC1-specific siRNA duplexes, a scrambled nonsilencing oligonucleotide and nontransfected cells. By transfecting breast cancer cell lines MCF-7 and BT-549 with these siRNA duplexes, we achieved a stable and reproducible reduction of constitutive PLAC1 mRNA expression by 80% to 90% compared with controls (Supplementary Fig. S3). Consistent with this observation, the 26-kDa band, detected in accordance with the predicted size of PLAC1 in Western blot, nearly completely disappeared in both cell lines (Fig. 1B), proving both robust knockdown of PLAC1 protein expression and specificity of the antibody.

Western Blot staining of PLAC1 protein in primary human tissue samples with rabbit anti-PLAC1/C-term confirmed that this gene is detectable in breast cancer specimens in levels comparable with placenta as the only normal tissue it is expressed in (Fig. 1C). Protein levels correlated well with transcript levels (data not shown). Immunohistochemistry with rabbit anti-PLAC1/C-term on human breast tumor sections showed specific immunoreactivity in specimens typed positive for PLAC1 mRNA expression by RT-PCR. Staining was confined to the neoplastic cell population, whereas adjacent stromal and nonneoplastic epithelial cells, as well as patient-matched normal tissues, were not reactive (Fig. 1D). Immunostaining of tumor cells was accentuated at the plasma membrane, raising the exciting possibility that PLAC1 may be a cell surface protein.

In silico analysis of membrane topology of the PLAC1 protein sequence predicted a type II membrane protein with one transmembrane helix spanning amino acids 5 to 22 followed by a large extracellular domain constituted by amino acids 23 to 212. Amino acids 29 to 119 of the extracellular part of PLAC1 represent a truncated zona pellucida (ZP) domain (Fig. 2A). The ZP domain is found in a variety of extracellularly exposed receptor-like proteins, including transforming growth factor- β (TGF- β) receptor type III, uromodulin, glycoprotein GP2, as well as the sperm receptors ZP2 and ZP3 (25), and is involved in polymerization (26).

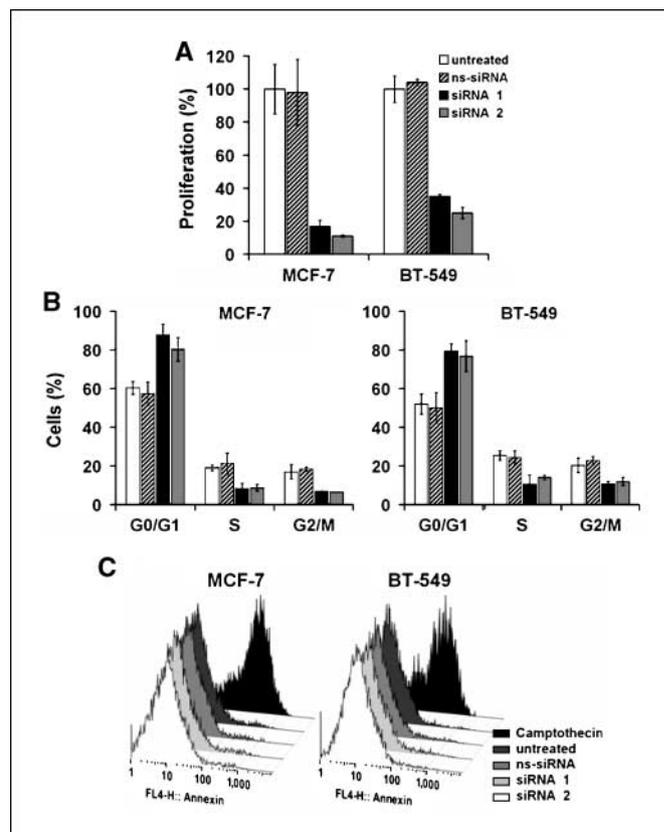


Figure 4. PLAC1 expression promotes proliferation of breast cancer cells. *A*, analysis of proliferation in MCF-7 and BT-549 cells 72 h after knockdown has been initiated by PLAC1-specific siRNA duplexes. *B*, cell cycle analysis of cells 72 h after initiation of PLAC1 silencing shown as bar chart of cell fractions in different cell cycle states. *C*, apoptosis of cells as determined by Annexin V staining 72 h after transfection with siRNA. As positive control for Annexin V, staining cells were treated with 6 μ Mol/L Camptothecin for 12 h.

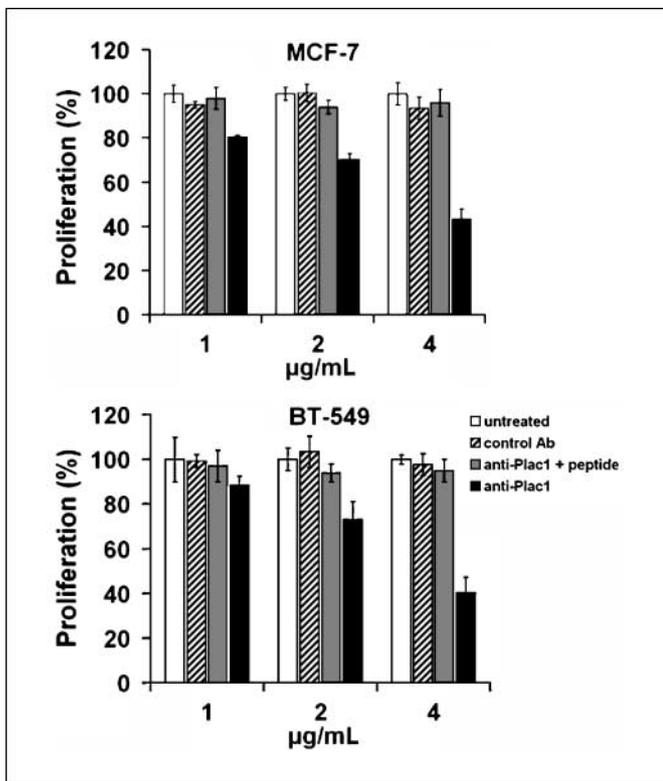


Figure 5. PLAC1 is drugable by function-antagonizing antibodies. Proliferation analysis of MCF-7 and BT-549 cells after incubation with different concentrations of anti-PLAC1 antibody for 48 h. Isotype control and anti-PLAC1 antibody preincubated with PLAC1 peptide used for immunizations were used as controls.

We assessed the subcellular localization of constitutively expressed PLAC1 by immunofluorescence microscopy of MCF-7 and BT-549 breast cancer cells stained with rabbit anti-PLAC1/C-term, which has its epitope (amino acids 117–127) in the presumably extracellular part of the protein. Both cell lines displayed distinct staining at the cell membrane (Fig. 2B). Loss of signal upon siRNA-induced knockdown of PLAC1 expression confirmed the specificity of the staining. Most importantly, specific membrane staining was observed not only on methanol-fixed, but also nonfixed, native cells (Fig. 2C), implying that the epitope of the antibody is accessible without permeabilization of the cell membrane and thus supporting the predicted topology with extracellular localization of the carboxy terminus.

To determine the biological significance of PLAC1 in tumor cells, we studied the effects of its siRNA-induced gene silencing on essential cell functions.

First, performance of breast cancer cell lines MCF-7 and BT-549 in transwell migration assays was investigated. Baseline motility (chemokinesis) of both cell lines assessed by adding 5% FCS as chemoattractant to both the upper and lower chambers of the system was substantially inhibited by PLAC1-specific siRNA duplexes (Fig. 3A). Consequently, we also observed a marked reduction of the directional chemotactic migratory capacity of the cells (Fig. 3B). Moreover, chemoinvasion activity of cells was profoundly affected by PLAC1 siRNA treatment, as cells were not able to migrate along chemoattractant gradients by breaking through a barrier of Matrigel (Fig. 3C).

Next, we observed that tumor cell proliferation, as measured by BrdUrd incorporation into DNA, was reduced by 80% to 90% in

both cell lines by PLAC1-specific siRNA duplexes (Fig. 4A). Cell cycle analysis revealed a distinct G₁-S arrest in the cells transfected with PLAC1 siRNA as the underlying cause for the proliferation block (Fig. 4B). Viability of the cells was not affected, and staining for Annexin V gave no indications for apoptotic cell death (Fig. 4C).

As the epitope of rabbit anti-PLAC1/C-term is localized in the extracellular portion of PLAC1 and thus freely accessible, we wondered whether treatment of cells with this antibody mediates inhibition of cell growth. We measured proliferation of MCF-7 and BT-549 cells incubated with rabbit anti-PLAC1/C-term and a nonreactive control antibody. Targeting of PLAC1 resulted in efficient inhibition of proliferation of both cell lines in a concentration-dependent manner. Importantly, antibody-mediated inhibition of tumor cell proliferation was abrogated in the presence of PLAC-1 peptide (Fig. 5).

Proliferation and cell cycle progression in eukaryotic cells is governed by cyclins and cyclin-dependent kinases. Individual cyclins act at different phases of the cell cycle by stimulating the activities of a series of cyclin-dependent kinases. Restriction point control is mediated by cyclin D-dependent and cyclin E-dependent kinase families (27, 28). To investigate whether PLAC1 silencing induces cell cycle dysregulation via alteration of cyclin expression, we determined expression of cyclins D1, D2, D3, and E in MCF-7 and BT-549 breast cancer cells treated with PLAC1 siRNA. Interestingly, a significant reduction of cyclin D1 transcripts as measured by real-time PCR (Fig. 6A), as well as cyclin D1 protein levels in Western blot (Fig. 6B), occurred as a consequence of PLAC1 knockdown. No change in transcription levels was observed for the other cyclins analyzed (Supplementary Fig. S4). Cyclin D1 is a major regulator of the G₁-S progression of the cell cycle. Interestingly, in tumorigenesis of sporadic breast cancer, overexpression of

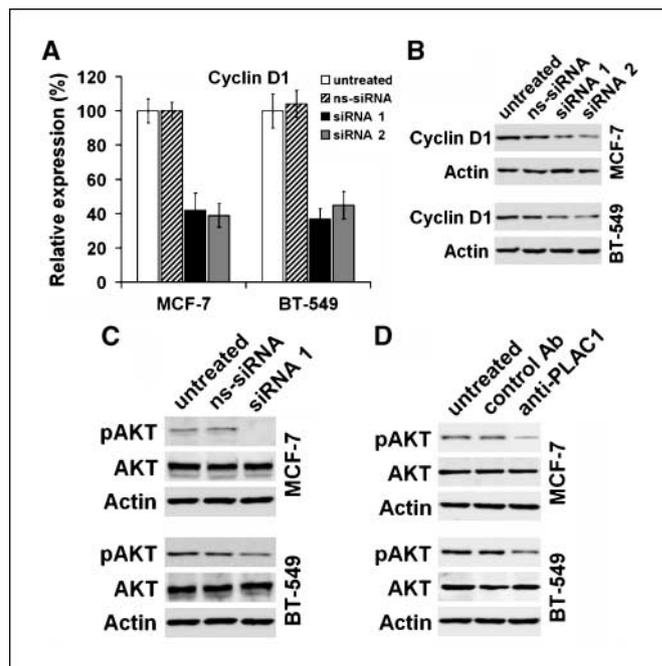


Figure 6. Cyclin D1 and AKT kinase are involved in PLAC1 function. Quantitative real-time RT-PCR analysis (A) and Western blot analysis (B) of cyclin D1 after cells were treated for 72 h with PLAC1-specific siRNA duplexes. Western blot analysis of AKT Ser-473 phosphorylation after 72 h of PLAC1 knockdown (C) and after 1 h of treatment with anti-PLAC1/C-term antibody (D).

cyclin D1 is regarded as an early event (29, 30). D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners depend on persistent mitogenic signaling. Thus, D-type cyclins act as growth factor sensors, forming active kinases in response to extracellular factors (30, 31). In breast cancer, it has been shown that cyclin D1 expression is controlled via a phosphatidylinositol 3-kinase (PI3K)/AKT-dependent pathway (32, 33). AKT inactivates glycogen synthase kinase-3 β , thereby increasing cyclin D1 transcription, as well as its proteolytic turnover and its protein levels in the nucleus (34, 35). In addition, the AKT pathway is an important regulator of cancer cell motility and migration (36, 37), two other cell functions in which PLAC1 is apparently involved. This prompted us to analyze whether PLAC1 has an effect on the regulation of AKT kinase in MCF-7 and BT-549 cells. Constitutive phosphorylation and hyperactivation of AKT consecutive to PI3K overactivation is frequently observed in tumor cells. Quantification of levels of Ser-473 phosphorylation of AKT subsequent to silencing of PLAC1 by siRNA technology and its functional antagonizing with antibody anti-PLAC1/C-term both resulted in a marked reduction of phosphorylated AKT levels in particular in MCF-7 cells (Fig. 6C), suggesting that AKT kinase activation is involved in execution of down-stream effects of PLAC1. Interestingly, down-regulation of phosphorylated AKT was less prominent in BT-549 cells, which lack PTEN and therefore have a higher level of PI3K overactivation.

In summary, our data disclose several properties that qualify PLAC1 as a highly attractive target for therapeutic antibodies. Being a differentiation antigen of a cell lineage which appears in the human body only in such an exceptional state as pregnancy, it is absent from toxicity-relevant normal tissues as a self-antigen can possibly be. Its high prevalence in a variety of tumor entities would make a broad number of patients eligible for treatment with PLAC1-targeting therapies. In the case of breast cancer, for example, >80% of patients carry this target. Her2/neu, in contrast, the target of Herceptin, the only mAb available for treatment of this cancer type, is overexpressed in only 20% to 25% of breast cancer patients (38). For lung and gastric cancer, in which PLAC1 is expressed in 42% and 58% of the cases, respectively, there is no approved mAb treatment thus far, owing to the lack of appropriate targets in these cancer types. Moreover, although antibody anti-PLAC1/C-term was not deliberately developed to be functionally active, it proofs the concept that PLAC1 is drugable by antibodies on living cells and that such antibodies may precipitate antitumoral effects, such as proliferation inhibition. PLAC1 seems to be involved not only in proliferation but also cell motility, migration, and invasion. Most interestingly, all these attributes do

not only substantially contribute to the tumor phenotype but are also inherent properties of the human trophoblast, which physiologic characteristics are to grow fast and to invade the uterus. It is likely that, in the frame of a state-of-the-art antibody development process, mAbs against PLAC1 can be engineered, which intervene with all these functions at once on top of their potential to mediate immune effector functions, such as ADCC and CDC.

It is not clear, thus far, which sort of activity may be disrupted by binding of an antibody to PLAC1. PLAC1 has been suggested to have a receptor-like function in the placental trophoblast, facilitating ligand-receptor interactions involving yet unknown constituents at the maternal-fetal interface (39). However, as it does not possess a discernible cytoplasmic domain for signal transduction, it is unlikely that PLAC1 acts as a receptor on its own. Rather, PLAC1 may represent an accessory receptor for a yet-to-be-defined receptor. Examples for accessory receptors that modulate or facilitate receptor-ligand interactions are β -glycan/TGF- β type III receptor and endoglin. Both molecules are discussed to operate in the TGF- β signaling network by binding TGF- β and presenting it to other members of the TGF- β receptor family (40, 41). Most intriguingly, β -glycan/TGF- β type III receptor, like PLAC1, also harbors a functional ZP domain. Alternatively, PLAC1 may operate as a receptor-activity modulating protein (RAMP). RAMPs are small membrane proteins that form stable complexes with a receptor resulting in either trafficking of receptor protein from an intracellular compartment to the cell surface or in alteration of receptor phenotype, presumably through a direct or indirect effect on ligand binding (42, 43). Consecutively, depending on which of these models is valid, an anti-PLAC1 antibody would interfere either with ligand binding or with receptor association.

Ongoing studies have to dissect in which receptor-ligand interactions and signal transduction paths PLAC1 is involved. Most interestingly, knockdown of PLAC1 transcription seems to counteract constitutive AKT kinase overactivation in tumor cells. This positions PLAC1 in a pathway which is already being addressed by a variety of antibody and small compound-based therapies either approved or currently in clinical development and adds to its attractiveness as drug target.

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