Suppression of Early Hematogenous Dissemination of Human Breast Cancer Cells to Bone Marrow by Retinoic Acid–Induced 2

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ABSTRACT
Regulatory pathways that drive early hematogenous dissemination of tumor cells (DTC) in the bone marrow to define patients with early disseminated breast cancer and identified low retinoic acid–induced 2 (RAI2) expression to be significantly associated with DTC status. Low RAI2 expression was also shown to be an independent poor prognostic factor in 10 different cancer datasets. Depletion of RAI2 protein in luminal breast cancer cell lines resulted in dedifferentiation marked by downregulation of ERα, FOXA1, and GATA3, together with increased invasiveness and activation of AKT signaling. Functional analysis of the previously uncharacterized RAI2 protein revealed molecular interaction with CtBP transcriptional regulators and an overlapping function in controlling the expression of a number of key target genes involved in breast cancer. These results suggest that RAI2 is a new metastasis-associated protein that sustains differentiation of luminal breast epithelial cells.

SIGNIFICANCE: We identified downregulation of RAI2 as a novel metastasis-associated genetic alteration especially associated with early occurring bone metastasis in ERα-positive breast tumors. We specified the role of the RAI2 protein to function as a transcriptional regulator that controls the expression of several key regulators of breast epithelial integrity and cancer.

See related commentary by Esposito and Kang, p. 466.

INTRODUCTION
In most patients with cancer, mortality is linked to metastasis. To prevent the occurrence of metastasis, different chemotherapeutic agents with severe side effects are currently administered systemically. However, many patients relapse after chemotherapy, most likely due to the activation of dormant disseminated tumor cells (DTC), which are non-proliferating and resistant to conventional chemotherapy (1). Using sensitive immunocytochemical assays, it has been shown that bone marrow is a common homing organ for DTCs of different origins, including breast, lung, and colon carcinomas (2–4). Several studies, including a large-scale pooled analysis (5), have shown that the presence of even a single DTC in the bone marrow of patients with breast cancer is an independent prognostic factor (3, 5).

Most cancer types are thought to be initiated and progress in a similar manner and share a common set of characteristics often called “the hallmarks of cancer,” which include genome instability and mutations. Also, a strong correlation between the metastatic potential of a primary tumor and reduced differentiation grade in breast cancer has been established (6). In addition, several wide-ranging studies of expression profiling have shown positive correlation between the expression of the luminal markers ERα (ESR1), GATA binding protein 3 (GATA3), and forkhead box protein A1 (FOXA1) and well-differentiated breast tumors, and favorable clinical outcome (7, 8).

Thus far, activation/inactivation of several important oncogenes and tumor-suppressor genes (TSG), such as HER2, EGFR, and TP53, have been described as driving breast cancer development and progression (9). For the dissemination and outgrowth of metastases, another set of metastasis-suppressor genes (MSG) and activator genes are required. These MSGs, in contrast with classic TSGs, are less well characterized and appear to be more cancer type–specific and rarely inactivated by mutations (10, 11). Notably, the time at which individual cells begin to spread from a primary tumor mass is unclear, and regulatory pathways that control early hematogenous dissemination into the bone marrow remain insufficiently defined (1).
In this study, we identified retinoic acid–induced 2 (RAI2) as a putative suppressor of early hematogenous dissemination of tumor cells to the bone marrow. RAI2 was initially described as a retinoic acid–inducible gene but its specific function has not yet been determined even though it has been assumed to play a role in development (12). Here, we show that depletion of RAI2 expression in luminal breast cancer cells is associated with a loss of epithelial differentiation, which leads to an aggressive tumor phenotype and increased invasiveness. Our findings indicate that early hematogenous dissemination of tumor cells, particularly in hormone receptor–positive breast cancer, is mediated by RAI2.

RESULTS
Gene Expression Profiling and DTC Signature

The gene signatures of 32 breast tumor samples were assessed, and 31 were classified as luminal A (n = 16) or B (n = 15) tumors, which correlated with the positive hormone receptor status of the tumors as determined by immunohistochemistry (Supplementary Table S1). This homogenous group of early-stage tumors was further divided into two subgroups based on DTC status, and the rank-sum test was used to identify genes that were associated with DTC status. After correcting for multiple testing, 28 genes corresponding to 54 Ensembl transcripts were found to be significantly downregulated, and only four transcripts were significantly upregulated in DTC-positive breast cancer samples, indicating a prominent role for potential suppressors of early dissemination (Fig. 1 and Supplementary Table S2). The genes characterizing the DTC signature are located on 17 different chromosomes without any clustering to specific regions or common chromosomal break points. We used the significant genes for clustering analysis with a large publicly available dataset (GSE3494). Interestingly, most of the downregulated genes were, in this dataset, downregulated in the basal and/or HER2-positive type of breast tumors and clustered as well with TP53 mutants, high grade, and proliferation (Fig. 1). The results indicate that our DTC signature obtained from patients with luminal breast cancer defines a more aggressive dedifferentiated tumor population within the luminal group usually found among basal and HER2-positive patients. The original results were not biased by an uneven distribution of grade, Ki67, or mutant p53 status of the study cohort (Supplementary Table S1).

Figure 1. Clustering analysis of DTC-associated genes. Heatmap of the 32 significant genes from the initial DTC analysis in 251 breast tumors, with genes and samples clustered separately. The color coding at the top indicates the breast cancer subtype. Below the heatmap, phenotypes are encoded such that black indicates a poorer value in terms of outlook. ER, estrogen receptor α; PgR, progesterone receptor.
RAI2 Is Downregulated in Early Metastasized Breast Tumors

RESEARCH ARTICLE

To provide further evidence of the prognostic impact of DTC-associated genes, we performed in silico validation of all significant genes using six publicly available breast datasets comprising a total of 3,613 breast cancer patients (Supplementary Table S3). The prognostic relevance of RAI2 was also verified in two lung cancer patient cohorts as well as one colon and one ovarian cancer patient cohort (Supplementary Fig. S1). Using appropriately preprocessed gene expression values, samples in the datasets were separated into high-expression and low-expression groups using the extreme quartiles. Differences in overall survival (OS) or disease-free survival (DFS), depending on data availability, between these groups were determined by the Kaplan–Meier estimates of survival and the log-rank test. A total of 27 genes showed a significant association in at least one dataset, whereas 16 genes showed an association in at least two datasets. Low RAI2 expression was significantly associated with shortened survival in all six tested breast cancer datasets (Fig. 2) as well as in all the other lung, ovarian, and colon cancer datasets (Supplementary Fig. S1). Among the four upregulated genes, high ABHD12 expression was associated with worse survival in two datasets.

Figure 2. Validation of RAI2 as a prognostic marker. Survival analysis using the extreme quartiles of RAI2 mRNA expression. RAI2 mRNA expression was determined in six published breast cancer datasets (for details, see Table S3) and analyzed using the Kaplan–Meier estimations. P values were calculated on the basis of the log-rank test.

Prognostic Impact of the DTC-Associated Genes

To provide further evidence of the prognostic impact of DTC-associated genes, we performed in silico validation of all significant genes using six publicly available breast datasets comprising a total of 3,613 breast cancer patients (Supplementary Table S3). The prognostic relevance of RAI2 was also verified in two lung cancer patient cohorts as well as one colon and one ovarian cancer patient cohort (Supplementary Fig. S1). Using appropriately preprocessed gene expression values, samples in the datasets were separated into high-expression and low-expression groups using the extreme quartiles. Differences in overall survival (OS) or disease-free survival (DFS), depending on data availability, between these groups were determined by the Kaplan–Meier estimates of survival and the log-rank test. A total of 27 genes showed a significant association in at least one dataset, whereas 16 genes showed an association in at least two datasets. Low RAI2 expression was significantly associated with shortened survival in all six tested breast cancer datasets (Fig. 2) as well as in all the other lung, ovarian, and colon cancer datasets (Supplementary Fig. S1). Among the four upregulated genes, high ABHD12 expression was associated with worse survival in two datasets.

Quantitative Real-Time RT-PCR Verification of RAI2 Expression

Because RAI2 downregulation was significantly associated with both positive DTC status and poor OS, it was chosen as a candidate gene for further validation and functional tests. The expression of RAI2 was tested in 76 mainly hormone receptor–positive primary tumor samples from 36 DTC-positive and 40 DTC-negative cases. We could validate that RAI2 mRNA expression was significantly downregulated in DTC-positive patients (P < 0.05; Supplementary Fig. S3A). These results provide evidence that significantly lower RAI2 gene expression is associated with an aggressive tumor phenotype and underlies the putative role of the RAI2 protein in the suppression of early tumor cell dissemination.

Prognostic Impact of RAI2 Expression

Multivariate Cox proportional hazards model analysis of the six different breast cancer datasets showed that RAI2 gene expression provides survival information independent of other prognostic factors, making this gene interesting for further validation and functional tests (Fig. 2 and Supplementary Table S4). Additional survival and clinical correlation analyses were performed for RAI2 using the large METABRIC breast cancer dataset (13) EGAS000000000083.
Figure 3. Analysis of RAI2 mRNA and protein expression in human breast cancer tissue and cell lines. A, RAI2 mRNA expression was determined in a large published expression dataset breast #6 (13) and correlated with the indicated clinicopathologic parameters. RAI2 mRNA expression according to ERα status, histologic grade, and breast carcinoma molecular subtype is shown. B, survival analysis in extreme quartiles of RAI2 expression in ER+ and ER− patients. RAI2 expression was determined in the dataset breast #6 and analyzed in the Kaplan–Meier estimations. P values are calculated on the basis of log-rank tests. C, RAI2 and ERα protein expression in a panel of human breast carcinoma cell lines was determined by Western blot analysis using two polyclonal RAI2-specific antibodies that recognize either a C-terminal or internal epitope. Equal loading was demonstrated using an antibody recognizing HSC70 protein. D, determination of RAI2 protein localization in the luminal breast cancer cell lines MCF-7 and CAMA-1 by immunofluorescence staining (blue, DAPI/DNA staining; green, α-RAI2). E, Western blot analysis of RAI2 protein expression in whole-cell extracts of the indicated cell lines following either hormone depletion or treatment with ATRA or ICI182,780 (ICI) for 5 days. A highly significant correlation between RAI2 mRNA expression and ERα status and the molecular luminal A subtype (both \( P < 0.001 \)) was found, which is particularly associated with well-differentiated tumors and good clinical outcome. Furthermore, a statistically significant correlation between decreased RAI2 mRNA expression and differentiation grade, mutant TP53, and advanced stage was found (all \( P < 0.001 \)), whereas no association was found to lymph node status. A strong association with hormone receptor status was furthermore detected in 12 other datasets using the Oncomine platform. In each dataset, a significant correlation could be found between high RAI2 status and hormone receptor-positive status (Supplementary Fig. S4).
To test whether the prognostic power of RAI2 expression in breast cancer is due to its association with ERα status, we carried out a survival analysis of the large dataset (13) grouping the patients according to ERα. RA12 status showed that the worst survival was found for patients with low RAI2 expressors with an ERα-negative status followed by RA12-low/ERα-positive patients, indicating a stronger impact of RA12 than ERα status with regards to poor patient outcome (Fig. 3B). These results indicate that low RAI2 mRNA expression is associated with less differentiated and more aggressive breast tumors, especially in the ERα-positive patient cohort.

Expression of RAI2 Protein in Breast Cancer Cell Lines

To further investigate the biologic relevance of RAI2 expression in breast cancer, we evaluated RAI2 expression at the protein level in a panel of breast cancer cell lines (Fig. 3C). Western blot analyses indicated that RAI2 protein was generally expressed in all tested breast cancer cell lines; however, a correlation between cancer subtype of cell lines and intensity of RAI2 protein expression was found. As in the patient data, we found that the RAI2 protein was most abundantly expressed in cell lines corresponding to the luminal ERα-positive subtype, whereas RAI2 protein expression was considerably lower in HER2-overexpressing and highly aggressive basal cell lines. The immortalized, nontransformed breast epithelial cell line MCF-10A showed the highest RAI2 protein expression. Immunofluorescence analyses demonstrated a speckled intranuclear localization of the RA12 protein in the different breast cancer cell lines (Fig. 3D), indicating potential function in transcriptional regulation. Because RA12 was found to be especially related to both the luminal subtype of ERα-positive breast cancer and to well-differentiated tumors, we analyzed the dynamics of RAI2 protein expression in the course of hormone depletion and pharmacologic treatment with either the ERα antagonist ICI182,780 (Fulvestrant) or all-trans retinoic acid (ATRA) in ERα-positive KPL-1 and CAMA-1 cells and ERα-negative MDA-MB-231 cells. We found that RAI2 protein expression was downregulated upon hormone depletion and induced in the course of ATRA treatment in KPL-1 and CAMA-1 cells. Also, ERα inhibition led to the induction of RA12 protein expression in CAMA-1 cells. In contrast, the different treatments had no effect on RAI2 expression in MDA-MB-231 cells (Fig. 3E).

Experimental Modulation of RAI2 Expression in Breast Cancer Cell Lines

The association between RA12 expression and histologic grade and ERα status led us to investigate whether RA12 sustains the cellular differentiation of luminal breast cancer cells. Therefore, we first expressed two shRNAs, which efficiently knocked down RA12 protein expression in MCF-7, KPL-1, and CAMA-1 cells (Fig. 4A). shRNA-mediated knockdown of RA12 protein expression caused downregulation of ERα protein expression in all three of the tested cell lines and a concomitant downregulation of the expression of GATA3, FOXA1, and GRHL2 proteins, which are known to be key regulators of breast epithelial differentiation (14–16). Furthermore, Western blot analysis further revealed decreased protein expression of the transcriptional coregulators carboxyl-terminal binding proteins (CtBP) 1 and 2 (Fig. 4A) in KPL-1 and CAMA-1 cells and decreased CtBP2 expression in MCF-7 cells.

Furthermore, loss of RA12 protein expression led to an apparent alteration in cellular morphology. The RA12-silenced, ERα-positive cell lines MCF-7, KPL-1, and CAMA-1 exhibited enlarged and less refractive cell bodies (Fig. 4B). In addition, some cells demonstrated microfilament branching at cellular edges. Furthermore, a subset of CAMA-1 cells with silenced RA12 also demonstrated a spindle shape form, which is characteristic of cellular plasticity (Fig. 4B). Although RA12 knockdown in MCF-7 cells did not affect the number or size of colonies in soft-agar assays, the formed colonies exhibited increased sprouting (Fig. 4C). When grown in 3D Matrigel, colonies formed from RA12 knockdown in MCF-7 cells did not differ in size or number of cells but demonstrated a cell polarization defect in the form of a notably diffuse distribution of the cis-Golgi matrix protein GM130 (Fig. 4D).

We could detect increased AKT protein phosphorylation at Ser473 as a consequence of RA12 depletion, which is indicative of activation of the AKT signaling cascade (Fig. 4A). Consequently, we also analyzed whether RA12 depletion has an influence on cell viability of cultured breast cancer cells that were treated with either MK-2206 or RAD001 that target the AKT or mTOR kinases, respectively. As shown in Fig. 4E, RA12 depletion caused a significant increase in cell viability in cells that were treated with one particular drug, whereas little combinatorial effect was seen in MCF-7 cells and none in KPL-1 and CAMA-1 cells.

Collectively, the data strongly suggest that the RA12 protein sustains epithelial traits and luminal differentiation in ERα-positive breast cancer cells because RA12 downregulation induces the loss of essential differentiation-sustaining transcription factors and morphologic changes.

Phenotypic Changes in RA12-Depleted Breast Cancer Cell Lines

Next, we assessed whether loss of RA12 protein expression is associated with epithelial-to-mesenchymal plasticity, as strongly suggested by the aberrant cell morphology of RA12-depleted CAMA-1 cells. We determined the number of mesenchymally transformed CAMA-1 cells by counting cells that stained for F-actin. Approximately 10% of the RA12-depleted CAMA-1 cells had a spindle-shape characteristic of epithelial-to-mesenchymal transition (EMT; Fig. 5A and Supplementary Fig. S5). Immunofluorescence staining for E-cadherin in MCF-7 and KPL-1 cells (Supplementary Fig. S6) after RA12 depletion also revealed a decreased E-cadherin expression at cell junctions, which is indicative of a disruption in adhesion junction formation and loss of epithelial properties. However, no spindle-shaped cells were observed in these two cell lines upon RA12 depletion. We also analyzed alterations in the mRNA expression of selected mesenchymal-specific genes (ZEB1, VIM, SNAI1, and SNAI2) in four RA12-depleted cell lines by qRT-PCR analysis. Data presented in Fig. 5B indicate significant changes in a variety of mesenchymal markers in the tested cell lines. To obtain further evidence for the potential role of RA12 in EMT, we examined RA12 protein expression after EMT induction by TGFβ stimulation in MCF-10A cells. In this experimental system, we found downregulation of RA12 protein expression after TGFβ stimulation, which was accompanied by...
downregulation of E-cadherin and upregulation of vimentin expression (Fig. 5C). We also assessed whether loss of RAI2 protein expression might have an impact on the motility and invasiveness of breast cancer cell lines. As shown in Fig. 5D and E, loss of RAI2 protein expression in the luminal cell lines MCF-7 and CAMA-1 promoted cell migration and invasion. In contrast, when we ectopically expressed HA-tagged RAI2 protein in the highly aggressive basal MDA-MB-231 cell line, we found that forced RAI2 overexpression impaired the migratory and invasive abilities of this cell line (Fig. 5F). Hence, we could show that loss of RAI2 expression contributes to epithelial-to-mesenchymal plasticity and induces a more aggressive phenotype.

**Analysis of Molecular Interaction between RAI2 and CtBP Proteins**

The RAI2 protein is a largely uncharacterized molecule with unknown molecular function. However, in the course
of systematically mapping the protein–protein interactions of RAI2, CtBPs were identified as potential interaction partners (17). The CtBP1 and CtBP2 (carboxyl-terminal binding) proteins have been shown to be highly conserved transcriptional corepressors that are important in development and epithelial differentiation (18) and also counteracting several TSGs (19). Proteins reported to bind CtBP usually contain a conserved “PXDLS” CtBP-interaction domain (19). Here, we performed a multiple sequence alignment analysis using Clustal (20) with the primary RAI2 amino acid sequence from different species and found two highly orthologously conserved sequences (ALDLS) in the internal region (amino acids 316–320 and 342–346 in human sequence) of the RAI2 protein (Fig. 6A). Furthermore, the RAI2 protein colocalizes with CtBP1 and CtBP2 in the nuclei of MCF-7 cells stably expressing HA-tagged RAI2 protein as shown by immunofluorescence staining (Fig. 6B). To validate the potential interactions of RAI2 and CtBP2 and analyze the relative importance of the identified potential binding sites, both sites were mutated to 316ALDAA 320 and 342ALDAA 346. In addition, a double mutant was established in which both sites were mutated (termed 4A; Fig. 6C). The subsequent pull-down experiments (Fig. 6D) demonstrated that when any of the sites were mutated, there was a marked decrease in coprecipitated CtBP2, with the first CtBP binding site being slightly less important for CtBP2 binding, which mirrors the results from subsequent fluorescence anisotropy analysis. When both sites were mutated, no CtBP2 binding could be detected. To further characterize the interaction between CtBP2 and RAI2, we used fluorescence anisotropy to quantify the binding of CtBP2 and two fluorescent peptides representing fragments 313–326 and 339–350 of RAI2, which comprise binding motifs 1 and 2, respectively. For both peptides, the experiments demonstrated specific binding with a dissociation constant in the low micromolar range (Fig. 6E).

Recent global profiling of CtBP protein activity in breast cancer cells revealed that CtBP proteins control epithelial differentiation following RAI2 depletion. F-actin filaments were stained with phalloidin 12 days after viral transduction of the cells (see Methods). Figure 5. Loss of RAI2 expression is associated with the EMT and an invasive phenotype. A, quantification of CAMA-1 cells with mesenchymal transformed morphology following RAI2 depletion. F-actin filaments were stained with phalloidin 12 days after viral transduction of the cells [see Methods]. Error bars, SD of the mean of three independent experiments. P values were calculated with a two-sided Student t test (*, P < 0.05). B, Western blot analysis of RAI2, E-cadherin, and vimentin in MCF-10A cells following 48-hour treatment with TGFβ1. C, quantitative gene expression analysis of four selected mesenchymal-specific markers in MCF-7, CAMA-1, KPL-1, and MCF-10A cells following RAI2 depletion by shRNA1 expression. Error bars, SD of the mean. P values were calculated with a two-sided Student t test (*, P < 0.05). D and E, migration and invasion analysis of MCF-7 and CAMA-1 cells following RAI2 depletion in a Boyden chamber assay with or without Matrigel coating using complete medium as a chemoattractant for 24 (migration) or 48 hours (invasion). Whole filters were counted after staining. F, analysis of the migration and invasion of MDA-MB-231 breast cancer cells following ectopic RAI2 protein expression. RAI2 overexpression was analyzed by Western blot following a Boyden chamber assay using complete medium as a chemoattractant for 24 hours (migration and invasion). Analysis was performed as described above except five randomly chosen fields per membrane were counted at ×200 magnification. Published OnlineFirst February 25, 2015; DOI: 10.1158/2159-8290.CD-14-1042.
**Figure 6.** The RAI2 protein interacts with CtBP transcriptional coregulators. 

**A,** ClustalW2 multispecies alignment of the RAI2 internal protein region obtained from ensembl.org, containing the consensus bipartite CtBP binding motifs (highlighted by a box).

**B,** analysis of the protein colocalization of RAI2-HA and CtBP1/2 by immunofluorescence staining.

**C,** schematic representations of the RAI2 proteins and mutants used in coimmunoprecipitation analysis.

**D,** coimmunoprecipitation analysis of RAI2 and CtBP2 interaction using cell lysates from 293T cells transfected with indicated RAI2 mutants.

**E,** titration curves of fluorescein-labeled RAI2 peptides with CtBP2. The reported anisotropy values are the average of three independent measurements (standard deviations are shown for each point) for which the baseline corresponding to the anisotropy of the free fluorescent probe was subtracted. The quality of the fit (represented by the curves) demonstrates a single specific saturating binding event.

**F,** quantitative gene expression analysis of selected direct transcriptional targets of CtBP in MCF-7, CAMA-1, and KPL-1 cells following RAI2 depletion. Data, average fold change normalized to RPLP0 and parental cell line expression of three independent experiments; error bars, SD of the mean. *P* values are calculated with a two-sided Student t test (*, *P* < 0.05).

**G,** Western blot and Boyden chamber analysis of MDA-MB-231 cells expressing either wild-type or CtBP binding–deficient proteins. Error bars, SD of the mean of three independent experiments. *P* values were calculated by a two-sided Student t test (**, *P* < 0.01).
differentiation in breast cancer cells (18). On the basis of these global binding and expression patterns, we analyzed multiple members of the CtBP-targeted gene network by quantitative PCR (qPCR) to validate whether RAI2 depletion in luminal cell lines affects the mRNA expression of direct CtBP transcriptional target genes. Data presented in Fig. 6E indicate significant changes in the number of tested CtBP-target genes, suggesting that loss of RAI2 expression directly affects the function of CtBP proteins as transcriptional regulators and the function of the RAI2 protein is most likely also associated with transcriptional regulation.

To test whether the decreased motility and invasiveness induced by forced RAI2 protein expression (Fig. 5E) is dependent on interaction with CtBP proteins, we overexpressed the RAI2-4A mutant and wild-type RAI2 proteins by retroviral transduction in MDA-MB-231 cells. Western blot analysis showed equivalent expression of both the wild-type and 4A-mutant proteins. Interestingly, overexpression of RA2-4A in this cell line resulted in downregulation of CtBP2 expression, whereas CtBP1 expression was unaffected (Fig. 6G). Migration analysis by Boyden chamber assays further demonstrated that the migration-inhibitory activity of the RAI2 protein was partially abolished when CtBP binding was depleted, indicating that the RA2 migratory effect is partially dependent on interaction with CtBP proteins (Fig. 6G).

To better understand the underlying molecular changes, we performed whole-genome expression profiling of all three cell lines. Under stringent conditions, we identified 23 genes whose expression is significantly deregulated in MDA-MB-231 cells overexpressing the RA2 protein in comparison with vector transduced cells (Supplementary Fig. S7). Twenty of those genes were found to be differentially expressed only in cells overexpressing the wild-type RA2 protein and not in cells overexpressing the CtBP binding–deficient RA2 mutant (Supplementary Fig. S7). On the other hand, only three genes, including RA2, were concordantly found to be differentially expressed in both cell lines (Supplementary Fig. S7). Subsequent pathway analysis of the differentially expressed genes showed that the tyrosine kinase receptor-α (P: 5.41E−05; BY-value: 0.246) and the integrin-2 pathways (P: 0.00015; BY-value: 0.352) were activated in MDA-MB-231 cells overexpressing RAI2 (Supplementary Fig. S7). Among those DTC-associated genes, RAI2 was the only gene associated with OS in all of the tested cancer datasets, suggesting an important role for this gene in metastasis. We found no association between RA2 mRNA expression and lymph node metastasis. Thus, RA2 expression seems to specifically affect hematogenous dissemination of tumor cells, which is consistent with the concept that lymphatic and hematogenous dissemination are governed by different sets of genes in breast cancer (21, 22).

Thus far, RA2 represents a virtually uncharacterized protein. The RA2 gene is located at Xp22.3 and contains a single coding exon for a 530 amino acid protein with a high degree of orthologic conservation. Except for a proline-rich domain that lies between amino acids 200 and 268 and which is hypothesized to function in protein binding (23), the RA2 protein sequence does not share any known protein domains with other proteins. Thus, RA2 represents a unique protein. Thus far, RA2 expression has been associated with neural differentiation (12), and RA2 transcripts have been detected in different human adult and fetal tissues (23).

Our expression analysis of breast tumors and breast cancer cell lines showed that RA2 transcript and protein expression was significantly increased in ERα-positive, luminal breast tumors and cell lines compared with basal or HER2 overexpressing tumors. Furthermore, we showed that increased RA2 expression was also associated with well-differentiated breast tumors and correlated with the expression of good prognostic factors such as low tumor grade. These results suggest that RA2 loss is a common characteristic of primary and cultured tumor cells that have acquired an aggressive phenotype, strengthening the hypothesis that RA2 downregulation might be functionally important. RA2 silencing led to reduced expression of the ERα receptor in luminal breast cancer cell lines. Although the exact meaning and molecular mechanism behind this observation remains to be elucidated, Gattelli and colleagues (24, 25) have reported that integration of viral mouse mammary tumor virus (MMTV) into the RA2 locus is associated with the emergence of recurrent and hormone-independent breast tumors, emphasizing a particular role for RA2 deletion in the progression from hormone-dependent to hormone-independent breast tumors. Concordantly, we found that RA2 protein expression is downregulated upon hormone depletion and upregulated in the course of pharmacologic reduction of ERα, further indicating that RA2 might be an active part in the transcriptional network of hormonal responses in breast cancer.

Silencing RA2 in luminal breast cancer cell lines induced morphologic changes together with the induction of mesenchymal marker expression and a more aggressive phenotype characterized by increased cell migration and invasiveness, indicating that loss of RA2 function induces epithelial-to-mesenchymal plasticity. In addition to the observed reduction in ERα protein expression, we found markedly reduced protein expression of the GATA3, FOXA1, and GRHL2 transcription factors, which are pivotal for determining the epithelial differentiation of breast cancer cells (14–16, 26–28). Importantly, mere loss of the transcription factor GATA3 has been shown to actively drive dedifferentiation and marks the onset of tumor dissemination and metastasis formation in different breast cancer models (29, 30). Because of the well-established association between metastatic progression and loss of differentiation (6–8, 16), this appears to be a plausible explanation for the correlation between the diminished RA2 expression in early-stage primary tumors and the presence of DTCs in the bone marrow. Also, treatment with ATRA, a regulator of differentiation, in both normal and tumor cells led to the induction of RA2. Our findings thus provide evidence that RA2 is a bona fide determinant of differentiation in breast cancer; therefore, loss of RA2 expression might represent a key event for early steps of the metastatic cascade by promoting dedifferentiation, cellular plasticity, and thus tumor cell dissemination. On the other hand, tyrosine kinase receptor-α and the integrin-2 pathways were the most significantly deregulated pathways in nonluminal MDA-MB-231 cell lines.
cells overexpressing RAI2, suggesting that in this cell line cellular adhesion and/or signal integration and not mesenchymal-to-epithelial transition might be important for the observed phenotypic changes. We therefore conclude that it is most likely that the RAI2 protein exhibits cell type–specific functions beyond the regulation of differentiation.

Our primary sequence analysis of the RAI2 amino acid sequence revealed a nonconsensus bipartite CtBP-interaction domain, which was shown to mediate RAI2 binding to the CtBP transcriptional repressor protein. Such nonconsensus bipartite CtBP-interaction domains are also found in some other, mostly viral, proteins, creating efficient binding to CtBP proteins (31). Binding of the adenoviral E1A protein to CtBP via PXDLS motifs has been reported to negatively modulate transformation, tumorigenicity, and metastasis in cell line models (32). Here, we have provided evidence that the inhibitory effects of the RAI2 protein on cellular motility are dependent on interaction between RAI2 and CtBP proteins and most of the RAI2-induced gene expression changes that occurred in MDA-MB-231 cells were not seen in cells overexpressing the CtBP binding-deficient RAI2 protein. CtBP1 and CtBP2 are closely related, evolutionarily conserved transcriptional corepressors functionally linked to tumorigenesis and tumor progression by promoting EMT and mediating the repression of several TSGs (19). It was recently shown that among the diverse direct transcriptional targets of CtBP in breast cancer cell lines are the epithelial-specific transcription factors GATA3, FOXA1, and GRHL2 (18). Moreover, CtBP proteins control epithelial-specific gene expression in different cell types (33, 34), indicating that these factors are acting as ubiquitous regulators of epithelial differentiation. Because we found a significant correlation of high RAI2 gene expression with prolonged survival not only in breast cancer but also in lung, colon, and ovarian cancers, the molecular interaction of RAI2 protein and CtBP factors might be important for maintaining epithelial traits in general. Clearly as a putative transcriptional regulator, RAI2 is envisaged to be able to regulate different cellular processes, some of which might partially be cell type–specific, in a similar way as the interaction partner CtBP.

Another interesting finding is that loss of CtBP protein expression is observed in both RAI2-silenced luminal breast cancer cells and in MDA-MD-231 cells overexpressing CtBP binding-deficient RAI2 protein. These results imply that RAI2 is directly involved in the regulation of CtBP expression. On the basis of the nuclear colocalization of RAI2 and CtBP, direct interaction with CtBP, and the overlap in regulated genes, we hypothesize that RAI2 might function as a transcriptional coregulator that sustains differentiation of breast epithelial cells by controlling the expression of several key regulators of breast epithelial integrity.

We also discovered in RAI2-depleted cells increased phosphorylation of AKT proteins at S473 and significant increase in cell viability in cells treated with MK-2206 or RAD001 (everolimus) that both target the AKT–mTOR pathway. Because RAD001 has already been approved in combination with exemestane for treating postmenopausal hormone-receptor–positive advanced breast cancer (35) and a clinical phase II trial of MK-2206 in treating patients with advanced breast cancer is ongoing (36), RAI2 might represent a predictive marker for response to AKT–mTOR targeted therapeutic strategies. Accordingly, RAI2 expression might be used in the future for optimizing patient selection and clinical benefit, respectively. Further studies are needed to analyze this clinical implication. Furthermore, different studies have identified the AKT pathway as a major source of survival signals for enabling latent DTCs and circulating tumor cells (CTC) to survive in the circulation and secondary organs (1, 37, 38). Interestingly, pAKT(S473) and AKT3-positive DTCs were detected in bone marrow samples from patients with lung cancer, and AKT1/ AKT3 regulated the proliferation and survival of these DTCs (39). It has been shown that the CtBP proteins, in addition to controlling epithelial gene expression, also modulate the cellular threshold for apoptotic responses (33, 34). These data lead to the hypothesis that RAI2 depletion is not only involved in the onset of dissemination but might also affect survival of DTCs in the bone marrow. Importantly, bone marrow represents a retinoic acid–rich microenvironment (40); also, retinoic acid signaling regulates differentiation and self-renewal of hematopoietic stem cells in the bone marrow (40, 41). Because DTCs may lodge in the hematopoietic stem cell niches in bone marrow (42), DTCs might be exposed to the same regulatory mechanisms and therefore the RAI2 protein might also be involved in the control of overt bone metastasis formation.

In summary, the results described here indicate that loss of RAI2 expression might represent a so far undiscovered key event at the onset of metastatic progression. Because of a swift recovery of RAI2 expression in the knockdown cell lines used here, their usage is thus restricted to \textit{in vitro} applications. Further studies, including molecular analysis of DTCs and CTCs, are certainly needed to dissect the exact role of RAI2 in individual steps of the metastasis formation. Understanding the biology of metastasis-suppressing proteins, such as RAI2, provides valuable mechanistic insights that may be translated to novel therapeutic strategies (10). Future studies will show whether the RAI2 protein is suitable to act as a druggable target or whether RAI2 expression might be used as a novel predictive marker.

**METHODS**

**Study Design**

To identify novel genetic lesions especially related to the onset of metastasis formation, we compared in this retrospective study whole-genome expression profiles of early metastasized primary breast tumors with nonmetastasized tumors using the presence of DTCs in the bone marrow as an indicator for early occurring metastasis. For gene-expression profiling, primary tumor samples were collected from 32 primary lymph node–negative, hormone receptor–positive untreated breast cancer patients. Patients underwent surgical resection at the University Medical Center, Hamburg-Eppendorf (UKE; Hamburg, Germany). Tumor samples were divided into two groups based on patient DTC status: (i) DTC-negative (n = 16) and (ii) DTC-positive (n = 16) samples, which were classified as cMO(i=16) according to tumor-node-metastasis (TNM) staging (43). The cases were matched for age, histology, and TNM status. The patient clinical data are summarized in Supplementary Table S1. For quantitative RT-PCR analysis, an additional set of 76 early-stage primary breast tumor samples from UKE and University Hospital Tübingen (Tübingen, Germany) were analyzed, including 36 DTC-positive and 40 DTC-negative cases (Supplementary Table S1). Fifteen of these patients overlapped with those used in the initial array experiments. This study received ethics review board approval, and sample donors gave informed written consent.
Bone Marrow Analysis

The procedures used for the isolation and immunocytochemical detection of tumor cells in the bone marrow have been described in detail (44). Bone marrow was aspirated from the upper iliac crest, and mononuclear cells isolated by density centrifugation were cyto-centrifuged onto glass slides (2 × 10⁶ cells/patient). DTCs were detected by immunocytochemical staining using the monoclonal antibody A45-B/B3 (Micromet). An isotype-matched, murine monoclonal antibody (MOPC 21, IgG1; Sigma-Aldrich) served as a negative control. Screening for CK-positive cells was performed in an automated fashion (ACIS system) using color-based imaging technology and microscopy to automatically scan and analyze immunohistochemically stained slides (45).

Gene Expression Profiling and In Silico Validation

The detailed procedure is provided in the Supplementary Methods. Microarray data of patients are available at ArrayExpress accession no. E-MTAB-5085. Authentication of cell lines used in functional analyses, 1 line, was conducted with short tandem repeat (STR) profiling to standards. Microarray data of MDA-MD-231 cells overexpressing RAI2 and CtBP binding–deficient mutant are available by GEO accession no. GSE 65489.

Quantitative Real-Time RT-PCR Analysis

qRT-PCR analysis of the patient samples was performed with 150 ng of total RNA isolated by the RNeasy Micro Kit as described above. The RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Thermo Scientific), and the mRNA quantity was estimated by fluorescence qPCR based on SYBR Green and random hexamers using the following primer sequences: RAI2-F: CGGTCATTAAGATGCGGAAGTGAG, RAI2-R: GAGGCTCGGATTTCCGGTG, RPLP0-F: ACCACGCCTGAGAAAGACTG, and RPLP0-R: TGAGGTCCTCCTTGGTGAAACA. The qRT-PCR reactions were run in triplicate and performed using the Mastercycler Eppendorf Realplex thermal cycler. Data were analyzed by applying the ΔΔCT method using RPLP0 expression for normalization. The results, expressed as fold changes, were set for normalization. The results, expressed as fold changes, were set in relation to the parental cell lines. Microarray data of MDA-MD-231 cells overexpressing RAI2 and CtBP binding–deficient mutant are available by GEO accession no. GSE 65489.

Cell Culture

MDA-MD-231 and SK-BR-3 cells were obtained from the ATCC. BT-549, BT-474, T-47D, and MDA-MD-468 cells were obtained from Cell Lines Service. MCF-7 and Phoenix amphoteric retroviral packaging cells were a kind gift from Dr. Volker Assmann (UKE). Authentication of data and use in functional assays was conducted with random tandem repeat (STR) profiling to exclude cross-contamination between the cell lines. Authentication was last confirmed on February 12, 2014. Cells were grown as monolayers according to standard conditions in either DMEM or RPMI supplemented with 10% fetal calf serum (FCS; PAA) and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 10% CO₂ or 5% CO₂, respectively. MCF-10A cells were cultured in 1:1 DMEM/F12 medium supplemented with 5% horse serum, 100 ng/mL cholera toxin, 20 ng/mL EGF, 500 ng/mL hydrocortisone, and 10 μg/mL insulin. For induction of EMT, cells were stimulated with 10 ng/mL TGFβ1 (R&D Systems) for 48 hours. For hormonal depletion, cells were cultured in medium containing charcoal-stripped instead of normal FCS for 5 days. Treatment with either 1 μmol/L ATRA or 100 nmol/L JC1182,780 was done by adding drugs to standard medium for 5 days. Analysis of cellular viability was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Five thousand cells of the analyzed cell line were plated in quadruplicate and treated with either MK-2206 or RAD001 for 72 hours. Relative viability was calculated by determining absorbance at 570 nm and normalization to carrier treated cells.

3D Culture

The detailed procedure was previously described (46) and is provided in the Supplementary Methods.

RAI2 Plasmid Construction and Viral Transduction

The RAI2 coding sequence was RT-PCR amplified from Hs578t cells with the oligonucleotides 5′-CAAGTGCGATCAGAAGGAGG-3′ and 5′-CTTCTTGGAAAAAAGGTGGGCCGC-3′ using Phi2 DNA polymerase (Agilent Technologies), and it was inserted into the pCMV3 expression plasmid (Genelantis) using EcoRI and KpnI restrictions sites. To generate a RAI2 protein incapable of CtBP binding, site-directed mutagenesis was performed with the oligonucleotides 5′-CGAGGGCCCCTGGATGCAGCCGAGTCGTC-3′ and 5′-GACATGCTTCTGATGGGCGGCACCCAG-3′, and it was inserted into the phCMV3 expression plasmid (Genelantis) using EcoRI and KpnI restrictions sites. To generate a RAI2 protein incapable of CtBP binding, site-directed mutagenesis was performed with the oligonucleotides 5′-CGAGGGCCCCTGGATGCAGCCGAGTCGTC-3′ and 5′-GACATGCTTCTGATGGGCGGCACCCAG-3′, and it was inserted into the phCMV3 expression plasmid (Genelantis) using EcoRI and KpnI restrictions sites. To generate a RAI2 protein incapable of CtBP binding, site-directed mutagenesis was performed with the oligonucleotides 5′-CGAGGGCCCCTGGATGCAGCCGAGTCGTC-3′ and 5′-GACATGCTTCTGATGGGCGGCACCCAG-3′, and it was inserted into the phCMV3 expression plasmid (Genelantis) using EcoRI and KpnI restrictions sites. To generate a RAI2 protein incapable of CtBP binding, site-directed mutagenesis was performed with the oligonucleotides 5′-CGAGGGCCCCTGGATGCAGCCGAGTCGTC-3′ and 5′-GACATGCTTCTGATGGGCGGCACCCAG-3′, and it was inserted into the phCMV3 expression plasmid (Genelantis) using EcoRI and KpnI restrictions sites.

shRNA-Mediated Knockdown of Gene Expression

Lentiviral pLKO.1.sh vectors targeted against human RAI2 (shRNA1 TRCN0000139927 and shRNA2 TRCN0000441623) were obtained from the RNAi Consortium. A pLKO.1 vector harboring a scrambled nontargeting shRNA sequence served as a negative control. Lentiviral supernatants were generated by transfecting HEK 293T cells with these plasmids by using a three-plasmid packaging system according to standard protocols. Supernatants were harvested, sterile filtered, and then used in the presence of 8 μg/mL of polybrene to infect target cells. Transduced cells were selected by puromycin. Because of the recovery of RAI2 expression, all experiments were performed within 8 to 14 days after the initial transduction.

Immunoprecipitation

HEK 293T cells were transfected with 4 μg of phCMV3 expression plasmids containing either the wild-type or mutated RAI2 cDNA sequence using Lipofectamine 2000 (Life Technology). For transfection, 500 μL of viral supernatant was added to 50% confluent recipient cultures in 6-well plates containing 1 mL DMEM plus 10% FCS. Positive selection was begun 24 hours after transduction using 2 μg/mL of puromycin containing medium. Cells were subsequently maintained under puromycin selection for 4 days.
Western Blotting
Whole-cell extracts from cultured cells were prepared by direct lysis and sonication of cells in 2% SDS sample buffer containing phosphatase and protease inhibitors. Cell extracts were separated in denaturing 8% or 10% polyacrylamide gels and blotted onto a nitrocellulose membrane. Detection of proteins was performed by incubation with the following specific antibodies: ERα (Cell Signaling Technology; D8F8B), FOXA1 (Abcam; 2F83), GATA3 (Cell Signaling Technology; D153C9), HSC70 (Santa Cruz Biotechnology; B-6), HA (Sigma-Aldrich; H6908), pan-AKT (Cell Signaling Technology; 11E7), phospho-AKT Ser473 (Cell Signaling Technology; D9E), E-cadherin (BD; 36), CtBP1 (BD; 3), CtBP2 (BD; 16), and vimentin (Cell Signaling Technology; R28). Detection of the GRHL2 protein was performed with a custom-made antibody (27). A protoplyc, polyclonal RAI2 antibody recognizing a C-terminal epitope (Cell Signaling Technology; BL12173/M11-7847) was used to detect the RAI2 protein. In addition, a second custom-made polyclonal antibody directed against the RAI2 protein was generated. The peptide EKDELKFPDIQLQPKQFYQ, corresponding to a central region of human RAI2 protein, was synthesized and coupled to keyhole limpet hemocyanin and then injected into rabbits. RAI2-specific antibodies were isolated by immunoaffinity purification using the corresponding immunizing peptide coupled to a solid support (Thermo Fisher Scientific).

Migration and Invasions Assays
For Transwell migration assays, 5 × 10⁴ MDA-MB-231 or 10⁵ MCF-7 and CAMA-1 cells were plated in serum-free DMEM media in the upper chambers of BD Cell Culture Inserts for 24-well plates with 8.0-µm pores (BD Falcon). In the lower chamber, DMEM containing 10% FCS was used as a chemotactic agent. Plates were incubated at 37°C under standard conditions, and migration was allowed to proceed for 24 hours. Nonmigrated cells in the upper chambers were removed with cotton swabs, and the remaining cells were fixed in 4% paraformaldehyde and stained with crystal violet. Four fields (MDA-MB-231) or whole filters (MCF-7 and CAMA-1) were counted under the microscope. For invasion assays, BD BioCoat Matrigel Invasion Chambers (BD Falcon) were used. A total of 5 × 10⁴ of MDA-MB-231 or 10⁵ MCF-7 and CAMA-1 cells were plated in serum-free DMEM media in upper chambers, and DMEM-containing 10% FCS was used as a chemotactic agent. Invasion was allowed to proceed for 24 (MDA-MB-231) or 48 hours (MCF-7 and CAMA-1). Assessment of invaded cells was performed as described above.

Immunofluorescence Staining
Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, washed three times with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. After incubation with PBS containing 1% BSA for 30 minutes, the cells were further incubated with primary antibodies diluted with 1% BSA for 1 hour. After three washes with PBS, secondary antibody binding was visualized with fluorochrome-conjugated anti-mouse IgG or anti-rabbit IgG (Dako) diluted with 1% BSA. After three washes with PBS, nuclei were stained with DAPI, and F-actin was stained using Rhodamine Phalloidin (Cytoskeleton) mounted in Mowiol (Sigma-Aldrich) according to the manufacturer’s instructions.

Protein Purification and Fluorescence Anisotropy
The detailed procedure is provided in the Supplementary Methods.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Suppression of Early Hematogenous Dissemination of Human Breast Cancer Cells to Bone Marrow by Retinoic Acid–Induced 2

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