Identification of a pharmacologically tractable Fra-1/ADORA2B axis promoting breast cancer metastasis

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Metastasis confronts clinicians with two major challenges: estimating the patient's risk of metastasis and identifying therapeutic targets. Because they are key signal integrators connecting cellular processes to clinical outcome, we aimed to identify transcriptional nodes regulating cancer cell metastasis. Using rodent xenograft models that we previously developed, we identified the transcription factor Fos-related antigen-1 (Fra-1) as a key coordinator of metastasis. Because Fra-1 often is overexpressed in human metastatic breast cancers and has been shown to control their invasive potential in vitro, we aimed to assess the implication and prognostic significance of the Fra-1–dependent genetic program in breast cancer metastasis and to identify potential Fra-1–dependent therapeutic targets. In several in vivo assays in mice, we demonstrate that stable RNAi depletion of Fra-1 from human breast cancer cells strongly suppresses their ability to metastasize. These results support a clinically important role for Fra-1 and the genetic program it controls. We show that a Fra-1–dependent gene-expression signature accurately predicts recurrence of breast cancer. Furthermore, a synthetic lethal drug screen revealed that antagonists of the adenosine receptor A_{2B} (ADORA2B) are preferentially toxic to breast tumor cells expressing Fra-1. Both RNAi silencing and pharmacologic blockade of ADORA2B inhibited filopodia formation and invasive activity of breast cancer cells and correspondingly reduced tumor outgrowth in the lungs. These data show that Fra-1 activity is causally involved in and is a prognostic indicator of breast cancer metastasis. They suggest that Fra-1 activity predicts responsiveness to inhibition of pharmacologically tractable targets, such as ADORA2B, which may be used for clinical interference of metastatic breast cancer.

epithelial-mesenchymal transition | invasion

The path toward improved management of metastatic tumors, the major cause of death among cancer patients, involves tackling of two major challenges: the development of therapies that combat the patient's metastatic disease and of means of reliably assessing the individual patient's risk of developing metastasis. In line with the second objective, patient stratification has received increasing attention as a way to improve the therapeutic management of cancer patients. In recent years, for example, gene-expression profiling of primary breast cancer has uncovered gene signatures that assist clinicians in classifying breast cancer subtypes more accurately (1, 2) and that provide predictions of tumor recurrence and clinical outcome (3–5). Such classifiers have proven useful for formulating prognosis and adjusting therapeutic management of breast cancer patients, complementing conventional histopathological criteria such as tumor size, nodal status, and estrogen receptor (ER) status (6–8) to predict clinical outcome better.

The most important challenge posed by metastatic cancer, blocking metastatic spread, has proven more troublesome. Indeed, most current anticancer treatments have been developed to treat primary disease; they often fail in blocking metastasis. Targeted therapies for metastatic breast cancer are emerging slowly but thus far have yielded mixed clinical results. Because metastasis accounts for up to 90% of cancer mortality, there is an undeniable need for novel therapies efficiently targeting metastatic cancer.

Metastatic spread of carcinoma cells is a highly complex process in which tumor cells must overcome a series of sequential rate-limiting steps often called the "metastatic cascade." First, malignant cells disseminate from the primary site and invade the neighboring tissue. Subsequently, they intravasate into blood or lymphatic vessels, survive in the circulation, and lodge and grow out at distant organs (9, 10). Several of the steps of the metastatic cascade require the acquisition of increased cell motility, which often coincides with disruption of the normal epithelial organization (11). Although it adds to the complexity of the problem, one might argue that the multistep process of metastasis also allows therapeutic interference at several levels in the cascade. Therefore, the identification of critical regulators of metastatic activity is key to improved clinical management of metastatic cancer.

In this study, we used rodent model systems to search for mediators of metastasis, ideally factors that link metastasis biology to prognosis, aiming to address simultaneously the two major challenges in breast cancer described above. Because transcription factors are key integrators of signaling pathways that connect biological characteristics to clinical outcome (12), we focused our attention on this class of proteins.

Results

Gene-Expression Profiling and Functional Perturbation in a Metastasis Model System Identify Fra-1 as an Essential Metastasis Gene. To identify transcription factors regulating metastasis, we used as model systems rat intestinal epithelial (RIE-1) and rat kidney epithelial (RK3E) cells engineered to express ectopically the neurotrophic receptor tyrosine kinase TrkB and its ligand brain-derived neurotrophic factor (BDNF) (hereafter called "RIE^{TB}" and "$RK3E^{TB}$," cells, respectively). Active TrkB transforms both types of epithelial, anoikis-sensitive, non-oncogenic cells into mesenchymal, anoikis-resistant, tumorigenic, and highly metastatic cells (13, 14). Microarray gene-expression profiling of both RIE^{TB}

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and RK3E^{TB} cells identified Fra-1 [encoded by the *FOS-like antigen 1* (*FOSL1*) gene] as the most up-regulated transcription factor in both model systems (Fig. 1*A*). Fra-1 was up-regulated up to 50-fold by active TrkB at the transcriptional level, and Fra-1 protein levels also were sharply induced (*SI Appendix*, Fig. S1*A*), conceivably because of TrkB-mediated activation of RAS signaling (15, 16). Fra-1 participates in various transcription factor complexes, including activator protein 1 (AP-1) (17). Accordingly, gel-shift experiments revealed that TrkB activity converted Fra-1 into a major component of AP-1 DNA-binding complexes (*SI Appendix*, Fig. S1*B*).

In vitro, Fra-1 has been shown to be involved in the migratory or invasive capabilities of various cancer cell lines (18–20). Therefore it has long been suspected that Fra-1 may play an important role in metastasis (21). In vivo, however, experimental evidence supporting a role for Fra-1 in promoting metastasis formation has been limited thus far to overexpression studies in a lung tumor cell line (22). Hence, the importance of previous studies notwithstanding, whether Fra-1 is essential for metastasis, a process that cannot be recapitulated in the in vitro motility and invasive studies, has remained unclear. This knowledge is an essential prerequisite for answering the next important question: Whether Fra-1 activity can be exploited for predicting prognosis and/or for targeted treatment of metastatic cancer. To address the functional relevance of Fra-1 in metastasis, we depleted it from RK3E^{TB} clonal cell populations using retroviral vectors encoding independent shRNAs, reducing its levels to those seen in parental cells (Fig. 1*B*). Upon s.c. inoculation into athymic nude mice, Fra-1–depleted RK3E^{TB} cells produced tumors that expanded with kinetics indistinguishable from those of control tumors (*SI Appendix*, Fig. S2). However, in sharp contrast to animals that had received control tumor cells, mice injected with Fra-1–silenced tumor cells did not develop visible lung metastases (Fig. 1 *C* and *D*).

Fra-1 silencing completely also reversed the induction by TrkB of a spindle-cell fibroblast-like phenotype, and cells readopted a typical epithelial cobble-stone morphology with extensive cell–cell junctions (*SI Appendix*, Fig. S3 *A* and *B*). This reversal was observed for both cell clones and polyclonal cell pools (*SI Appendix*, Fig. S4 *A* and *B*). An RNAi-resistant *FOSL1* allele reverted these properties (*SI Appendix*, Fig. S4 *C* and *D*). Taken together, these results all strongly argue against an RNAi off-target effect. Fra-1 depletion also restored the expression and correct subcellular localization of E-cadherin (Fig. 1*B* and *SI Appendix*, Fig. S3*B*), and this restoration was paralleled by cancellation of both the migratory and invasive properties of TrkB-expressing tumor cells (*SI Appendix*, Fig. S3*C*).

These changes were reminiscent of a reversion of an epithelial–mesenchymal transition (EMT)-like phenotype. We have demonstrated previously that the critical EMT regulators Twist, Snail, and zinc finger E-box binding homeobox 1 (Zeb1) act in a network essential for driving the EMT-like transformation of RK3E^{TB} cells, in which they control a common gene-expression signature comprising 52 genes (*SI Appendix*, Fig. S5*A*) (23, 24). Interestingly, 40 (77%) of these EMT signature genes were also regulated by Fra-1, an enrichment that was not random ($P < 0.001$, Fisher's exact test; *SI Appendix*, Fig. S5 *B* and *C*). This result is consistent with the regulation by Fra-1 of EMT properties and suggests an important contribution of this program to Fra-1–dependent metastasis.

Suppression of Fra-1 Abrogates the Metastatic Potential of Human Breast Cancer Cells and Restores Epithelial Characteristics. Fra-1 frequently is overexpressed in human solid tumors as well as in many cell lines derived from such tumors (20, 21, 25–28). To investigate whether Fra-1 has a more general role in metastasis, we focused on human breast cancer, in which several lines of evidence suggest a role for Fra-1 in metastatic disease. Indeed, Fra-1 commonly is overexpressed in human breast cancer and derived cell lines, including the triple [ER/ progesterone receptor/ human epidermal growth factor receptor 2 (Her2)]-negative and metastatic cell line MDA-MB-231 (*SI Appendix*, Fig. S6*A*), and its expression has been shown to correlate with, and regulate, the cell lines' invasive potential in vitro (19, 21, 25, 27).

We stably depleted *FOSL1* mRNA from LM2 cells, an MDA-MB-231–derived cell line that has a high proclivity to metastasize to the lungs of mice (29) (Fig. 2*A*). Noninvasive in vivo bioluminescence imaging revealed that Fra-1–depleted cells were more than 1,000-fold less successful than parental breast cancer cells in developing experimental pulmonary metastases (Fig. 2 *B* and *C*), allowing the mice to survive almost twice as long (Fig. 2*D*). Immunohistochemical analysis revealed that the lung macrometastases that developed in mice inoculated with Fra-1–depleted cells at very late time points had regained Fra-1 expression (*SI Appendix*, Fig. S7), confirming the need for Fra-1 for metastatic activity. A similar requirement for Fra-1 in experimental metastasis was observed when 10^6 or 10^5 LM2 cells or 10^6 parental MDA-MB-231 cells were inoculated into mice (*SI Appendix*, Fig. S6 *B*–*G*).

Analysis of lung sections 5 wk after mice had been inoculated with tumor cells revealed that although parental LM2 cells had seeded successfully throughout the lungs, the Fra-1–depleted counterparts had failed to do so but had not extinguished. They were still present and were confined to the lung alveolar septum (Fig. 2*E*). In contrast to control cells, they did not interrupt the preexisting lung structure and failed to form multiple nodular tumor masses in the lung parenchyma, an observation typical of nonaggressive tumor cells.

To analyze whether Fra-1 also is required for metastasis of breast cancer cells to other organs than the lungs, control and Fra-1–depleted MDA-MB-231 cells were injected into the left cardiac ventricle. Mice inoculated with control cells developed metastases to various organs, including the adrenal glands and bones and in lymph nodes. In contrast, Fra-1 depletion strongly reduced metastasis formation and increased the survival of recipient mice (*SI Appendix*, Fig. S8 *A*–*D*), demonstrating that Fra-1 is essential for the seeding of breast tumor cells to multiple sites.

Because in this experimental metastasis system i.v. inoculation bypasses the need for tumor cells to invade and intravasate, we recapitulated these results in an orthotopic model in which GFP-

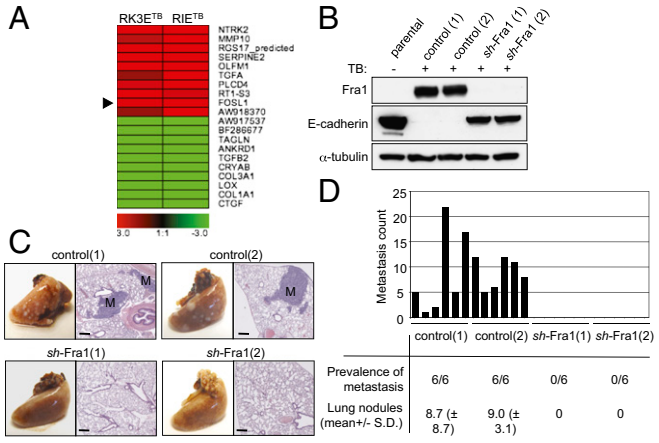

Fig. 1. Gene-expression profiling of a metastasis model system identifies Fra-1 as a candidate metastasis gene. (*A*) Microarray gene-expression analysis of RK3E^{TB} and RIE^{TB} cells. The top 10 genes that are up- or down-regulated in both cell systems are shown in a heat map. (*B*) Fra-1 and E-cadherin expression levels measured by Western blotting in RK3E^{TB} cells expressing independent shRNAs targeting Fra-1 as indicated. α-Tubulin serves as loading control. (*C*) Representative images of macroscopic pulmonary metastases and hematoxylin-eosin staining of histological lung sections from mice injected s.c. with control or Fra-1–depleted RK3E^{TB} cells analyzed 3 wk postinoculation. (Scale bars: 200 μm.) M, metastasis. (*D*) Macroscopic quantification of pulmonary metastases in mice described in *C*. Data in *B*–*D* are representative of three independent experiments.

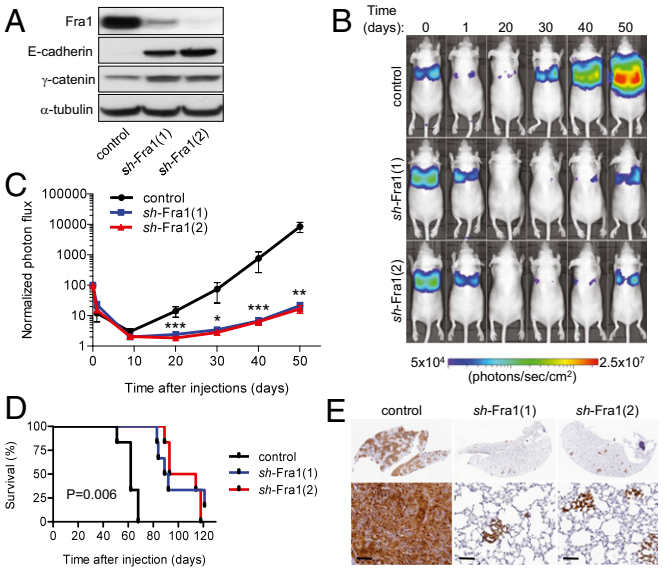

Fig. 2. Suppression of Fra-1 abrogates the metastatic potential of human breast cancer cells and restores epithelial characteristics. (*A*) Expression levels of Fra-1 and epithelial proteins in LM2 cells as a function of Fra-1 depletion measured by Western blotting. α-Tubulin serves as loading control. (*B*) Representative bioluminescence images of mice injected i.v. with 2×10^5 LM2 cells expressing a control or sh-Fra1 vector at different time points as indicted. (*C*) Quantification of the luminescence signal in the lungs of mice described in *B* as a function of time. $n = 6$. Error bars indicate SE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. control based on a two-tailed Wilcoxon signed-rank test. (*D*) Kaplan–Meier curves for the survival of the mice described in *B* and *C*. Mice were killed when clinical symptoms became apparent. Displayed *P* value is based on a log-rank test. (*E*) Immunohistochemical analysis of human vimentin expression (which was retained by Fra-1–depleted cells) identifying metastases formed in the lungs of recipient mice by control or Fra-1–depleted LM2 cells. (*Upper*) Composite image of the entire left lung. (*Lower*) Image taken at 20× magnification. (Scale bars: 100 μm.) Data are representative of two (*B–E*) or three (*A*) independent experiments.

labeled LM2 cells were introduced into the inguinal mammary fat pad of mice. Control tumor cells developed primary tumors that metastasized to the lungs in most of the animals (*SI Appendix*, Fig. S8 *E* and *F*). In contrast, Fra-1–depleted cells developed tumors that grew more slowly (*SI Appendix*, Fig. S8*G*) and, again, were unable to develop detectable lung metastases (*SI Appendix*, Fig. S8 *E* and *F*).

Consistent with our observations in the TrkB metastasis model system, Fra-1 depletion also restored E-cadherin expression and localization in LM2 and MDA-MB-231 human breast cancer cells (Fig. 2*A* and *SI Appendix*, Fig. S9*A*). Extending previous observations in non-oncogenic breast epithelial cells (30), we found that induction of E-cadherin upon treatment of breast cancer cells with a MAP kinase-ERK kinase (MEK) inhibitor was dependent on Fra-1 (*SI Appendix*, Fig. S9 *B* and *C*).

Fra1-Associated Gene-Expression Profile Accurately Predicts Clinical Outcome of Human Breast Cancer. We next aimed to determine whether Fra-1 activity has prognostic value in estimating breast cancer recurrence. Immunohistochemical assessment of Fra-1 expression, although consistently reporting higher Fra-1 expression in neoplastic lesions, has delivered ambiguous results regarding its prognostic value (25–27). This ambiguity might result either from the technical limitations of current detection techniques or from the extensive regulation of Fra-1 by posttranslational modifications, so that its expression may not strictly correlate with its activity. We thus asked instead whether the Fra-1–associated transcriptome has prognostic power for breast cancer recurrence. Indeed, it has been suggested that in a data-driven approach to finding connections between gene-expression patterns and tumor behavior, the target genes of a given transcription factor often represent better biomarkers than the transcription factor itself (31).

To uncover the Fra-1–dependent transcriptome of metastatic breast cancer cells, we compared the gene-expression profiles of Fra-1–depleted LM2 cells with those of control cells by microarray analysis (Fig. 3*A*). The resulting set of 1,234 probes to genes identified as being highly significantly up- or down-regulated by both Fra-1 shRNAs, referred to herein as the "Fra-1 transcriptome," showed prognostic value on publicly available patient datasets of breast cancers (Fig. 3*B* and *SI Appendix, Methods*). This result demonstrated that the Fra-1–dependent transcriptome has prognostic potential in human breast cancer. We next aimed to narrow this list to obtain a smaller set of genes more amenable to translation into a diagnostic test that also eventually might help identify the downstream Fra-1 targets most relevant to metastases in humans. Probes in the Fra-1–dependent transcriptome showing prognostic value in a cohort of 509 patients were used to generate an Fra-1–dependent gene-expression signature comprising 183 probes, which we called the "Fra-1 classifier" (Fig. 3*A*, *SI Appendix, Methods*, and *SI Appendix*, Table S1). Importantly, a set of this size could not have been generated by selecting probes associated with outcome from a randomly selected set of 1,234 probes (hypergeometric test, $P < 1 \times 10^{-6}$).

The Fra-1 centroid classifier was validated on publicly available patient datasets of breast cancers (*SI Appendix*, Fig. S10), and both the Fra-1 classifier and the Fra-1 transcriptome were compared with other published gene-expression classifiers (Fig. 3*B* and *SI Appendix, Methods*). The analyses indicate that the Fra-1 transcriptome and the Fra-1 classifier can predict the clinical outcome of breast cancer patients with accuracy at least

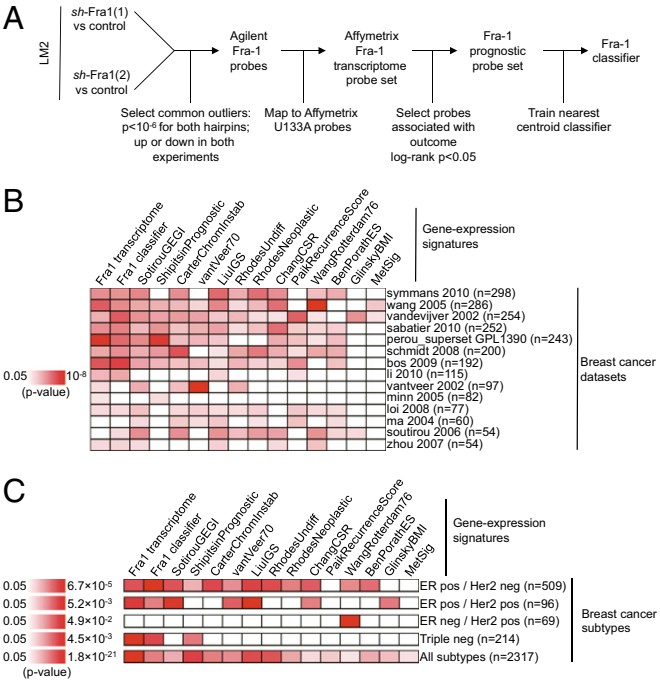

Fig. 3. A Fra-1–associated gene-expression profile accurately predicts clinical outcome of human breast cancer. (*A*) Outline of the procedure used to identify a Fra-1–dependent transcriptome signature in LM2 cells and to derive a prognostic Fra-1 classifier. (*B*) A heat map representing one-sided Cox proportional hazards model *P* values for time to distant metastasis (if available) or relapse on breast cancer datasets not used to train the Fra-1 classifier (*SI Appendix, Methods*) for the indicated gene-expression signatures. (*C*) A heat map representing one-sided log-rank *P* values between signature-high samples and signature-low samples for time to distant metastasis (if available) or relapse on samples of indicated breast cancer subtypes from datasets not used to train the Fra-1 classifier (*SI Appendix, Methods*).

similar to that of other signatures including those currently used in the clinic.

To analyze the prognostic power of the Fra-1 transcriptome and the Fra-1 classifier specifically in different breast cancer subtypes, samples from publicly available datasets were stratified according to their molecular subtype (Fig. 3*C*, *SI Appendix, Methods*, and *SI Appendix*, Fig. S10). We observed that the Fra-1 transcriptome and the Fra-1 classifier could predict the clinical outcome of ER-positive and/or Her2-positive patients with accuracy similar to that of other signatures. Moreover, the precision of the Fra-1 transcriptome and the Fra-1 classifier was slightly better than that of the other signatures in predicting clinical outcome of triple-negative breast cancers.

Adenosine Receptor A_{2B} Is a Pharmacologically Tractable Fra-1 Target Gene in Breast Cancer Metastasis. Together, these observations predict that it may be beneficial to interfere with Fra-1 function in breast cancer. Currently, however, Fra-1 is not readily amenable to pharmacologic inhibition by small molecules. Therefore, we designed a high-throughput pharmacological "synthetic lethality" screen to identify molecules that display selective cytotoxicity to metastasizing Fra-1–expressing (relative to Fra-1–depleted non-metastatic) breast cancer cells. We screened a library of 1,280 clinically active compounds with known biological activity (Fig. 4*A*). Of 144 compounds that are cytotoxic to MDA-MB-231 cells, we identified four that are significantly less toxic to Fra-1–depleted cells: the nonspecific DNA-damaging agents methotrexate and 5-fluorouracil (5-FU), and, more interestingly, two adenosine receptor antagonists (7-chloro-4-hydroxy-2-phenyl-1,8-naphthyridine and CGS-15953) (Fig. 4*B*). We validated the observations obtained with the two adenosine receptor antagonists in LM2 cells (Fig. 4*C*).

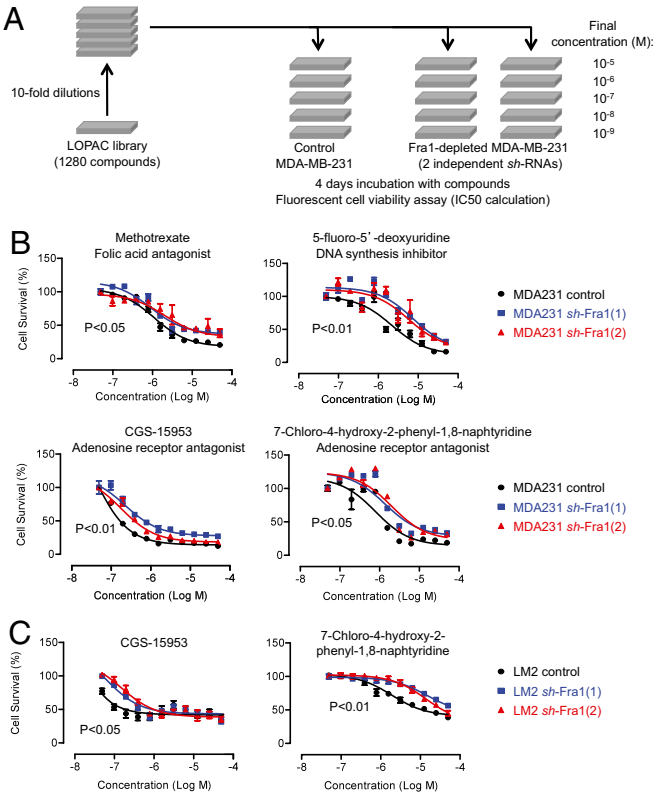

Fig. 4. A screen for small molecules identifies adenosine receptor inhibitors as compounds preferentially targeting Fra-1–expressing breast tumor cells. (*A*) Screen design and protocol. (*B*) Dose–response curves of control and Fra-1–depleted MDA-MB-231 cell lines treated with the indicated compounds. $n = 4$. Error bars indicate SD. (*C*) Dose–response curves of control and Fra-1–depleted LM2 cell lines treated with the indicated compounds. $n = 4$. Error bars indicate SD. The *P* values shown in *B* and *C* are for the two sh-Fra-1 groups vs. control based on a repeated measures ANOVA followed by Bonferroni's multiple comparison test. Data in *B* and *C* are representative of three independent experiments.

Four G protein-coupled adenosine receptors have been described, namely A_1, A_{2A}, A_{2B}, and A_3 (32). We focused our attention on adenosine receptor A_{2B} (ADORA2B) for three reasons. First, ADORA2B is expressed at higher levels in ER-negative breast cancer cell lines, especially in the highly tumorigenic and metastatic MDA-MB-231 and LM2 cell lines (*SI Appendix*, Fig. S11*A*) (33), whereas the other ADORA genes are expressed at low levels in these cell lines (*SI Appendix*, Fig. S11*B*). Second, in agreement with these results, *ADORA2B* mRNA levels generally are higher in tumors from triple-negative breast cancers than in other breast cancer subtypes (*SI Appendix*, Fig. S11*C*). Third, *ADORA2B* mRNA expression is highly correlated with *FOSL1* mRNA expression in breast cancer cell lines (*SI Appendix*, Fig. S12*A*) and is down-regulated significantly upon Fra-1 silencing in MDA-MB-231 and LM2 cells (*SI Appendix*, Fig. S12*B*). ChIP experiments corroborated these findings, indicating that Fra-1 binds to regulatory elements in the promoter and first intron of the *ADORA2B* gene in human breast cancer cells (*SI Appendix*, Fig. S12*C*).

In vivo bioluminescence imaging revealed that ADORA2B-depleted cells (*SI Appendix*, Fig. S13*A*) were significantly delayed in developing metastases (Fig. 5*A* and *SI Appendix*, Fig. S13 *A–D*). Similar to Fra-1, depletion of ADORA2B had a less pronounced effect on cell proliferation in culture and on primary tumor growth at orthotopic sites (*SI Appendix*, Fig. S13 *E* and *F*). Further, ADORA2B depletion significantly inhibited the migratory and invasive capacities of LM2 cells (Fig. 5*B*). This inhibition coincided with impaired formation of filopodia, membrane protrusions involved in the sensing of chemotactic cues, cell migration, and cell–cell contact (Fig. 5*C* and *SI Appendix*, Fig. S14*C*). In contrast, ADORA2B silencing failed to restore epithelial cell characteristics or E-cadherin expression (*SI Appendix*, Fig. S14 *A* and *B*). Taken together, these observations indicate that *ADORA2B* is a direct Fra-1 target gene whose product contributes to the metastatic capacity of breast cancer cells by regulating migration and invasion in an E-cadherin-independent fashion.

Unlike Fra-1, ADORA2B is amenable to pharmacological suppression. Although the adenosine receptor antagonists identified in our chemical screen cannot be used in vivo, the adenosine receptor antagonist theophylline already is used clinically as an inhibitor of adenosine monophosphate-induced bronchoconstriction in asthmatic patients (34). Theophylline recently has been shown to inhibit experimental metastasis of mouse melanoma cells in combination with the taxane antimitotic paclitaxel (35). We observed that theophylline potentiated the cytotoxic effect of paclitaxel on LM2 cells in vitro (*SI Appendix*, Fig. S15). Of note, like ADORA2B depletion, theophylline inhibited filopodia formation, invasion, and anchorage-independent growth (Fig. 5*C* and *SI Appendix*, Fig. S14 *C–E*). Thus, although we cannot exclude the possibility that theophylline also may have adenosine receptor-independent activities, these data strongly suggest that ADORA2B inhibition is an important activity of theophylline in the suppression of metastatic activity.

We subsequently addressed whether theophylline could potentiate the ability of the paclitaxel-related taxane docetaxel to inhibit the outgrowth of experimental breast cancer metastases in vivo. We observed that docetaxel and theophylline had a synergistic effect in reducing the ability of LM2 cells to produce tumors in the lungs, because docetaxel was up to sevenfold more effective upon cotreatment with theophylline (Fig. 5*D* and *SI Appendix*, Fig. S16*A*). Of note, no significant weight loss was observed in any group (*SI Appendix*, Fig. S16*B*). These results support the notion that pharmacological interference with the Fra-1 target gene product ADORA2B, when combined with a chemotherapeutic agent, suppresses breast cancer colonization in mice lungs.

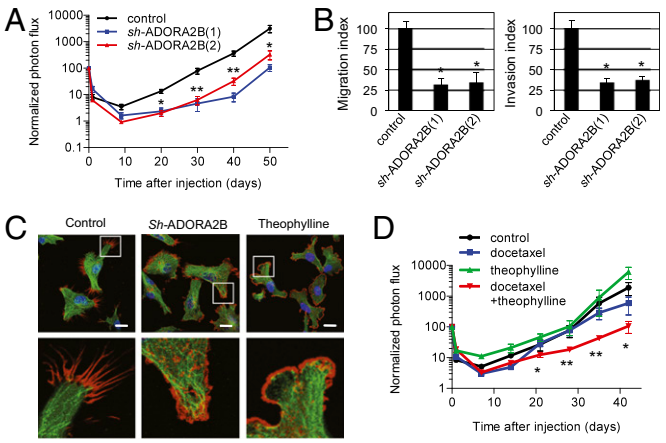

Fig. 5. ADORA2B is a Fra-1 target gene contributing to metastatic activity of breast cancer cells. (*A*) Quantification of the luminescence signal in the lungs of mice injected i.v. with 2×10^5 LM2 cells expressing a control or sh-ADORA2B vector at different time points as indicated. $n = 6$. Error bars indicate SE. $*P < 0.05$, $**P < 0.01$ for the two *sh*-ADORA2B groups vs. control based on a two-tailed Wilcoxon signed-rank test. (*B*) Migration (*Left*) and invasion (*Right*) capacities as a function of ADORA2B depletion. $n = 3$. Error bars indicate SD. $*P < 0.002$ vs. control based on a one-way ANOVA followed by a partial least-squares difference test. (*C*) Representative immunofluorescence imaging of filopodia in LM2 cells depleted for ADORA2B or treated with 100 μM theophylline. Control cells were treated with excipient. Merged images of DNA (blue), F-actin (red), and α-tubulin (green) staining are shown. Individual images are provided in *SI Appendix*, Fig. S14*C*. (Scale bars: 10 μm.). (*D*) Quantification of the luminescence signal in the lungs of mice injected i.v. with 2×10^5 LM2 cells and receiving the indicated treatment (docetaxel, 4 mg/kg i.p. weekly, and/or theophylline, 10 mM in drinking water) at different time points as indicated. $n = 6$. Error bars indicate SE. $*P < 0.05$, $**P < 0.005$, combined treatment vs. docetaxel-only group based on a two-tailed Wilcoxon signed-rank test. Data are representative of two (*A*–*C*) or three (*D*) independent experiments.

Discussion

Understanding the actual requirement for Fra-1 in the complex process of metastasis in vivo is an essential prerequisite for the potential exploitation and targeting of Fra-1, or rather targets thereof, in the clinic. Consistent with previous suggestions based on in vitro studies (17–20, 22, 36–39), we show here in several independent metastatic tumor cell systems in vivo that the presence of Fra-1 is an essential requirement to establish metastases. This requisite coincides with the need for Fra-1 expression to allow tumor cell migration and invasion in vitro as well as in vivo. Fra-1 depletion perturbed EMT-like programs in several types of cancer cells and restored expression and correct subcellular localization of E-cadherin. These observations indicate that Fra-1 is involved causally in the down-regulation of epithelial characteristics in metastatic cancer cells. This effect is consistent with the previous findings that Fra-1 expression levels correlate negatively with E-cadherin expression (40) and that Fra-1 positively regulates the expression of pro-EMT microRNAs (41) and transcription factors (42).

In line with the idea that Fra-1 controls gene-expression programs mediating metastasis, the Fra-1–dependent transcriptome of human breast carcinoma cells and a gene-expression signature derived from the transcriptome showed high prognostic power. Thus these potential tools for patient stratification functionally connect prognostic power in breast cancer recurrence with a defined set of genes whose expression is regulated by a single transcription factor (Fra-1) that is functionally validated as a causal element for the disease.

To begin mining the Fra-1-dependent pathway for pharmacologically tractable targets, we performed a synthetic lethality drug screen and identified the adenosine receptor ADORA2B to be required for survival and metastasis as a function of Fra-1 expression. The corresponding gene is a direct Fra-1 transcriptional target. We show that ADORA2B contributes to the migratory and invasive activity of breast tumor cells. As expected for a master regulator–target gene relationship, depletion of Fra-1 was more effective than silencing of *ADORA2B* in blocking experimental metastasis. Unlike observations following Fra-1 depletion, silencing or pharmacological inhibition of ADORA2B did not result in a reversion of the EMT-like phenotype of breast cancer cells, although it efficiently blocked tumor cell invasion. Instead, impairment of ADORA2B activity inhibited filopodia formation by tumor cells, which is consistent with the recently proposed role of adenosine receptor signaling in chemotaxis (43).

We observed that ADORA2B depletion inhibited the growth of primary tumors considerably less than it suppressed metastatic activity. This differential effect may result from the fact that, although ADORA2B conceivably modestly promotes tumor cell survival and primary tumor growth, it also is required for the migratory/invasive activity of breast cancer cells, and this latter effect is likely to be more relevant for metastatic dissemination than for growth in situ. Recent evidence indicates that tumor cells may hijack immunosuppressive adenosine signaling to evade destruction by the immune system, notably by leading to activation of A_{2A} or A_{2B} receptors on leukocytes and especially T cells through enhanced adenosine production (44–47). Our data suggest that activation of adenosine receptor A_{2B} in tumor cells also contributes to tumor cell-intrinsic prosurvival and prometastatic signaling. Taken together, these observations suggest that pharmacological inhibition of adenosine receptors may be an attractive therapeutic strategy for metastatic breast cancer and could act both by promoting immune surveillance and by impairing metastatic dissemination.

In conclusion, a model emerges from our study in which Fra-1 is a pleiotropic regulator of the invasive potential of breast cancer cells that is required at several steps of the metastatic cascade to allow full-blown metastatic activity. Our evidence suggests that Fra-1 controls both early (i.e., stimulating an EMT-like genetic program) and later (i.e., promoting filopodia formation through ADORA2B) events and also contributes to the establishment of distant metastases (i.e., by allowing extravasation and anchorage-independent growth). We also demonstrate that the Fra-1–associated genetic program has high prognostic significance for breast cancer outcome. Furthermore, Fra-1 predicts the responsiveness of breast tumor cells to small-molecule inhibitors of pharmacologically tractable targets, such as ADORA2B. Therefore the Fra-1 signature may be used to stratify patients according to their likelihood of responding to the inhibition of such Fra-1–associated targets. We propose that systematic intervention of Fra-1–dependent genes may be explored for screening therapeutic options in metastatic breast cancer.

Methods

Cell Culture. RIE-1 (a gift from R. D. Beauchamp, Vanderbilt-Ingram Cancer Center, Nashville, TN, and K. D. Brown, Babraham Institute, Cambridge, UK), RK3E (American Type Culture Collection), MDA-MB-231 (a gift from L. Smit, Netherlands Cancer Institute, Amsterdam), and LM2 (subline #4173, a gift from J. Massagué, Memorial Sloan-Kettering Cancer Center, New York) cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS (Greiner Bio-One), 2 mM glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (all from Gibco).

Tumor Xenografts and Bioluminescence Analysis. All animal work was done in accordance with a protocol approved by the Netherlands Cancer Institute Animal Experiment Ethics Committee. Female BALB/c nude mice aged 6–8 wk were used for all xenografting experiments. RK3E cells (10^5 viable cells in PBS) were injected s.c. in each flank. LM2 cells (10^6 cells in a 1:1 mixture of PBS and growth factor-reduced Matrigel) were injected into the fourth mammary fat pad of nude mice. Tumors were removed surgically after 1 mo, and mice were kept for an additional 6 wk. For experimental lung metastasis, MDA-MB-231 and LM2 cells were injected into the lateral tail vein (2×10^5, 10^5, or 10^6 viable cells). Bioluminescence imaging was performed as described previously (12).

Migration and Invasion Assays. Migration and invasion assays were performed as described previously (23). RK3E clones (2.5×10^5 cells per well) and MDA-MB-231 clones (3×10^5 cells per well) were used.

Microarray Gene-Expression Profiling, Classifier Generation, and Analysis. A full description of the methods used in microarray gene-expression profiling, classifier generation, and analysis is available in the *SI Appendix*.

Synthetic Lethal Drug Screen. Screening of the LOPAC 1280 compounds library (Sigma) was performed as previously described (48). For the follow-up validation experiments, 11 twofold concentrations of the compounds were used ranging from 50 μM to ~50 nM. Each compound concentration was tested in quadruplicate, and values from quadruplicate measurements were averaged and normalized against DMSO-only controls. Data were represented as percent cell viability and were normalized against DMSO-only controls. IC_{50} values were calculated using GraphPad Prism5 software.

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