Reduced tumorigenesis in mouse mammary cancer cells following inhibition of Pea3- or Erm-dependent transcription

Virginie Firlej[1,*], Franck Ladam[1,*], Guillaume Brysbaert[2], Patrick Dumont[1], François Fuks[3], Yvan de Launoit[1], Arndt Benecke[2] and Anne Chotteau-Lelievre[1,‡]

[1]UMR 8161, Institut de Biologie de Lille, CNRS Universités de Lille 1 and 2, Institut Pasteur de Lille, IFR 142, BP 447, 1 rue Calmette, 59021 Lille Cedex, France
[2]Institut des Hautes Études Scientifiques and Institut de Recherche Interdisciplinaire, CNRS USR3078, Université de Lille 1, 35 route de Chartres, 91440 Bures sur Yvette, France
[3]Laboratoire d'Epigénétique du Cancer, Faculté de Médecine, Université Libre de Bruxelles, CP 614, 808 Route de Lennik, 1070 Brussels, Belgium

*These authors contributed equally
‡Author for correspondence (e-mail: anne.chotteau@ibl.fr)

Accepted 28 May 2008
Journal of Cell Science 121, 3393-3402 Published by The Company of Biologists 2008
doi:10.1242/jcs.027201

Summary

Pea3 and Erm are transcription factors expressed in normal developing branching organs such as the mammary gland. Deregulation of their expression is generally associated with tumorigenesis and particularly breast cancer. By using RNA interference (RNAi) to downregulate the expression of Pea3 and/or Erm in a mammary cancer cell line, we present evidence for a role of these factors in proliferation, migration and invasion capacity of cancer cells. We have used different small interfering RNAs (siRNAs) targeting *pea3* and *erm* transcripts in transiently or stably transfected cells, and assessed the physiological behavior of these cells in in vitro assays. We also identified an in vivo alteration of tumor progression after injection of cells that overexpress *pea3* and/or *erm* short hairpin RNAs (shRNAs) in immunodeficient mice. Using transcriptome profiling in Pea3- or Erm-targeted cells, two largely independent gene expression programs were identified on the basis of their shared phenotypic modifications. A statistically highly significant part of both sets of target genes had previously been already associated with the cellular signaling pathways of the 'proliferation, migration, invasion' class.

These data provide the first evidence, by using endogenous knockdown, for pivotal and complementary roles of Pea3 and Erm transcription factors in events crucial to mammary tumorigenesis, and identify sets of downstream target genes whose expression during tumorigenesis is regulated by these transcription factors.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/20/3393/DC1

Key words: Pea3/Erm, Transcription factor, Mammary tumor, Transcriptome, RNAi

Introduction

The three members of the PEA3 group of ETS transcription factors, Pea3 (also known as ETV4), Er81 (also known as ETV1) and Erm (also known as ETV5), share one functionally highly conserved DNA-binding and two transactivation-regulation domains. They are generally characterized as transcriptional activators by binding to DNA through the consensual core sequence GGA[A/T] (for a review, see de Launoit et al., 2006).

In situ hybridization studies performed at various stages of murine embryogenesis have shown that the *pea3*, *erm* and *er81* genes are expressed in multiple organs and present a specific expression pattern associated with branching morphogenesis in lung, mammary gland, salivery gland or kidney (Chotteau-Lelievre et al., 1997; Chotteau-Lelievre et al., 2001; Chotteau-Lelievre et al., 2003). Their roles and functions are not precisely known but deregulation of their expression has been associated with carcinogenesis. For example, Pea3 overexpression is involved in different cancers such as lung, ovarian, colorectal, oral and gastric cancer, and is generally correlated to a poor prognosis and the presence of metastases (de Launoit et al., 2006). Transcription factors from the PEA3 group have been also implicated in mammary oncogenesis (Kurpios et al., 2003; Shepherd and Hassell, 2001). Pea3 is expressed in more than half of the studied human breast cancers and in 75% of the analyzed pleural breast effusions (de Launoit et al., 2006). It is thus suggested to have a role in tumor aggressiveness (Bieche et al., 2004) and could be considered as a marker for malignant progression to effusions (Davidson et al., 2004). Correlation between the expression of the tyrosine kinase receptor Her2 and Pea3 has also been shown, since this latter is expressed in the majority of tumors expressing Her2 (Benz et al., 1997; Fleming et al., 2004). Expression of another PEA3 group member, Erm, is detectable in more than 80% of human breast cancers, thus also demonstrating a role of this transcription factor in breast cancer and, more particularly, as an independent adverse prognosis factor for overall survival (Chotteau-Lelievre et al., 2004). In experimental mammary models, it has been demonstrated that ectopic overexpression of Pea3 in non-metastatic human breast-cancer cells increases their invasiveness and their metastasis potential in nude mice (Kaya et al., 1996). Moreover, in mouse mammary tumor virus (MMTV)-*neu*-induced tumors, a dominant form of the PEA3 group members delays the onset of these tumors and reduces their number and size (Shepherd et al., 2001). Altogether, these findings suggest that, when

aberrantly regulated, PEA3 group transcription factors can contribute to tumorigenesis.

Although, modulation of the expression of the PEA3 group members is generally correlated to the expression of certain matrix metalloproteinases (MMP), such as MMP1, MMP3, MMP7 and MMP9, MT1-MMP or the intercellular adhesion molecule as ICAM1, as well as other molecules, such as cyclooxygenase 2 or osteopontin (de Launoit et al., 2006; Shindoh et al., 2004), the precise molecular mechanism by which they act in mammary tumorigenesis is currently unknown. To comprehend Pea3-and Erm-induced tumorigenesis in MMT mouse mammary tumor (MMT) cells, inhibition of *pea3* and/or *erm* expression using RNA interference (RNAi) was performed. Downregulation of *pea3* and *erm* expression decreases cell proliferation, migration, invasion in vitro and MMT-derived tumor growth in vivo. A transcriptome analysis reveals a very limited overlap between the Pea3- and Erm-target genes, indicating that both factors can regulate alternate, and maybe complementary, gene expression programs in mouse mammary tumorigenesis.

Results

Knockdown of *pea3* and *erm* inhibits proliferation, migration, anchorage-independent growth and morphogenetic capacity of MMT cells

To determine the role of Pea3 and Erm factors on mammary tumorigenesis, we used RNAi to downregulate the expression of these molecules in MMT cells, aggressive cancer cells derivated from a spontaneous mouse mammary gland tumor (ATCC CCL-51) that express high protein levels of members of the PEA3 group (supplementary material Fig. S1B,C and data not shown). For this purpose, we first tested the efficiency of the siRNA targeting *pea3* (si *pea3* 1-2) and *erm* (si *erm* 1-2), as well as a control siRNA (si *ctrl*). Forty-eight hours after transfection, *pea3*-targeting siRNA substantially inhibited *pea3* mRNA expression by ~80% (si *pea3* 1) and ~65% (si *pea3* 2) (Fig. 1A), and dramatically reduced the expression of Pea3 protein, detectable in a western blot as an ~65 kDa band (Fig. 1C). *erm* mRNA expression was also repressed by ~65% (si *erm* 1) and ~85% (si *erm* 2) when using *erm*-siRNA (Fig. 1B). Using the commercially available antibodies against Erm, we were not able to detect endogenous Erm protein in MMT cells, because this protein is highly unstable (Baert et al., 2007). Given the fact that we targeted only ~80% of the cells using this transfection technique, we assumed that neither endogenous *erm* nor endogenous *pea3* mRNA remained in the specific-siRNA-transfected cells at this time. After 96 hours, downregulation is still effective (40-50%) as assessed by semi-quantitative reverse transcriptase (RT)-PCR for si *pea3* 1 and si *erm* 2 (supplementary material Fig. S1A). Altogether, these results suggest that the chosen *pea3*- and *erm*-siRNA can efficiently downregulate the expression of *pea3* and *erm* in MMT cells.

MMT cells transfected with *pea3* and *erm* siRNA have been used to assess the effect of Pea3 and Erm factors on in vitro cell proliferation by counting assays. The proliferation rate of the si *pea3* 1-2 (Fig. 2A) and si *erm* 2 (Fig. 2B) transfected MMT cells was inhibited by ~40% and that of si *erm* 1 by ~30% (Fig. 2B), compared with the MMT control cells 96 hours post transfection.

The effect of Pea3 and Erm transcription factors on MMT cell migration was tested using Boyden chambers. MMT cells transfected with si *pea3* 1-2 or si *erm* 1-2 siRNA were seeded on the upper part of the chamber. Cells that migrated to the lower surface were fixed, stained and counted under microscope. Inhibition of *pea3* and *erm* expression in siRNA-transfected MMT cells resulted at mean in 80% (si *pea3* 1 and si *erm* 1), 90% (si *pea3* 2) and 75% (si *erm* 2) decrease, when compared with the control cells (si *ctrl*) (Fig. 2C). We thus concluded that Pea3 and Erm factors have a role in the migratory behavior of MMT cells, because their downregulation in MMT cells resulted in an inhibition of the migration ability.

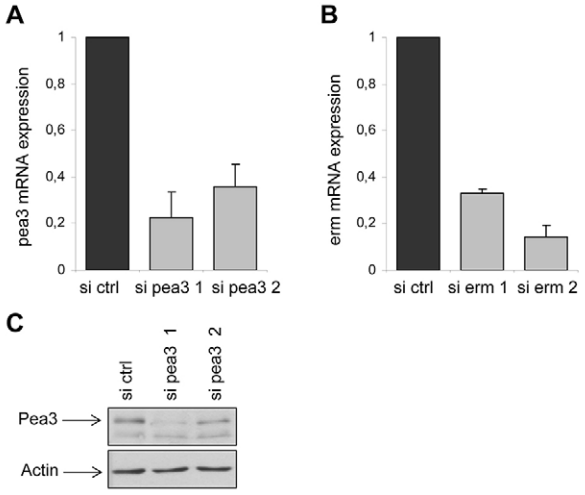

Fig. 1. Quantification of siRNA-mediated *pea3* and *erm* knockdown. (A,B) Relative mRNA expression levels of *pea3* in si *pea3* 1-2 (A) and *erm* in si *erm* 1-2 (B) versus si *ctrl* transfected MMT cells, assessed by quantitative RT-PCR, 48 hours post transfection. Results are expressed as ratios of mRNA levels of *pea3* or *erm* to *cyclophilin* (endogenous control standard) (1=si *ctrl*) and are the mean ± s.d. of three experiments in duplicate. (C) Western blot analysis of Pea3. Extracts of total protein of transfected MMT cells were analyzed using anti-Pea3 and anti-actin (control) antibodies 48 hours post transfection.

The effect of Pea3 and Erm factors on anchorage-independent growth was tested by using agar gel cloning assays. MMT cells transfected with si *pea3* 1-2 and si *erm* 1-2 were grown in agar with growth medium for 15 days, and then fixed and counted. Repression of *pea3* and *erm* expression reduced anchorage-independent growth of MMT cell by ~70% (si *pea3* 1) and ~60% (si *pea3* 2 and si *erm* 1-2) (Fig. 2D), suggesting an important role of Pea3 and Erm transcription factors in the events that regulate the anchorage capacity of cancer cells.

MMT cells transfected with si *ctrl*, si *pea3* 1-2 and si *erm* 1-2 were then cultured on a three-dimensional matrix mimicking gel which provide to the cells an appropriate microenvironment allowing the rebuilding of tissue specific form (Nelson and Bissell, 2005). MMT control cells underwent branching and 'morphogenesis', which resemble morphogenesis that occurrs in the normal mammary gland. Compared with control MMT cells, those cells transfected with si *pea3* 1-2 and si *erm* 1-2 formed less branched structures and were less developed and organized (Fig. 3). This 3D cell organization implies the interweaving interaction between proliferation, migration and invasion events. Altogether, the results show a role for Pea3 and Erm in the morphogenetic abilities of the MMT cells, probably through the modulation of proliferation, migration and invasion properties of the cells.

Knockdown of *pea3* and *erm* inhibits tumor growth in immunodeficent mice

To follow in immunodeficient mice the behavior of MMT cells in which the PEA3 group members are knocked-down, we used a

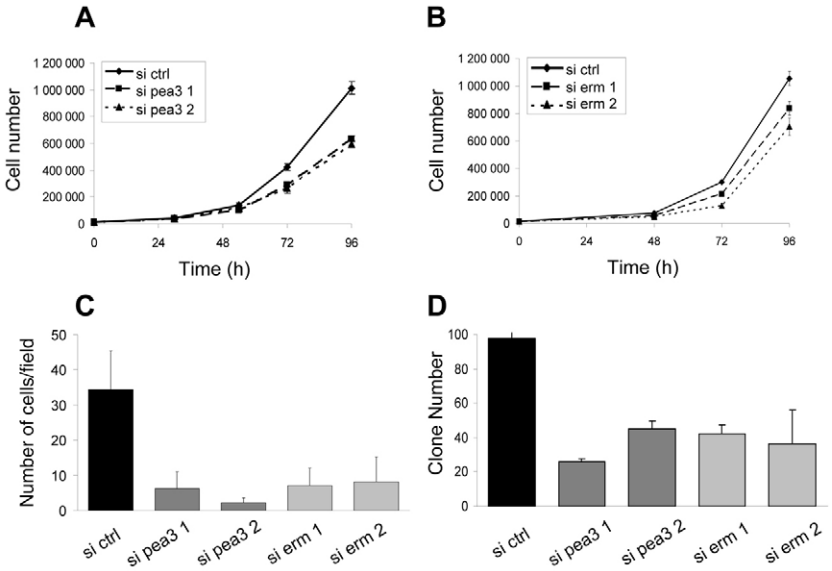

Fig. 2. *pea3* and *erm* knockdown impairs proliferation, migration and anchorage-independent growth ability of MMT cells. (A,B) Proliferation assays. si *pea3* 1-2 and si *ctrl* (A) or si *erm* 1-2 and si *ctrl* (B) transfected MMT cells were trypsinized and counted with a cell counter every day. Data are the mean ± s.d. of a representative experiment performed in triplicate. Experiments were reproduced four times in triplicate and gave the same results for each assay. (C) Migration assay. 24 hours post transfection, si *pea3* 1-2, si *erm* 1-2 or si *ctrl* transfected cells were seeded into the upper part of a Boyden chamber. After 18 hours, cells which have migrated through the membrane are counted. Experiments were done at least three times. Data are the mean ± s.d. of a representative experiment performed in triplicate. (D) Anchorage-independent growth assay. 24 hours post-transfection, si *pea3* 1-2, si *erm* 1-2 or si *ctrl* transfected cells were mixed with agar 0.65% and cultivated during 15 days. Experiments were done three times. Data are the mean ± s.d. of a representative experiment performed in triplicate.

retroviral strategy with viruses expressing shRNA. MMT cells were infected with the retroviral vector pSUPER.retro (pRS), pRS-*pea3* A, pRS-*pea3* B or pRS-*erm* retroviruses produced in HEK 293 cells or successively infected with both pRS-*pea3* A and pRS-*erm* (see Materials and Methods). Under selection conditions, this permits to generate populations expressing long-term *erm* and/or *pea3* shRNA or none, named MMT Rs, MMT Ri *erm*, MMT Ri *pea3* A, MMT Ri *pea3* B and MMT Ri *pea3/erm* respectively. These cells have been tested for downregulated expression of *pea3* or *erm* by quantitative RT-PCR. *pea3* expression was repressed by ~60% in MMT Ri *pea3* A and ~50% in MMT Ri *pea3* B and MMT Ri *pea3*/*erm* (Fig. 4A and supplementary material Fig. S2A), whereas *erm* expression was repressed by ~40% in MMT Ri *erm* and ~50% in MMT Ri *pea3/erm* (Fig. 4A) as compared with the control MMT Rs cells. In order to control the physiological effect of the shRNA mediated downregulation of *pea3* and *erm* expression, and to confirm that they have the same effect as the corresponding siRNA, proliferation and migration assays have been performed with MMT Rs, MMT Ri *pea3* A and MMT Ri *pea3* B infected cells as experiments done with siRNA-transfected MMT cells presented in Fig. 2. The proliferation rate of MMT Ri *pea3* A and MMT Ri *pea3* B 72 hours post-spreading was inhibited respectively by ~70% and ~35% when compared with the control MMT Rs cells (supplementary material Fig. S2B). The migration rate of MMT Ri *pea3* A and B is reduced by ~40% (supplementary material Fig. S2B). The cloning assay performed with MMT Rs, MMT Ri *pea3* A and MMT Ri *pea3/erm* cells depicted that the number of clones is significantly reduced in MMT Ri *pea3* A and MMT Ri *pea3/erm* cells when compared with the control cells (60 and 90% respectively; supplementary material Fig. S2C). These data indicate that the shRNA induce the same effect in these functional assays as the corresponding siRNA – even if it is quantitatively different – and de facto can be used for long-term in vivo analysis.

MMT cells stably expressing shRNA, MMT Rs, MMT Ri *erm*, MMT Ri *pea3* A and MMT Ri *pea3/erm* were next injected subcutaneous in each flank of female SCID mice (Fig. 4B). Tumors were readily detectable in each group 3-4 days after grafting and no difference is discernible at this stage (data not shown). All along the experiment, the tumor volume remained lower in the *pea3* and *erm* groups than in the control group. From day 9 and more clearly day 12, a significant difference of tumor growth could be observed. On day 12, the most efficient tumor volume reduction was observed in the MMT Ri *pea3* A and in the MMT Ri *pea3/erm* groups (~50% of inhibition). At day 15, tumors from MMT Ri *pea3* A infected cells remained about twofold smaller than control tumors, whereas tumors induced by MMT Ri *erm* and MMT Ri *pea3/erm* infected cells were 30% smaller than tumors induced by MMT Rs infected cells. Later on, tumor growth was hampered by the emergence of necrosis and animals were sacrificed. Thus, Pea3 and Erm participate to the progression of in vivo induced mammary tumors.

pea3 and *erm* target-gene programs

We performed global gene expression analysis using mouse genome survey microarrays, which measure the expression of 28,218 validated mouse genes, to elucidate the target gene programs underlying the *pea3*- and *erm*-siRNA phenotypes described above. We compared gene-expression profiles between MMT cells transfected with si *pea3* 1 or si *erm* 2 and MMT cells transfected with *ctrl* siRNA. We also studied the transcriptome profile of untransfected cells. To this end total RNA was extracted from three independent biologic replicates of control, specific and untransfected cells, and labeled using the RT-IVT reaction from Applied Biosystems (Materials and Methods). After image analysis, subtraction profiles were calculated from the resulting transcriptome profiles. We thereby did not only compare the effect of the specific siRNAs versus the control siRNA to calculate the statistical significance, but also versus the mock-transfected cells- and the two controls amongst each other, thereby better controlling for false-positive gene-expression estimates. Genes that showed significant variation in their expression between untransfected MMT cells and MMT cells transfected with si *ctrl* were thus eliminated from the genes selected in the si *pea3* 1 and si *erm* 2 versus si *ctrl* experiments. 130 genes are significantly (fold change >2, $P<0.01$) down- or upregulated when comparing MMT cells transfected with si *pea3* 1 and si *ctrl* (supplementary material Table S1). 117 genes are significantly (fold change >1.5, $P<0.02$) regulated when we compared MMT cells transfected with si *erm* 2 and si *ctrl* (supplementary material Table S2). Notice the slight decrease in

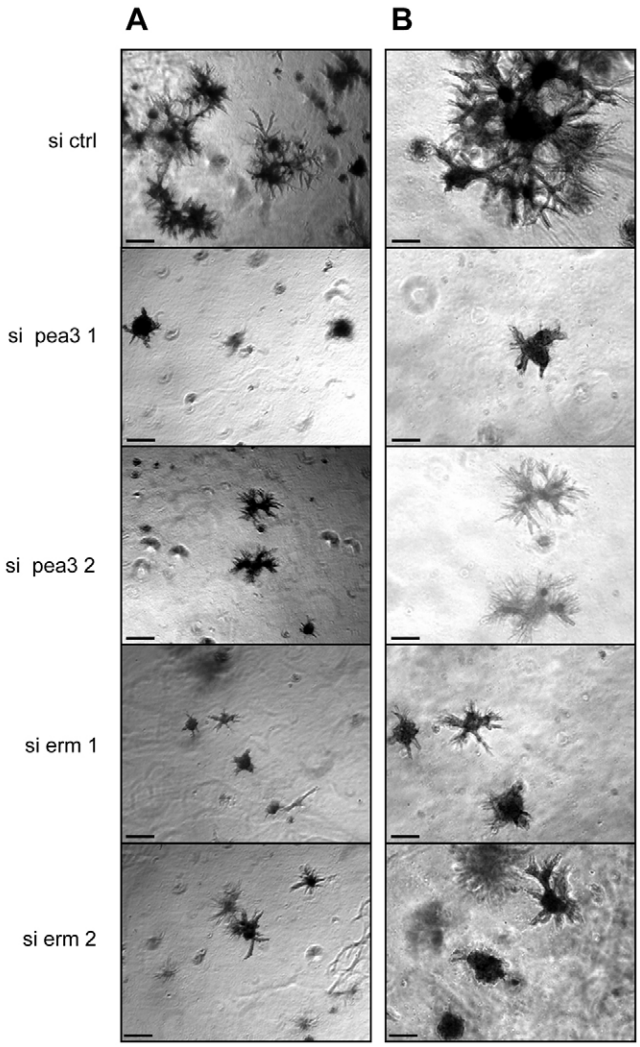

Fig. 3. *pea3* and *erm* knockdown affects morphogenetic capacity of MMT cells. si *pea3* 1-2, si *erm* 1-2 and si *ctrl* transfected MMT cells were seeded at low density on a Matrigel. 3D cultures were treated with HGF/SF (20 ng/ml). After 7 days, cells were stained with Neutral Red and fixed. Whole-mount pictures were taken at 50× (A) or 100× (B) using a microscope. Scale bars, 100 μm (A) or 100X (B). Images are representative of three experiments performed in duplicate.

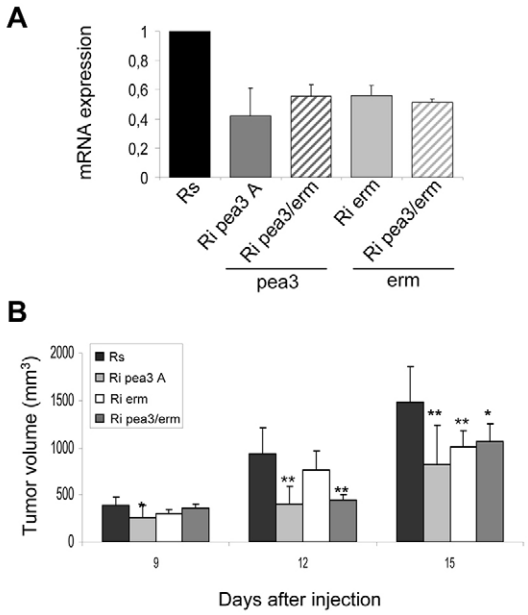

Fig. 4. In vivo tumor growth of MMT cells stably expressing shRNA, MMT Rs, MMT Ri *erm*, MMT Ri *pea3* and MMT Ri *pea3/erm*. (A) Validation of shRNA-mediated *pea3* and *erm* knockdown. Relative mRNA expression levels of *pea3* in MMT cells infected with pRS-*pea3* A (dark gray bars) or pRS-*pea3/erm* (dark striped bars) and *erm* in MMT cells infected with pRS-*erm* (light gray bars) or pRS-*pea3/erm* (light striped bars) versus control pRS retroviruses, as assessed by quantitative RT-PCR. Results are expressed as ratios of mRNA levels of *pea3* or *erm* to *cyclophilin* (endogenous control standard) (1=Rs) and are the mean ± s.d. of 3 experiments in duplicate. (B) Tumor growth of the MMT cells stably expressing shRNA. MMT cells infected with pRS-*pea3* A, pRS-*erm*, pRS-*pea3/erm* or pRS retroviruses were subcutaneously injected into SCID mice. Three days after injection, tumors were measured every 3 days for 15 days. Values represent the mean ± s.d. of one representative experiment (five mice per group) from the three experiments under identical conditions. Error bars indicate + s.d. * $P<0.05$, ** $P<0.01$.

statistical significance owing to the use of only three biologic replicates in the case of the *erm* knockdown. Only a common subset of ten genes is shown to be regulated by both Pea3 and Erm (supplementary material Table S3).

Fig. 5 summarizes the transcriptome analysis and results. These transcriptome studies have been subsequently validated either by semi-quantitative PCR for the following subset of genes: *Has2* (regulated in *pea3* condition), *Hgf* (regulated in *erm* condition) or *Ascl4* and *Fgfr1* (regulated in *pea3* and *erm* conditions) (Fig. 6A) or by quantitative PCR: *Fst* (regulated in *pea3* condition), *Suz12* (regulated in *erm* condition), *Has2* or *Stip1* (regulated in *pea3* condition) (Fig. 6B).

The quantitative comparison between the qPCR data and the microarray analysis was performed by calculating the Pearson correlation coefficients (R^2) of both datasets, the results of which are shown in Table 1. Joint correlations between both datasets are high (*erm*) to very high (*pea3*), confirming the concordance between the microarray and the qPCR analyses. These semi-quantitative and quantitative PCR analyses have been confirmed using also the other siRNAs targeting *pea3* and *erm* (data not shown).

We furthermore illustrate the microarray results obtained for previously known Pea3 and/or Erm gene targets (Fig. 7). These genes are not necessarily included in the target gene repertoires shown in Table 1 and the supplementary material because of the stringency of the statistical analysis. For most of them, the transcriptome measurements are in perfect agreement with the previously in the literature reported results. Pea3 and/or Erm have been described to be positive regulators for MMP1, MMP2, MMP3, MMP9 and MMP14, vimentin, cyclooxygenase 2, caveolin 1, heparanase, cyclin D3, Zhx1 or transcriptional repressors, e.g. for urokinase-type plasminogen activator (uPAR) of gene expression.

Table 1. Pearson correlations between the microarray experiments and corresponding quantitative RT-PCR results over the ensemble of genes shown in Fig. 6B

R^2	MA-siRNA-pea3	MA-siRNA-erm
qPCR-siRNA-pea3	0.939	0.613
qPCR-siRNA-erm	0.280	0.634

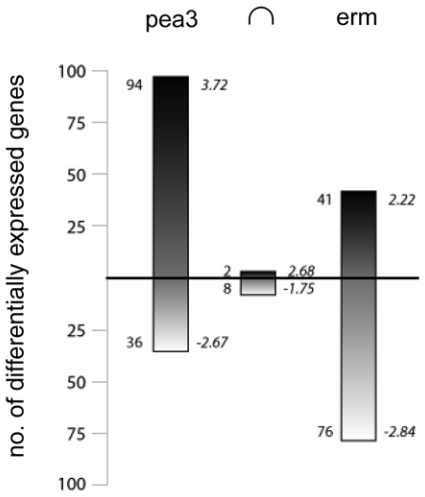

Fig. 5. Summary of transcriptome analysis and results. Inhibition of either *pea3* or *erm* induces significant changes in the cellular transcriptome of MMT cells. Expression levels of mRNAs from *pea3* or *erm* siRNA-treated cells were individually compared with control oligonucleotide-treated MMT samples by genome-wide microarray analysis. The absolute number of probes detecting statistically significant up- or downregulation following inhibition of either of the two Ets factors is shown to the left of each bar; the maximum positive or negative logarithmic (base two) fold-change is shown to the right. The black to gray gradient indicates positive, and the white to gray gradient negative fold changes in expression. The subset of genes regulated in both cases is also shown (∩).

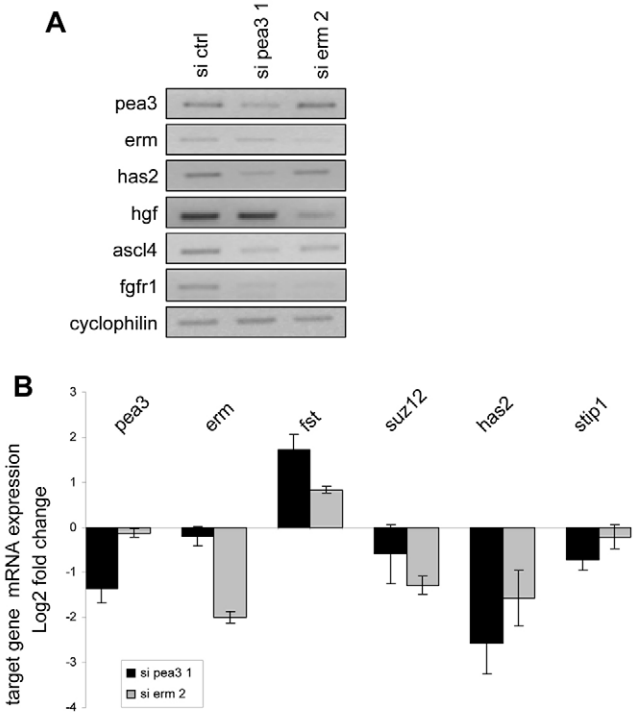

Fig. 6. Validation of the microarray experiments by semi- and quantitative RT-PCR. (A) mRNA expression levels of *Pea3*, *Erm*, *Has2*, *Hgf*, *Ascl4*, *Fgfr1* and *cyclophilin* (endogenous control standard), in si *ctrl* (lane 1) versus si *pea3* 1 (lane 2) and si *erm* 2 (lane 3) transfected MMT cells, assessed by semi-quantitative RT-PCR. (B) Logarithmic fold-changes for *pea3, erm*, *Fst*, *Suz12*, *Has2* and *Stip1* genes as determined by quantitative RT-PCR for *pea3* (black bars) and *erm* (gray bars) siRNA-mediated inhibition are shown. Results are expressed as ratios to *cyclophilin* mRNA levels. Error bars indicate ±s.e.m. of at least three independent biological replicates.

The microarray data obtained for these genes show that they are all downregulated (MMP1, MMP2, MMP3, MMP9 and MMP14, vimentin, cyclooxygenase 2, caveolin 1, heparanase, cyclin D3, Zhx1) or upregulated (uPAR) in si *pea3* and/or si *erm* targeted cells, and thus confirm the published data. For Erbb2 (also known as Her2, Neu), for which Pea3 regulation data are controversial, we observe a diminution of its expression in si *pea3* and si *erm* conditions. In a few cases our transcriptome measurements for Pea3 and Erm do not exactly match the observations reported in the literature. Vim, cyclooxygenase 2 and heparanase are, in accordance with the transactivating role of Pea3 on these genes, downregulated in the si *pea3* targeted cells; however, the modulation observed in si *erm* targeted cells is very weak and less significant. The weak effects observed by Erm on these genes are a further indicator of distinct regulatory capacities of Pea3 and Erm in this particular mammary tumor model, and is in agreement with the little overlap observed between the target-gene-expression programs of both transcription factors.

We have then mapped the ensemble of MGS V1.0 probe-IDs to GO, Kegg and PANTHER derived signaling pathways. We determined to which of the derived signaling pathways the Pea3- and Erm-target genes mapped to. Furthermore, we calculated the probability of observing those numbers of target genes from random samples of identical size using a binominal distribution. We thereby can show that the 'migration, proliferation and invasion pathway' we have defined (25 genes of 130 genes for Pea3 and 25 genes of 117 genes for Erm) is significantly (P<0.01) over-represented in both the Pea3- and Erm-target gene lists (Table 2).

Discussion

Expression of the transcription factors Pea3 and Erm is often associated with poor prognosis in cancer. They have been shown to be overexpressed in human and mouse mammary metastasis carcinomas (de Launoit et al., 2006). Nevertheless, the mechanisms by which they regulate gene expression and how they contribute to the physiological and physiopathological phenotypes are currently unknown. In the present study, we have elucidated some of their functions by knocking down their gene expression using RNAi. This study provides the first evidence for a pivotal role of Pea3 and Erm transcription factors in events that lead to the progression of cancer in mammary cancer cells by endogenously repressing these factors.

To assess the effect of Pea3 and Erm on in vitro and in vivo cell behavior, we used MMT cells that were transfected with *pea3*-siRNA and *erm*-siRNA or MMT cells infected with *pea3*-shRNA- and *erm*-shRNA-producing retroviruses. This cell line is currently used as a tumorigenic model because of its capacity to induce tumors and metastasis in vivo and to establish organized 3D structures in vitro (Delannoy-Courdent et al., 1998; Furlan et al., 2008). It expresses substantial amounts of *pea3* and *erm* mRNA and proteins when compared with wild-type epithelial mammary cells (TAC cells) (Soriano et al., 1995). This fact makes MMT cells a model of choice because the levels of Pea3 and Erm are comparable to the physiological overexpression in cancerous cells as previously demonstrated for other tumor-derived cell lines (supplementary material Fig. S1B,C) (Baert et al., 1997). Moreover, to prevent the potential off-target effects of these siRNA sequences and also to ensure

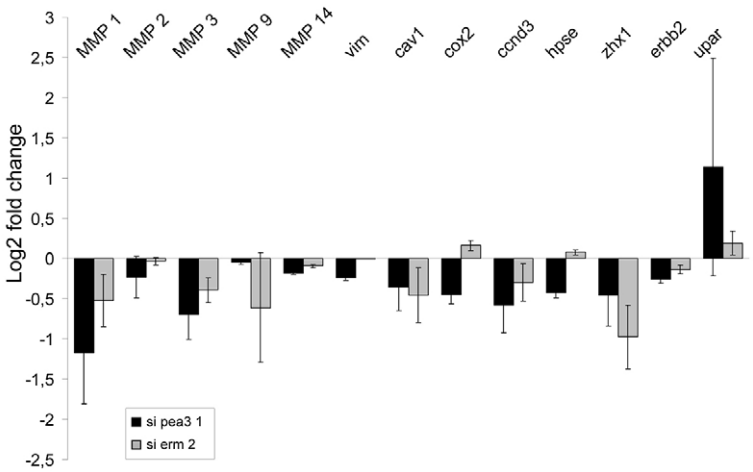

Fig. 7. Effects of *pea3* or *erm* siRNA-mediated inhibition on known target genes as determined by microarray analysis. For selected, known *pea3*- or *erm*-target genes, the average logarithmic fold-change and standard deviation recovered from the microarray experiments are shown. Black bars indicate the fold-change between *pea3* siRNA treated and control siRNA treated MMT cells, gray bars correspond to the *erm* siRNA-treated versus control-treated cells.

the physiological relevance of the modifications observed, most of the experiments were done with the different *pea3*- and *erm*-targeting siRNA or shRNA (in all, three distinct sequences for *pea3* and two for *erm*). Invasive and proliferative phenotypes are fundamental components of malignant disease, and cell multiplication in the absence of integrin-derived adhesive signals (anchorage-independent growth) is the phenotypic hallmark of neoplasic transformation. We showed in vitro in a 3D-matrix assay, that *pea3*- and *erm*-targeting siRNA or shRNA repression resulted in the inhibition of cancer-cell proliferation, migration, morphogenetic organization and cell growth independently from cell anchorage.

We extended the data obtained in the in vitro transformation assays to tumorigenesis in vivo by implanting subcutaneous xenografts of MMT cells and their derivatives in immunodeficient mice. In accordance with the in vitro results, *pea3* and/or *erm* knockdown MMT cancer cells form tumors that grew less rapidly than control tumors.

Normal morphogenetic and tumorigenic events require a number of biological processes, such as proliferation, migration and invasion of the surrounding tissue. We have previously established that Pea3 and Erm facilitate branching morphogenesis of wild-type mammary epithelial cells as well as promoting invasive properties in vitro (Chotteau-Lelievre et al., 2003). According to these data, the present in vitro and in vivo findings on cancerous mammary cells provide evidence for a role of the transcription factors Pea3 and Erm in different events that are interwoven regarding their morphogenetic organization abilities or tumor development and evolution. We thus suggest that the growth delay of tumors from *pea3*- and *erm*-knockdown cells is due to the modification of Pea3- and Erm-induced proliferation, migration, invasion and 3D-organization capacities observed in vitro.

These results are consistent with previous findings that demonstrate the involvement of PEA3 group members in proliferation, migration, invasion and in vivo tumor-formation events, particularly in mammary tumor formation (Habelhah et al., 1999; Hakuma et al., 2005; Hanzawa et al., 2000; Hida et al., 1997; Hiroumi et al., 2001; Kaya et al., 1996; Moss et al., 2006; Shepherd et al., 2001; Upadhyay et al., 2006). These data also add to the weight of evidence that Pea3 and Erm are tumor-enhancing in mice and highlight a crucial role of these factors in breast-cancer progression.

Different genes that are defined as PEA3-group-member target genes display functions known to be associated to the phenotype modulations we have observed here. The first targets that have been described were matrix metalloproteinases (MMPs), which are implicated in the degradation of extracellular matrix, and adhesion molecules, which have an important role during different steps of cancer progression. Other genes for which expression has been correlated with metastasis or invasion are known to be regulated by members of the PEA3 transcription factor group, for example heparanase (encoding a protease) (Lu et al., 2003), cyclooxygenase 2 (Upadhyay et al., 2006), vimentin (Chen et al., 1996) or mucin

Table 2. Significance of Pea3- and Erm-target gene distribution into defined pathways

	Pea3			Erm		
Pathway	Count	±	*P*-value	Count	±	*P*-value
Migration, proliferation, invasion	25	+	**1.09×10^{-6}**	25	+	**1.43×10^{-7}**
Fatty acid, lipid, steroid metabolism	8	+	2.54×10^{-2}	5	+	1.33×10^{-1}
apoptosis	5	+	8.12×10^{-2}	4	+	1.30×10^{-1}
Nucleic acid metababolism and transcription	11	+	1.25×10^{-2}	19	+	8.90×10^{-2}
Structure and adhesion molecule	10	+	1.23×10^{-1}	4	–	1.71×10^{-2}
Cellular transport	6	+	1.52×10^{-1}	5	+	1.76×10^{-1}
Protein metabolism	4	–	**2.36×10^{-3}**	7	–	5.07×10^{-2}
Other	61	+	4.17×10^{-2}	48	–	**6.03×10^{-3}**
Total	130			117		

Pea3 and Erm target gene distribution into defined biological pathways. The ensemble of MGS V1.0 probe-IDs was mapped to a signaling pathway list derived through GO, Kegg and PANTHER. Given are relative values as well as significance ($P<0.01$) of over- and under-represented (bold) pathways among the 130 (*pea3*) or 117 (*erm*) selected probes.

4 (encoding a transmembrane mucin) (Fauquette et al., 2005) and osteopontin (El-Tanani et al., 2004) in mammary cancer cell models. Furthermore, some Pea3-target genes belong to pathways implicated in proliferation or cell cycle regulation, such as *Neu* (Xing et al., 2000), *WT1* (Discenza et al., 2004), *cyclin D3* (Jiang et al., 2007) or *cyclin D1* (a gene implicated in G1-S transition) for which a correlation has been established with *pea3* expression in different mammary tissues and tumors (Galang et al., 2004). These findings fit well with the potential role of PEA3 members in tumorigenesis events.

To further explore the molecular mechanism of Pea3-knockdown- and Erm-knockdown-induced modulation of mammary-cancer-cell phenotypes, we have performed a transcriptome microarray analysis. Among the Pea3- and/or Erm-regulated genes that come out from the statistical analysis, a representative part of them can be classified in a 'proliferation, migration, invasion' group, which is significantly over-represented in our experiments when compared with the ensemble of probe-IDs, confirming the significance of our observations. For example, we observed a significant regulation of genes encoding proteins that are directly implicated in proliferation or cell-cycle regulation, such as FGF receptor 1 (*Fgfr1*), cyclin D1 (*Ccnd1*), cyclin D2 (*Ccnd2*), sestrin 2 (*Sesn2*; which encodes a p53-induced molecule), RAD52 motif 1 (*Rdm1*), or insulin like growth factor binding protein 7 (*Igfbp7*). Furthermore, this microarray study revealed many genes that are known to be implicated in migration or invasion, such as follistatin (*Fst*), melanoma antigen family D 2 (*Maged2*) or hyaluronane synthetase 2 (*Has2*) (see Table 3).

Table 3. Pea3- and/or Erm-target genes relevant in migration, proliferation or invasion

Repression	Probe number	Gene symbol	Gene Name	Fold change	*P*-value
pea3	664595	*Prl3d1*; *Prl3d2*	Prolactin family 3 subfamily d, member 1; prolactin family 3 subfamily d, member 2	0.16	0.0009
pea3	578632	*Prl3d1*; *Prl3d3*	Prolactin family 3 subfamily d, member 1; prolactin family 3 subfamily d, member 3	0.17	0.0012
pea3	337659	*Has2*	Hyaluronan synthase 2	0.22	0.0005
pea3	*715233*	*Tuba3b*	Tubulin alpha 3b	0.30	0.0045
pea3	*814680*	*Fgfr1*	Fibroblast growth factor receptor 1	*0.38*	*0.0005*
pea3	400066	*Eps8*	Epidermal growth factor receptor pathway substrate 8	0.45	<0.00005
pea3	764223	*Snn*	Stannin	0.45	0.0011
pea3	905667	*Stip1*	Stress-induced phosphoprotein 1	0.47	0.0064
pea3	401001	*Rras2*	Related RAS viral (r-ras) oncogene homolog 2	0.48	0.0032
pea3	529365	*Hsp90dl*	Tumor rejection antigen gp96	0.48	0.0009
pea3	716324	*Socs4*	Suppressor of cytokine signaling 4	2.01	0.0004
pea3	299973	*Csf1*	Colony-stimulating factor 1 (macrophage)	2.11	0.0020
pea3	401591	*Fgfr1op*	Fgfr1 oncogene partner	2.11	0.0077
pea3	891697	*Rrm2b*	Ribonucleotide reductase M2 B (TP53 inducible)	2.15	0.0059
pea3	690255	*Ppm1d*	Protein phosphatase 1D magnesium-dependent. delta isoform	2.17	0.0001
pea3	932873	*Nusap1*	Nucleolar and spindle associated protein 1	2.19	0.0001
pea3	893596	*LOC435791*	Interferon zeta	2.26	0.0047
pea3	839098	*Sesn2*	Sestrin 2	2.34	0.0052
pea3	825625	*Fst*	Follistatin	2.50	<0.00005
pea3	906306	*Trp53inp1*	Transformation-related protein 53 inducible nuclear protein 1	2.60	0.0033
pea3	402835	*Notch3*	Notch gene homolog 3	2.80	<0.00005
pea3	339138	*Hmmr*	Hyaluronan mediated motility receptor (RHAMM)	3.20	0.0014
pea3	*921450*	*Igfbp7*	Insulin-like growth factor binding protein 7	*4.12*	*<0.00005*
pea3	336643	*Myh7*	Myosin heavy polypeptide 7, cardiac muscle beta	4.22	<0.00005
pea3	829347	*Rdm1*	RAD52 motif 1	4.24	<0.00005
erm	*715233*	*Tuba7*	Tubulin alpha 7	*0.15*	*0.0030*
erm	590238	*Elmo2*	Engulfment and cell motility 2, ced-12 homolog	0.18	0.0073
erm	532042	*Maged2*	Melanoma antigen family D. 2	0.26	0.0158
erm	657515	*Acvr1b*	Activin A receptor type 1B	0.32	0.0108
erm	548855	*Mgea6*	Meningioma expressed antigen 6	0.33	0.0009
erm	895165	*Tuba2*	Tubulin alpha 2; tubulin alpha 6	0.34	0.0198
erm	*814680*	*Fgfr1*	Fibroblast growth factor receptor 1	*0.35*	*0.0067*
erm	298518	*Kif2c*	Kinesin family member 2C	0.37	0.0079
erm	414982	*Cdca5*	Cell division cycle associated 5	0.39	0.0084
erm	766102	*Gnai2*	Guanine nucleotide binding protein, alpha inhibiting 2	0.40	0.0001
erm	862934	*Hgf*	Hepatocyte growth factor	0.41	0.0169
erm	710361	*Hmga1*	High-mobility group AT-hook 1	0.43	0.0065
erm	608492	*Actb*	Actin beta, cytoplasmic	0.58	0.0001
erm	895170	*Cdc2l; Serf2*	Cell division cycle 2-like 1; small EDRK-rich factor 2	0.58	0.0044
erm	526436	*Igfbp4*	Insulin-like growth factor binding protein 4	0.58	0.0028
erm	684806	*Ccnd1*	Cyclin D1	0.62	0.0018
erm	862729	*Sp1*	Trans-acting transcription factor 1	0.63	0.0062
erm	588217	*Mobk1b*	Mps One Binder kinase activator-like 1B	0.63	0.0038
erm	789567	*Smc4l1*	SMC4 structural maintenance of chromosomes 4-like 1	0.63	0.0083
erm	453309	*Ccnd2*	Cyclin D2	0.65	0.0135
erm	526767	*Tspyl2*	TSPY-like 2	1.63	0.0152
erm	577094	*Lims2*	LIM and senescent cell antigen like domains 2	1.83	0.0040
erm	918786	*Nhlh2*	Nescient helix loop helix 2	2.00	0.0034
erm	625696	*Mfap5*	Microfibrillar associated protein 5	2.21	0.0030
erm	*921450*	*Igfbp7*	Insulin-like growth factor binding protein 7	*2.31*	*0.0036*

Our microarray data have shown several MMP genes and other previously described gene targets to be regulated by Pea3 and/or Erm but, because of the variability in the microarray experiments and the stringency of the statistical analysis, they are not encountered in the listing of the target genes (supplementary material Figs S3-S5, Tables S1-S3). For the target genes previously characterized in mammary cell lines (MMP1, MMP3, MMP9, vimentin, cyclin D3), the microarray data are in concordance with the type of regulation defined for Pea3 and/or Erm. Pea3 has been implicated in the transcriptional regulation of Erbb2. Direction of its modulation remains controversial; i.e. upregulator (Benz et al., 1997; Matsui et al., 2006) or repressor (Fauquette et al., 2005; Xing et al., 2000). Erbb2 is a tyrosine kinase receptor which contributes to the malignant phenotype of breast tumors and a correlation between Pea3 and Erbb2 overexpression has been depicted in primary breast tumors (Myers et al., 2006). Here, the microarray data suggest Pea3 and Erm as positive regulators of *Erbb2* transcription (Fig. 7), corroborating a recent report by Matsui et al. in breast cancer cell lines (Matsui et al., 2006).

We have also found that the *Hgf* gene is substantially regulated by Erm in the present microarray experiments. HGF increases cell migration and invasion capacity and it has been shown that HGF enhances Pea3-induced motility, invasion, and tumorigenesis and/or metastasis in lung-cancer cells (Hakuma et al., 2005). Moreover, HGF increased *pea3* expression in a dose-dependent manner (Hanzawa et al., 2000). It could thus be interesting to explore whether Erm directly regulates *Hgf* in a feed-back loop with Pea3 and, altogether, allows control of cell migration, proliferation and tumorigenic properties.

Interestingly, only ten genes (when using the significance thresholds we applied to the individual comparisons) were identified as targets in both *erm*- and *pea3*-knockdown cells. This coincides with the observation that both factors are generally expressed in the same organs and tissues, although not exactly at the same spatio-temporal localization (Chotteau-Lelievre et al., 1997; Chotteau-Lelievre et al., 2001; Chotteau-Lelievre et al., 2003). Whereas Pea3 and Erm contribute to similar cellular functions, as observed in malignant phenotypes, they apparently target distinct, but complementary molecular networks. It will be of great interest to understand how this differential targeting is achieved, because both molecules bind the same consensus-binding sites, and because apparent ability of Pea3 and Erm to compensate for each other in mouse gene inactivation studies (Chen et al., 2005; Laing et al., 2000). Most probably, co-regulatory molecules intervene in the selection of specific target genes. The present transcriptome analysis, thereby, could provide a systematic basis for future elucidation of the selection of Pea3- and Erm-target genes.

In addition to the proliferation, migration, invasion pathway, other Pea3/Erm regulated gene pathways are highlighted in the microarray experiments. Interestingly, we observed the regulation of genes that encode the water-channels, such as aquaporin 3 (*Aqp3*) that belongs to the 'cellular transport' pathway, or genes implicated in the 'fatty acid, lipid, steroid metabolism' pathway, such as, for example, acetyl-CoA synthetase long-chain-family member 4 (*Ascl4*), or genes implicated in the 'nucleic acid metabolism and transcription' pathway, such as nuclear transcription factor-Y beta (*Nfyb*) gene. These genes are until now not known to be target genes of PEA3-group members. It would be interesting to investigate the molecular mechanisms by which members of the PEA3 transcription factor groups regulate these genes and to explore the role of PEA3 factors in these newly defined pathways.

In conclusion, we have shown in vitro and in vivo the implication of Pea3 and Erm transcription factors in mechanisms involved in tumorigenesis, and depicted by microarray analysis the regulation of genes that are potentially involved in the Pea3- and/or Erm-induced phenotypic modifications. Our work provides for the first time evidences for a common and pivotal role of Erm and Pea3 in tumorigenesis by regulating complementary target-gene programs. Furthermore, we provide a basis for the dissection of the molecular mechanisms leading to Pea3- and/or Erm-induced tumorigenesis in mammary cancer cells.

Materials and Methods

Cell culture

Wild-type mouse mammary tumor (MMT) cells (ATCC CCL-51) and their derivatives were cultured in BD Falcon™ cell culture dishes in DMEM (Gibco, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Invitrogen), gentamycin (100 IU/ml) and non-essential amino acids (Gibco, Invitrogen).

Transfection

8×10^4 cells were seeded in 6-well plates under standard conditions. 200 pmol of each siRNA were mixed with 8 µl of Jetsi (PolyPlus transfection) and added drop by drop as recommended by the manufacturer. Cells were incubated for another 24 hours or more under standard conditions before being assayed.

Retroviral production and MMT infection

For production of the virus, 3×10^6 HEK 293 GP cells were transfected with 5 µg of pSUPER.retro (OligoEngine) or pSUPER.retro including a *pea3*- or *erm*-shRNA and 1 µg of pVPack-VSV-G (BD Biosciences Clontech) in the presence of 24 µl of ExGen 500 (Euromedex).

After 2 days of incubation with fresh medium, the supernatants containing viral particles were collected, filtered and used for infection of MMT cells. 2×10^6 per 100-mm dish MMT cells were incubated with supernatant mixed with 8 µg/ml of Polybrene (Sigma). The selection procedure started the next day using 2 µg/ml of puromycin (Gibco, Invitrogen).

RNA interference

The oligonucleotides used for RNA interference (RNAi) have the following sequences: si *pea3* 1 (siGENOME duplex (2), D-048237-02, ETV4, Dharmacon), si *pea3* 2 5′-GCAGGAAGGGAUUGGAGCU-3′ (Firlej et al., 2005), si *erm* 1 (siGENOME duplex (3), D-062952-03, ETV5, Dharmacon), si *erm* 2 5′-GUUCCUGAUGAUGAGCAGU-3′ and si *ctrl* (siCONTROL Non-Targeting siRNA#1, D-001210-01, Dharmacon).

The oligonucleotide DNA sequences of *pea3* and *erm* used for RNAi were hort hairpin RNAs (shRNAs) that are identical to si *pea3* 2 and si *erm* 2. They were cloned in the pSUPER.retro expression vector (Oligoengine) by following the procedure previously described (Firlej et al., 2005). These constructs were then named as pRS (empty), pRS-*pea3* A and pRS-*erm*. pRS-*pea3* B included the *pea3*-targeting DNA sequence 5′-GGTGATGGAGTGATGGGTT-3′.

RNA preparation and RT-PCR

Total RNA was isolated from Pea3- or Erm-downregulated, or control MMT cells using the RNeasy kit (Qiagen) as described by the manufacturer. cDNA were synthesized using High Archive cDNA kit (Applied Biosystems). RT- or semi-quantitative PCR were performed using the following specific primers: *pea3* (sense 5′-CGACTCAGATGTCCCTGGAT-3′ and antisense 5′-GAAAGCTCCAATC-CCTTCCT-3′), *erm* (sense 5′-GGGAAATCTCGATCAGAGGACTG-3′ and antisense 5′-GGAGCATGAAGCACCAAGTT-3′), *has2* (sense 5′-ACGGTGGGATGATGTCTTGGCTTA-3′ and antisense 5′-GCCAGTGGCTT-TCCAACTTTCCTT-3′), *hgf* (sense 5′-TGACCCAAACATCCGAGTTGGCTA-3′ and antisense 5′-GGAATGCCATTTACAACCCGCAGT-3′), *ascl4* (sense 5′-CTGTCTGACCAGTCCAGCAA-3′ and antisense 5′-GGTGTGTCTGAG-GGGACAGT-3′), *fgfr1* (sense 5′-AGATGAAGAGCGGCACCAAGAAGA-3′ and antisense 5′-CACTTTGGTCACACGGTTGGGTTT-3′), *fst* (sense 5′-CCTCCTG-CTGCTGCTACTCT-3′ and antisense 5′-CATTCGTTGCGGTAGGTTTT-3′), *suz12* (sense 5′-ATGGACAGGAGAAACCAACG-3′ and antisense 5′-CCGGTCCATT-TCGACTAAAA-3′), *stip1* (sense 5′-CAAGGAACTGGACCCTACCA-3′ and antisense 5′-TATAAGCCAAGCGCTCCTGT-3′) and *cyclophilin A* (sense 5′-GCATACAGGTCCTGGCATCTTGTCC-3′ and antisense 5′-ATGGTGATCTTCT-TGCTGGTCTTGC-3′).

Quantitative PCR was performed using the brilliant SYBRgreen qPCR master mix (Stratagene) on a MX4000 instrument as described by the provider .

Immunoblotting

Western blot analyses were done as previously described (Firlej et al., 2005) using 20 µg of whole-cell extracts and the following antibodies: anti-Pea3 (sc-113), anti-actin (sc-1616), secondary anti-goat antibody coupled to horseradish peroxydase

(HRP; sc-2020) (all Santa Cruz Biotechnology) and secondary anti-mouse antibody coupled to HRP (AP192P; Chemicon).

Morphogenetic assays

Proliferation assays

1×10^4 MMT cells were seeded on 12-well plates and were transfected the next day with 100 pmol of each siRNA and 4 μl JetSi (PolyPlus transfection). Time of transfection is referred as t_0. Each well was counted every day, after 24, 48, 72 and 96 hours.

Migration assays

Boyden chamber cell invasion was assayed using a cell culture-chamber-insert system (BD Biosciences) with an 8 μm polyethylene terephthalate (PET) membrane. Twenty-four hours post-transfection 4×10^4 cells were seeded on the upper chamber in DMEM 10% FBS. The same medium was added in the lower chamber. After 18 hours, cells that did not cross the membrane were scraped off the upper side of the membrane with a cotton swab. Cells that had migrated to the lower side were fixed with methanol at –20°C and stained with Hoechst 33258 (Sigma). The membrane was excised from its support and mounted on a glass side with Glycergel (DAKO).

Three-dimensional culture assays

After 24 hours of transfection, 4×10^3 cells per well were seeded on 200 μl of Matrigel® in a 48-well plate. After gel formation, 500 μl of 10% FBS DMEM were added. After overnight incubation, 10% FBS DMEM supplemented with hepatocyte growth factor/scatter factor (HGF/SF; 20 ng/ml) was added to 3D cultures. Cultures were maintained for 7 days, stained with Neutral Red 0.5% w/v (Sigma), fixed in PBS containing 4% paraformaldehyde and washed in PBS. Whole-mount pictures were taken using a light-microscope at 50× or 100× magnification.

Anchorage-independent growth

3×10^5 cells were seeded in 500 μl of medium mixed with 1 ml of 0.65% agar in growth medium (MEM supplemented with 10% FBS). The cell suspension was cast onto 12-well plates with 1 ml of 0.65% agar in growth medium, which was used as an underlay. DMEM supplemented with 10% FBS was added onto the agar layer and changed weekly. Colonies were counted after 15 days using a light-microscope.

In vivo tumor growth assay

MMT cells were trypsinized, resuspended in PBS (8×10^6 cells/ml). 8×10^5 cells were injected subcutaneously into SCID-deficient mice at both flanks (five mice per clone in each assay). Tumor size was assessed by measuring the length and width of tumor every 3-4 days. Tumor volume was estimated using the formula: (length × width^2) ÷2. Results are expressed as the mean of tumor volume for each experimental group.

AB1700 Microarray Technology

All experimental data referred to as MGS V1.0 (Mouse Genome Survey Version 1, ProdNo: 4337467) in this manuscript were generated on Applied Biosystems AB1700 transcriptome platform (ProdNo: 4338036). These arrays contain probes for 28,218 validated mouse genes, and have been demonstrated to cover an increased dynamic signal range, to display higher sensitivity and provide more robust gene expression estimates when compared with the leading competing technologies (Calderwood et al., 2006; Noth et al., 2006b).

RNA labeling, hybridization and detection

RNA amplification, labeling, hybridization, and detection were done following the protocols supplied by Applied Biosystems together with the corresponding kits. 2 μg of total RNA were submitted to RT-IVT amplification and labeling (Applied Biosystems, ProdNo: 4339628). Labeled cRNAs were then hybridized and detected according to the supplied protocols (Applied Biosystems, ProdNo: 4346875). Three independent biological replicates were performed for each condition (mock-transfected and siRNA-transfected cells).

Data preprocessing and primary analysis

Applied Biosystems Expression Array System Software v1.1.1. (ProdNo: 4364137) has been used to acquire the chemiluminescence and fluorescence images and primary data analysis. Notice that we once more normalized the resulting data according to the median after probes had been removed for which the Applied Biosystems Software has set flags equal or greater 2^{12}, indicating compromised or failed measurements (as recommended by Applied Biosystems).

Data processing and statistical analysis

Calculation of subtraction profiles was performed according to standard procedures with the following modifications: data from different biological conditions were compared in an 'everyone-against-everyone' scheme and log2 quotients (logQ, L) where then determined as averages of weighted individual logQ values. The weights were anti-proportional to the variance over the individual logQ values. For these inter-assay comparisons the NeONORM method was used for normalization, using k=0.2 (Noth et al., 2006a). *P*-values were determined on the basis of a normal distribution hypothesis of signal intensities by using the standard ANOVA method. Multiple probes recognizing a single gene, cross-reactivity of a single probe with several genes, as well as the resolution of probe-ID annotations was done according to the standards defined previously (Noth and Benecke, 2005).

Pathway analysis

Combining GO, Kegg, and PANTHER annotations (see supplementary material Figs S3-S5), we assigned all probes present on the MGS V1.0 array to the pathways shown in the figures. We then calculated the relative representation of those probes detected as significantly regulated by the siRNA as compared with a random set of probes drawn from the ensemble of probes. *P*-values for over- and under-representation of pathways were calculated using a binominal distribution.

The authors warmly thank Isabelle Damour for excellent technical assistance, Maud Merchez, Didier Monté and Nathalie Marchand for their contribution to this work, and Corine Glineur for help with the qPCR experiments. François-Xavier Pellay is thanked for help with the pathway analysis. This work was made possible through financial support from Ligue Nationale Contre le Cancer (Comité Aisne, France), GEFLUC Flandre Artois, Association pour la Recherche sur le Cancer (France), Fondation pour la Recherche Médicale, Conseil Régional Nord/Pas-de-Calais (France), European Regional Development Fund (Intergenes program), Centre National pour la Recherche Scientifique (CNRS, France), Institut des Hautes Études Scientifiques, the Fonds National pour la Recherche Scientifique (FNRS) (Belgium), Action de Recherche Concertée de la Communauté Française de Belgique (Belgium) and European Hematology Association – José Carreras Foundation. The transcriptome data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE6588.

References

Baert, J. L., Monte, D., Musgrove, E. A., Albagli, O., Sutherland, R. L. and de Launoit, Y. (1997). Expression of the PEA3 group of ETS-related transcription factors in human breast-cancer cells. *Int. J. Cancer* **70**, 590-597.

Baert, J. L., Beaudoin, C., Monte, D., Degerny, C., Mauen, S. and de Launoit, Y. (2007). The 26S proteasome system degrades the ERM transcription factor and regulates its transcription-enhancing activity. *Oncogene* **26**, 415-424.

Benz, C. C., O'Hagan, R. C., Richter, B., Scott, G. K., Chang, C. H., Xiong, X., Chew, K., Ljung, B. M., Edgerton, S., Thor, A. et al. (1997). HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. *Oncogene* **15**, 1513-1525.

Bieche, I., Tozlu, S., Girault, I., Onody, P., Driouch, K., Vidaud, M. and Lidereau, R. (2004). Expression of PEA3/E1AF/ETV4, an Ets-related transcription factor, in breast tumors: positive links to MMP2, NRG1 and CGB expression. *Carcinogenesis* **25**, 405-411.

Calderwood, S. K., Khaleque, M. A., Sawyer, D. B. and Ciocca, D. R. (2006). Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem. Sci.* **31**, 164-172.

Chen, J. H., Vercamer, C., Li, Z., Paulin, D., Vandenbunder, B. and Stehelin, D. (1996). PEA3 transactivates vimentin promoter in mammary epithelial and tumor cells. *Oncogene* **13**, 1667-1675.

Chen, C., Ouyang, W., Grigura, V., Zhou, Q., Carnes, K., Lim, H., Zhao, G. Q., Arber, S., Kurpios, N., Murphy, T. L. et al. (2005). ERM is required for transcriptional control of the spermatogonial stem cell niche. *Nature* **436**, 1030-1034.

Chotteau-Lelievre, A., Desbiens, X., Pelczar, H., Defossez, P. A. and de Launoit, Y. (1997). Differential expression patterns of the PEA3 group transcription factors through murine embryonic development. *Oncogene* **15**, 937-952.

Chotteau-Lelievre, A., Dolle, P., Peronne, V., Coutte, L., de Launoit, Y. and Desbiens, X. (2001). Expression patterns of the Ets transcription factors from the PEA3 group during early stages of mouse development. *Mech. Dev.* **108**, 191-195.

Chotteau-Lelievre, A., Montesano, R., Soriano, J., Soulie, P., Desbiens, X. and de Launoit, Y. (2003). PEA3 transcription factors are expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells in vitro. *Dev. Biol.* **259**, 241-257.

Chotteau-Lelievre, A., Revillion, F., Lhotellier, V., Hornez, L., Desbiens, X., Cabaret, V., de Launoit, Y. and Peyrat, J. P. (2004). Prognostic value of ERM gene expression in human primary breast cancers. *Clin. Cancer Res.* **10**, 7297-7303.

Davidson, B., Goldberg, I., Tell, L., Vigdorchik, S., Baekelandt, M., Berner, A., Kristensen, G. B., Reich, R. and Kopolovic, J. (2004). The clinical role of the PEA3 transcription factor in ovarian and breast carcinoma in effusions. *Clin. Exp. Metastasis* **21**, 191-199.

de Launoit, Y., Baert, J. L., Chotteau-Lelievre, A., Monte, D., Coutte, L., Mauen, S., Firlej, V., Degerny, C. and Verreman, K. (2006). The Ets transcription factors of the PEA3 group: transcriptional regulators in metastasis. *Biochim. Biophys. Acta* **1766**, 79-87.

Delannoy-Courdent, A., Mattot, V., Fafeur, V., Fauquette, W., Pollet, I., Calmels, T., Vercamer, C., Boilly, B., Vandenbunder, B. and Desbiens, X. (1998). The expression of an Ets1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells. *J. Cell Sci.* **111**, 1521-1534.

Discenza, M. T., Vaz, D., Hassell, J. A. and Pelletier, J. (2004). Activation of the WT1 tumor suppressor gene promoter by Pea3. *FEBS Lett.* **560**, 183-191.

El-Tanani, M., Platt-Higgins, A., Rudland, P. S. and Campbell, F. C. (2004). Ets gene PEA3 cooperates with beta-catenin-Lef-1 and c-Jun in regulation of osteopontin transcription. *J. Biol. Chem.* **279**, 20794-20806.

Fauquette, V., Perrais, M., Cerulis, S., Jonckheere, N., Ducourouble, M. P., Aubert, J. P., Pigny, P. and Van Seuningen, I. (2005). The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells: implications for the proliferation/differentiation balance in the cells. *Biochem. J.* **386**, 35-45.

Firlej, V., Bocquet, B., Desbiens, X., de Launoit, Y. and Chotteau-Lelievre, A. (2005). Pea3 transcription factor cooperates with USF-1 in regulation of the murine bax transcription without binding to an Ets-binding site. *J. Biol. Chem.* **280**, 887-898.

Fleming, F. J., Myers, E., Kelly, G., Crotty, T. B., McDermott, E. W., O'Higgins, N. J., Hill, A. D. and Young, L. S. (2004). Expression of SRC-1, AIB1, and PEA3 in HER2 mediated endocrine resistant breast cancer; a predictive role for SRC-1. *J. Clin. Pathol.* **57**, 1069-1074.

Furlan, A., Vercamer, C., Desbiens, X. and Pourtier, A. (2008). Ets-1 triggers and orchestrates the malignant phenotype of mammary cancer cells within their matrix environment. *J. Cell Physiol.* **215**, 782-793.

Galang, C. K., Muller, W. J., Foos, G., Oshima, R. G. and Hauser, C. A. (2004). Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. *J. Biol. Chem.* **279**, 11281-11292.

Habelhah, H., Okada, F., Kobayashi, M., Nakai, K., Choi, S., Hamada, J., Moriuchi, T., Kaya, M., Yoshida, K., Fujinaga, K. et al. (1999). Increased E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. *Oncogene* **18**, 1771-1776.

Hakuma, N., Kinoshita, I., Shimizu, Y., Yamazaki, K., Yoshida, K., Nishimura, M. and Dosaka-Akita, H. (2005). E1AF/PEA3 activates the Rho/Rho-associated kinase pathway to increase the malignancy potential of non-small-cell lung cancer cells. *Cancer Res.* **65**, 10776-10782.

Hanzawa, M., Shindoh, M., Higashino, F., Yasuda, M., Inoue, N., Hida, K., Ono, M., Kohgo, T., Nakamura, M., Notani, K. et al. (2000). Hepatocyte growth factor upregulates E1AF that induces oral squamous cell carcinoma cell invasion by activating matrix metalloproteinase genes. *Carcinogenesis* **21**, 1079-1085.

Hida, K., Shindoh, M., Yoshida, K., Kudoh, A., Furaoka, K., Kohgo, T., Fujinaga, K. and Totsuka, Y. (1997). Expression of E1AF, an ets-family transcription factor, is correlated with the invasive phenotype of oral squamous cell carcinoma. *Oral Oncol.* **33**, 426-430.

Hiroumi, H., Dosaka-Akita, H., Yoshida, K., Shindoh, M., Ohbuchi, T., Fujinaga, K. and Nishimura, M. (2001). Expression of E1AF/PEA3, an Ets-related transcription factor in human non-small-cell lung cancers: its relevance in cell motility and invasion. *Int. J. Cancer* **93**, 786-791.

Jiang, J., Wei, Y., Liu, D., Zhou, J., Shen, J., Chen, X., Zhang, S., Kong, X. and Gu, J. (2007). E1AF promotes breast cancer cell cycle progression via upregulation of Cyclin D3 transcription. *Biochem. Biophys. Res. Commun.* **358**, 53-58.

Kaya, M., Yoshida, K., Higashino, F., Mitaka, T., Ishii, S. and Fujinaga, K. (1996). A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. *Oncogene* **12**, 221-227.

Kurpios, N. A., Sabolic, N. A., Shepherd, T. G., Fidalgo, G. M. and Hassell, J. A. (2003). Function of PEA3 Ets transcription factors in mammary gland development and oncogenesis. *J. Mammary Gland Biol. Neoplasia* **8**, 177-190.

Laing, M. A., Coonrod, S., Hinton, B. T., Downie, J. W., Tozer, R., Rudnicki, M. A. and Hassell, J. A. (2000). Male sexual dysfunction in mice bearing targeted mutant alleles of the PEA3 ets gene. *Mol. Cell. Biol.* **20**, 9337-9345.

Lu, W. C., Liu, Y. N., Kang, B. B. and Chen, J. H. (2003). Trans-activation of heparanase promoter by ETS transcription factors. *Oncogene* **22**, 919-923.

Matsui, K., Sugimori, K., Motomura, H., Ejiri, N., Tsukada, K. and Kitajima, I. (2006). PEA3 cooperates with c-Jun in regulation of HER2/neu transcription. *Oncol. Rep.* **16**, 153-158.

Moss, A. C., Lawlor, G., Murray, D., Tighe, D., Madden, S. F., Mulligan, A. M., Keane, C. O., Brady, H. R., Doran, P. P. and Macmathuna, P. (2006). ETV4 and Myeov knockdown impairs colon cancer cell line proliferation and invasion. *Biochem. Biophys. Res. Commun.* **345**, 216-221.

Myers, E., Hill, A. D., Kelly, G., McDermott, E. W., O'Higgins, N. J. and Young, L. S. (2006). A positive role for PEA3 in HER2-mediated breast tumour progression. *Br. J. Cancer.* **95**, 1404-1409.

Nelson, C. M. and Bissell, M. J. (2005). Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation. *Semin. Cancer Biol.* **15**, 342-352.

Noth, S. and Benecke, A. (2005). Avoiding inconsistencies over time and tracking difficulties in Applied Biosystems AB1700/Panther probe-to-gene annotations. *BMC Bioinformatics* **6**, 307.

Noth, S., Brysbaert, G. and Benecke, A. (2006a). Normalization using weighted negative second order exponential error functions (NeONORM) provides robustness against asymmetries in comparative transcriptome profiles and avoids false calls. *Genomics Proteomics Bioinformatics* **4**, 90-109.

Noth, S., Brysbaert, G., Pellay, F. X. and Benecke, A. (2006b). High-sensitivity transcriptome data structure and implications for analysis and biologic interpretation. *Genomics Proteomics Bioinformatics* **4**, 212-229.

Shepherd, T. and Hassell, J. A. (2001). Role of Ets transcription factors in mammary gland development and oncogenesis. *J. Mammary Gland Biol. Neoplasia* **6**, 129-140.

Shepherd, T. G., Kockeritz, L., Szrajber, M. R., Muller, W. J. and Hassell, J. A. (2001). The pea3 subfamily ets genes are required for HER2/Neu-mediated mammary oncogenesis. *Curr. Biol.* **11**, 1739-1748.

Shindoh, M., Higashino, F. and Kohgo, T. (2004). E1AF, an ets-oncogene family transcription factor. *Cancer Lett.* **216**, 1-8.

Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L. and Montesano, R. (1995). Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J. Cell Sci.* **108**, 413-430.

Upadhyay, S., Liu, C., Chatterjee, A., Hoque, M. O., Kim, M. S., Engles, J., Westra, W., Trink, B., Ratovitski, E. and Sidransky, D. (2006). LKB1/STK11 Suppresses Cyclooxygenase-2 Induction and Cellular Invasion through PEA3 in Lung Cancer. *Cancer Res.* **66**, 7870-7879.

Xing, X., Wang, S. C., Xia, W., Zou, Y., Shao, R., Kwong, K. Y., Yu, Z., Zhang, S., Miller, S., Huang, L. et al. (2000). The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis. *Nat. Med.* **6**, 189-195.