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

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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.

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, salivary 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 derived 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.

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 occurs 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 immunodefficient mice**

To follow in immunodefficient mice the behavior of MMT cells in which the PEA3 group members are knocked-down, we used a
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. 2. pea3 and erm](image-url)
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**

|                  | MA-siRNA-pea3 | MA-siRNA-erm |
|------------------|---------------|--------------|
| qPCR-siRNA-pea3  | 0.939         | 0.613        |
| qPCR-siRNA-erm   | 0.280         | 0.634        |

**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.
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 regulatory capacities of Pea3 and Erm in this particular mammary tumor model, and 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 material Fig. S1B,C) (Baert et al., 1997). Moreover, to prevent the potential off-target effects of these siRNA sequences and also to ensure 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 regulatory capacities of Pea3 and Erm in this particular mammary tumor model, and in agreement with the little overlap observed between the target-gene-expression programs of both transcription factors.

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In accordance with the in vitro results, xenografts of MMT cells and their derivatives in immunodeficient assays to tumorigenesis in vivo by implanting subcutaneous in vivo. siRNA or shRNA repression resulted in the inhibition of cancer-cell proliferation, migration, morphogenetic organization and cell growth. The absence of integrin-derived adhesive signals (anchorage-independent growth) is the phenotypic hallmark of neoplastic 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; Kaye 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

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

| Pathway                        | Pea3 | Erm |
|-------------------------------|------|-----|
| Migration, proliferation, invasion | 25  | ±   | 1.09×10^{-4} | 25  | ±   | 1.43×10^{-5} |
| 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 metabolism and transcription | 11  | +   | 1.25×10^{-1} | 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^{-2} |
| 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.
A correlation has been established with cyclin D1 (Jiang et al., 2000), implicated in proliferation or cell cycle regulation, such as FGF receptor 1 (FGFR1), cyclin D1 (Ccnld1), cyclin D2 (Ccnld2), sestrin 2 (Sesn2), and other genes. For example, we observed a significant regulation of genes encoding proteins that are directly implicated in proliferation or cell-cycle regulation, such as follistatin (Fst), melano antigen family D 2 (Maged2) or hyaluronan synthetase 2 (Has2).

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       | Tub3a6      | Tubulin alpha 3b | 0.30 | 0.0045 |
| pea3       | 814680       | Fgfr1       | Fibroblast growth factor receptor 1 | 0.38 | 0.0005 |
| pea3       | 640066       | Eph8        | Epidermal growth factor receptor pathway substrate 8 | 0.45 | <0.00005 |
| pea3       | 764223       | Snn         | Stanniain | 0.45 | 0.0011 |
| pea3       | 905667       | Stip1       | Stress-induced phosphoprotein 1 | 0.47 | 0.0064 |
| pea3       | 410010       | 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       | Rrn2b       | 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       | 825265       | Est         | 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       | Hmnr        | 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       | Mmp7        | Myosin heavy polypeptide 7, cardiac muscle beta | 4.22 | <0.00005 |
| pea3       | 829347       | Rdm1        | RAD52 motif 1 | 4.24 | <0.00005 |
| erm        | 715233       | Tubu7       | 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       | Acrv1b      | Activin A receptor type 1B | 0.32 | 0.0108 |
| erm        | 548855       | Mga6        | Meningioma expressed antigen 6 | 0.33 | 0.0009 |
| erm        | 895165       | Tuba2       | Tubulin alpha 2; tubulin alpha 6 | 0.34 | 0.0198 |
| erm        | 814660       | Fgfr1       | Fibroblast growth factor receptor 1 | 0.35 | 0.0067 |
| erm        | 298518       | Kif2c       | Kinesin family member 2C | 0.37 | 0.0079 |
| erm        | 414982       | Cdx5a       | Cell division cycle associated 5 | 0.39 | 0.0084 |
| erm        | 766102       | Gna2        | Guanine nucleotide binding protein, alpha inhibiting 2 | 0.40 | 0.0001 |
| erm        | 862934       | Hgf         | Hepatocyte growth factor | 0.41 | 0.0169 |
| erm        | 710336       | 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        | 684086       | Ccd1        | Cyclin D1 | 0.62 | 0.0018 |
| erm        | 862729       | Sp1         | Trans-acting transcription factor 1 | 0.63 | 0.0062 |
| erm        | 588217       | Mobs1b      | Mps One Binder kinase activator-like 1B | 0.63 | 0.0138 |
| erm        | 789567       | Smc4l1      | SMC4 structural maintenance of chromosomes 4-like 1 | 0.63 | 0.0083 |
| erm        | 453309       | Ccd2        | Cyclin D2 | 0.65 | 0.0135 |
| erm        | 526767       | Tspyl2      | TSPY-like 2 | 1.16 | 0.0152 |
| erm        | 577094       | Lim2        | LIM and senescent cell antigen like domains | 1.83 | 0.0040 |
| erm        | 918786       | Nhlh2       | Nescent helix loop helix 2 | 2.00 | 0.0034 |
| erm        | 625696       | Mjaps5      | 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 (Fauquet 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), 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 Ermbinding 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 35-mm 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

8×10⁵ cells were seeded in 6-well plates under standard conditions. 200 pmol of each siRNA were mixed with 8 μl of Jets (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, 3×10⁶ 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 (Euromex). After 2 days of incubation with fresh medium, the supernatants containing viral particles were collected, filtered and used for infection of MMT cells. 2×10⁶ 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'-GACGAGAAAGAUAGAGCGU-3' (Firel et al., 2005), si erm 1 (siGENOME duplex (3), D-062952-03, ETV5, Dharmacon), si erm 2 5'-GUUCCUAGUGAUGAGCU-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 (Firel et al., 2005). These constructs were then named as pRS, pRS-pea3 A or pRS-erm, pRS-pea3 B included the pea3-targeting DNA sequence 5'-GGTGTAGGTGATGTTGTTT-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'-GGAAAGTCTCCACTCCCTTCTT-3'), errm (sense 5'-GGGAAATCTCGATCAGAGGACTG-3' and antisense 5'-GGGACAGTAAAGCACAAGATGTT-3'), hat2 (sense 5'-ACGGTGAGGATGCTTGGCTT-3' and antisense 5'-GCCAGTGCTTCTCAACCTTTTCT-3'), hgf (sense 5'-TGACCCAAAATCAGGAGCAGTTGCT-3' and antisense 5'-GGAAATGGCCATTACACCCCGACGAT-3'), ascl4 (sense 5'-CTGGTGACGGACTCGGAAGGAAAGGATA-3' and antisense 5'-GGTGTGCTGCTGGAGGACAGT-3'), gfrl (sense 5'-AGATGAGGAGGCGACCAAGGAAGA-3' and antisense 5'-CACTTTTGTCAGACCTGGTTT-3'), fat (sense 5'-CCTCGTCTGCTGCTGATCTTCT-3' and antisense 5'-CCTCTGTTGCCCTGACAGAATGTTT-3'), ssc12 (sense 5'-ATGGACAGAGAGAAGGCAAGCG-3' and antisense 5'-CCTGGTCATTCTGACTAA-3'), stpl (sense 5'-CAAGGAACCTGACCCCTACCA-3' and antisense 5'-TATAAGCCAAGGCGTCCGTTT-3'), cyclophilin A (sense 5'-GCTACAGGCTGCCGTCTGCTGTCG-3' and antisense 5'-ATGTTGATCTTCTCTGCTGTCG-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 (Firel 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 peroxidase.
(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 JetSti (PolyPlus transfection). Time of transfection is referred as t=D. 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 hour 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, 4×10^4 cells per well were seeded on 200 μl of Matrigel® in a 48-well plate. After gel formation, 500 μl H11003 was excised from its support and mounted on a glass side with Glycergel (DAKO). 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).

**In vivo tumor growth assay**

MMT cells were trypsinned, resuspended in PBS (8×10^5 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: \( V = \frac{4}{3} \pi r^2 \times \text{width}^2 \). 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 transcription probe platform (ProdNo: 4538036). 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., 2006).

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 binomial distribution.

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