MFG-E8/lactadherin regulates cyclins D1/D3 expression and enhances the tumorigenic potential of mammary epithelial cells

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Milk fat globule-EGF factor 8 (MFG-E8) is a glycoprotein highly expressed in breast cancer that contributes to tumor progression through largely undefined mechanisms. By analyzing publicly available gene expression profiles of breast carcinomas, we found that MFG-E8 is highly expressed in primary and metastatic breast carcinomas, associated with absent estrogen receptor expression. Immunohistochemistry analysis of breast cancer biopsies revealed that MFG-E8 is expressed on the cell membrane as well as in the cytoplasm and nucleus. We also show that increased expression of MFG-E8 in mammary carcinoma cells increases their tumorigenicity in immunodeficient mice, and conversely, its downregulation reduces their *in vivo* growth. Moreover, expression of MFG-E8 in immortalized mammary epithelial cells promotes their growth and branching in three-dimensional collagen matrices and induces the expression of cyclins D1/D3 and N-cadherin. A mutant protein unable to bind integrins can in part exert these effects, indicating that MFG-E8 function is only partially dependent on integrin activation. We conclude that MFG-E8-dependent signaling stimulates cell proliferation and the acquisition of mesenchymal properties and contributes to mammary carcinoma development.

Keywords: MFG-E8; mammary tumorigenesis; neoplastic transformation; cyclin D; N-cadherin

Introduction

Milk fat globule-EGF factor 8 (MFG-E8), also known as lactadherin in humans, is a secreted glycoprotein

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originally identified as a membrane constituent of MFGs (Ceriani *et al*., 1977, 1982). It is expressed in several cell types, including mammary epithelial and myoepithelial cells, macrophages, dendritic cells, endothelial cells, intestinal and retinal epithelial cells (Raymond *et al*., 2009). Structurally, mouse MFG-E8 contains two N-terminal EGF-like domains and two C-terminal discoidin-like domains. The second EGF repeat includes the integrin-binding motif RGD that can interact with integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, whereas the discoidin-like domains promote binding to membrane phospholipids. The binding of MFG-E8 to integrins on macrophages and to phosphatidylserine and phosphatidylethanolamine residues on apoptotic cells establishes intercellular interactions that promote the subsequent engulfment of apoptotic cells (Hanayama *et al*., 2002, 2004). Similarly, MFG-E8 is required for the removal of apoptotic cells by mammary epithelial cells during mammary gland involution (Atabai *et al*., 2005; Hanayama and Nagata, 2005). MFG-E8 binding to integrins regulates a variety of signaling pathways. In endothelial cells, this interaction activates Akt, thus inducing VEGF-dependent angiogenesis (Silvestre *et al*., 2005), and it promotes tumor angiogenesis in a mouse model of pancreatic cancer (Neutzner *et al*., 2007). In melanoma cells, MFG-E8 was found to promote a 'mesenchymal', motile phenotype by inducing the expression of the transcription factors Twist and Snail and to enhance cell survival through the activation of Akt (Jinushi *et al*., 2008). Moreover, when these cells were injected in mice, MFG-E8 enhanced tumor growth through the inhibition of antitumor immune responses (Jinushi *et al*., 2008). In line with this, functionally blocking antibodies against MFG-E8 potentiated the effects of chemotherapeutic agents on experimental melanomas, colon carcinomas and lymphomas (Jinushi *et al*., 2009). In the mammary gland, MFG-E8 expression increases during pregnancy, lactation and subsequent mammary gland involution (Oshima *et al*., 1999; Hanayama and Nagata, 2005). It was demonstrated that MFG-E8 is required for mammary gland branching morphogenesis (Ensslin and Shur, 2007). During this process, the interaction of MFG-E8, secreted by luminal

cells, with the integrins αvβ3/αvβ5, expressed on myoepithelial cells, activates mitogen-activated protein kinases in the latter and promotes cell proliferation and duct development (Ensslin and Shur, 2007). Years ago MFG-E8, originally called BA46, was found to be expressed in some breast carcinomas and breast carcinoma cell lines and to be present in the sera of patients with advanced breast cancer, but not in that of healthy individuals (Ceriani *et al.*, 1982; Peterson *et al.*, 1990; Larocca *et al.*, 1991). Schmidt and co-workers recently confirmed and extended these observations, showing that MFG-E8 is highly expressed in triple-negative breast cancers, but that it is downregulated during progression in estrogen receptor (ER)-positive carcinomas, suggesting that it may perform different functions in different breast cancer subtypes (Yang *et al.*, 2011). These findings underscore the putative value of MFG-E8 as a biomarker and likely therapeutic target in breast carcinomas, and the need to better understand its functional properties. In this work, we show that MFG-E8 induces the expression of cyclins D1 and D3, and of N-cadherin in non-transformed mammary epithelial cells, by a mechanism only partly dependent on integrin activation, and stimulates their proliferation and branching in collagen gels. In addition, we show that upregulation of MFG-E8 expression in mammary cancer cells enhances their ability to form tumors in mice, whereas its downregulation inhibits tumor growth *in vivo*. Our results suggest that MFG-E8 deregulation plays an important role in mammary carcinogenesis.

Results

MFG-E8 is highly expressed in breast cancer

We evaluated the expression of MFG-E8 in breast cancer by analyzing the gene expression profiles of 808 breast carcinomas obtained by combining three data sets from the Gene Expression Omnibus database (Supplementary Table S1). MFG-E8 was found to be highly expressed in both primary and metastatic breast carcinomas and its expression was significantly higher in ER-negative samples (Figure 1a). When examining a possible correlation between MFG-E8 and ErbB2 expression, we found that MFG-E8 was expressed independently of ErbB2; nonetheless, patients with higher MFG-E8 levels were usually among those who did not have the highest ErbB2 levels (log intensity ErbB2 >12, possibly corresponding to tumors with ErbB2 amplification) (Figure 1b). Notably, patients with high MFG-E8 expression (log intensity MFG-E8 >9) and levels of ErbB2 characterized by log intensity <12 (included in the rectangular area in Figure 1b) showed a tendency to shorter survival, although the difference with respect to patients having tumors with low MFG-E8 levels was not statistically significant ($P = 0.057$) (Supplementary Figure S1A). This tendency did not reflect the patients' ER status because in the patient cohort analyzed, we could not detect an association between low ER levels and decreased

survival, probably due to the small number of ER-negative patients present in this cohort (Supplementary Figure S1B). Despite the small number of patients, we divided them according to their ER status and examined their survival with respect to MFG-E8 expression. Again, we observed a tendency to shorter survival for MFG-E8-high patients in both ER-positive and -negative groups, even if the differences were not statistically significant (Supplementary Figure S1C).

MFG-E8 is expressed in in situ *and in invasive breast carcinomas*

We then analyzed MFG-E8 expression by immunohistochemistry in a random series of human breast cancer samples. MFG-E8 was detected in 33 out of 46 samples, including *in situ* and invasive carcinomas, with 13 biopsies characterized by high expression and 20 by low expression (Figure 2A). MFG-E8 appeared to be localized in the plasma membrane and the cytoplasm, and in some samples also in the nucleus (Figure 2B (a–d, g)). Normal mammary epithelial cells express MFG-E8 (Figure 2B (f)), as reported (Ensslin and Shur, 2007). We also noticed that in several samples, MFG-E8 expression was high at the tumors' borders, particularly in cells invading the stroma (Figure 2B (h, i)).

MFG-E8 promotes in vivo *growth of mammary cancer cells*

To understand if MFG-E8 influences mammary cancer cell growth, we modulated its expression in the cell lines 4T1 and 168FARN, which were derived from a spontaneous mouse mammary carcinoma after several passages *in vivo* and *in vitro*. Both cell lines form tumors *in vivo* when injected into the mouse fat pad, but while 4T1 cells are systemically metastatic, 168FARN cells locally invade lymph nodes and do not metastasize systemically (Aslakson and Miller, 1992). Endogenous expression levels of MFG-E8 are high in 4T1 cells and much lower in 168FARN cells (Figure 3a). We downregulated or increased MFG-E8 expression in 4T1 and 168FARN cells, respectively, by infection with lentiviruses containing either short hairpin RNAs (shRNAs) for MFG-E8 or MFG-E8 cDNA. Of the four constructs tested, sh95, sh97 and sh98 efficiently reduced MFG-E8 levels in 4T1 cells (Figure 3b); on the other hand, MFG-E8 expression was highly increased in 168FARN cells after transduction (Figure 3a). We observed that upon MFG-E8 downregulation, 4T1 cells acquired an epithelial morphology and tended to grow in cohesive islets (Figure 3c). However, these features were lost after a few passages and cells re-expressed MFG-E8 (not shown). These results suggest that high expression of MFG-E8 is necessary for normal *in vitro* growth of 4T1 cells.

In contrast, overexpression of MFG-E8 in 168FARN cells did not induce morphological changes (not shown) and did not affect cell growth *in vitro* (Figure 4a). To test whether MFG-E8 might influence cell behavior *in vivo*, we injected control and MFG-E8-expressing 168FARN cells into the fat pads of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.
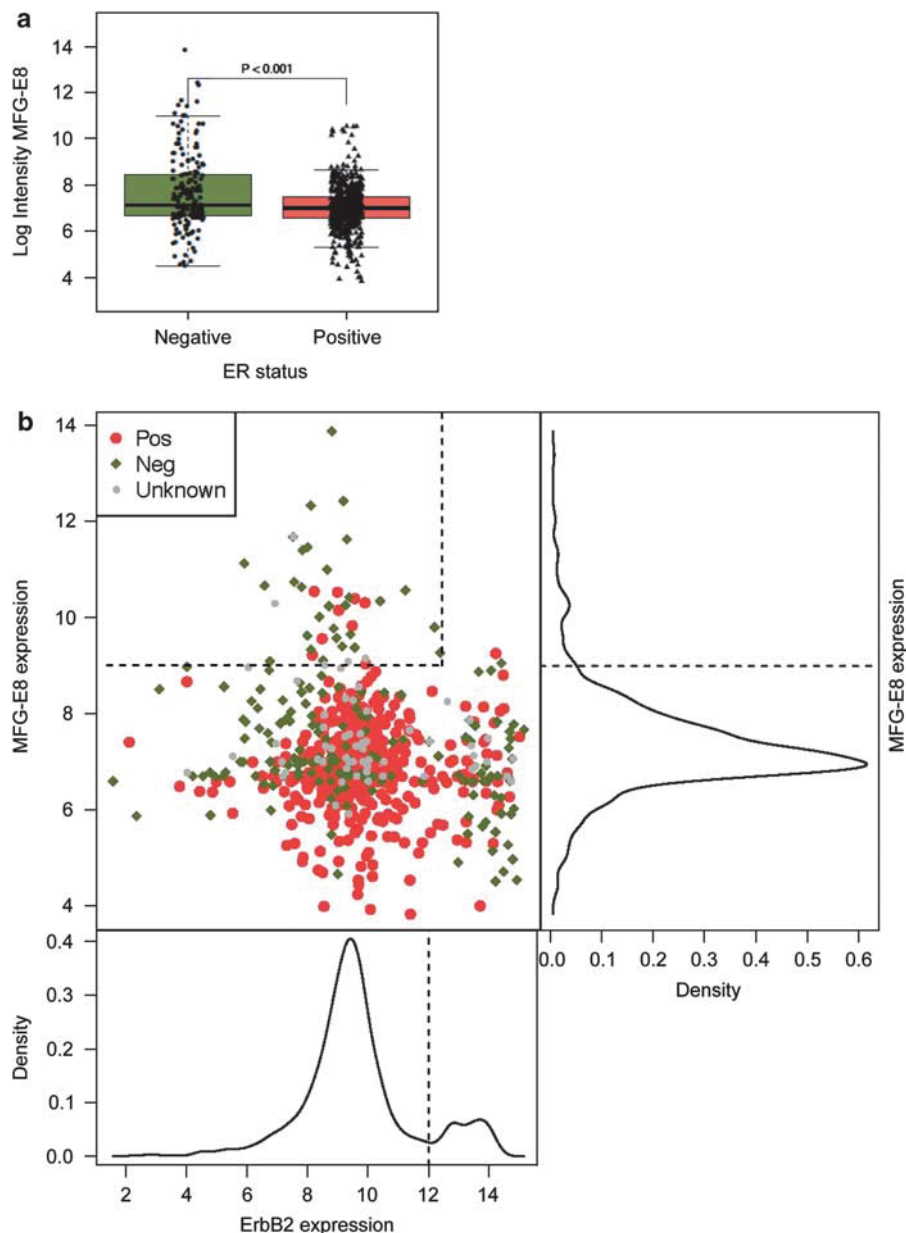
Figure 1 MFG-E8 is highly expressed in breast carcinomas in association with lack of ER expression. (**a**) Box and whiskers plot showing that MFG-E8 expression is negatively correlated to ER expression ($P < 0.001$). (**b**) Expression of MFG-E8 in relation to ER status and ErbB2 levels. MFG-E8 and ErbB2 expression is represented as log intensity; red and green dots represent ER-positive and -negative patients, respectively; expression density plots shown for MFG-E8 and ErbB2 indicate a bimodal distribution for ErbB2, with a group of patients having very high ErbB2 expression (log intensity > 12), possibly due to ErbB2 amplification. Higher MFG-E8 levels are found in patients with log intensity ErbB2 between 6 and 12. The rectangular area delimited by a dashed line defines patients indicated as MFG-E8 high whose survival was compared with that of the remaining patients (see Supplementary Figure S1A).

We observed that within 10 days after cell injection, all mice that received 168FARN-MFG-E8 cells had developed palpable tumors, whereas only one mouse injected with control cells presented a small tumor. Within 20 days after injection, all mice injected with control cells had developed tumors, which were much smaller than the MFG-E8-expressing ones (Figure 4b). To analyze if MFG-E8 expression can also promote systemic metastasis formation of 168FARN cells, we repeated the experiment with 168FARN-control and -MFG-E8 cells expressing luciferase. Tumor growth and metastasis formation was monitored over a period of 20 days during which time no metastases were observed in any mice, despite the presence of large tumors in mice injected with 168FARN-MFG-E8 cells. The experiment could not be protracted beyond 20 days because of the large tumors, and mice had to be killed (Figure 4c). As MFG-E8 was shown to promote tumor angiogenesis (Neutzner *et al.*, 2007), we tested whether tumors derived from 168FARN-MFG-E8 cells were more vascularized than control tumors. Staining of tumor sections with anti-CD31 antibodies revealed no differences in the vascular density
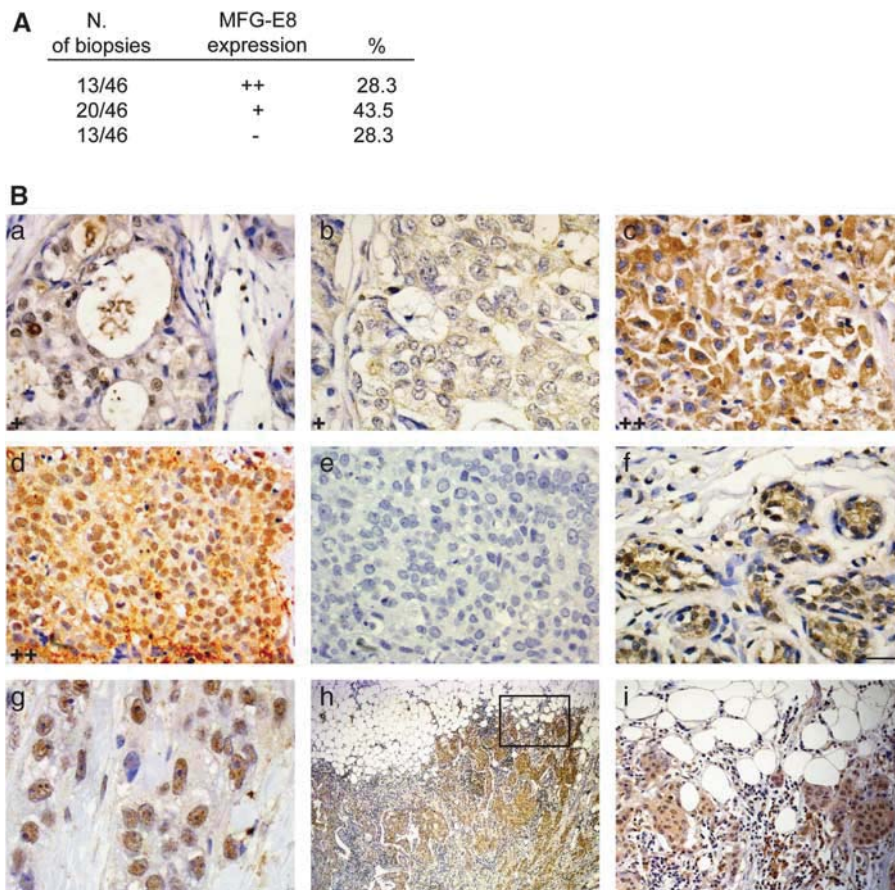
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N. of biopsies	MFG-E8 expression	%
13/46	++	28.3
20/46	+	43.5
13/46	-	28.3

Figure 2 MFG-E8 is expressed in *in situ* and in invasive breast carcinomas. (**A**) Immunostaining of formalin-fixed breast cancer specimens with anti-MFG-E8 antibodies reveals expression of the protein in 33 out of 46 cases (71%), with 13 of the cases showing strong staining intensity (28%). Samples with different staining intensities (+, + +) are shown in (**B**): a, *in situ* carcinoma; b–d, invasive carcinomas; e, negative control without primary antibody; f, normal mammary gland. MFG-E8 was detected both in the cytoplasm and nucleus (a–d); g, higher magnification of a sample presenting strong nuclear staining; h, more intense MFG-E8 staining was often detected at the invasion front of the tumors; and i, higher magnification of the region enclosed in the rectangular area in h. Scale bars: a–f, i, 50 μm; g, 30 μm; h, 200 μm.

in 168FARN-MFG-E8 tumors compared with controls, despite the differences in tumor size (Figure 4d). This observation suggests that increased *in vivo* proliferation of cells expressing MFG-E8 is not due to enhanced recruitment of blood vessels.

Taken together, these results indicate that MFG-E8 promotes *in vivo* growth of mammary cancer cells and its loss impairs *in vitro* cell proliferation and/or survival.

MFG-E8 downregulation in Ras-transformed mammary epithelial cells impairs their in vivo *growth*

As MFG-E8 loss is not compatible with the *in vitro* growth of 4T1 cells, we hypothesized that its down-regulation could be maintained in transformed cells expressing an oncogene that would sustain their proliferation/survival in the absence of MFG-E8. We used Ras-transformed mouse mammary epithelial cells EpRas that stably express the v-Ha-Ras oncogene (Oft *et al.*, 1996). As shown in Figure 5a, these cells express MFG-E8, whose levels were successfully decreased by MFG-E8-directed shRNAs. Similarly to 4T1shMFG-E8 cells, EpRas-shMFG-E8 cells acquired an epithelial

morphology and tended to grow in cohesive groups (Figure 5b). These features, along with the correlated downregulation of MFG-E8, could be maintained in culture after several passages, allowing us to inject two EpRas-shMFG-E8 cell populations orthotopically into nude mice. We found that downregulation of MFG-E8 decreased tumor growth (Figure 5c). The difference in tumor size was significant for both sh cell populations until day 12 ($P = 0.021$ for sh95 tumors, $P = 0.004$ for sh98 tumors); at day 15, only EpRas-sh98 grew significantly less than control cells ($P = 0.010$), whereas at day 19, the difference in tumor growth was no longer significant for either population ($P > 0.05$). Similar results with significant slower growth for the shMFG-E8 tumors in the first 15 days compared with control tumors were obtained in two independent experiments. Analysis of MFG-E8 expression in five shMFG-E8 tumors that were excised at day 19 showed that three tumors had regained the expression of MFG-E8 (Figure 5d). This may be either due to loss of shRNAs' efficacy in silencing MFG-E8 expression *in vivo* or to the fact that cells with higher levels of MFG-E8 proliferate faster and hence outgrow the MFG-E8-negative cell

population. Expression levels of MFG-E8 correlated to tumors size for sh tumors 1, 2, 3 and 4 at day19; on the other hand, sh tumor 5, which did not appear to regain expression of MFG-E8, was as large as sh tumor 3,
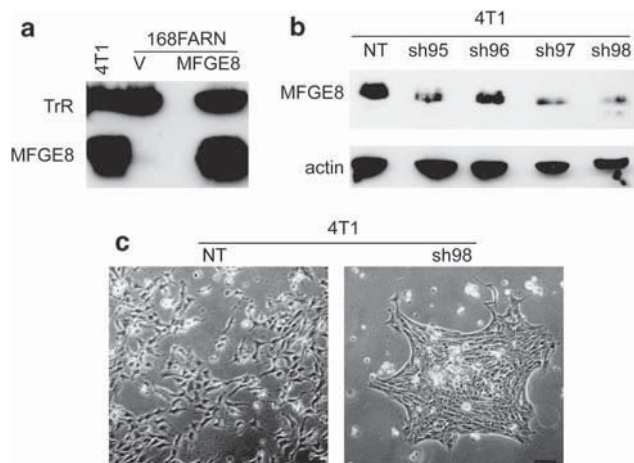

Figure 3 Downregulation of MFG-E8 induces an epithelial morphology in 4T1 cells. (**a**) Expression of endogenous MFG-E8 in 4T1 cells and in control (V) or MFG-E8-transduced (MFG-E8) 168FARN cells; transferrin receptor (TrR) expression was analyzed as a loading control. (**b**) Decreased expression of MFG-E8 in 4T1 cells stably expressing different shRNAs for the protein. (**c**) Partial silencing of MFG-E8 expression induces the acquisition of an epithelial morphology in 4T1 cells, which tend to grow in compact islets. Scale bar, 20 μm.

which displayed the highest levels of MFG-E8 (Figure 5e), and NT tumor 3 grew slowly, despite expressing high levels of MFG-E8. If the differences in the growth of NT tumors are probably due to stochastic experimental variability, the fast growth of sh tumor 5 after day 15 suggests that other mechanisms, possibly dependent on oncogenic Ras, circumvent growth inhibition due to decreased MFG-E8 expression. The results obtained in this experiment indicate that partial loss of MFG-E8 expression decreases the tumorigenicity of EpRas cells in the initial phases of *in vivo* growth, and suggest that MFG-E8 cooperates with Ras in promoting their proliferation.

MFG-E8 expression in immortalized mammary epithelial cells promotes their proliferation and the formation of cord-like structures in collagen gels

We then wondered if increased expression of MFG-E8 could affect the growth of immortalized, non-trans-formed mammary epithelial cells. We chose immortalized normal murine mammary epithelial cells NMuMG in which we overexpressed wild-type MFG-E8, or a mutant version of the protein carrying a point mutation in the integrin-binding motif RGD, and thus unable to interact with integrins (Andersen *et al.*, 1997; Taylor *et al.*, 1997) (Figure 6A). We first verified that the interaction of MFG-E8 with NMuMG cells' surface receptors requires the RGD motif by plating the cells on dishes coated with recombinant mouse MFG-E8
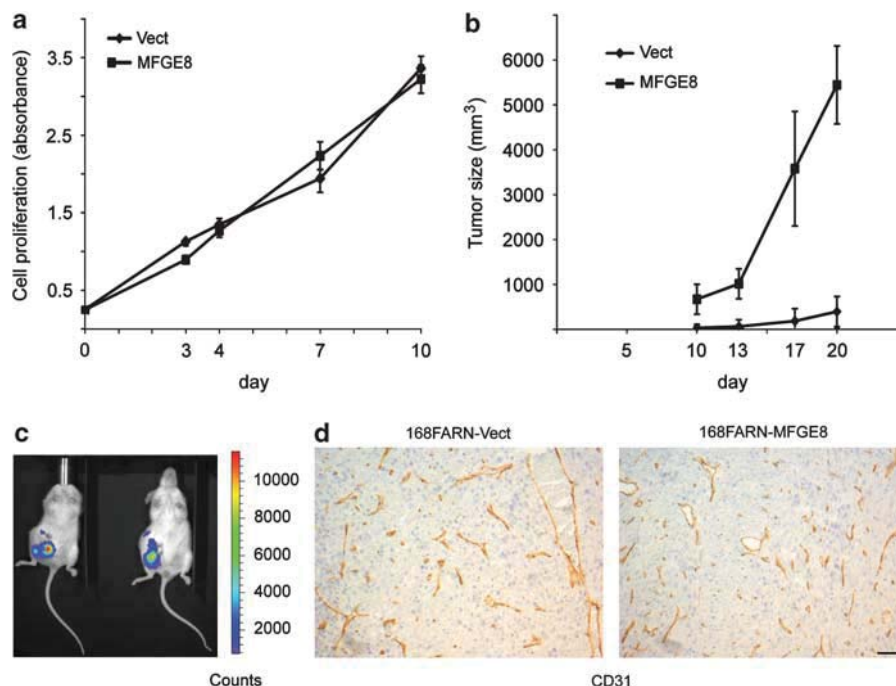

Figure 4 Overexpression of MFG-E8 in 168FARN cells promotes their *in vivo* growth. (**a**) MFG-E8 expression does not stimulate cell proliferation *in vitro*. Cells were transduced with an empty vector (Vect) or with a vector driving the expression of MFG-E8. (**b**) Analysis of *in vivo* growth of control and MFG-E8-expressing 168FARN cells after orthotopic injection in NOD/SCID mice (6 mice per group). Each point represents the average tumor size. MFG-E8 significantly enhances experimental tumor formation already during the first days after injection. (**c**) Xenogen images of mice injected orthotopically with 168FARN cells expressing MFG-E8 and luciferase. After 20 days from injection, no metastases were detectable in mice injected with either control or MFG-E8-expressing cells (6 mice per group). (**d**) CD31 staining of experimental tumors derived from control (168FARN-Vect) and MFG-E8-expressing cells shows similar vascular densities. Scale bar, 100 μm.
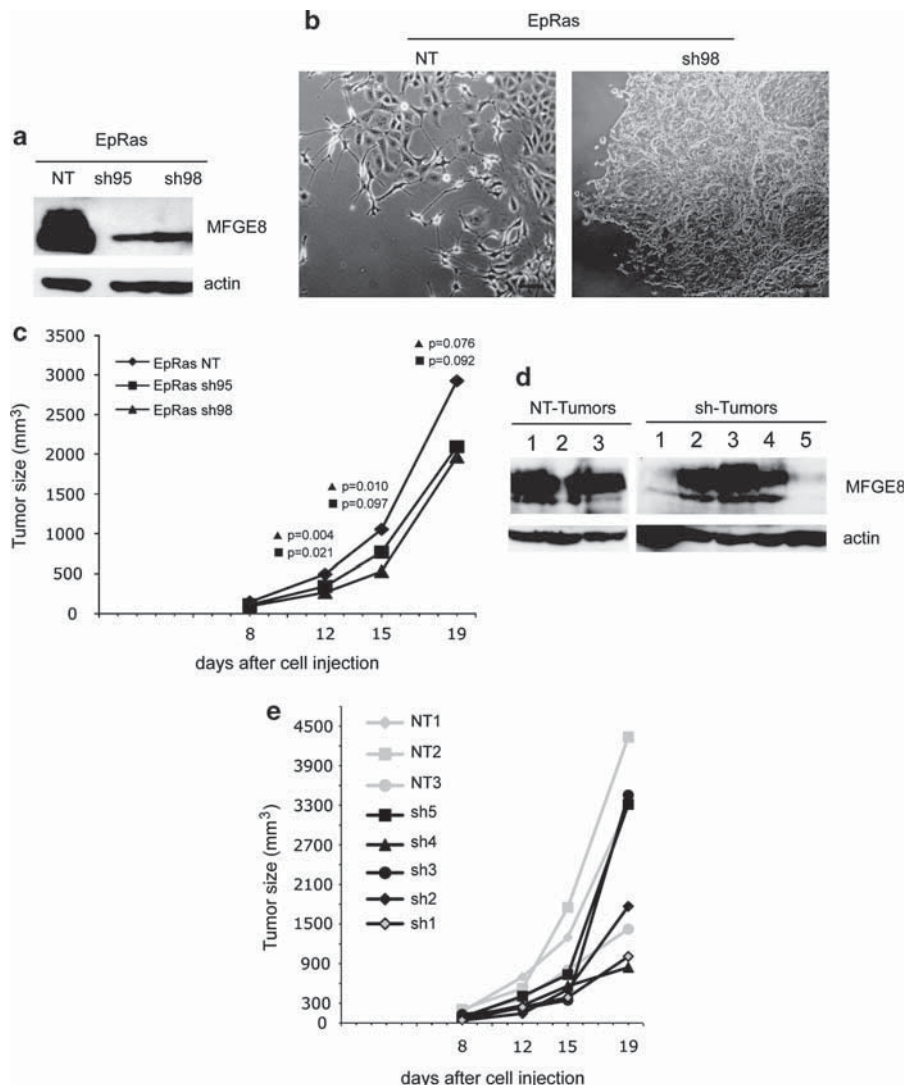
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Figure 5 Downregulation of MFG-E8 in cells expressing activated Ras induces epithelial features and reduces their *in vivo* growth. (**a**) Western blot showing the expression of MFG-E8 in EpRas cells after transduction with a non-targeting (NT) or two different MFG-E8-silencing (sh95, sh98) RNAs. (**b**) EpRas cells acquire an epithelial morphology upon reduction of MFG-E8 expression. Scale bars, 20 μm (NT) and 40 μm (sh98). (**c**) *In vivo* growth of experimental tumors derived from control and MFG-E8-silenced EpRas cells injected orthotopically in nude mice. MFG-E8 silencing partially inhibited tumor growth during the first 12–15 days; at later times, the difference in growth between control and silenced tumors was no longer statistically significant (6 mice per cell population; each point represents the average tumor size); *P*-values refer to comparisons of each sh cell population to control cells at different time points. (**d**) Western blots showing re-expression of MFG-E8 in tumors originally silenced for its expression, excised after 19 days from cell injection. (**e**) Growth of individual NT and sh tumors shown in (**d**). Correlation between MFG-E8 levels and tumor size is seen for sh tumors 1–4, whereas sh tumor 5 grew fast despite low expression levels of MFG-E8.

(recMFG-E8) in the presence of soluble RGD peptides or control RAD peptides. As a control, cells were also plated on collagen I that mediates binding through β1 integrins in an RGD-independent manner (Taubenberger *et al.*, 2010), and on poly-lysine that promotes cell attachment through electrostatic interactions with the cell membranes without engaging integrins. As shown in Supplementary Figure S2, cells spread on recMFG-E8 and collagen I, but not on poly-lysine, suggesting that MFG-E8 binds to adhesion receptors expressed in NMuMG cells; RGD peptides, but not RAD, inhibited cell spreading on recMFG-E8, but not on collagen I, indicating that MFG-E8 binds NMuMG surface

receptors through its RGD motif. Expression of MFG-E8 and of the RGE mutant in NMuMG cells promoted cell proliferation *in vitro* with no evident morphological change (Figure 6B and data not shown). To better understand the effect of MFG-E8 in NMuMG cells, we examined their growth pattern in collagen gels. Embedding of cells in a gel of an extracellular matrix protein allows studying their morphology and underlying biochemical properties in a three-dimensional (3D) environment that mimics the situation encountered by the cells *in vivo* (Janda *et al.*, 2002). After 6 days in collagen gels, we observed that whereas control cells tended to form elongated structures with minimal side
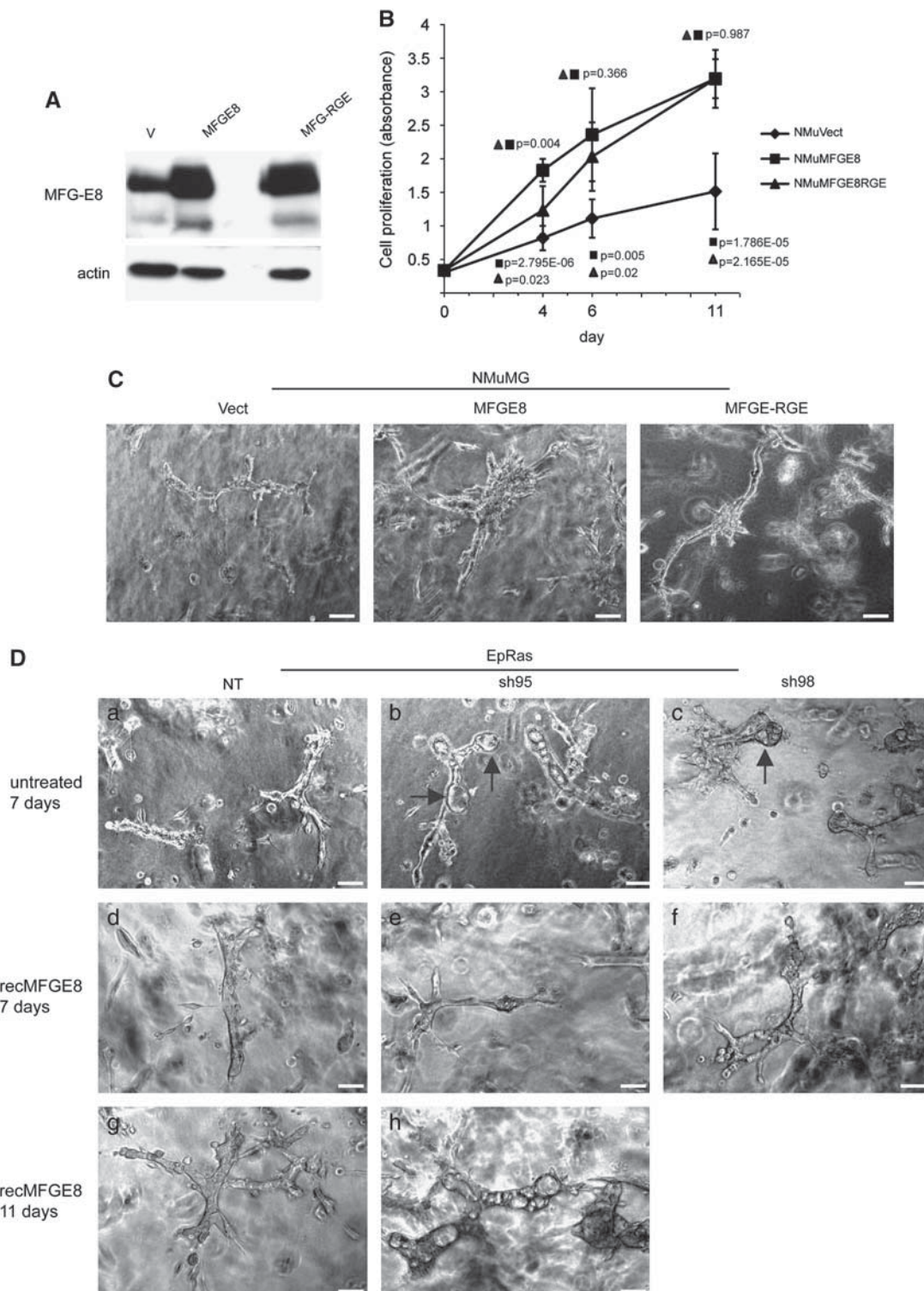
Figure 6 Modulation of MFG-E8 expression affects cell proliferation and branching in collagen I matrices. (**A**) Expression levels of MFG-E8 and MFG-E8-RGE in non-transformed NMuMG cells after viral transduction (V: control cells). (**B**) MFG-E8 and MFG-E8-RGE increase the *in vitro* proliferation of NMuMG cells. In the upper part of the graph, *P*-values refer to comparisons between NMuMG-MFG-E8 and NMuMG-MFG-E8-RGE cells; in the lower part, they refer to comparisons between cell populations expressing MFG-E8 or MFG-E8-RGE and control cells. (**C**) Morphology of NMuMG cells in collagen I gels. Control cells (Vect) and cells expressing MFG-E8 or the RGE mutant were embedded in collagen I matrices for 6 days. The expression of MFG-E8 and, to a lesser extent, MFG-E8-RGE promotes the formation of cell clusters that tend to form several protrusions invading the gel. (**D**) EpRas control (NT) and sh cells embedded in collagen I gels in the absence (a–c) or presence (d–h) of recMFG-E8. In 7 days, NT cells formed elongated structures, whereas sh cells formed round structures with lumina (b, c, arrows) whose establishment was only retarded by treatment with recMFG-E8 (e, f, h). The morphology of control cells was apparently not affected by recMFG-E8 treatment (d, g). Scale bars, 20 μm.

branching, possibly precursors of tubular structures, cells expressing MFG-E8 grew in colonies, forming multiple protrusions and lateral branching that extended into the gels. Cells expressing the RGE mutant showed an intermediate phenotype, with a tendency to grow in small clusters and form protrusions (Figure 6C). These observations suggest that MFG-E8 promotes prolifera-tion of NMuMG cells and formation of cord-like structures in 3D collagen, and that this effect is only partially dependent on its interaction with integrins.

When injected in NOD/SCID mice, NMuMG cells expressing MFG-E8 or MFG-E8-RGE did not form tumors, indicating that MFG-E8 alone is not sufficient to induce neoplastic cell transformation.

EpRas-control and -shMFG-E8 cells were also seeded in collagen gels. After 7 days, control cells had formed elongated structures, whereas EpRas-shMFG-E8 cells had a tendency to form round, empty structures suggestive of ongoing differentiation (Figure 6D). When the cells were grown in the presence of recMFG-E8 for 7 days, shMFG-E8 cells tended to produce somewhat more elongated, cord-like structures, while no morphological changes were evident in control cells. However, longer incubation in the presence of recMFG-E8 for 11 days could not completely reverse the morphology of shMFG-E8 cells to that of control cells in these conditions, and several empty structures eventually formed; control cells maintained the same morphology after exposure to recMFG-E8 for 11 days (Figure 6D).

MFG-E8 signaling function in NMuMG cells
To gain some insight into the biochemical mechanisms underlying MFG-E8 function, NMuMG cells expres-sing MFG-E8 or MFG-E8-RGE and control cells were lysed after 3 days in collagen I gels. We observed that MFG-E8 and, to a slightly lesser extent, MFG-E8-RGE increased the expression levels of cyclins D1 and D3. When examining the activation state of the phosphati-dylinositol 3-kinase pathway and of the extracellular signal-regulated kinases, we found no differences in the activation of Akt and extracellular signal-regulated kinases in the three cell populations (Figure 7a). We also found that MFG-E8 and MFG-E8-RGE increased the expression of N-cadherin, whereas E-cadherin levels remained unchanged. In agreement with this result, cyclins D1 and D3 expression was decreased in 4T1 cells silenced for MFG-E8 expression compared to control cells, whereas E-cadherin levels remained unchanged, and N-cadherin was undetectable (Figure 7b). The expression of cyclin D3, but not of cyclin D1, appeared reduced also in EpRas-sh tumors in relation to MFG-E8 levels, but this only partially correlated to tumor growth (Supplementary Figure S3). We also detected a slight increase in N-cadherin levels in EpRas-sh tumors that had regained expression of MFG-E8 (Supplementary Figure S3). These results indicate that MFG-E8 may promote cell cycle progression and the acquisition of mesenchymal features by inducing D cyclins and N-cadherin, respectively.
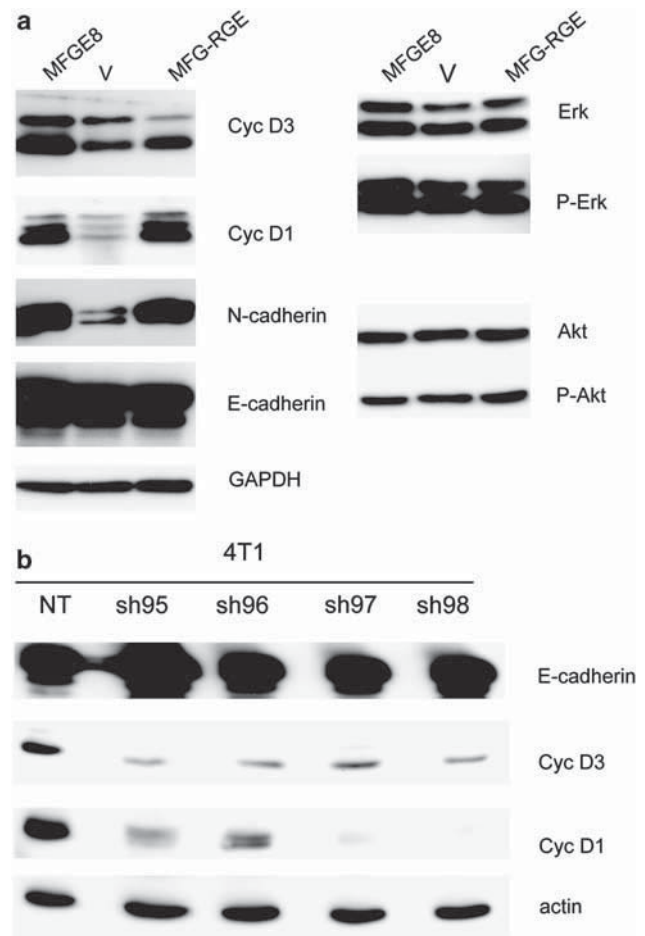

Figure 7 MFG-E8 induces cyclins D1 and D3 and N-cadherin. (**a**) NMuMG cells expressing MFG-E8 or the RGE mutant, and control cells (V) were cultured in collagen gels for 3 days. Western blots show increased levels of cyclins D1 and D3, and of N-cadherin in MFG-E8- and MFG-E8-RGE-expressing cells. No changes in E-cadherin expression and in extracellular signal-regulated kinases and Akt phosphorylation were detected. (**b**) Expression of cyclins D1 and D3 and of E-cadherin in control (NT) 4T1 cells and in cells silenced for MFG-E8 expression (sh cells).

Discussion

In this work, we asked whether increased expression of MFG-E8 may promote mammary epithelial cell trans-formation and contribute to breast carcinoma progres-sion. Our analysis of gene expression data revealed that MFG-E8 is highly expressed in primary and metastatic breast cancers and that its high expression is significantly associated with lack of ER expression as reported previously (Yang *et al.*, 2011). We also found that high levels of MFG-E8 can be present in tumors expressing ErbB2, but not in those with the highest levels of ErbB2 (possibly bearing gene amplification). By modulating MFG-E8 expression in mammary tumor cells, we found that MFG-E8 upregulation promotes tumorigenesis *in vivo* and that its downregulation induces an epithelial morphology and impairs cell growth in mice. In addition, we show that overexpression of MFG-E8 in non-transformed mammary epithelial cells increases their

in vitro proliferation and the expression of cyclins D1 and D3, but it is not sufficient to promote their growth *in vivo*, indicating that MFG-E8 *per se* cannot induce neoplastic cell transformation; conversely, MFG-E8 silencing in 4T1 cells decreases cyclin D1 and D3 levels.

D cyclins regulate cell cycle progression through the G1 phase and their expression is often increased in tumors as a result of oncogene activation (Lee and Yang, 2003). In particular, cyclin D1 is overexpressed in over 50% of breast carcinomas (Roy and Thompson, 2006), and in some cases along with increased cyclin D3 expression (Russell *et al.*, 1999). Targeted expression of cyclin D1 in the mammary gland of transgenic mice causes hyperplasia, followed by neoplastic transformation after a latent period during which oncogenic mutations are presumably acquired (Wang *et al.*, 1994). In contrast, its ablation in transgenic mice expressing Neu or ras oncogenes prevents mammary carcinoma development (Yu *et al.*, 2001). Our finding that MFG-E8 induces cyclin D expression in non-transformed cells suggests that it may cooperate with oncogenes to initiate mammary epithelial cell transformation, whereas its ability to increase *in vivo* growth of cancer cells indicates that in later stages of tumor development, it may enhance cell aggressiveness and sustain tumor progression.

We have also observed that in EpRas cells expressing oncogenic Ras, reduction of MFG-E8 expression initially impairs tumor growth and is correlated in most tumors with decreased cyclin D3 (but not cyclin D1) expression, which could contribute to tumor growth inhibition; restoration of sh tumors' growth at later times correlates in most cases with re-expression of MFG-E8. It thus appears that at least in the initial phases of tumor growth MFG-E8 silencing decreases the tumorigenicity of EpRas cells, but this effect is rapidly counteracted by still undefined mechanisms, probably functionally related to oncogenic Ras, which are readily switched on and that over time make EpRas cell growth independent of MFG-E8 levels. These observations suggest that MFG-E8 and Ras can cooperate in increasing cell tumorigenic potential.

We have also found that MFG-E8 and the RGE mutant induce the expression of N-cadherin in NMuMG cells. Even if there is no clear evidence for a role of N-cadherin in promoting breast carcinoma progression (Cowin *et al.*, 2005), several studies have demonstrated that N-cadherin expression in breast cancer cells induces the acquisition of mesenchymal properties and facilitates invasion and metastases in experimental models (Hazan *et al.*, 2000; Suyama *et al.*, 2002). In support of these findings, increased N-cadherin levels were found to be associated with the progression of highly invasive micropapillary breast carcinomas (Nagi *et al.*, 2005).

MFG-E8 is known to activate integrin-dependent signaling by interacting with $\alpha v \beta 3/5$ integrins through its RGD motif (Taylor *et al.*, 1997; Raymond *et al.*, 2009). In this work, we show that inactivation of the RGD motif does not impair MFG-E8 ability to stimulate cell proliferation *in vitro* and it only partially reduces cell proliferation and branching in a 3D collagen matrix.

The RGE mutant is also able to induce cyclins D1 and D3 and N-cadherin. Even if we cannot exclude that the expression of the RGE mutant might increase the secretion of the endogenous protein that would then be responsible for the effects we observe, it is also possible that MFG-E8 function in mammary epithelial cells is only in part mediated by its binding to integrins. Other yet unidentified membrane receptors might be able to interact with MFG-E8 independently of the RGD motif and activate cell signaling. Alternatively, MFG-E8 might function intracellularly. In this respect, our observation that it may localize in the nuclei of breast carcinomas and also normal breast epithelial cells suggests a possible nuclear function. An intracellular function for MFG-E8 is also supported by the fact that recMFG-E8 can only partially revert the morphology of EpRas cells in which MFG-E8 was silenced. This observation suggests that the interaction of exogenous MFG-E8 with membrane receptors is not sufficient to restore the morphological features of the parental cells when intracellular levels of the protein are low. Experiments to clarify MFG-E8 activity in different cellular compartments are currently ongoing in the lab.

Ensslin and Shur (2007) showed that in the developing mammary gland, MFG-E8 is expressed in both luminal and myoepithelial cells and that it promotes mammary gland branching morphogenesis by activating mitogen-activated protein kinases in the myoepithelial cells. They also showed that primary mammary gland organoids lacking MFG-E8 have reduced proliferation and branching in collagen gels. Our results demonstrate that MFG-E8 can activate signaling pathways also in mammary epithelial cells even in the absence of interacting myoepithelial cells and stimulate their proliferation and branching in 3D collagen. This observation supports a role for MFG-E8 in promoting mammary cancer cell growth when normal cell–cell interactions are progressively lost. The fact that MFG-E8 increases the tumorigenicity of 168FARN cells in NOD/SCID mice without an apparent induction of tumor angiogenesis suggests that this protein has additional functions in a developing mammary tumor, independent of, or not limited to, endothelial cell activation (Silvestre *et al.*, 2005; Neutzner *et al.*, 2007) and Treg-lymphocyte-mediated immune suppression (Jinushi *et al.*, 2008).

Yang *et al.* (2011) have reported that whereas MFG-E8 may promote the progression of triple-negative breast carcinomas, it appears to inhibit the progression of ER- and ErbB2-positive tumors that in their study are characterized by a reduced basal expression of this protein. They also found that MFG-E8 downregulation in ER-positive cells stimulates their proliferation. These observations are not consistent with our results in ER-expressing NMuMG and 168FARN cells (Supplementary Figure S4 and Joel *et al.*, 1998; Lincoln *et al.*, 2003), where MFG-E8 exerted growth-promoting effects *in vitro* and *in vivo*, respectively. However, it is possible that both NMuMG and 168FARN cells have acquired mutations or signaling abnormalities during the immortalization process (NMuMG) and sequential *in vivo*

and *in vitro* passages (168FARN) that cooperate with MFG-E8 in inducing cell proliferation, irrespective of the presence of functional ERs. It is hence conceivable that the ER status *per se* is not sufficient to determine MFG-E8 function in breast carcinoma cells and that the overall effect of MFG-E8 signaling will depend on its interactions with other signaling pathways that may become deregulated during malignant progression.

It has been shown that functionally blocking anti-MFG-E8 antibodies induced the regression of experimental breast cancers (Ceriani *et al.*, 1987) and acted synergistically with conventional chemotherapy to reduce experimental colon carcinomas, melanomas and lymphomas (Jinushi *et al.*, 2009); in addition, triple-negative breast cancer cells in which MFG-E8 expression was silenced were more sensitive to cisplatin treatment (Yang *et al.*, 2011). Taken together with this published evidence, our present results support the idea that combinatorial therapies that include MFG-E8 blockade may be operative in the treatment of some breast cancer patients. However, as MFG-E8 function might be only partly mediated by its interaction with membrane receptors upon secretion, functionally blocking antibodies may only dampen its activity, without completely abrogating it. For this reason, it is critical to better elucidate MFG-E8 signaling properties and intracellular partners to identify novel mechanisms that can be therapeutically targeted. Moreover, as MFG-E8 function appears to vary in different breast cancer subtypes (Yang *et al.*, 2011), it is important to identify those cancer types that will respond to anti-MFG-E8 therapies.

In conclusion, we have shown that MFG-E8 enhances the growth and tumorigenic potential of mammary epithelial cells by an autocrine mechanism that may imply the induction of cyclins D1 and D3 and the acquisition of mesenchymal properties, and that only in part requires its interaction with integrins. Our results emphasize the importance of MFG-E8 as a biomarker in the diagnosis of breast carcinomas and also as a putative molecular target in breast cancer therapy.

Materials and methods

MFG-E8 gene expression analysis
The gene expression profile of 808 carcinomas performed with the Affymetrix platform hgu133a were obtained from three data sets contained in the Gene Expression Omnibus database, namely GSE20194 (230 samples) (Symmans *et al.*, 2003; Ayers *et al.*, 2004; Hess *et al.*, 2006; Peintinger *et al.*, 2007), GSE3494 (251 samples) (Miller *et al.*, 2005) and GSE6532 (327 samples) (Loi *et al.*, 2007). The raw CEL files were combined and normalized by Robust Multiarray Averaging using the Affymetrix power tools software. The output file was imported in the R software for statistical analysis. The difference in MFG-E8 expression between ER-positive or -negative samples was tested using non-parametric Wilcoxon's rank-sum test. The test was performed also within each batch of data to rule out any bias effect.

Cell culture and lentivirus transduction
4T1 and 168FARN cells were kindly provided by F Miller (Wayne State University, Detroit, MI, USA), EpRas cells by E Reichmann (University Children's Hospital Zurich, Zurich, Switzerland) and NMUMG cells by G Christofori (University of Basel, Basel, Switzerland). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified CO_2 atmosphere. 168FARN cells were supplemented with 1% non-essential amino acids. For over-expression experiments, a synthetic clone coding for MFG-E8 was used (ID: NM_008594; Source Bioscience imaGenes, Berlin, Germany). MFG-E8 cDNAs was subcloned into the lentiviral vector psd44 (from R Iggo, University of St Andrews, Edinburgh, UK). The RGD/RGE mutation was introduced by using the QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) using the primers: forward, 5′-ACACCCAGCGGGGCGAGATCTTCACCGAGTACAT-3′; reverse, 5′-ATGTACTCGGTGAAGATCTCGCCCCGCTGGGTGT-3′, and psd44-MFG-E8 as a template. The resulting construct was sequenced to verify the presence of the mutation. For silencing experiments, we used the lentiviral shRNA expression system Mouse pLKO.1-puro, consisting of several shRNAs for MFG-E8 and a control, non-targeting shRNA (shRNA set: RMM4534-NM_008594; Open Biosystems, Huntsville, AL, USA). Infected cells were selected by puromycin.

Animals and tumorigenesis experiments
Engineered 168FARN and EpRas cells (10^6) were implanted into the mammary fat pad of 4- to 6-week-old NOD/SCID or Hsd:athymic nude-Foxn1nu females (Harlan Laboratories, Venray, the Netherlands), respectively. Six mice per group were analyzed in two independent experiments. Mice were inspected daily and tumors were measured with calipers twice a week. Bioluminescence images of mice injected with cells stably expressing luciferase were acquired by using the *in vivo* Imaging System (Xenogen Biosciences, Cranbury, NJ, USA) 10 min after intraperitoneal injection of D-luciferin at 150 mg/kg (Biosynth, Staad, Switzerland). All animal studies were conducted in accordance with the regulations of the Service of Consumables and Veterinary Affairs—Division of Animal Protection (SCAV-EXPANIM) and the Federal Veterinary Office.

Proliferation assay
Cells were seeded in 96-well plates, stained with crystal violet and lysed in sodium citrate 0.1 M. Absorbance was measured using a Modulus Microplate Multimode Reader (Promega, Madison, MI, USA). Results shown are the average of three experiments performed in quadruplicate.

Collagen gel cultures
Cultures of NMuMG and EpRas cells in 3D collagen were performed as described with minor modifications (Janda *et al.*, 2002). Cells were resuspended in 1.5 mg/ml rat-tail collagen I (BD Biosciences, Franklin Lakes, NJ, USA) in serum-free Dulbecco's modified Eagle's medium containing the Mammary Epithelial Cell Growth Medium Supplement Mix (PromoCell, Heidelberg, Germany). The gels were overlaid with the same medium and placed at 37 °C in a CO_2 incubator; 2% fetal bovine serum in the upper medium was added for NMuMG cell cultures. In some experiments, recombinant mouse MFG-E8 (5 μg/ml; R&D Systems, Minneapolis, MN, USA) was added to the gels and the above medium.

Cell and tissue lysis and western blots
Frozen tumors or cells cultured on plastic were lysed in a modified radioimmunoprecipitation buffer (25 mM HEPES, 150 mM NaCl, 1.5 mM. $MgCl_2$, 1 mM EGTA, 10 mM Na

pyrophosphate, 100 mM NaF, 1% Triton X-100, 1% sodium deoxycolate, 0.1% sodium dodecyl sulfate, Protease Inhibitor Cocktail (Sigma, St Louis, MO, USA). Collagen gels were resuspended in lysis buffer (25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40) containing protease and phosphatase inhibitors as above, and the suspensions were passed several times through 25- and 29-G needles sequentially before centrifugation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blots were performed using standard techniques. The following antibodies were used: anti-N-cadherin, -E-cadherin (BD Transduction Laboratories, Franklin Lakes, NJ, USA); -cyclin D1 (Lab Vision; NeoMarkers, Fremont, CA, USA); -cyclin D3 (Santa Cruz, Santa Cruz, CA, USA), -Akt, -phospho-Akt (Ser473), -p44/p42 mitogen-activated protein kinase, -phospho-p44/42 (Thr202/Tyr204) (Cell Signaling, Danvers, MA, USA); -MFG-E8 (R&D Systems); -transferrin receptor (Zymed Laboratories, San Francisco, CA, USA); and -GAPDH (Sigma).

Immunohistochemistry
After surgical resection, tumors were fixed in 4% formalin and embedded in paraffin. Sections of experimental tumors and of human breast carcinoma specimens (obtained in a codified manner from the tissue bank of the Institute of Pathology,

Locarno, Switzerland) were stained with anti-CD31 (NeoMarkers) or anti-MFG-E8 (Sigma Prestige, St Louis, MO, USA) antibodies, respectively, according to standard protocols.

Statistical analysis
Statistical significance of the *in vitro* and *in vivo* growth assays was determined by Student's *t*-tests.

Conflict of interest

The authors declare no conflict of interest.

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