ADAM12 redistributes and activates MMP-14, resulting in gelatin degradation, reduced apoptosis and increased tumor growth

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Summary

Matrix metalloproteinases (MMPs), in particular MMP-2, MMP-9 and MMP-14, play a key role in various aspects of cancer pathology. Likewise, ADAMs (a disintegrin and metalloproteinases), including ADAM12, are upregulated in malignant tumors and contribute to the pathology of cancers. Here, we show that there is a positive correlation between MMP-14 and ADAM12 expression in human breast cancer. We demonstrated that in 293-VnR and human breast cancer cells expressing ADAM12 at the cell surface, endogenous MMP-14 was recruited to the cell surface, resulting in its activation. Subsequent to this activation, gelatin degradation was stimulated and tumor cell apoptosis was decreased, with reduced expression of the pro-apoptotic proteins BCL2L11 and BIK. The effect on gelatin degradation was abrogated by inhibition of the MMP-14 activity and appeared to be dependent on cell surface αVβ3 integrin localization, but neither the catalytic activity of ADAM12 nor the cytoplasmic tail of ADAM12 were required. The significance of ADAM12-induced activation of MMP-14 was underscored by a reduction in MMP-14-mediated gelatin degradation and abolition of apoptosis-protective effects by specific monoclonal antibodies against ADAM12. Furthermore, orthotopic implantation of ADAM12-expressing MCF7 cells in nude mice produced tumors with increased levels of activated MMP-14 and confirmed that ADAM12 protects tumor cells against apoptosis, leading to increased tumor progression. In conclusion, our data suggest that a ternary protein complex composed of ADAM12, αVβ3 integrin and MMP-14 at the tumor cell surface regulates the function of MMP-14. This interaction might point to a novel concept for the development of MMP-14-targeting drugs in treating cancer.

Key words: ADAM12, MMP-14, αVβ3 integrin, Gelatin degradation, Apoptosis

Introduction

Studies on the biological and pathological functions of ADAMs (a disintegrin and metalloproteinases) provide evidence that several ADAMs are overexpressed in malignant tumors and contribute to the pathogenesis of many cancers (Duffy et al., 2011; Edwards et al., 2008). Over the past few years, it has become clear that ADAMs play a major role in the processing of cell surface receptors that undergo cleavage near the transmembrane domain (Blobel, 2005; Saftig and Reiss, 2011). However, the functions of ADAMs appear to rely not only on their metalloproteinase activity, but also on functions mediated through exosites, such as the extracellular disintegrin-like and cysteine-rich domains, that interact with other transmembrane proteins (such as integrins) at the cell surface (Edwards et al., 2008). It is thought that through these various domain activities, ADAMs could stimulate cancer-related processes (such as cell adhesion, growth and invasion) with or without engagement of the catalytic activity (Fröhlich et al., 2011; Roy et al., 2011). We have recently described that ligation to a monoclonal antibody against ADAM12 results in the formation of clusters of invadopodia containing both ADAM12 and matrix metalloproteinase (MMP)-14 (Albrechtsen et al., 2011). Gelatin degradation, observed in these invadopodia, was independent of the catalytic activity of ADAM12, suggesting that other proteinases (e.g. MMP-14), mediate the ADAM12-induced gelatin degradation.

MMP-14 is a classical transmembrane metalloproteinase and, like ADAM12, it is upregulated in human cancer (Egeblad and Werb, 2002; Figueira et al., 2009; Jiang et al., 2006; Seiki, 2003; Strongin, 2010) and accelerates tumor progression in mouse models of cancer (Fröhlich et al., 2011; Kveiborg et al., 2005; Maquoi et al., 2012; Perentes et al., 2011; Roy et al., 2011). MMP-14 degrades extracellular matrix components (i.e. fibrillar collagen and gelatin), activates enzymes such as MMP-2 and MMP-13, sheds cell surface proteins (Chan et al., 2012; Egeblad and Werb, 2002; Itoh and Seiki, 2006; Koshikawa et al., 2011; Koshikawa et al., 2010), and was recently shown to prevent collagen-induced apoptosis (Maquoi et al., 2012). The activity of MMP-14 is regulated by multiple mechanisms, including activation by cell surface proteins such as tetraspanin CD151 and αVβ3 integrins (Deryugina et al., 2004; Gonzalo et al., 2010; Hadler-Olsen et al., 2011; Itoh and Seiki, 2004; Yañez-Mó et al., 2008).

In the present study, we investigated whether ADAM12 influences the activity of MMP-14 and how this might impact on tumor progression. We found that upon increased expression of

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ADAM12, MMP-14 was redistributed to the cell surface and activated, resulting in gelatin degradation and reduction of collagen-induced apoptosis. The gelatin degradation appeared to be strongly dependent on the presence of αVβ3 integrin at the cell surface. Interestingly, the MMP-14 proteolytic activity enhanced by ADAM12 could be significantly reduced by monoclonal antibodies against ADAM12. Finally, we tested the effect of increased ADAM12 in a mouse model of human breast cancer and found that, in fact, ADAM12 increased tumor progression and correlated with reduced tumor cell apoptosis and increased levels of activated MMP-14, supporting the notion that ADAM12 and MMP-14 promote tumor aggressiveness.

Results

MMP-14 recruitment to the tumor cell surface is stimulated by ADAM12

To investigate whether ADAM12 affects recruitment of MMP-14 to the cell surface, we took advantage of a HEK293 cell line that stably expresses αVβ3 integrin, called 293-VnR (Sanjay et al., 2001). Immunostaining of endogenous MMP-14 in 293-VnR cells demonstrated a dot-like localization close to the nucleus in nearly 90% of the cells, whereas very few cells exhibited MMP-14 staining at the cell surface (Fig. 1A, upper panels; Fig. 1B). However, upon transfection with ADAM12Δcyt, which lacks the cytoplasmic tail and is therefore readily directed to the cell

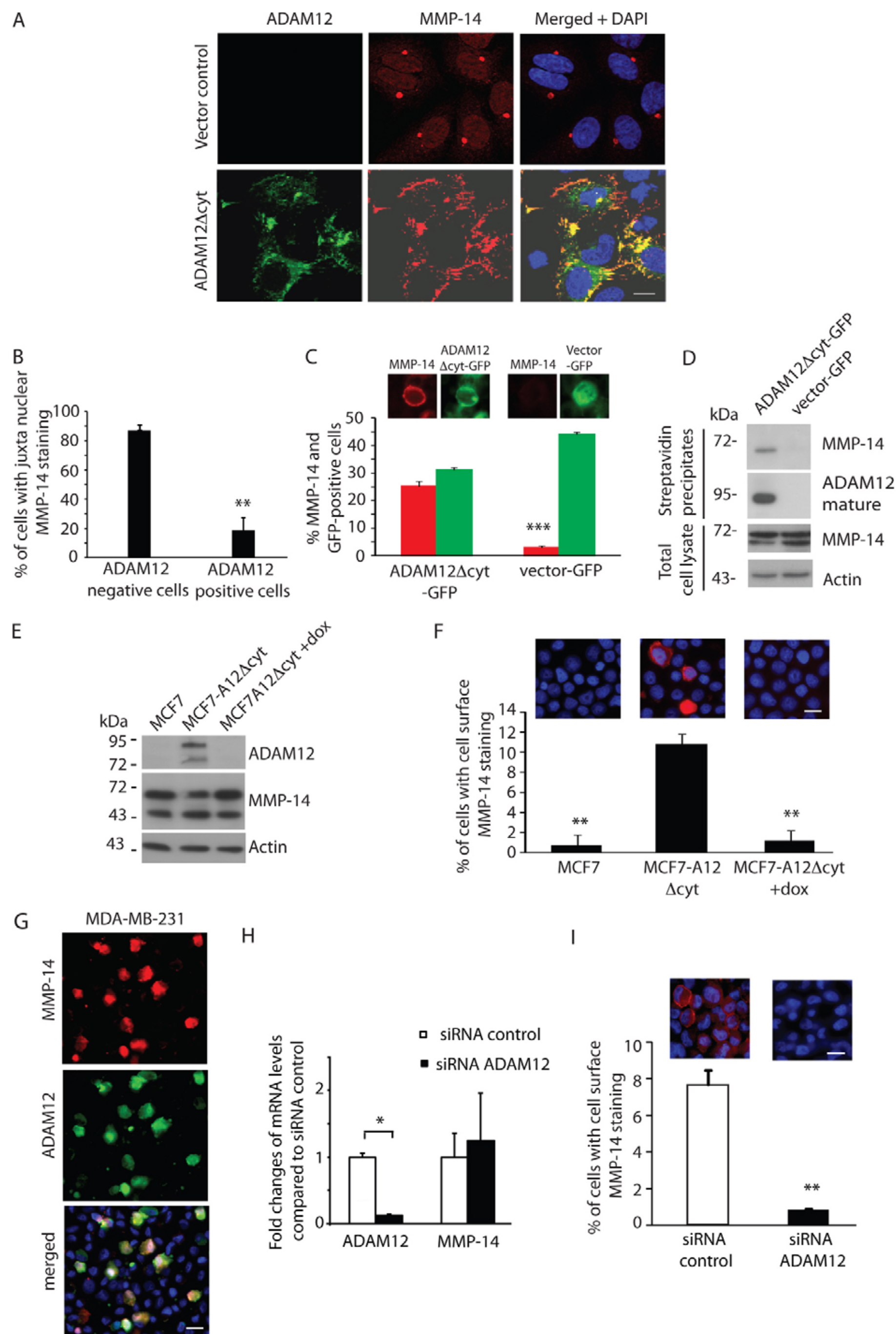

Fig. 1. Recruitment of MMP-14 to the cell surface is stimulated by ADAM12. (A–D) 293-VnR cells were transfected with either vector control or ADAM12Δcyt-GFP. (A) Cells were permeabilized and stained for MMP-14 (red), ADAM12 (green) and the nucleus (blue). Confocal microscopy was used to visualize protein expression. (B) Cells were stained as described in A and >500 cells per experiment were counted for localization of MMP-14 to the juxta nuclear region. The means±s.d. are expressed as the percentage of total cells. (C) Cytospin experiments detected surface MMP-14 in non-permeabilized 293-VnR cells. The graph shows the distribution of MMP-14 (red bars) and GFP (green bars) cells as a mean±s.d. percentage of the total cells. For each experiment, >1000 cells were counted. (D) Cell surface biotinylation assay. Streptavidin precipitates were analyzed for MMP-14 and ADAM12Δcyt-GFP by western blotting. MMP-14 protein is also shown in total cell lysates. (E) Total cell lysates from wild-type MCF7, MCF7-A12Δcyt, MCF7-A12Δcyt+dox cells were analyzed for MMP-14 and ADAM12 by western blotting. (F) Cytospin experiments of non-permeabilized MCF7 cells. Cells that immunostained for cell surface MMP-14 and counted as described in C. The means±s.d. are expressed as the percentage of total cells. (G) Cytospin of non-permeabilized MDA-MB-231 cells immunostained for cell surface ADAM12 (green), MMP-14 (red) and DAPI (blue). (H) MDA-MD-231 cells were treated with ADAM12 siRNA or control siRNA for 48 hours, mRNA was extracted, and then subjected to qPCR to detect *ADAM12* and *MMP14*. (I) Cytospin of siRNA-treated MDA-MB-231 cells immunostained and counted as described in C. Means±s.d. are expressed as the percentage of total cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$; Student's *t*-test. Scale bars: 6 µm (A); 12 µm (F,G,I).

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surface (Hougaard et al., 2000), cells exhibited MMP-14 immunostaining at the cell surface (Fig. 1A, lower panels), and the number of cells with dot-like MMP-14 immunostaining decreased significantly (Fig. 1B). The effect of ADAM12 on MMP-14 localization was subsequently assessed in cytospin experiments, where specifically cell surface localization is analyzed. Cells transfected with GFP-tagged ADAM12Δcyt exhibited an ~10-fold increase in the number of cells with MMP-14 cell surface staining compared with control vector-GFP-expressing cells (Fig. 1C). Finally, in a biotinylation assay and fluorescence-activated cell sorting (FACS) analysis, we demonstrated that cell surface MMP-14 could be detected in ADAM12Δcyt-transfected 293-VnR cells, but not in control-transfected cells (Fig. 1D; supplementary material Fig. S1A). We did not observe changes in the expression levels of endogenous MMP-14 in total cell lysate when overexpressing ADAM12Δcyt compared with control cells (Fig. 1D). Taken together, these data support the finding that ADAM12 stimulates recruitment of MMP-14 to the cell surface of 293-VnR cells.

To explore the functional relevance of combined expression of ADAM12 and MMP-14 in a cancer setting, we first sought to determine whether ADAM12 also stimulates MMP-14 translocation to the cell surface in cancer cell lines, using the low-invasive and non-metastatic breast-cancer cell line MCF7. Wild-type MCF7 cells express little or no ADAM12 (Fig. 1E); hence, we induced the expression of ADAM12 (MCF7-A12Δcyt) using a Tet-Off system. MCF7-A12Δcyt cells growing without doxycycline expressed ADAM12 (Fig. 1E), and adding doxycycline to the growth medium switched off ADAM12 expression (MCF7-A12Δcyt+dox). In contrast to earlier publications (Deryugina et al., 2000; Figueira et al., 2009; Maquoi et al., 2012), we were able to detect equal levels of MMP-14 under all three experimental conditions (Fig. 1E). MMP-14 localization at the cell surface was analyzed without membrane permeabilization, and the number of cells exhibiting MMP-14 cell surface staining was increased by more than 10-fold in MCF7-A12Δcyt cells as compared with MCF7 and MCF7-A12Δcyt+dox cells (Fig. 1F). These data was confirmed by FACS analysis (supplementary material Fig. S1B).

Next, we investigated whether endogenously expressed ADAM12 would influence the cellular distribution of MMP-14. For this purpose, we used the invasive breast cancer cell line MDA-MB-231, which has been previously shown to express ADAM12 (Solomon et al., 2010). We confirmed that this line had cell surface expression of ADAM12 by cytospin experiments, and furthermore showed the presence of MMP-14 in juxtaposition to ADAM12 at the cell surface (Fig. 1G). siRNA knockdown of ADAM12 in MDA-MB-231 cells caused a significant reduction in *ADAM12* mRNA levels (Fig. 1H) MMP-14 immunolocalization at the cell surface was analyzed without membrane permeabilization, and the number of cells exhibiting MMP-14 cell surface staining was significantly lower in MDA-MB-231 cells treated with ADAM12 siRNA than control treated cells (Fig. 1I). These data were confirmed by FACS analysis (supplementary material Fig. S1C). Notably, the level of *MMP14* mRNA was not affected by knockdown of ADAM12 (Fig. 1H). These data further confirm that ADAM12 might regulate the recruitment of MMP-14 to the tumor cell surface.

ADAM12 and MMP-14 colocalization at the tumor cell surface induces gelatin degradation

The spatial relationship between ADAM12 and MMP-14 at the cells surface was analyzed using the Duolink technique (briefly described in the Materials and Methods) in the three cell lines examined in Fig. 1. The Duolink experiments were performed on non-permeabilized cells, and colocalization of ADAM12 and MMP-14 was visualized as bright red dots at the cell surface (Fig. 2A). ADAM12 and MMP-14 colocalized at the cell surface independently of whether ADAM12 was exogenously or, as in MDA-MB-231 cells, endogenously expressed (Fig. 2A).

Next, we asked whether the ADAM12-mediated change in the subcellular localization of MMP-14 resulted in altered biological activities characteristic of MMP-14. A gelatin degradation assay was used to test the matrix degradation activity of 293-VnR cells under various experimental conditions. Gelatin degradation was defined as disappearance of gelatin fluorescence, leaving behind black areas underneath the cells. ADAM12Δcyt-transfected cells were able to degrade gelatin, whereas cells not expressing ADAM12 (i.e. non-transfected cell or cells transfected with vector control) showed low or no gelatin degradation (Fig. 2B). The gelatin degradation increased substantially when increasing amounts of ADAM12 plasmid were used for the transfection (supplementary material Fig. S2A). In cultures expressing ADAM12, gelatin degradation was detectable after 4 hours in culture, but increased about five times after 20 hours (Fig. 2C). The need for ADAM12 and MMP-14 to translocate to the cell surface for the gelatin degradation to occur was further tested by transient transfecting 293-VnR cells with full-length ADAM12-L, which is mostly retained intracellularly (Hougaard et al., 2000), and showed less efficient gelatin degradation than cells with ectopic expression of ADAM12Δcyt, which readily locates to the cell surface (supplementary material Fig. S2B).

We next analyzed whether the ADAM12-dependent change in the subcellular localization of MMP-14 in breast carcinoma cell lines, as shown in Fig. 1, influenced their ability to degrade gelatin. MCF7-A12Δcyt cells exhibited significant gelatin degradation after 48 hours, whereas the wild-type MCF7 and MCF7-A12Δcyt+dox cells showed little tendency to degrade gelatin in this timeframe (Fig. 2D,E). MDA-MB-231 cells are known to degrade gelatin per se (Yamaguchi et al., 2009). Here, we demonstrated that siRNA knockdown of endogenous ADAM12 in MDA-MB-231 cells significantly decreased gelatin degradation (Fig. 2F,G).

Taken together, these data suggest that ADAM12 and MMP-14 colocalize at the tumor cell surface and that ADAM12 is an important regulator of gelatin degradation in breast cancer cell lines.

ADAM12-induced gelatin degradation is caused by MMP-14

We next asked which proteinase was responsible for the ADAM12-induced gelatin degradation. To test whether the catalytic activity of ADAM12 itself was required for the observed gelatin degradation, we transfected 293-VnR cells with ADAM12Δcyt containing a catalytic site mutation (E351Q). Gelatin degradation was similar after transfection with wild-type ADAM12Δcyt and ADAM12ΔcytE351Q, indicating that ADAM12 is not itself responsible for the gelatin degradation (Fig. 3A). In order to confirm that the gelatin degradation results from a metalloproteinase activity, we treated cells grown on gelatin with GM6001 or TAPI-2, which are both broad inhibitors of metalloproteinases (Black et al., 1997; Gijbels et al., 1994; Moss and Rasmussen, 2007). Both GM6001 and TAPI-2 caused a significant reduction in gelatin degradation (Fig. 3B).

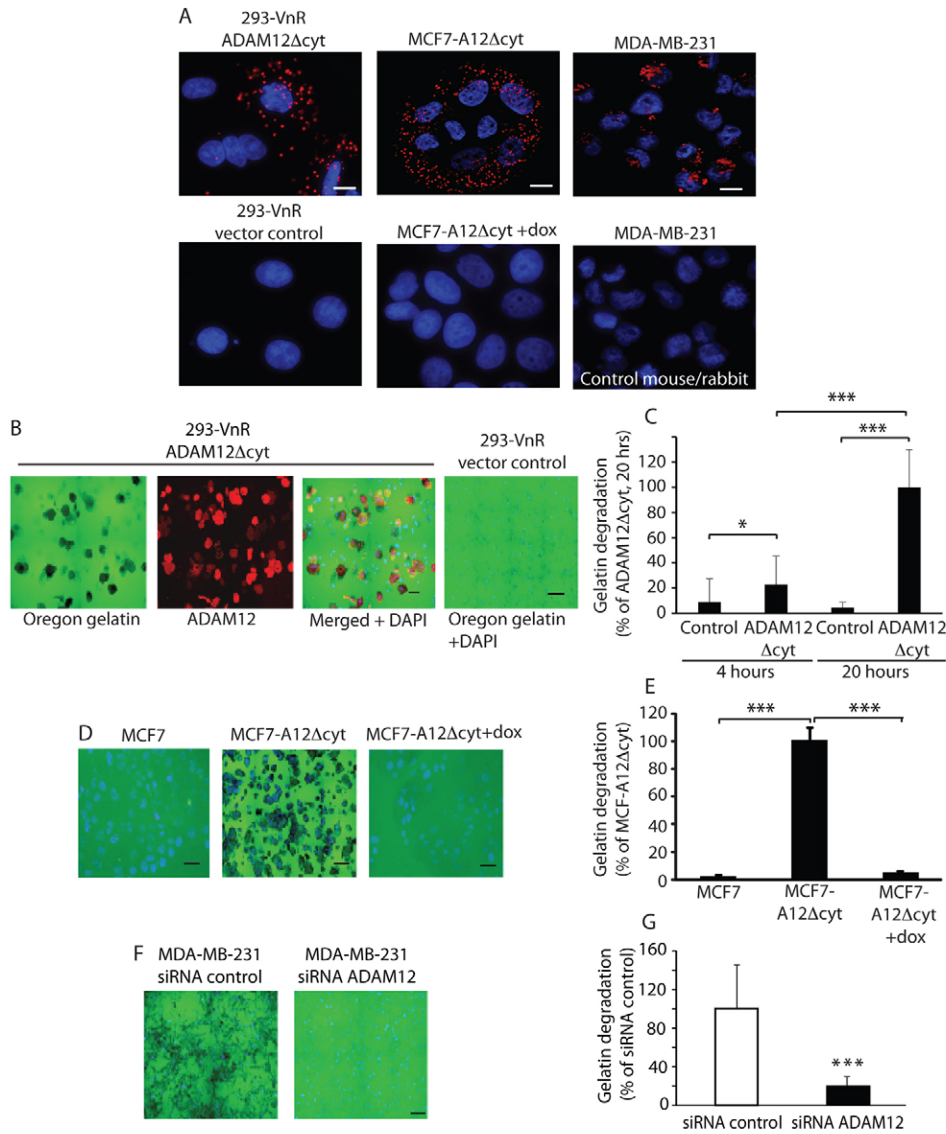

Fig. 2. ADAM12 and MMP-14 colocalization at the cell surface induces gelatin degradation. (A) Non-permeabilized 293-VnR cells transfected with ADAM12Δcyt or control vector (left panels), non-permeabilized MCF7-A12Δcyt and MCF7-A12Δcyt+dox cells (middle panels), and cytospin of non-permeabilized MDA-MB-231 cells (right panels) were subjected to Duolink® reagents with antibodies to ADAM12 (6E6) and MMP-14. Red spots indicate colocalization at the cell surface. (B) *In situ* solid-phase gelatinase assay using 293-VnR cells transfected with vector control or ADAM12Δcyt. Cell cultures were tested for ADAM12 expression (red) and the nucleus (blue). (C) Percentage of gelatin degradation after 4 and 20 hours. Areas without green fluorescence were measured (μm^2) from experiments in B and the gelatin degradation per cell was calculated. Mean percentage data (±s.d.) are expressed relative to the mean ADAM12Δcyt 20 hour percentage degradation value (set at 100%). (D) *In situ* solid-phase gelatinase assay of MCF7, MCF7-A12Δcyt and MCF7-A12Δcyt+dox cells. (E) Mean percentage of gelatin-degradation (±s.d.) for the MCF7 cells shown in D expressed relative to mean MCF7-A12Δcyt degradation value (set at 100%). (F) *In situ* solid-phase gelatinase assay (20 hours) of MDA-MB-231 cells treated with control siRNA or ADAM12 siRNA. (G) Mean percentage of gelatin-degradation (±s.d.) for the MDA-MB-231 cells shown in F expressed relative to the mean siRNA control degradation value (set at 100%). *P<0.05, **P<0.01, ***P<0.001, Student's t-test. Scale bars: 12 μm (A, left and middle panels), 8 μm (A, right panels); 40 μm (B, ADAM12Δcyt), 100 μm (B, vector control; D, MCF7-A12Δcyt); 20 μm (D, MCF7, MCF7-A12Δcyt+dox); 10 μm (F).

Subsequently, to determine whether MMP-14 is involved, we performed siRNA knockdown of MMP-14 in ADAM12Δcyt-transfected 293-VnR cells (Fig. 3C). Gelatin degradation was significantly decreased upon MMP-14 knockdown compared with control siRNA (Fig. 3C), indicating that either MMP-14 itself or an MMP-14-activated metalloproteinase (for example, MMP-2) is the major contributor to gelatin degradation under these experimental conditions.

It is well established that upon MMP-14 recruitment to the cell surface, MMP-2 becomes activated and degrades gelatin and collagen (Itoh et al., 2008). This led us to test whether MMP-2 was responsible for the observed gelatin degradation. First, serum-free cell culture supernatants from transfected 293-VnR cells were tested for gelatin degradation and examined for the presence and activity of MMP-2 by zymography (Fig. 3D, upper panel). Only the presence of the inactive proform of MMP-2 could be detected in medium from untransfected 293-VnR cells and no mature form of MMP-2 was observed when the cells were transfected with ADAM12Δcyt or an empty vector control. In contrast, when cells were transfected directly with MMP-14, the active (mature) form of MMP-2 could be detected as evidenced by its size shift in the zymogram (Fig. 3D, upper panel). As seen from the corresponding gelatin degradation experiments (lower panel), gelatin degradation takes place in the ADAM12Δcyt-transfected culture without the activation of MMP-2, whereas in cells transfected with MMP-14 both MMP-2 activation and gelatin degradation are observed. Zymography performed on serum-free cell culture supernatants from MCF7-A12Δcyt and MCF7-A12Δcyt+dox also showed no activation of MMP-2 (Fig. 3E). Thus, these data together indicate that MMP-2 is not the proteinase that is responsible for the ADAM12-induced gelatin degradation.

αVβ3 integrin provides a link between ADAM12 and MMP-14-mediated gelatin degradation

We have previously shown that the secreted form of ADAM12 binds αVβ3 integrin on tumor cells (Thodeti et al., 2005b). Moreover, it is well established that MMP-14 associates with αVβ3 integrin at the cell surface, and that the cell surface MMP-14 activity can be regulated by αVβ3 integrin (Borrirukwanit et al., 2007; Deryugina et al., 2004; Gálvez et al., 2002). On the

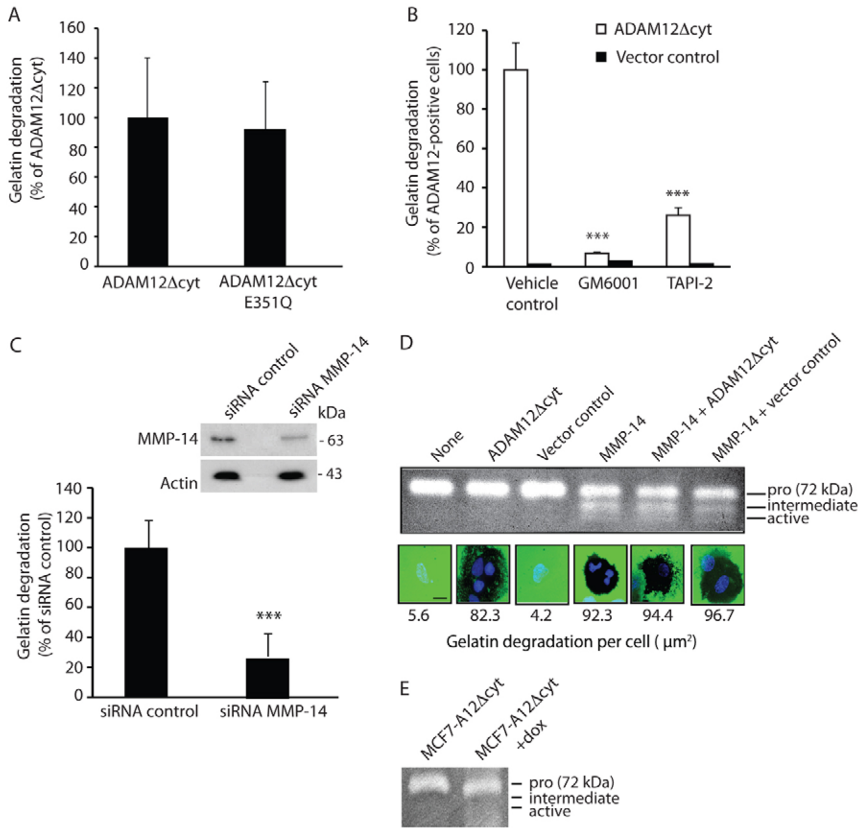

Fig. 3. ADAM12-induced gelatin degradation is mediated by MMP-14. (A) *In situ* solid-phase gelatinase assay using 293-VnR cells transfected with ADAM12Δcyt or its catalytic inactive form ADAM12Δcyt-E351Q. Data are expressed as the mean gelatin degradation (±s.d.) relative to the mean ADAM12Δcyt degradation value (set at 100%). (B) *In situ* solid-phase gelatinase assay of 293-VnR cells transfected with ADAM12Δcyt or control vector and treated with GM6001 (10 μM), TAPI-2 (10 μM) or vehicle only (control) overnight. Cells were stained for ADAM12 and for the nucleus (DAPI). The mean number of gelatin-degrading ADAM12-positive cells (±s.d.) is presented as a percentage of the total number of ADAM12-positive cells. (C) *In situ* solid-phase gelatinase assay of 293-VnR cells co-transfected with ADAM12Δcyt and control or MMP-14 siRNA. Mean data (±s.d.) are expressed relative to the mean siRNA control degradation value (set at 100%). Inset, western blots of total cell lysates analyzed for MMP-14.
(D) MMP-2 zymography for untransfected 293-VnR cells or 293-VnR cells transfected with ADAM12Δcyt, vector control, or MMP-14, or co-transfected with MMP-14 and ADAM12Δcyt or MMP-14 and vector control (upper panel). The images in the lower panel illustrate gelatin degradation and nuclear staining (DAPI) in the respective cultures. Gelatin degradation was quantified by measuring the degraded area in μm^2 and correlated to the number of cells. Scale bar: 10 μm. (E) MMP-2 zymography for MCF7-A12Δcyt and MCF7-A12Δcyt+dox. ***$P<0.001$, Student's *t*-test.

basis of these data we undertook experiments with parental HEK293 cells, which unlike 293-VnR cells do not exhibit αVβ3 integrin expression, but express MMP-14 to the same extent as 293-VnR cells (supplementary material Fig. S3A). We were unable to detect surface localization of MMP-14 in non-permeabilized HEK293 expressing ADAM12Δcyt (supplementary material Fig. S3B) and found no gelatin degradation (Fig. 4A). To test the role of αVβ3 integrin on gelatin degradation in ADAM12Δcyt-transfected 293-VnR cells, we added the inhibitory LM609 antibody against αVβ3 integrin to the cells. Gelatin degradation was significantly decreased in the presence of LM609 (Fig. 4B). These results suggest a role for αVβ3 integrin in the ADAM12–MMP-14 axis. Therefore, to analyze whether membrane-anchored ADAM12 associates with αVβ3 integrin and MMP-14 in a larger complex, we performed immunoprecipitation studies using monoclonal antibodies (mAbs) against ADAM12. ADAM12 coimmunoprecipitated with both MMP-14 and αVβ3 integrin (Fig. 4C). Interestingly, even though we were unable to detect cell surface MMP-14 in HEK293 expressing ADAM12, we still observed a coimmunprecipitation between ADAM12 and MMP-14 proteins (supplementary material Fig. S3C). This suggests that a direct or indirect interaction between ADAM12 and MMP-14 occurs in the lysate; however, αVβ3 integrin seems to be the key-player for correct transport of the protein triple complex to the cell surface. In addition, to visualize the colocalization between ADAM12, MMP-14 and αVβ3 integrin at the cell surface, we employed the Duolink technique on non-permeabilized 293-VnR cells transfected with ADAM12Δcyt. As shown in Fig. 4D, colocalization at the cell surface was observed between ADAM12 and αVβ3 integrin and between αVβ3 integrin and MMP-14 (Fig. 4D). Because we used ADAM12Δcyt to transfect the cells examined, these data reveal that the interaction between ADAM12, MMP-14 and αVβ3 integrin relates to the extracellular portion of the three molecules.

Several reports have demonstrated low or no levels of αVβ3 integrin at the cell surface of MCF7 cells (Deryugina et al., 2000; Figueira et al., 2009; Taherian et al., 2011); however, both FACS analysis and immunostaining showed that overexpression of ADAM12 in MCF7 cells increases cell surface levels of αVβ3 integrin (Fig. 4E,F). In addition, we confirm previous reports (Borrirukwanit et al., 2007; Taherian et al., 2011) showing that MDA-MB-231 cells express moderate levels of αVβ3 integrin (Fig. 4E). Therefore, to analyze whether ADAM12-dependent activation of MMP-14 was related to αVβ3 integrin function, we treated MCF7-A12Δcyt cell cultures with the αVβ3 integrin inhibitory LM609 antibody. We observed an 80% reduction of gelatin degradation in LM609-treated MCF7-A12Δcyt cell cultures compared with IgG-treated cultures (Fig. 4G). Taken together, these data strongly implicate αVβ3 integrin in the ADAM12-induced activation of MMP-14.

Monoclonal antibodies against the ADAM12 prodomain inhibit gelatin degradation

We have previously developed monoclonal antibodies (mAbs) to human ADAM12, and a series of these were tested for function-blocking activity in the gelatin degradation assay. The mAb 6E6, which has previously been shown to cluster ADAM12 at the cell surface (Albrechtsen et al., 2011), had no effect on gelatin degradation in 293-VnR cells transfected with ADAM12Δcyt. Interestingly, two other mAbs against ADAM12 (7B8 and 8F8)

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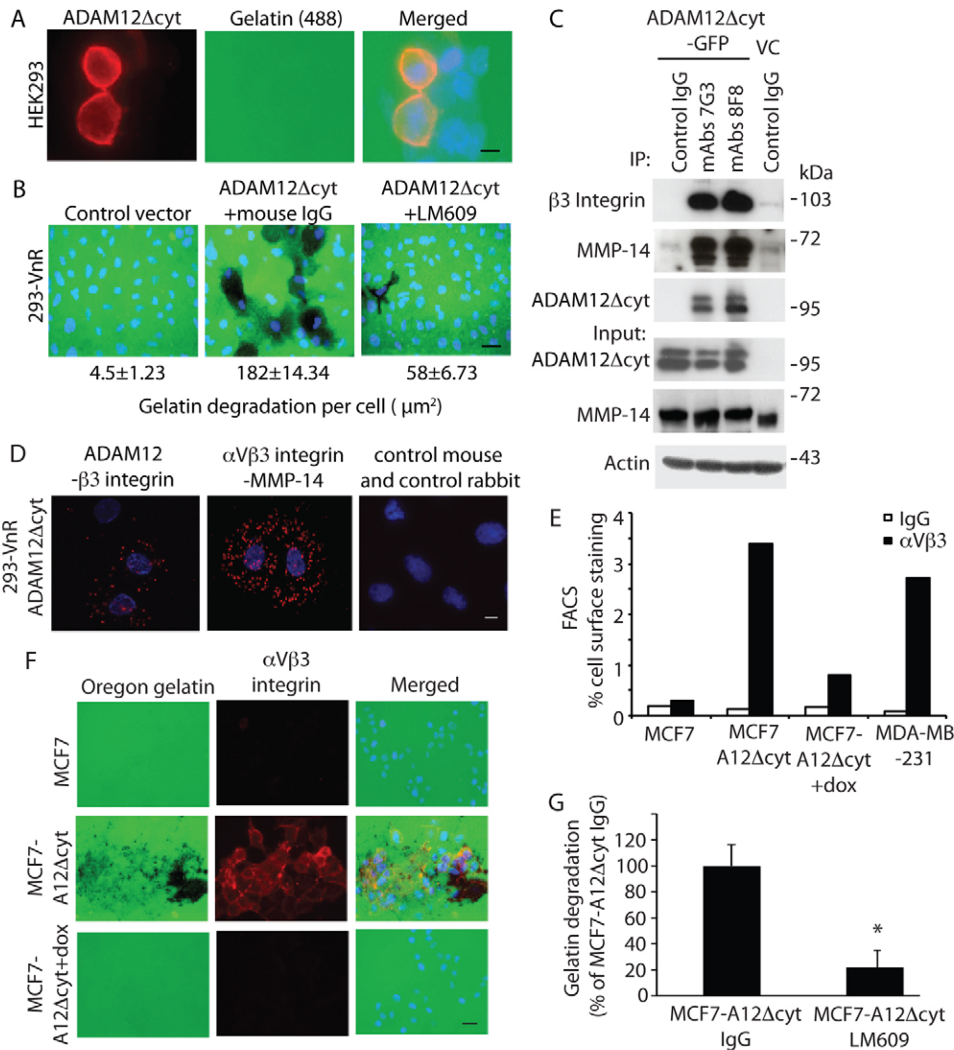

Fig. 4. ADAM12-induced gelatin degradation is dependent on αVβ3 integrin. (A) *In situ* solid-phase gelatinase assay of HEK293 cells transfected with ADAM12Δcyt and immunostained for ADAM12, and DAPI staining. (B) *In situ* solid-phase gelatinase assay of 293-VnR cells transfected with ADAM12Δcyt or control vector and treated overnight with normal mouse IgG or an inhibitory antibody against αVβ3 integrin (10 μg/ml LM609). The mean gelatin degradation per cell (μm^2) (±s.d.) is presented below the images. (C) 293-VnR cells were transfected with ADAM12Δcyt-GFP or vector control, and immunoprecipitated with mouse IgG or mAbs against ADAM12 (7G3 or 8F8). Precipitates and input samples were analyzed by western blotting with the indicated antibodies. (D) Non-permeabilized 293-VnR cells transfected with ADAM12Δcyt were subjected to Duolink® reagents with antibodies to ADAM12 (6E6) and β3 integrin, αVβ3 integrin and MMP-14, and negative control mouse and rabbit IgG. (E) Mean percentage cell surface staining of αVβ3 integrin (LM609 antibody staining) for MCF7, MCF7-A12Δcyt, MCF7-A12Δcyt+dox and MDA-MB-231 cell lines analyzed by FACS. Normal mouse IgG was used as a staining control. (F) *In situ* solid-phase gelatinase assay of MCF7, MCF7-A12Δcyt and MCF7-A12Δcyt+dox cells, immunostained for αVβ3 integrin (LM609 antibody) with the nuclei visualized by DAPI staining. (G) Mean percentage of gelatin degradation (±s.d.) for MCF7-A12Δcyt cells treated overnight with normal mouse IgG or inhibitory αVβ3 integrin antibody (LM609), expressed relative to mean MCF7-A12Δcyt cells + IgG degradation value (set at 100%). *$P<0.001$, Student's *t*-test. Scale bars: 8 μm (A,D); 20 μm (B); 15 μm (F).

clearly inhibited gelatin degradation (Fig. 5A,B). It appeared that 8F8 and 7B8 mAbs recognize an epitope in the prodomain domain of ADAM12 (supplementary material Fig. S4A). Using an *in vitro* quenched-fluorescent peptide cleavage assay, as well as a cell-based ectodomain shedding assay, we demonstrated that there was no inhibitory effect of 8F8 and 7B8 on the catalytic activity of ADAM12 (data not shown). Importantly, these antibodies also did not exert any inhibitory effect on gelatin degradation in 293-VnR cells that were transfected with MMP-14 instead of ADAM12 (supplementary material Fig. S4B).

In addition, we tested the effect of mAbs against human ADAM12 on the ability of MCF7-A12Δcyt and MDA-MB-231 cells to degrade gelatin. Using the MCF7-A12Δcyt cells, the mAbs 7B8 and 8F8 exhibited a significant inhibitory effect on the gelatin degradation, as compared with mouse control IgG (Fig. 5C,D). Similarly, in MDA-MB-231 cells, gelatin degradation was significantly inhibited when using 7B8 and 8F8 mAbs (Fig. 5E,F).

ADAM12 protects breast tumor cell lines from collagen-induced apoptosis

A recent study demonstrated that MMP-14 protects breast cancer cells from apoptosis induced by type I collagen (Maquoi et al., 2012). We have previously shown that overexpression of ADAM12, both *in vivo* and *in vitro*, confers decreased tumor

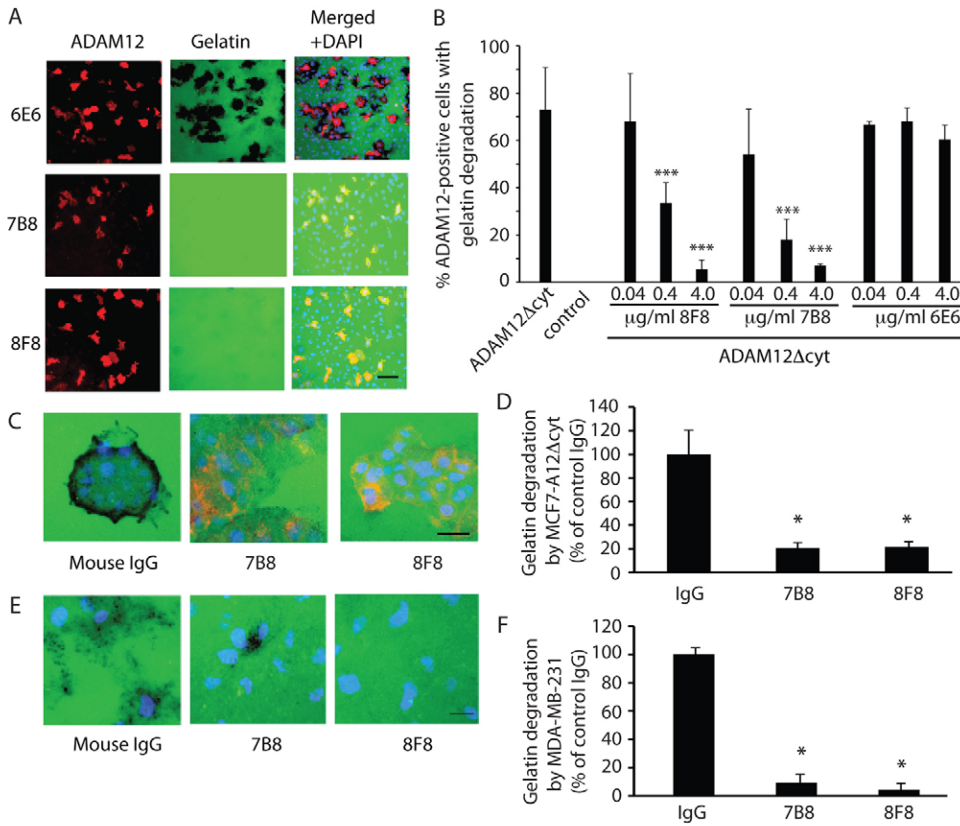

Fig. 5. Monoclonal antibodies against ADAM12 inhibit ADAM12-induced gelatin degradation. (A) *In situ* solid-phase gelatinase assay of 293-VnR cells transfected with ADAM12Δcyt and treated overnight with 4 μg/ml of mAbs against ADAM12 (6E6, 7B8 and 8F8). Dishes were stained using the ADAM12 monoclonal antibody 6E6 (red) and DAPI (blue). (B) *In situ* solid-phase gelatinase assay of 293-VnR cells transfected with ADAM12Δcyt (or control vector) and treated overnight with mAbs against ADAM12 in increasing concentration (0.04–4.0 μg/ml). Data represent the mean percentage (±s.d.) of ADAM12-positive cells with gelatin degradation. (C–F) *In situ* solid-phase gelatinase assay of MCF7-A12Δcyt (C,D) and MDA-MB-231 (E,F) cells treated overnight with control mouse IgG or mAbs 7B8 and 8F8 (10 μg/ml). (D,F) Mean percentage of gelatin degradation (±s.d.) for MCF7-A12Δcyt (D) and MDA-MB-231 (F) cells treated with 7B8, 8F8 or control mouse IgG (10 μg/ml) relative to the mean control IgG degradation value (set at 100%). *$P<0.05$, **$P<0.01$, ***$P<0.001$, ANOVA (B) and Student's *t*-test (D,F). Scale bars: 45 μm (A); 20 μm (C); 10 μm (E).

cell apoptosis (Kveiborg et al., 2005). In light of these results, we wanted to test whether ADAM12-mediated activation of MMP-14 could protect MCF7 cells against apoptosis induced by type I collagen. To this end, MCF7, MCF7-A12Δcyt, and MCF7-A12Δcyt+dox cells were submerged in type I collagen for 6–7 days and examined for morphological characteristics of apoptotic cells (e.g. membrane blebbing). MCF7 cells expressing ADAM12 (MCF7-A12Δcyt) remained round with a distinct cell border, whereas MCF7 cells not expressing ADAM12 (MCF7 and MCF7-A12Δcyt+dox) displayed membrane blebbing, indicating occurrence of apoptosis (data not shown). To analyze the frequency of apoptotic cells in the three MCF7 cell lines, apoptotic bodies were counted in cells recovered from the 3D collagen gels. A representative image of apoptotic bodies in MCF7 cells is shown in supplementary material Fig. S5A. The percentage of apoptotic bodies was significantly reduced in MCF7-A12Δcyt compared with MCF7 and MCF7-A12Δcyt+dox cells (Fig. 6A). The observation that overexpression of ADAM12 protects MCF7 cells from apoptosis was confirmed using ApopTag staining (supplementary material Fig. S5B).

MMP-14 has been previously shown to prevent upregulation of the proapoptotic Bcl-2–interacting killer protein (BIK) (Maquoi et al., 2012). Initially, we analyzed the protein level of BIK and another pro-apoptotic Bcl-2-interacting protein (BCL2L11; BIM) in the three MCF7 cell lines grown in 2D cultures. Although no changes in BCL2L11 levels were observed in the three MCF cell lines, we found a slight downregulation of BIK in MCF7-A12Δcyt (supplementary material Fig. S5C). However, consistent with previous results (Maquoi et al., 2012), we observed a significant downregulation of BIK, as well as BCL2L11 in MCF7-A12Δcyt cells grown in 3D collagen gels compared to MCF7 and MCF7-A12Δcyt+dox controls cells. This suggests that ADAM12 in 2D cultures primes the cells to obtain an anti-apoptotic phenotype, which seems even more pronounced when the cells are challenged, such as when grown in 3D collagen gels (Fig. 6C). Taken together, this suggests that ADAM12, through regulation of MMP-14, BIK and BCL2L11 activity, protects MCF7 cells from programmed cell death (Fig. 6B). The apoptosis-protective effect induced by ADAM12 in MCF7-A12Δcyt cells could be decreased by incubating those cells with mAb 8F8 against ADAM12 (Fig. 6C; supplementary material Fig. S5B). Similarly, using the metalloproteinase inhibitor GM6001, which has no effect on ADAM12 but inhibits MMP-14 activity, significantly increased the fraction of apoptotic bodies in MCF7-A12Δcyt cells, whereas no effects of mAb 8F8 or GM6001 were observed in wild-type MCF7 and MCF7-A12Δcyt+dox cells (Fig. 6C; supplementary material Fig. S5B).

Maquoi et al. have previously shown that MDA-MB-231 cells exhibit very low levels of apoptotic cells in 3D collagen cultures (Maquoi et al., 2012). Thus, we asked whether inhibition of ADAM12 activity would influence apoptosis of MDA-MB-231 cells grown in 3D collagen gel. Indeed, MDA-MB-231 cells incubated with mAb against ADAM12 (8F8) had significantly increased levels of apoptotic bodies compared with control cells (Fig. 6D). Also ApopTag staining showed a decrease in the number of apoptotic cells in MCF7-A12Δcyt compared to control cells (supplementary material Fig. S5D). GM6001 similarly significantly increased the fraction of apoptotic bodies in MDA-MB-231 cells (Fig. 6D, supplementary material Fig. S5D). To further dissect a role for MMP-14 in ADAM12-mediated protection against apoptosis, we transiently transfected MDA-MB-231 cells with siRNA against MMP-14. 3D collagen cultures

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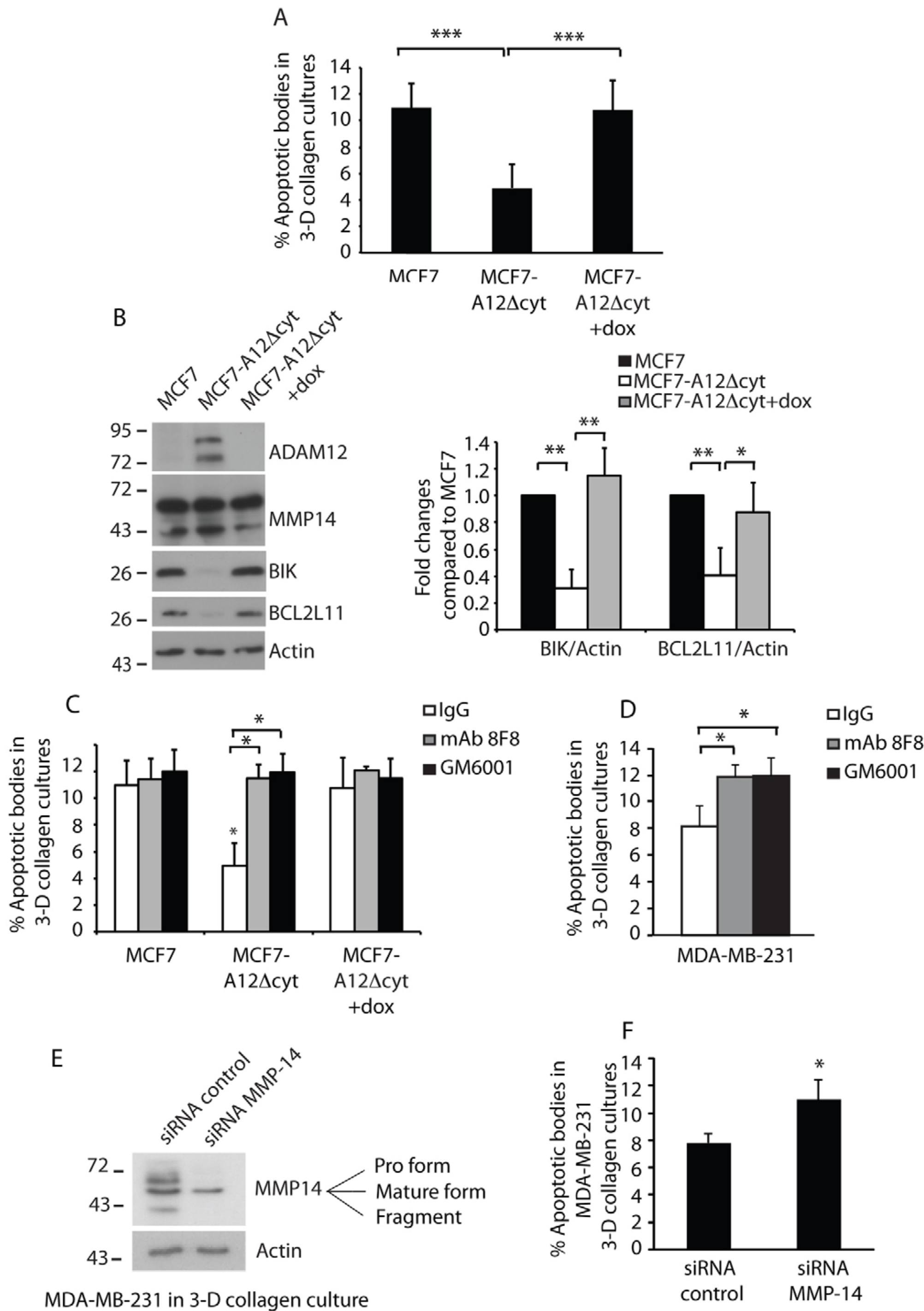

Fig. 6. ADAM12 protects tumor cells against apoptosis *in vitro*. (A) MCF7, MCF7-A12Δcyt and MCF7-A12Δcyt+dox cell lines were embedded in 3D collagen and the mean percentage of apoptotic bodies (±s.d.) was determined from >500 cells per experiment. (B) Western blot analysis of ADAM12, MMP-14, BIK and BCL2L11 from cells recovered from 3D collagen. The bar graph depicts the levels of BIK and BCL2L11 determined by quantification of the band intensities (using ImageJ software), normalized to actin. The MCF7 level was set to 1. (C,D) Mean percentage of apoptotic bodies (±s.d.) from 3D collagen cultures of MCF7, MCF7-A12Δcyt and MCF7-A12Δcyt+dox (C) and MDA-MB-231 (D) cells treated every second day with control mouse IgG, 10 µg/ml 8F8 or 10 µM GM6001. More than 500 cells were used in each experiment. (E) Western blot of cells recovered from 3D cultures of MDA-MB-231 cells analyzed for MMP-14 after siRNA treatment. (F) Mean percentage of apoptotic bodies (±s.d.) from >500 cells of 4-day 3D collagen cultures of MDA-MB-231 cells treated with MMP-14 siRNA or control siRNA prior to growth in collagen gels. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test.

of MDA-MB-231 cells, which showed efficient knockdown of MMP-14 (Fig. 6E), displayed a significant increase in the percentage of apoptotic bodies (Fig. 6F). Collectively, these data suggest that ADAM12-induced MMP-14 activity protects cancer cells from undergoing programmed cell death.

ADAM12 decreases the apoptotic capacity of breast tumor cells *in vivo*

On the basis of previous studies demonstrating that both ADAM12 and MMP-14 influence tumor cell apoptosis *in vivo* (Kveiborg et al., 2005; Maquoi et al., 2012; Roy et al., 2011), we hypothesized that ADAM12 could regulate the apoptotic capacity in a mouse model of breast cancer through regulation of MMP-14 activity. Hence, MCF7-A12Δcyt cells were orthotopically injected into the mammary glands of 6–8-week-old NOD.Cg-Prkdc mice. Overexpression of ADAM12 in MCF7 cells resulted in a significantly higher tumor burden compared with control MCF7-A12Δcyt+dox mice (which were also injected with MCF7-A12Δcyt cells, but then had doxycycline administered in their drinking water) (Fig. 7A). Western blotting confirmed the

expression of ADAM12 in MCF7-A12Δcyt tumors but not in MCF7-A12Δcyt+dox tumors (Fig. 7B). Verifying previous studies with ADAM12-S and ADAM12-L (Roy et al., 2011), increased expression of ADAM12Δcyt in MCF7 tumors did not affect tumor cell proliferation, as determined by Ki67 staining (Fig. 7C). In contrast, ApopTag staining revealed a significant decrease in the number of apoptotic tumor cells in MCF7-A12Δcyt-inoculated mice compared with MCF7-A12Δcyt+dox mice (Fig. 7D). To evaluate the influence of ADAM12 on MMP-14 activation, we analyzed the level of the partially autodigested 43 kDa fragment of MMP-14, which is indicative of active MMP-14 (Cho et al., 2008; Ellerbroek et al., 2001; Itoh, 2006), by western blotting (Fig. 7E). As determined by quantification of the band intensities, we observed a significantly increased level of the 43 kDa fragment of MMP-14 in MCF7-A12Δcyt tumors compared with control MCF7-A12Δcyt+dox tumors (Fig. 7F). These *in vivo* data suggest that the effect of ADAM12 on tumor progression is mediated through decreased apoptosis, possibly through an increased activity of MMP-14.

Expression of ADAM12 correlates with MMP-14 and MMP-2 expression in human breast cancer

Our present results, obtained from cell cultures and mouse studies, suggest that increased levels of both ADAM12 and MMP-14 in breast tumors would be an advantage for tumor progression. Therefore, we aimed to investigate the correlation between ADAM12 and MMP-14 expression in human breast tumors. We combined gene expression profile datasets from four different cohorts, as described in Materials and Methods, for a total of 733 human breast tumor samples. When all tumors, which were taken from lymph-node-negative patients who had not received adjuvant chemotherapy, were analyzed together, we found a positive correlation between ADAM12 and MMP-14 expression (Fig. 8A). This positive correlation was retained in estrogen receptor (ER)-positive tumors and in triple-negative breast tumors (TNBC), a subtype of breast cancer that lacks expression of ER, progesterone receptor and epidermal growth factor receptor (ERBB2) (Fig. 8A). Interestingly, we found an even stronger correlation between ADAM12 and MMP-2. This positive correlation was found in all tumors, in ER-positive tumors and in TNBC (Fig. 8B). These data suggest that ADAM12, as a modulator of MMP-14 activity, might be biologically relevant in human breast tumors.

Discussion

Malignant tumor cells are characterized by their ability to grow and invade directly from tumor islets into the surrounding stroma, which is composed of a variety of extracellular matrix proteins and cell types (Hanahan and Weinberg, 2011). In this process, multiple enzymes are in action (López-Otin and Matrisian, 2007), but only a few (e.g. MMP-14) are able to degrade basement membrane components and collagens, the main structural components of the stroma (Egeblad and Werb, 2002; Ellerbroek et al., 2001; Hadler-Olsen et al., 2011; Sato et al., 2005; Seiki, 2003; Strongin, 2010). In fact, MMP-14 is considered to be perhaps the most potent proteinase in tumor progression (Hadler-Olsen et al., 2011; Seiki, 2003; Szabova et al., 2008). Here, we demonstrate that ADAM12 can induce gelatin degradation and reduce apoptotic activity by recruiting and activating MMP-14 at the cell surface. This is the first time a

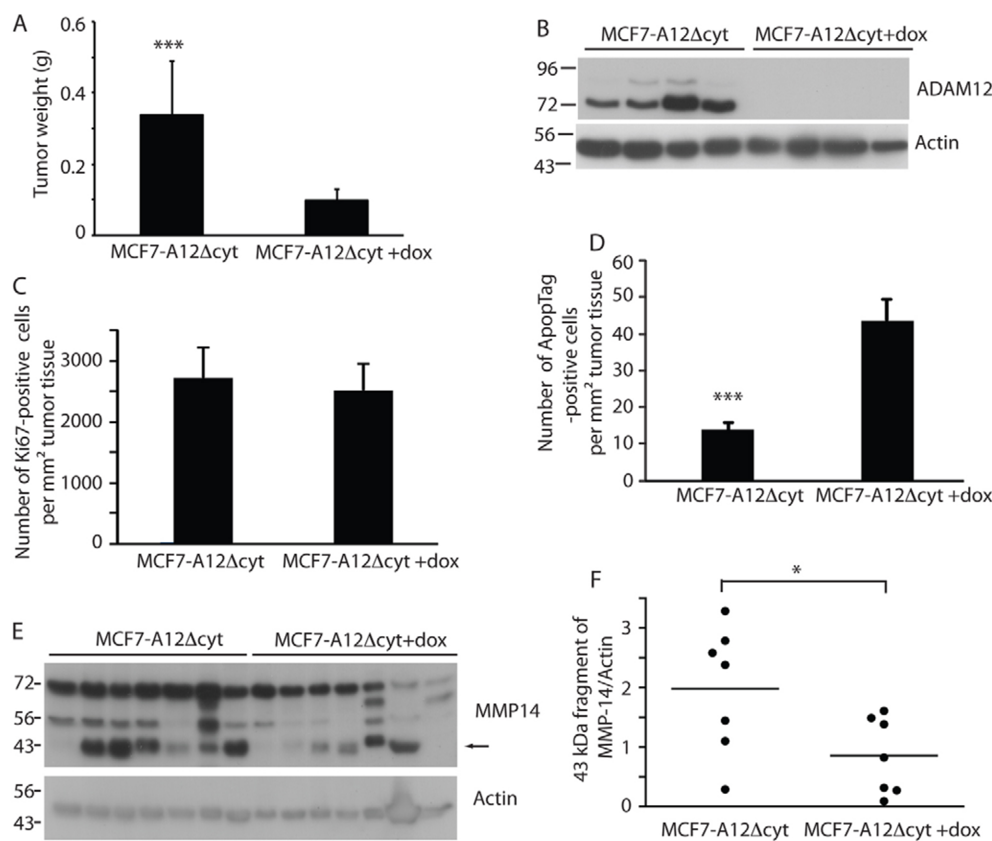

Fig. 7. ADAM12 accelerates tumor growth and inhibits tumor apoptosis *in vivo*. MCF7-A12Δcyt tumor cells were orthotopically implanted in the mammary glands of female mice. Some mice received doxycycline in their drinking water (MCF7-A12Δcyt+dox tumors, $n=8$), whereas other mice (MCF7-A12Δcyt tumors, $n=8$) did not. (A). Data represent mean tumor mass (±s.d.). (B) Western blot analysis of tumor extracts for ADAM12. (C) Mean cell proliferation (±s.d.) was calculated using the Metamorf software program for nuclei counting of images from five areas of each MCF7-A12Δcyt and MCF7-A12Δcyt+dox tumor tissue immunostained for Ki67 staining. (D) A similar counting method to that for C was used to estimate the mean number of ApopTag-positive cells (±s.d.) in tumor tissue. (E) Western blot analysis of MCF7-A12Δcyt and MCF7-A12Δcyt+dox tumor extracts for MMP-14. (F) Graphical representation of the levels of the 43 kDa fragment of MMP-14 (arrow in E) determined by quantification of the western blot band intensities (using ImageJ software) normalized to actin. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test.

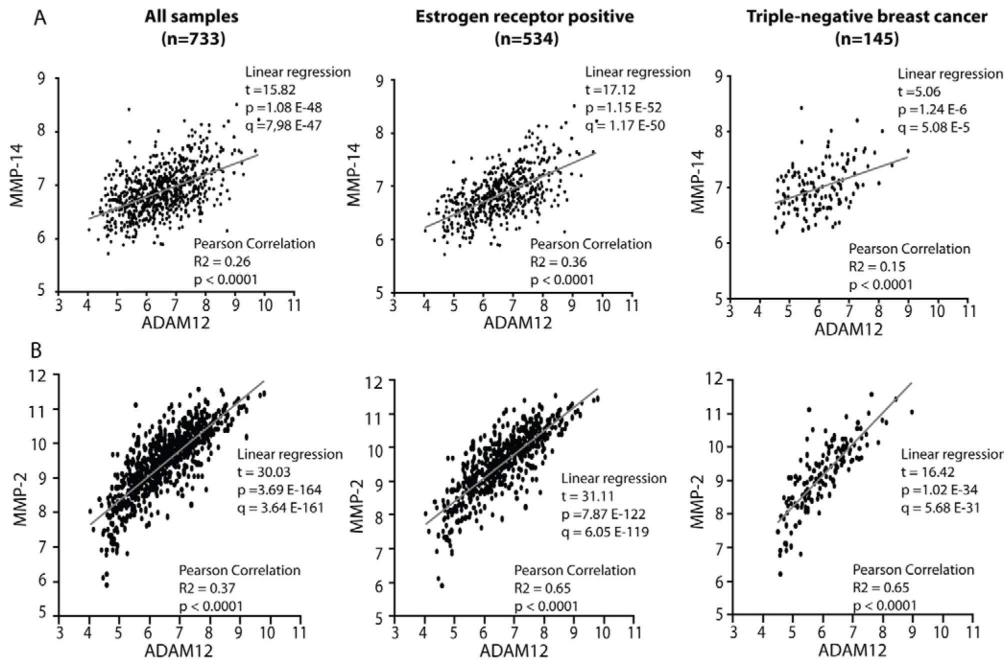

Fig. 8. Positive correlation between expression of ADAM12 and expression of MMP-14 and MMP-2. Correlation analysis of ADAM12-L, MMP-14 and MMP-2 gene expression in 733 human breast tumors from four different cohorts: EMC286, Erasmus, TRANSBIG and Mainz. The gene expression profile for estrogen-receptor-positive tumors (n=534) and triple-negative breast cancers (n=145) was also assessed by a simple linear regression analysis and Pearson correlation. (A) Correlation between ADAM12 and MMP-14. (B) Correlation between ADAM12 and MMP-2.

direct interaction between ADAM12 and MMP-14 has been described, with this interaction resulting in an apparent striking effect on tumor cell behavior *in vitro* and *in vivo*.

Individually, MMP-14 and ADAM12 are considered important cancer proteinases; that is, both are highly upregulated in many types of human cancer, including breast carcinoma (Figueira et al., 2009; Fröhlich et al., 2011; Iba et al., 1999; Lendeckel et al., 2005; Roy et al., 2004; Ueno et al., 1997), and both accelerate tumor progression in mouse models of cancer (Fröhlich et al., 2011; Kveiborg et al., 2005; Maquoi et al., 2012; Peduto et al., 2006; Perentes et al., 2011; Roy et al., 2011; Szabova et al., 2008; Zarrabi et al., 2011). We report here for the first time a positive correlation between ADAM12 and MMP-14 expression in human breast cancer, which was present in all samples analyzed as well as in ER-positive tumors and in TNBC samples. Until recently, MMP-14 was thought to mainly affect tumor cell dissemination by degrading extracellular matrix, with little or no effect on tumor growth per se (Perentes et al., 2011; Szabova et al., 2008; Zarrabi et al., 2011). However, a new exciting report from the laboratory of Agnes Noël demonstrated that MMP-14 also has the capacity to accelerate primary tumor growth by circumventing tumor cell apoptosis (Maquoi et al., 2012). More specifically, MMP-14 was shown to confer apoptotic resistance to breast cancer cells when embedded in a collagen gel, as well as after orthotopic implantation in mice. The suggested underlying molecular mechanism was that, by cleaving the collagen matrix, MMP-14 decreased the expression of the pro-apoptotic protein BIK. Our data now extend their finding by pointing to a direct link between ADAM12 and MMP-14 in reducing tumor cell apoptosis, as well as reporting an accompanying decreased expression of both BIK and BCL2L11 in ADAM12-expressing tumor cells. The reduced tumor cell apoptosis could be rescued by siRNA-mediated knockdown of MMP-14, further supporting the notion that MMP-14 is a key driver in this process. The metalloproteinase inhibitor GM6001, which inhibits MMP-14 but not ADAM12, significantly increased apoptosis in collagen-embedded ADAM12-expressing tumor cells. Importantly, we report that monoclonal antibodies to ADAM12 also increased tumor cell apoptosis, which indicates that they can inhibit the effect of the ADAM12–MMP-14 axis on apoptosis. These data provide an important mechanistic explanation to observations in previous *in vivo* studies (Kveiborg et al., 2005; Roy et al., 2011) that showed increased expression of ADAM12 accelerated tumor growth apparently by diminishing the apoptotic capacity of tumor cells. Because a high number of malignant tumors in humans simultaneously express both ADAM12 and MMP-14, we hypothesize that ADAM12 also can function as an important key regulator for MMP-14 activation in clinical cases.

In the present study, we revealed that ADAM12 induces gelatin degradation independently of its own catalytic activity. Our data indicate that MMP-14 directly degrades gelatin rather than it being an indirect effect of an MMP-14-mediated activation of the gelatinase MMP-2, an otherwise well-described effect of active MMP-14 (d'Ortho et al., 1998; Itoh et al., 2011; Itoh et al., 2001; Strongin, 2010). It should be noted that the increase in MMP-14 activity was not due to an increased amount of MMP-14 at the mRNA or protein level, but relies on its appropriate localization at the plasma membrane in accordance with earlier findings (Seiki 2003). Processing of MMP-14 at the cell surface is an event in which the active MMP-14 proteinases is autocatalytically cleaved to generate a membrane-anchored fragment of 43 kDa and a soluble 18 kDa inactive fragment of the catalytic domain (Cho et al., 2008; Ellerbroek et al., 2001; Itoh, 2006). Here, we detected the 43 kDa MMP-14 fragment in both tumor cell and tumor tissue extracts, further demonstrating that MMP-14 was present in an active form.

MMP-14 activity is regulated at several different levels, and interaction with cell surface integrins seems to play an important role for activation of MMP-14 (Ellerbroek et al., 2001; Gálvez et al., 2002; Gonzalo et al., 2010). In particular, the interaction between MMP-14 and αVβ3 integrin has been implicated

in a variety of processes involved in tumor progression (Borrirukwanit et al., 2007; Deryugina et al., 2002; Deryugina et al., 2004). Like MMP-14, ADAM12 has been shown to interact with several integrins (i.e. α9β1, α7β1, α5β1, and αVβ3), thereby affecting the actin cytoskeleton, cell migration and myotube formation (Lafuste et al., 2005; Thodeti et al., 2005a; Thodeti et al., 2005b; Zhao et al., 2004). We recently found that ADAM12-antibody-induced formation of MMP-14-positive invadopodia-like clusters required αVβ3 integrin (Albrechtsen et al., 2011). In the present study, we demonstrated that ADAM12 immunoprecipitated both MMP-14 and β3 integrin, and that ADAM12 colocalized with both MMP-14 and αVβ3 integrin at the cell surface. Moreover, ADAM12-producing 293-VnR cells, which express αVβ3 integrin, induced cell surface localization of MMP-14 and subsequent gelatin degradation, whereas the parental HEK293 cells, which express MMP-14 at levels similar to 293-VnR cells but do not express αVβ3 integrin, were incapable of redistributing MMP-14 to the cell surface and degrading gelatin, even upon ADAM12 expression. Interestingly, activation of integrin has previously been shown to relocate ADAM12 to the cell surface through interactions with RACK1 (Bourd-Boittin et al., 2008). Further evidence to support the involvement of αVβ3 integrin in ADAM12-induced gelatin degradation was provided by the finding of decreased gelatin degradation after treatment with inhibitory antibodies against αVβ3 integrin in both ADAM12-producing 293-VnR and MCF7-A12Δcyt cells. On the basis of these findings, we suggest that ADAM12, probably through a clustering of the integrin, initiates an activation of MMP-14 and thus becomes a part of a ternary protein complex with αVβ3 integrin and MMP-14 at the cell surface, with all three together responsible for profound effects on tumor cell behavior.

The membrane-anchored form of human ADAM12-L is a multidomain protein. From the N-terminus, it consists of a prodomain, a catalytic domain, a disintegrin-like domain, a cysteine-rich domain, an epidermal growth factor-like domain, a transmembrane domain and a cytoplasmic tail (Gilpin et al., 1998). The prodomain is thought to primarily keep the ADAM12 molecule in an inactive form until after cleavage at the furin site between the prodomain and the catalytic domain. Interestingly, after furin cleavage, the prodomain remains associated with the mature molecule through noncovalent bonds (Wewer et al., 2006). Our data indicated that the monoclonal antibodies against the prodomain (7B8 and 8F8) of ADAM12 disrupt the functional activities of the ternary protein complex consisting of ADAM12, MMP-14 and αVβ3 integrin at the cell surface. Although 8F8 was able to coimmunoprecipitate ADAM12 together with MMP-14 and αVβ3 integrin, we hypothesize that mAbs 7B8 and 8F8 somehow change the conformation of the ADAM12 molecule and/or alter its refined interaction with the MMP-14–αVβ3-integrin complex, resulting in disruption of the functional activities of the complex at the cell surface. We cannot draw any conclusions on the role of the other domains of ADAM12, but, interestingly, neither the catalytic activity of ADAM12 nor the cytoplasmic tail appeared to be required for gelatin degradation. These data let us propose that, at the cell surface, ADAM12, probably through its prodomain, provides a key signal for MMP-14 and αVβ3 integrin to promote tumor progression.

In conclusion, the data presented here demonstrate that ADAM12 regulates gelatin degradation and tumor cell apoptosis by enhancing cell surface levels and subsequent proteolytic activity of MMP-14. Interestingly, formation of a ternary complex of ADAM12, MMP-14 and αVβ3 integrin at the cell surface appeared to be critical, implying that these molecules, at least in part, act together in exerting their protumorigenic functions. Future studies should be designed to test whether inhibitory antibodies against ADAM12 could be used as a new paradigm to develop potential tumor-targeting agents that are active at the tumor cell surface.

Materials and Methods

Antibodies and chemicals

Antibodies against ADAM12 (mouse 8F8, 6E6 and rabbit rb122) were as previously described (Albrechtsen et al., 2011; Fröhlich et al., 2006; Gilpin et al., 1998; Iba et al., 1999; Kveiborg et al., 2005; Sundberg et al., 2004). Furthermore, not previously described mouse monoclonal antibodies against ADAM12 (7B8 and 7G3) were generated as described (Sundberg et al., 2004). Other antibodies used in the study include mouse monoclonal antibodies against GFP (Clontech Laboratories, Mountain View, CA, USA), αVβ3 integrin (LM609) (Chemicon/Millipore, Ballerica, MA, USA), and actin (Calbiochem, Ballerica, MA, USA), a goat polyclonal antibody against BIK and rabbit polyclonal antibodies against BCL2L11 (Santa Cruz Biotechnology, CA, USA; Nordic Biosite, Täby, Sweden), MMP-14 (Abcam, Cambridge, MA, USA and LifeSpan Bioscience, Seattle, WA, USA) and β3 integrin (Santa Cruz Biotechnology Inc.). Ki67, horseradish-peroxidase-conjugated secondary goat anti-mouse, goat anti-rabbit, and rabbit anti-goat immunoglobulins were from Dako (Glostrup, Denmark). Alexa-Fluor®-488-conjugated rabbit anti-goat IgG and goat anti-mouse IgG, and the Alexa-Fluor®-546-conjugated $F(ab)_2$ fragment of goat anti-mouse IgG and $F(ab)_2$ fragment of goat anti-rabbit IgG were from Invitrogen (Naerum, Denmark). GM6001 and TAPI-2 were from Calbiochem.

Plasmids

Mammalian expression constructs encoding full-length human ADAM12-L, human ADAM12-L fused to GFP or human ADAM12-L lacking the cytoplasmic tail (ADAM12Δcyt) used for transfections were as previously described (Hougaard et al., 2000; Kawaguchi et al., 2003). A point mutation in the catalytic site (E351Q) of ADAM12 was introduced by Mutagenex (Hillsborough, NJ, USA) to generate an expression construct encoding ADAM12Δcyt-E351Q. For retroviral transduction, cDNA encoding ADAM12-Δcyt was inserted into pRevTRE (Clontech BD Sciences, Brøndby, Denmark).

Cell culture, transfections, FACS and biotinylation of cell surface proteins

The HEK293 cell line stably expressing αVβ3 integrin, called 293-VnR, was as previously described (Sanjay et al., 2001). HEK293, MCF7 and MDA-MB-231 were from ATCC (LGC Standards AB, Boras, Sweden), cultured as previously described (Albrechtsen et al., 2011; Fröhlich et al., 2011), and transiently transfected using FuGENE® 6 Transfection Reagent (Roche Applied Science, Hvidovre, Denmark). Gelatinase-depleted FBS was used in some culture media and was used as previously described (Kang et al., 2000). ADAM12Δcyt in the pRevTRE vector was stably transduced into MCF7 Tet-Off (Clontech BD Sciences) as described previously (Rønnov-Jessen et al., 2002). The stable MCF7-A12Δcyt cell line was kept in growth medium supplied with 50 μg/ml hygromycin B (Roche Applied Science) and 100 μg/ml geneticin (Sigma). To silence ADAM12 expression in the MCF7-A12Δcyt cell line, 100 ng/ml doxycycline (Sigma-Aldrich) was added to the growth medium. Small interfering RNAs (siRNAs) against MMP-14 and ADAM12 were obtained as siGENOME® SMARTpool reagents from Thermo Scientific Dharmacon® (Lafayette, CO, USA), and siRNA universal negative control was from Sigma-Aldrich. siRNA transfection was performed using OPTI-MEM® I and Lipofectamine™ 2000 (Invitrogen). FACS analysis and biotinylation of cell surface proteins was performed as previously described (Lydolph et al., 2009; Stautz et al., 2012).

Immunofluorescence staining

Visualization of ADAM12 was as described previously (Albrechtsen et al., 2011). For visualization of MMP-14 and αVβ3 integrin, the cells were fixed in paraformaldehyde, blocked (1% BSA and 1% normal goat serum), and permeabilized or not (0.5% Triton-X 100) before primary and secondary antibodies and 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen; 1:5000) were added. In cytospin experiments, the cell surface staining for ADAM12 and MMP-14 was performed using a similar method to that described by Kawaguchi et al. (Kawaguchi et al., 2003). In brief, the cells were trypsinized and stained without permeabilization, fixed in 4% paraformaldehyde and spun in a cytospin centrifuge (Sandon, Thermo Fisher Scientific Inc., IL 60133, USA). ADAM12- and MMP-14-staining cells were counted using the MetaMorph software with multi wavelength cell scoring program.

For colocalization at the cell surface, Duolink® reagents from Olink (Uppsala, Sweden) were used on non-permeabilized cells. In brief, the Duolink assay is based on the *in situ* proximity ligation assay (PLA) technique, where two primary antibodies raised in different species are allowed to bind to their respective target antigen (i.e. ADAM12, MMP-14 or αVβ3 integrin) Species-specific secondary antibodies, each with a unique short DNA strand attached to it, bind to the primary antibodies and, when in close proximity, the DNA strands interact, get amplified and labeled with complementary fluorescent probe, which are visible as a distinct dots in the fluorescence microscope. Fluorescence imaging was performed using a confocal laser-scanning microscope (LSM510 Meta, Carl Zeiss, Oberkochen, Germany) equipped with a 63×/1.4 Plan-Apochromat water immersion objective or an inverted Zeiss Axiovert 220 Apotome system with the same type of objectives. The images were processed using the Axiovision program (Carl Zeiss) and MetaMorph software.

In situ gelatinase, 3D collagen assays and gelatin zymography

Cells were seeded on dishes coated with gelatin (10 μg/ml) coupled to Oregon Green® 488 dye (G-13186) from Molecular Probes (Life Technologies, Naerum, Denmark). At 24 hours after cell seeding (unless otherwise stated), gelatin degradation was quantified by measuring the degraded area in μm^2 (observed as black holes in the gelatin fluorescence) by use of MetaMorph software and correlated to the number of cells as well as the number of cells stained for ADAM12. For each experiments more than 1000 cells were counted and same type of experiment was repeated independently at least three times. PureCol (Advanced BioMatrix, San Diego, CA, USA) solution was used for making the 3D collagen gels according to the manufacturer's protocol and cells were embeded and grown as described previously (Maquoi et al., 2012). Gelatin zymography was performed as previously described (Tatti et al., 2008).

Detection of apoptotic and proliferative cells

The MetaMorph® microscopy automation and image analysis software was used for automatic nuclei counting for detection of apopototic cell bodies and cell proliferation (Universal Imaging Corporation, Downingtown, PA, USA). The ApopTag® peroxidase ISOL apoptosis detection kit (Millipore) was used on cultured cells or on paraffin sections from mouse tumor tissue. Apoptosis was also evaluated by counting the percentage of cells stained with DAPI that displayed chromatin condensation and nuclear fragmentation (apoptotic bodies). At least 500 cells were examined in each sample to quantify apoptosis. Parallel paraffin sections of mouse tumors were stained for Ki67 (Dako) to estimate cell proliferation.

Immunoprecipitation and western blot analysis

Immunoprecipation and western blots of cell lysates and tumor tissue were performed as described previously (Fröhlich et al., 2011; Stautz et al., 2012).

Quantitative PCR

Total RNA was extracted and isolated from cell lines and a quantitative PCR (qPCR) was performed with primers as previously described (Fröhlich et al., 2011; Pennington and Edwards, 2010).

In vivo tumorigenic assay

Equal amounts of MCF7-A12Δcyt cells were injected orthotopically into the mammary gland of 6–8-week-old NOD.Cg-Prkdc ^{scid} Il2rg^{tm1Wjl}/SzJ mice (The Jackson Laboratory, Bar Harbor, ME, USA). Two experiments were performed using two different concentrations of tumor cells: 1×10^6 and 3×10^6 cells per mouse. One week prior to tumor cell injection and during the rest of the experiment, mice were given 0.667 μg/ml estradiol-17β (Sigma-Aldrich) in their drinking water. Some mice injected with MCF7-A12Δcyt cells also received 2 mg/ml doxycycline (Sigma-Aldrich) in their drinking water. Tumor size (length and width) was measured over time. Mice were killed as soon as one mouse displayed a 1.2 cm^2 tumor, and tissue was dissected as described previously (Fröhlich et al., 2011; Kveiborg et al., 2005). All experiments were conducted in accordance with the guidelines of the Animal Experiment Inspectorate, Denmark.

Patient population and data analysis

Raw data (CEL files) from the following datasets were downloaded from Array Express (GSE2034, GSE5327, GSE7390, GSE11121) and are available at Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). The four datasets from four different cohorts with appropriate ethical consents (EMC286, Erasmus, TRANSBIG, and Mainz; Desmedt et al., 2007; Minn et al., 2007; Schmidt et al., 2008; Wang et al., 2005) were selected as described previously (Li et al., 2012). All data were normalized together using the RMA (Robust Multichip Average) approach, and calculations were performed using the Partek Genomics Suite 6.6 (Saint Louis, MO, USA). All calculations and representation of data are on log2 transformed values. ER status and triple-negative breast cancer were assessed by the gene expression profile (supplementary material Fig. S6). A simple linear regression analysis was performed in Qlucore Omics Explorer 2.2 (Lund, Sweden), where the expression level of ADAM12 (Affymetrix probe 213790_at) was correlated to the expression of MMP-14 (Affymetrix probe 202828_s_at) and MMP-2 (Affymetrix probe 201069_at). Pearson correlations were performed using GraphPad Prism.

Statistical analysis

All assays were performed in at least three independent experiments. Statistical analysis was performed using Excel or GraphPad Software with the Student's *t*-test for comparing two independent groups or Fisher's exact test. The association between gelatin degradation and antibody inhibition was analyzed using the analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

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Author contributions

R.A. designed the study, performed the main research, and contributed to writing of the manuscript. D.S., J.B.N. and A.K. performed additional experiments. J.V. and F.C.N. analyzed the gene expression profiles obtained from the patient material. M.K. and U.W. helped to design the study, contributed with interpretation of data and wrote/edited the manuscript prior to submission. C.F. designed the study, contributed to experiments and wrote the manuscript.

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