

Short Communication

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Overexpression of the urokinase receptor splice variant uPAR-del4/5 in breast cancer cells affects cell adhesion and invasion in a dose-dependent manner and modulates transcription of tumor-associated genes

Abstract: mRNA levels of the urokinase receptor splice variant uPAR-del4/5 are associated with prognosis in breast cancer. Its overexpression in cancer cells affects tumor biologically relevant processes. In the present study, individual breast cancer cell clones displaying low vs. high uPAR-del4/5 expression were analyzed demonstrating that uPAR-del4/5 leads to reduced cell adhesion and invasion in a dose-dependent manner. Additionally, matrix metalloproteinase-9 (MMP-9) was found to be strongly upregulated in uPAR-del4/5 overexpressing compared to vector control cells. uPAR-del4/5 may thus play an important role in the regulation of the extracellular proteolytic network and, by this, influence the metastatic potential of breast cancer cells.

Keywords: adhesion; breast cancer; differential gene expression; invasion; MMP-9; uPAR splice variant.

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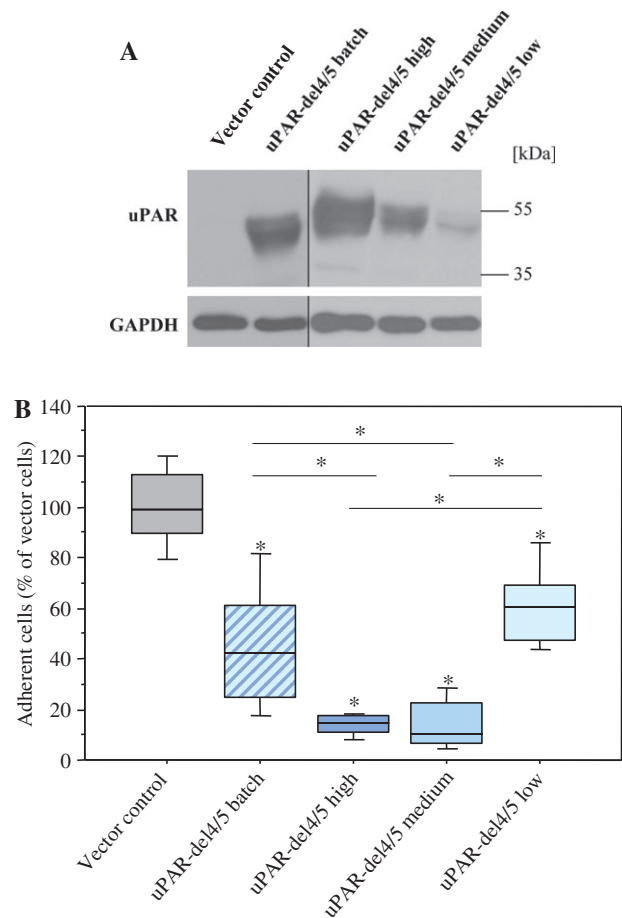
The urokinase-type plasminogen system mainly consists of the serine protease uPA, its serpin-type inhibitor PAI-1, and its cell surface receptor uPAR (CD87) and is intimately involved in several (patho-)physiological processes such as thrombolysis, inflammation, fertility, cell migration, tissue re-modeling, cancer cell invasion and metastasis, and vascularization (Montuori et al., 2005; Mengele et al., 2010). Furthermore, these factors represent important biomarkers in a number of malignancies, including breast cancer (Høyer-Hansen and Lund, 2007; Schmitt et al., 2010). uPAR, which is linked to the cell membrane via its glycosyl phosphatidyl inositol (GPI) moiety, is an ubiquitously expressed 45–60 kDa highly glycosylated protein. The receptor is composed of three homologous domains designated DI, DII, and DIII, from the amino- to the carboxy-terminus (Kriegbaum et al., 2011; Xu et al., 2012). Upon binding of its ligand uPA, uPAR

Figure 1 Characterization of uPAR-del4/5 overexpressing CAMA-1 cell clones.

CAMA-1 breast cancer cells were stably transfected with an expression plasmid encoding uPAR-del4/5 (pRcRSV-uPAR-del4/5) or the vector only (pRcRSV; Invitrogen, Karlsruhe, Germany), and individual cell clones isolated by limited dilution. (A) Western blot analysis. Cell pellets were lysed in TBS-T (Tris-buffered saline, 1% Triton X-100) containing the protease inhibitor cocktail 'Complete with EDTA' (Roche Diagnostics, Mannheim, Germany). The cleared lysate was applied to a polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore Corporation, Bedford, MA) using a semidry blotting device. Domain I of uPAR was detected using the monoclonal antibody IIIIF10 (Luther et al., 1997), diluted in TBS-T supplemented with 1% (w/v) dried skimmed milk. After several washes in TBS-T, binding of the antibodies was visualized by incubation of the membranes with a horseradish peroxidase-conjugated secondary goat antibody against mouse Ig (Jackson ImmunoResearch Lab, West Grove, PA) followed by chemoluminescence reaction using ECL (Thermo Scientific, Pierce, Stonehouse, UK). (B) Adhesion of CAMA-1 breast cancer cells to collagen type IV. CAMA-1 breast cancer cells were resuspended in culture medium containing 0.5% (w/v) BSA + HEPES (Invitrogen), seeded at a density of 20 000 cells/well on Col IV-coated 96-well plates (5 µg/ml; Sigma-Aldrich, Saint Louis, MO), and allowed to adhere for 2 h at 37°C. Non-adherent cells were subsequently removed by washing three times with phosphate-buffered saline (PBS). The number of adherent cells was quantified by a hexosaminidase activity assay. For this, cells were incubated with *p*-nitrophenyl-*N*-acetyl- α -D-glucosaminide (Sigma-Aldrich) diluted to 15 mM in a 100-mM sodium citrate buffer, pH 5.0, 0.5% (v/v) Triton X-100, for 90 min at 37°C. The reaction was terminated by the addition of stop buffer (0.2 M NaOH, 5 mM EDTA) and the optical density recorded at 405 nm. At least three independent experiments were performed in triplicates each. The results are given in % relative to the cell number of adherent vector-transfected control cells. Whisker box plots indicate the 25th and 75th percentile, the vertical bars indicate the 10th and 90th percentile. The median value is indicated by a bar within the box. Statistically significant differences ($p < 0.05$) are indicated by an asterisk.

focuses the extracellular proteolytic network, including plasmin and matrix metalloproteinases (MMPs), on the cell surface. In addition to its proteolysis-related functions, uPAR can act as a signaling receptor that modulates cell adhesion, migration, and proliferation through interactions with other membrane proteins such as several integrins, epidermal growth factor receptor (EGFR), G protein-coupled receptors, low-density lipoprotein receptor-related protein, and the extracellular matrix protein vitronectin (Tang and Wei, 2008; Blasi and Sidenius, 2010; Smith and Marshall, 2010).

Several mRNA splice variants of uPAR have been identified and their expression analyzed in human cells and tissues. One of these splice variants, uPAR-del4/5 (lacking exons 4 and 5), encodes a form of uPAR lacking the domain DII and was found to be overexpressed in breast cancer cells (Luther et al., 2003). Interestingly, high uPAR-del4/5 mRNA expression levels were found to be significantly associated



with shorter disease-free survival of breast cancer patients (Luther et al., 2003; Kotzsch et al., 2005). Moreover, uPAR-del4/5 mRNA was shown to be a highly sensitive, statistically independent prognostic factor for distant metastasis-free survival in untreated node-negative breast cancer patients (Kotzsch et al., 2008). Recently, we described the characteristics of breast cancer cell lines overexpressing the urokinase receptor splice variant (Sato et al., 2011). Batch-transfected, uPAR-del4/5 overexpressing breast cancer cells showed a reduced adhesion toward the extracellular matrix proteins as well as a decreased invasive capacity in Matrigel™ invasion assays *in vitro*. Accordingly, the ability of batch-transfected cells to form lung colonies upon tail vein injection in an *in vivo* athymic mouse model was significantly reduced (Sato et al., 2011). In the present study, individual cell clones of two different breast cancer cell lines expressing homogeneous high, medium, or low levels of uPAR-del4/5 were isolated and analyzed whether tumor biologically relevant characteristics of tumor cells are affected in a dose-dependent manner. Furthermore, we investigated whether overexpression of uPAR-del4/5 in breast cancer cells modulates gene expression of other tumor-relevant factors.

First, individual cell clones were isolated by limited dilution from the breast cancer cell line CAMA-1, which has been stably transfected with an expression plasmid encoding uPAR-del4/5 or the vector alone as described previously (Sato et al., 2011). In Western blot analysis, the vector control cells do not display any uPAR-related signal, whereas the uPAR-del4/5 batch-transfected cells display moderate expression levels (Figure 1A). Additionally, cell clones expressing uPAR-del4/5 protein at low, medium, or high uPAR-del4/5 levels could be generated (Figure 1A). Densitometric analysis of the Western blot indicated a relative uPAR-del4/5 expression level of 1 (low):3.7 (moderate):6.1 (high). Cell growth of batch-transfected cells as well as individual cell clones, analyzed by cell counting, was not affected by uPAR-del4/5 overexpression. However, the adhesive capacity of CAMA-1 cells toward different extracellular matrix (ECM) proteins distinctly changed. As seen in Figure 1B, uPAR-del4/5 high- and moderate-expressing CAMA-1 cell clones display a very low binding capacity toward collagen type IV (Col IV). The low-expressing cell clone, on one hand, is characterized by an about 40% significantly lower binding capacity compared to the vector control cells. On the other hand, this clone binds about fourfold better to collagen type IV compared to the uPAR-del4/5 high-/moderate-expressing clones. Similar results were obtained when cell culture plates were precoated with collagen type I (Col I) or, to a lesser extent, vitronectin (VN) (data not shown). These results clearly demonstrate that uPAR-del4/5 controls cell adhesion in CAMA-1 cells in a dose-dependent manner.

These findings were confirmed in a second breast cancer cell line, MDA-MB-231. These cells endogenously express low levels of wild-type uPAR, whereas uPAR-del4/5 expression is not detectable even on the mRNA level by quantitative PCR. Again, individual cell clones were isolated by limited dilution, and low- and high-expressing clones were selected by flow cytometry, applying monoclonal antibody IIIF10 directed against domain I of uPAR (Luther et al., 1997) detecting both wild-type uPAR and uPAR-del4/5. These analyses indicated a relative cell surface-associated uPAR-reactive protein of 1 (vector control, endogenously expressing wild-type uPAR):1.5 (low uPAR-del4/5-expressing clone):16 (high uPAR-del4/5-expressing clone). Alike CAMA-1 cells, we did not observe any changes in cell proliferation upon stable transfection of the uPAR-del4/5 expression vector, neither in low- nor in high-expressing cell clones. In contrast, adhesion to the ECM proteins Col IV, Col I, and VN is significantly reduced by about 30% in the high-expressing clone (Figures 2A and 3). These results clearly validate previous results obtained with batch-transfected MDA-MB-231 cells

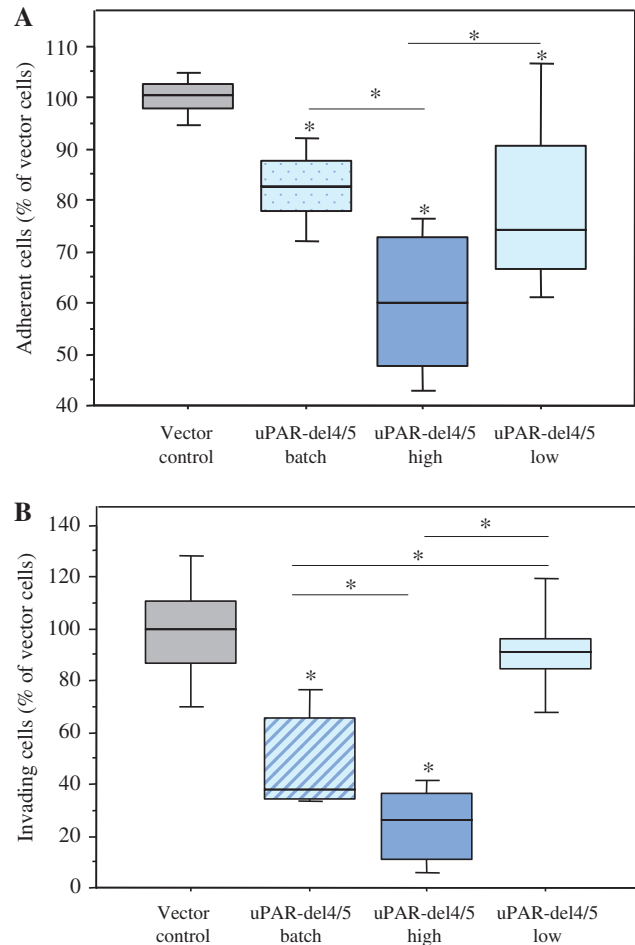


Figure 2 Characterization of uPAR-del4/5-overexpressing MDA-MB-231 cell clones.

(A) Adhesion of MDA-MB231 breast cancer cells to vitronectin. Ninety-six-well plates were coated with VN (2 $\mu\text{g}/\text{ml}$; BD Biosciences, Franklin Lakes, NJ) and cell adhesion quantified as described in Figure 1B. (B) Characterization of the invasive capacity of uPAR-del4/5-overexpressing MDA-MB-231 cell clones. Transwell inserts (8 μm pore size; Corning Costar, Amsterdam, The Netherlands) were coated with 30 μg basement membrane complex growth factor-reduced Matrigel™ (BD Biosciences). After incubation for 3 h at 37°C followed by overnight incubation in a laminar hood at room temperature, inserts were rehydrated with 200 μl FCS-free DMEM, containing 0.1% (w/v) BSA, 2 h at 37°C. MDA-MB-231 cells were resuspended in culture medium and seeded in the upper chamber of the device at a density of 40 000 cells/chamber. The lower chambers were filled with 600 μl DMEM supplemented with 10% (v/v) FCS as a chemoattractant. After a 24-h incubation at 37°C, Matrigel™ and non-invaded cells, located on the upper side of the insert, were wiped off, whereas invaded cells on the lower side of the insert were fixed, stained, and counted. At least three independent experiments were performed in triplicates each. The results are given in % relative to the cell number of adherent vector-transfected control cells (A) or normalized to the number of invaded vector-transfected cells (B). Whisker box plots indicate the 25th and 75th percentile, the vertical bars indicate the 10th and 90th percentile. The median value is indicated by a bar within the box. Statistically significant differences ($p < 0.05$) are indicated by an asterisk.

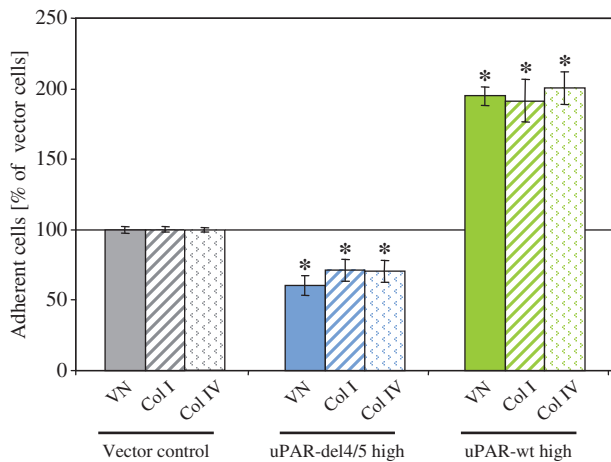


Figure 3 Adhesion of uPAR-del4/5 or uPAR-wt-overexpressing MDA-MB-231 cell clones to extracellular matrix proteins. Ninety-six-well plates were coated with either VN (2 μ g/ml; BD Biosciences), Col I or Col IV (5 μ g/ml; Sigma-Aldrich), and cell adhesion was quantified as described in Figure 1B. At least three independent experiments were performed in triplicates each. The results are given in % relative to the cell number of adherent vector-transfected control cells (mean \pm SEM). Statistically significant differences ($p < 0.05$) to the vector control cells are indicated by an asterisk.

(Sato et al., 2011). Interestingly, already low expression of uPAR-del4/5 protein in MDA-MB-231 cells significantly affects the binding of the cells to VN (Figure 2A) and Col IV (data not shown).

To verify that the observed effects on cell adhesion were specific to uPAR-del4/5 expression, and not merely due to cellular alterations resulting from protein overexpression, we also analyzed the overexpression of uPAR-wt in MDA-MB-231 cells. Wild-type uPAR has been shown to be involved in the regulation of cell adhesion, e.g., through interactions with integrins (Tang and Wei, 2008). In fact, in contrast to uPAR-del4/5, overexpression of uPAR-wt protein led to an about twofold increased adhesion of MDA-MB-231 cells to the various ECM proteins (Figure 3). These results strongly suggest that upon overexpression, uPAR-del4/5 and uPAR-wt, respectively, exert different effects on cell adhesion in breast cancer cells. In addition, our data indicate that the decrease of adhesion in uPAR-del4/5-overexpressing cells correlates with the respective uPAR-del4/5 protein levels.

Furthermore, the invasive capacity of the cell clones overexpressing uPAR-del4/5 was determined using Matrigel™ Transwell assays. For this, only the highly metastatic cell line MDA-MB-231 was used because parental CAMA-1 cells do not display any invasion through Matrigel™. Batch-transfected uPAR-del4/5 expressing MDA-MB-231 cells showed a significant reduction in their invasive capacity, which corresponds to previous findings

(Sato et al., 2011). This effect, however, was much stronger pronounced in uPAR-del4/5 high-expressing cell clones (Figure 2B). The invasive capacity of cells with low expression of uPAR-del4/5 was not significantly different from that of vector control cells (Figure 2B). It is of note that breast cancer cells overexpressing uPAR-wt are characterized by a strong increase in its invasive capacity [data not shown; see also Sato et al. (2011)]. The observed effects on *in vitro* invasion appear to be dependent on the level of uPAR-del4/5 protein, as the number of invaded cells, overexpressing high levels of uPAR-del4/5, was significantly lower compared to the number of invaded cells in the low-expressing cell clones (Figure 2B).

As processes such as tumor cell adhesion and invasion depend on the expression and activity of adhesion molecules or matrix-degrading proteolytic enzymes, we investigated whether overexpression of uPAR-del4/5 in breast cancer cells modulates the expression of related tumor biologically relevant proteins. For this, gene expression of a series of tumor-associated proteases, inhibitors, and extracellular matrix proteins was analyzed in stably transfected vector control vs. uPAR-del4/5 high-expressing MDA-MB-231 breast cancer cells using a low-density microarray (Hauser et al., 2011). Nine genes were found to be upregulated, and 11 genes downregulated more than tenfold in uPAR-del4/5 high-expressing cell clones compared to vector control cells (Table 1). mRNA expression levels of genes encoding the members of the plasminogen activation system (uPA, uPAR-wt, and PAI-1) were not affected.

Expression of one of the upregulated genes encoding the matrix metalloproteinase MMP-9 was further analyzed at the protein level (Ries et al., 2007). For this, MMP-9 production and secretion into the cell culture supernatant of uPAR-del4/5 and uPAR-wt high-expressing MDA-MB-231 clones as well as vector control cells was determined in gelatin zymogram gels indicating a distinctly increased MMP-9 level in the cell culture supernatant of the uPAR-del4/5 high-expressing clone (Figure 4A). Western blot analysis, using a monoclonal antibody, IE-5E9-B1, directed to pro-MMP-9, in fact, confirmed enhanced secretion of MMP-9 into the supernatant of cultured breast cancer cells overexpressing uPAR-del4/5, but not uPAR-wt or vector control cells (Figure 4B).

In the present study, we found that the urokinase receptor splice variant uPAR-del4/5 modulates unique tumor biological characteristics of breast cancer cells, depending on its protein expression level. Whereas, on one hand, overexpression of uPAR-del4/5 in MDA-MB-231 and CAMA-1 cells did not have striking effects on cell growth, on the other, adhesive properties as well as the invasive capacity of cells were significantly impaired in a dose-dependent

uPAR-del4/5 overexpressing vs. vector control cells

Upregulation, more than 10-fold	Downregulation, more than 10-fold
MIP-1 β	EMMPRN
Cadherin-1	MIP-1 alpha
VEGFR	Cyclin D2
HGF	GCSF
Hepsin	FAP
IL-1RA	aFGF
Integrin α 4	IL-6R
MMP-9	MMP-13
Vitronectin	MMP-3
	Selectin
	VCAM-1

Table 1 Impact of uPAR-del4/5 overexpression on gene expression of tumor-associated genes.

mRNA was extracted from uPAR-del4/5 over-expressing or vector control cells and subjected to quantitative PCR-based low-density array analyses. mRNA isolation from cell lines and reverse transcription to cDNA were done as described (Hauser et al., 2011). All quantitative real-time PCRs on the array were performed with inventoried primers and probes from Applied Biosystem (Darmstadt, Germany). Data were normalized to human 18S rRNA (4319413E, RefSeq: X03205.1). Only genes that were either more than 10-fold upregulated or repressed are listed.

manner in both breast cancer cell lines. Even very low uPAR-del4/5 protein levels clearly affected adhesion and invasion of breast cancer cells, which strongly indicates that uPAR-del4/5 expression leads to a pathophysiological modulation of cellular processes. Notably, overexpression of wild-type uPAR in MDA-MB-231 cells displays converse effects, i.e., increased cellular adhesion and invasion.

In clinical studies, quantification of uPAR-del4/5 mRNA levels in breast cancer tissue revealed that higher

uPAR-del4/5 expression is significantly associated with a shorter disease-free survival of breast cancer patients (Luther et al., 2003; Kotzsch et al., 2005, 2008). uPAR expression has been shown to be regulated by the RNA-binding protein HuR *in vitro* and *in vivo* (Tran et al., 2003; Heinonen et al., 2011). Thus, the observed higher uPAR-del4/5 mRNA expression in metastasizing breast cancer may be due to higher HuR expression in these tumors. Although high expression levels of both HuR and uPAR-del4/5 in tumor tissue are clearly associated with poor prognosis of breast cancer patients, respectively, *in vitro* overexpression of uPAR-del4/5, surprisingly, resulted in a reduced tumorigenic potential of transfected tumor cells. The observed phenotype of uPAR-del4/5-overexpressing cells resembles the phenotype of uPAR-wt-silenced cells, which are known to have a reduced invasive capacity *in vitro* and *in vivo* (Subramanian et al., 2006; Kunigal et al., 2007). As uPAR-del4/5 protein does not interact with uPA and vitronectin (Sato et al., 2010), it is tempting to speculate that uPAR-del4/5 acts as a dominant-negative receptor by replacing/competing with endogenous uPAR-wt, which may lead to altered cell adhesion properties and cell signaling events.

Overexpression of uPAR-del4/5 in MDA-MB-231 breast cancer cells resulted in upregulation of tumor-related proteins, including MMP-9. MMPs are indeed upregulated in virtually all human and animal tumors as well as in most tumor cell lines [reviewed in Coussens et al. (2002)]. Similar to the plasminogen activator system, members of the MMP family have been implicated in tumor cell invasion and metastasis by ECM degradation (Schmalfeldt et al., 2001; Kessenbrock et al., 2010). Moreover, MMPs promote initiation and sustained growth of tumor cells (Overall and López-Otín, 2002; Bauvois, 2011). Most

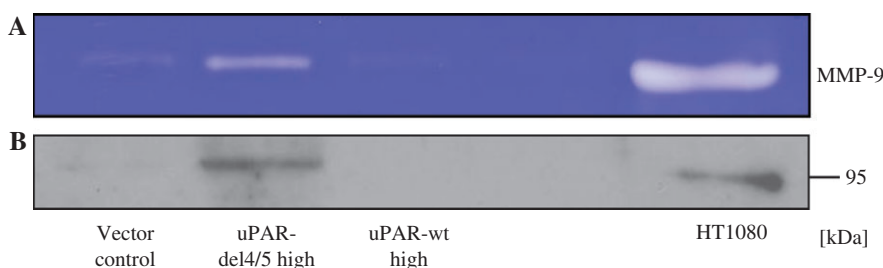


Figure 4 MMP-9 expression in uPAR-del4/5-overexpressing MDA-MB-231 cells.

MDA-MB-231 cells transfected with the empty vector (pRcRSV), pRcRSV-uPAR-wt, or pRcRSV-uPAR-del4/5 were seeded onto untreated plates. Conditioned serum-free medium was collected after 48 h of incubation. (A) Zymographic analysis and quantification of gelatinases in zymograms. Zymography was performed in 10% polyacrylamide minigels containing 0.1% gelatin as substrate (Invitrogen). Samples were run under nonreducing conditions without prior boiling. Conditioned medium from PMA-treated HT1080 fibrosarcoma cells containing pro-MMP-9 (95 kDa) was used as marker. After electrophoresis, gels were washed twice for 15 min in renaturing buffer (Invitrogen) and then incubated in developing buffer (Invitrogen) for 72 h at 37°C. The zymograms were stained for 90 min with 0.02% Coomassie Brilliant Blue R250 (Carl Roth, Karlsruhe, Germany) in a 30% (v/v) methanol/10% (v/v) acetic acid solution. (B) Western blot analysis. Immunoblotting was performed as described in Figure 1A, using the mouse monoclonal antibody IE-5E9-B1 directed to pro-MMP-9 as primary antibody.

MMPs are not expressed under normal quiescent conditions, but their transcription can be induced in tumor or stromal cells by various signals, such as cytokines, growth factors, and oncogene products released by tumor cells themselves, by the stroma or infiltrating host defense cells [reviewed in Overall and López-Otín (2002)]. Interestingly, uPAR-mediated signaling events trigger expression of MMP-9 during macrophage differentiation (Rao et al., 1995). MMP-9 expression in tumor cells plays a crucial role in cell signaling by controlling the bioavailability and bioactivity of molecules that target receptors, which regulate cell growth and migration. Thus, concerning the elevated pro-MMP-9 levels monitored in conditioned medium of MDA-MB-231 cells overexpressing uPAR-del4/5, one would expect an enhanced proteolytic activity and, subsequently, a more invasive phenotype

of these cells. Surprisingly, the opposite effect, impaired adhesive properties as well as decreased invasive capacity of these cells was observed. This phenomenon might be explained by either alterations in the activation of pro-MMP-9, an overexpression of MMP-9 inhibitors, or by additional nonproteolytic functions of MMP-9 in uPAR-del4/5-over-expressing breast cancer cells. In fact, there is growing evidence that MMPs under several circumstances do exhibit tumor-suppressing effects (Kessenbrock et al., 2010; Bauvois, 2011; Klein and Bischoff, 2011). All in all, our results point to a pathophysiological role of uPAR-del4/5 expression in cancer cells among others *via* modulation of gene expression of further tumor-associated factors such as MMP-9.

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