Matrix metalloproteinase-9 (MMP9) is involved in the TNF-α-induced fusion of human M13SV1-Cre breast epithelial cells and human MDA-MB-435-pFDR1 cancer cells

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Abstract

Background: In addition to physiological events such as fertilisation, placentation, osteoclastogenesis, or tissue regeneration/wound healing, cell fusion is involved in pathophysiological conditions such as cancer. Cell fusion, which applies to both the proteins and conditions that induce the merging of two or more cells, is not a fully understood process. Inflammation/pro-inflammatory cytokines might be a positive trigger for cell fusion. Using a Cre-LoxP-based cell fusion assay we demonstrated that the fusion between human M13SV1-Cre breast epithelial cells and human MDA-MB-435-pFDR1 cancer cells was induced by the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α).

Methods: The gene expression profile of the cells in the presence of TNF-α and under normoxic and hypoxic conditions was analysed by cDNA microarray analysis. cDNA microarray data were verified by qPCR, PCR, Western blot and zymography. Quantification of cell fusion events was determined by flow cytometry. Proteins of interest were either blocked or knocked-down using a specific inhibitor, siRNA or a blocking antibody.

Results: The data showed an up-regulation of various genes, including claudin-1 (CLDN1), ICAM1, CCL2 and MMP9 in M13SV1-Cre and/or MDA-MB-435-pFDR1 cells. Inhibition of these proteins using a blocking ICAM1 antibody, CLDN1 siRNA or an MMP9 inhibitor showed that only the blockage of MMP9 was correlated with a decreased fusion rate of the cells. Likewise, the tetracycline-based antibiotic minocycline, which exhibits anti-inflammatory properties, was also effective in both inhibiting the TNF-α-induced MMP9 expression in M13SV1-Cre cells and blocking the TNF-α-induced fusion frequency of human M13SV1-Cre breast epithelial cells and human MDA-MB-435-pFDR1 cancer cells.

Conclusions: The matrix metalloproteinase-9 (MMP9) is most likely involved in the TNF-α-mediated fusion of human M13SV1-Cre breast epithelial cells and human MDA-MB-435-pFDR1 cancer cells. Likewise, our data indicate that the tetracycline-based antibiotic minocycline might exhibit anti-fusogenic properties because it inhibits a cell fusion-related mechanism.

Keywords: Cell fusion, TNF-α, Minocycline, MMP9, Breast cancer
Background

Even though cell fusion has a pivotal role in several physiological and pathophysiological conditions such as fertilisation, placentaion, muscle development, osteoclastogenesis, wound healing, tissue regeneration, infection with enveloped viruses, and cancer (for review see: [1–4]), the conditions that favour and the detailed mechanisms of how the plasma membranes of two or more cells merge are not fully understood.

During evolution different cell fusion strategies developed using different proteins and protein-protein interactions. Some cell fusion-mediating proteins, such as EFF-1 in *C. elegans* and members of the syncytin family (syncytin-1 and syncytin-2 in humans or syncytin-A and syncytin-B in mice) are of viral and retroviral origin (for review see: [1, 5, 6]). Because of the high homology of these proteins to viral fusion proteins, the cell fusion mechanisms might conceivably be similar. Syncytin-1 and syncytin-2 are expressed in trophoblasts and mediate their fusion to multi-nucleated syncytiotrophoblasts [5]. Trophoblast fusion depends on cyclic AMP (cAMP)/PKA- and PKC-dependent mechanisms [7]. In contrast, the fusion of myoblasts to multinucleated myofibres chiefly depends on the reorganisation of the actin cytoskeleton and the interplay of several actin regulatory proteins (for review see: [8]). In order to fuse with osteoclasts, the macrophages first have to adopt a fusion-competent status, which is induced through several cytokines, including IL-4, IL-13, M-CSF and RANKL, and surface molecules such as TREM-2 and DAP12 (for review see: [9]). Fusion-competent macrophages are characterised by the expression of several markers, including E-cadherin, DC-STAMP/OC-STAMP, CD200, SIRPα and CD81, CCL2, and MMP9 (for review see: [9]).

The fusion of mesenchymal stem cells with MDA-MB-231 breast cancer cells depends on S100A4 [10].

The induction of a fusion-competent status indicates that the cells per se are not fusogenic, but rather, have to acquire the ability to fuse, which has been termed priming [4]. In addition to priming, four additional steps have been proposed for the entire cell fusion process, namely, chemotaxis, adhesion, fusion and post-fusion (an excellent and detailed overview is given in [4]). Fusion-competent cells have to migrate towards their cellular fusion partners by chemotaxis and close cell-cell contact is a prerequisite for the subsequent fusion step. As important as the induction of a fusion-competent status is, fused cells must finally acquire a fusion-incompetent status to prevent further cell fusion events.

Even though factors have been identified that induce a fusion-competent status in macrophages, the regulation of the entire process but particularly basal cell fusion events that occur between different body cell types, is not understood. In addition to macrophages [11], basal cell fusion events were also observed for bone marrow-derived stem cells (BMDCs) [12], intestinal cells [13], and haematopoietic cells [14]. It is unclear how these spontaneous cell fusion events are regulated. However, the data from several studies indicated an increased cell fusion frequency under injurious/inflammatory conditions. For instance, the fusion frequency of bone marrow-derived stem cells (BMDCs) with epithelial intestinal cells and Purkinje neurons is markedly increased in response to chronic inflammation [13, 15]. Likewise, an increased fusion frequency for different cell types, including macrophages and tumour cells, was observed in the presence of the pro-inflammatory cytokine TNF-α [16–19]. In this context, it is worth speculating whether the chronically inflamed tumour microenvironment [20, 21] would also provide a fusion-friendly milieu. It is well-known that tumour cells could fuse with tumour cells and normal cells, like macrophages and stem cells, thereby giving rise to hybrid cells that could exhibit novel properties, such as an enhanced metastatic capacity and an increased drug resistance (for review see: [22–24]).

To clarify whether the fusion of tumour cells and normal cells is triggered by cytokines, chemokines and/or growth factors, we developed a Cre-LoxP-based cell fusion assay [18]. Human breast cancer cells were stably transfected with a fluorescence double reporter (FDR) plasmid containing a *loxP* flanked HcRed/DsRed cassette followed by an EGFP cassette and were co-cultured with Cre recombinase-expressing human breast epithelial cells [18]. The Cre-mediated recombination in fused cells led to a switch from red fluorescence to green fluorescence that could be easily quantified by flow cytometry [18]. Using this assay we were able to show that the fusion of human breast cancer cells and human breast epithelial cells was positively triggered by TNF-α in a dose-dependent manner [18].

Here, we investigated the impact of TNF-α-induced protein expression on cell fusion. MMP9 was markedly up-regulated in TNF-α treated cells and the inhibition of MMP9 activity was correlated with a significantly impaired TNF-α-induced fusion rate. Similar results were achieved using the tetracycline-based antibiotic minocycline, which also has anti-inflammatory properties (for review see: [25]).

Methods

Cell culture

M13SV1-Cre-Puro human breast epithelial cells and MDA-MB-435-pFDR1 human cancer cells were generated as previously described [18]. The pFDR1 vector [26] was kindly provided by Frank Edenhofer (University of Würzburg, Würzburg, Germany). M13SV1-mCherry-Cre human breast epithelial cells were derived from M13SV1 human breast epithelial cells [27] by stable transduction
with the pcDNA-mCherry-P2A-Cre vector. This vector was derived by exciting the mCherry-P2A-Cre sequence of pLM-CMV-R-Cre (a gift from Michel Sadelain (Addgene plasmid #27546)) with NheI and Sall (both restriction enzymes were purchased from ThermoFisher Scientific GmbH, Schwerte, Germany) and cloning it into the pcDNA3.1 vector digested with NheI and Xhol (vector and restrictions enzymes were obtained from ThermoFisher Scientific GmbH, Schwerte, Germany). Xhol and Sall have identical cohesive ends. M13SV1-Cre-Puro and M13SV1-mCherry-Cre human breast epithelial cells (M13SV1-Cre cells) were cultivated in MSU-1 basal media (Biochrom GmbH, Berlin, Germany) supplemented with 10% foetal calf serum (FCS; Biochrom GmbH, Berlin, Germany), 1% penicillin/streptomycin (100 U/ml penicillin, 0.1 mg/ml streptomycin; Sigma-Aldrich, Taufkirchen, Germany), 1% penicillin/streptomycin (100 U/ml penicillin, 0.1 mg/ml streptomycin; Sigma-Aldrich, Taufkirchen, Germany), 10 μg/ml human recombinant EGF, 5 μg/ml human recombinant insulin, 0.5 μg/ml hydrocortisone, 4 μg/ml human transferrin, 10 nM β- oestrogen (all chemicals were purchased from Sigma-Aldrich, Taukirchen, Germany) and 1 μg/ml puromycin (InvivoGen, Toulouse, France). MDA-MB-435-pFDR1 cells were cultivated in DMEM media (Sigma-Aldrich, Taukirchen, Germany) supplemented with 10% FCS (Biochrom GmbH, Berlin, Germany), 1% penicillin/ streptomycin (100 U/ml penicillin, 0.1 mg/ml streptomycin; Sigma-Aldrich, Taufkirchen, Germany), and 2 μg/ml puromycin (InvivoGen, Toulouse, France). All cells were maintained in a humidified atmosphere at 37 °C and 5% CO₂.

**Cell fusion assay**

Quantification of fusion events between M13SV1-Cre cells and MDA-MB-435-pFDR1 cells was performed as previously described [18]. Briefly, M13SV1-Cre human breast epithelial cells and MDA-MB-435-pFDR1 human breast cancer cells were co-cultured in a ratio of 1:3 per well of a 96-well plate for 72 h in a humidified atmosphere at 37 °C and 5% CO₂. Depending on the experiment 100 ng/ml TNF-α (Bio-Technie GmbH, Wiesbaden-Nordenstadt, Germany), up to 10 μg/ml minocycline (Sigma-Aldrich, Taufkirchen, Germany), up to 10 μM SB-3CT (Sigma Aldrich, Taufkirchen, Germany), 10 μg/ml anti-ICAM1/CD54 (mouse monoclonal, clone 11C81; Bio-Technie GmbH, Wiesbaden-Nordenstadt, Germany) and appropriate combinations of inhibitors and TNF-α were added to the cell culture media. Hypoxia was induced by culturing the cells in stepwise oxygen-deprivation conditions (24 h with 10% O₂, 24 h with 5% O₂, 24 h with 1% O₂) in hypoxia chambers (Billups-Rothenberg, Del Mar, CA, USA) with a constant flow of 5% CO₂ and the remainder of nitrogen, as previously described [18]. All co-cultured cells were harvested, washed once with PBS and the amount of EGFP-expressing cells was quantified by flow cytometry (FACScalibur; Becton Dickenson, Heidelberg, Germany). Freshly harvested M13SV1-Cre cells and MDA-MB-435-pFDR1 cells mixed in a ratio of 1:3 served as a negative control to adjust the flow cytometer. The relative fold change was calculated in relation to untreated co-cultured cells, which was set to 1. Each condition was assayed in triplicate.

**Total RNA preparation**

Total RNA from all the cell lines/cells used in this study was isolated using the NucleoSpin® RNA Kit (Macherey Nagel, Düren, Germany) according to the manufacturer’s instructions. For PCR and qPCR, RNA concentration and purity was determined by UV spectrophotometric analysis. For subsequent cDNA microarray studies, the RNA integrity number (RIN) was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The samples were diluted to a final concentration of 500 ng/μl and applied to an RNA chip according to the manufacturer’s instructions. RNA samples with a RIN of 8 to 10 were used for microarray analysis.

**Single-colour microarray analysis**

To investigate differential gene expression after the TNF-α stimulation of MDA-MB-435-pFDR1 and M13SV1-Cre-Puro cells under normoxia and hypoxia, a single colour microarray analysis (Agilent Human 4×44K v2 Microarray (Agilent Technologies, Waldbronn, Germany)) was performed. The total RNA of the cells (three independent experiments were pooled) matching the RIN criteria of 8 to 10 were sent on dry ice to Source BioSciences (Nottingham, UK), which performed synthesis and Cy3 labelling of the cDNA and hybridisation of the microarrays. Microarray gene expression data were analysed using GeneSpring GX v14.8 software (Agilent Technologies, Waldbronn, Germany). Expression data were normalized based on quartiles with a threshold of 1 and no baseline transformation was performed. For further analysis, data were filtered by flags (not detected flags and compromised spots were removed) and fold changes (2-fold up and down). All microarray data were deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6084.

**RT-PCR and qPCR**

Synthesis of cDNA from mRNA by reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific GmbH, Schwerte, Germany) as referred to in the instruction manual. Conventional PCR (total reaction volume of 25 μl) was performed with ready to use 5× Master Mix containing Bio&Sell Taq-Polymerase, dNTPs and MgCl₂ (Bio&Sell GmbH Nuremberg, Germany) and 10 μM primers
Cycling conditions comprised of an initial denaturation for 5 min at 94 °C and 30 cycles of 30 s at 94 °C, 30 s at the appropriate annealing temperature and 30 s at 72 °C followed by final elongation for 7 min at 72 °C. Primer pairs used in this study are summarised in Table 1. PCR products were separated on a 1.5% agarose gel and the bands were visualized with GelRed™ stain (.VWR International GmbH, Darmstadt, Germany) and the GFel-Doc™ EZ Imager system (Bio-Rad, Munich, Germany). For qPCR (total volume of 10 μl per reaction), the SYBR Green Super Mix with ROX (Quanta Bioscience, Beverly, CA, USA) and 10 μM primers (Table 1) were used according to the manufacturer's instructions. The StepOne Plus Real-Time PCR System (ThermoFisher Scientific GmbH, Schwerte, Germany) was used for qPCR. The relative target gene expression level was determined in relation to GAPDH using the 2−ΔΔCT method.

Western blot
M13SV1-Cre cells and MDA-MB-435-pFDR1 cells were cultivated for 72 h at 37 °C and 5% CO2 under normoxic and hypoxic conditions in the presence of TNF-α (100 ng/ml), minocycline (10 μg/ml) and a combination of both. Subsequently, cells were harvested and were lysed in ice-cold RIPA buffer (50 mM Tris-HCl pH 8.0; 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate) supplemented with complete, mini, EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich, Taufkirchen, Germany) and Pierce Phosphatase Inhibitor Mini Tablets (ThermoFisher Scientific GmbH, Schwerte, Germany). The samples were sonicated three times (10 s on and 30 s off) and the total protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific GmbH, Schwerte, Germany) according to the manufacturer's instructions. For Western blots, 40 μg of total protein lysate was mixed with 3× Laemmli Sample Buffer and was incubated for 6 min at 95 °C. The samples were separated by 10% or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon polyvinyl difluoride (PVDF) nitrocellulose membrane (Merck Millipore, Darmstadt, Germany) under semi-dry conditions. The membranes were blocked in 5% (w/v) non-fat milk powder in PBS-T (phosphate-buffered saline) with 0.1% (v/v) Tween 20 (PBS-T) for 1 h at room temperature. The following antibodies were used for Western blot analysis: anti-MMP9 (rabbit monoclonal; Abcam, Cambridge, UK), anti-CCL2 (MCP-1; rabbit...
Fig. 1 (See legend on next page.)
polyclonal; Abcam, Cambridge, UK), anti-claudin-1 (mouse monoclonal; Abcam, Cambridge, UK), anti-ICAM1/CD54 (rabbit polyclonal; Cell Signaling, Leiden, Netherlands), β-actin (rabbit monoclonal; Cell Signaling, Leiden, Netherlands); anti-mouse-IgG-HRP-linked (Cell Signaling, Leiden, Netherlands), and anti-rabbit-IgG-HRP-linked (Cell Signaling, Leiden, Netherlands). The bands were visualized using the Pierce ECL Western blot substrate (Thermo Fisher Scientific, Bonn, Germany), according to the manufacturer’s instructions, and the Aequoria Macroscopic Imaging System (Hamamatsu Photonics Germany, Herrsching am Ammersee, Germany).

Zymography
The proteolytic activity of MMP9 was measured using a gelatin zymography assay. M13SV1-Cre cells and MDA-MB-435-pFDR1 cells were plated at a density of 5x10^5 cells/well in a 6-well plate. After a 72 h incubation with 100 ng/ml TNF-α, 10 μg/ml minocycline, and a combination of both, the cell culture supernatants were collected and were mixed with non-reducing Laemmli sample buffer (250 mM Tris-HCl (pH 6.8), 10% (v/v) SDS, 25% (v/v) glycerol, 0.01% (w/v) bromophenol blue) without boiling. The samples were separated on a 10% sodium dodecyl sulphate polyacrylamide gel containing 0.1% gelatin (Sigma Aldrich, Taufkirchen, Germany). After separation by electrophoresis, the gels were washed four times in wash buffer (50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, 2.5% (v/v) Triton X-100, 0.02% NaN₃) for 2 h at room temperature to remove the SDS. Then, the gel was incubated in incubation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃) overnight at 37 °C. Subsequently, the gel was stained with Coomassie brilliant blue R-250 (Sigma Aldrich, Taufkirchen, Germany) for 1 h at room temperature with gentle agitation. Finally, the bands were destained until clear bands appeared visible, which were indicative of the proteolytic activity of proteases, including MMP9.

XTT assay
To determine the appropriate minocycline concentration for the quantification of cell fusions, M13SV1-Cre and MDA-MB-435-pFDR1 cells were cultured in triplicate at a ratio of 1:3 for up to 3 days in the presence of different concentrations of minocycline. Non-treated cells served as a control. After 24 h, 48 h, and 72 h the media was removed and the plates were analysed using the XTT reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Absorption of the XTT-formazan derivative that formed was measured using a BioTek EL800 microplate reader (BioTek, Bad Friedrichshall, Germany).

siRNA experiments
Expression of claudin-1 was knocked-down using a specific claudin-1 siRNA (Santa Cruz Biotechnology, Heidelberg, Germany). For control purposes, a non-targeting negative control siRNA was used (QIAGEN GmbH, Hilden, Germany). M13SV1-Cre cells (2.5x10^5) were resuspended in Opti-Mem (ThermoFisher Scientific, Schwerte, Germany) and were subsequently transfected with claudin-1 siRNA or control siRNA to a final concentration of 100 nM by lipofection (Lipofectamine™ 2000; ThermoFisher Scientific, Schwerte, Germany) as described in the user’s manual. The cells were seeded in serum-free MSU medium in 6-well plates. After 6 h, the serum-free media was replaced with complete MSU medium containing FCS, antibiotics and additional supplements. The knock-down of claudin-1 expression in M13SV1-Cre cells was confirmed by Western blot.

Co-immunoprecipitation
M13SV1-Cre cells and MDA-MB-435-pFDR1 cells were stimulated with 100 ng/ml TNF-α, 10 μg/ml minocycline, or a combination of both, for 72 h at 37 °C and 5% CO₂ in a humidified atmosphere. Subsequently, the cells were washed once with PBS and lysed in ice-cold lysis buffer (1% (v/v) NP-40, 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and proteinase inhibitors (see above)) for 30 min on ice. The samples were sonicated three times (5 s on/20 s off) on a low pulse frequency and the total protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific GmbH, Schwerte, Germany) according to the manufacturer’s instructions. The lysates were cleared by centrifugation (10 min, 12,000 rpm) and the supernatants were transferred to a new tube. Prior to immunoprecipitation, the lysates were pre-treated with 25 μl of Protein A Magnetic Beads (Cell Signaling, Leiden, Netherlands) for 2 h at 4 °C to remove any proteins that might bind non-specifically to Protein A. The samples were incubated with anti-ICAM1/CD54 (5 μg/ml; mouse monoclonal, clone 11C81; Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany) or IgG1 (5 μg/ml; Beckman Coulter, Krefeld, Germany) overnight.
| GenBank Accession | Gene Symbol | Gene Symbol | Gene Name | Normoxia M13SV1-Cre + TNF-α vs. M13SV1-Cre | Hypoxia M13SV1-Cre + TNF-α vs. M13SV1-Cre | Normoxia MDA-MB-435-pFDR1 + TNF-α vs. MDA-MB-435-pFDR1 | Hypoxia MDA-MB-435-pFDR1 + TNF-α vs. MDA-MB-435-pFDR1 |
|------------------|-------------|-------------|----------|------------------------------------------|-------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| NM_182920        | ADAMT59     | ADAMT59     | ADAM metallopeptidase with thrombospondin type 1 motif, 9 | 15.34                                    | 25.45                                      | 3.05                                              | 10.59                                              |
| NM_058172        | ANTXR2      | ANTXR2      | anthrax toxin receptor 2 | 2.64                                      | 4.43                                       | 3.61                                              | 5.63                                              |
| NM_001165        | BIRC3       | BIRC3       | baculoviral IAP repeat containing 3 | 4.55                                      | 13.11                                      | 23.74                                             | 97.65                                             |
| NM_001734        | C1S         | C1S         | complement component 1, s subcomponent | 5.66                                      | 6.97                                       | 6.91                                              | 7.03                                              |
| NM_000064        | C3          | C3          | complement component 3 | 4.31                                      | 4.24                                       | 182.13                                             | 115.58                                             |
| NM_002982        | CCL2        | CCL2        | chemokine (C-C motif) ligand 2 | 25.36                                     | 39.20                                      | 179.73                                             | 62.92                                             |
| NM_001252        | CD70        | CD70        | CD70 molecule | 3.15                                      | 6.99                                       | 4.76                                              | 4.52                                              |
| NM_001710        | CFB         | CFB         | complement factor B | 2.41                                      | 2.28                                       | 11.21                                             | 5.74                                              |
| NM_032888        | COL27A1     | COL27A1     | collagen, type XVII, alpha 1 | 2.70                                      | 4.45                                       | 2.09                                              | 5.29                                              |
| NM_004405        | Dlx2        | Dlx2        | distal-less homeobox 2 | 2.39                                      | 7.50                                       | 5.38                                              | 4.42                                              |
| NM_014888        | FAM3C       | FAM3C       | family with sequence similarity 3, member C | 2.08                                      | 2.50                                       | 3.27                                              | 4.06                                              |
| NM_001024071     | GCH1        | GCH1        | GTP cyclohydrolyase 1 | 2.20                                      | 3.07                                       | 2.93                                              | 3.83                                              |
| NM_001300919     | HMG2        | HMG2        | high mobility group AT-hook 2 | 2.26                                      | 2.35                                       | 3.27                                              | 3.66                                              |
| NM_000201        | ICAM1       | ICAM1       | intercellular adhesion molecule 1 | 4.46                                      | 6.91                                       | 5.76                                              | 3.58                                              |
| NM_000873        | ICAM2       | ICAM2       | intercellular adhesion molecule 2 | 2.53                                      | 4.04                                       | 4.71                                              | 46.87                                             |
| NM_022377        | ICAM4       | ICAM4       | intercellular adhesion molecule 4 (Landsteiner-Wiener blood group) | 4.47                                      | 15.53                                      | 7.15                                              | 11.04                                             |
| NM_003897        | IER3        | IER3        | immediate early response 3 | 2.02                                      | 2.84                                       | 6.61                                              | 2.96                                              |
| NM_022168        | IFIH1       | IFIH1       | interferon induced with helicase C domain 1 | 2.43                                      | 3.47                                       | 2.01                                              | 2.48                                              |
| NM_004843        | IL27RA      | IL27RA      | interleukin 27 receptor, Alpha | 2.65                                      | 2.31                                       | 3.47                                              | 2.56                                              |
| NM_001012633     | IL32        | IL32        | interleukin 32 | 4.47                                      | 4.23                                       | 45.05                                             | 61.04                                             |
| NM_001012631     | IL32        | IL32        | interleukin 32 | 3.53                                      | 5.65                                       | 174.41                                             | 103.38                                             |
| NM_152899        | IL41        | IL41        | interleukin 4 induced 1 | 13.76                                     | 46.63                                      | 50.44                                             | 74.89                                             |
| NM_002185        | IL7R        | IL7R        | interleukin 7 receptor | 2.81                                      | 3.65                                       | 23.52                                             | 8.37                                              |
| NM_001570        | IRAK2       | IRAK2       | interleukin-1 receptor-associated kinase 2 | 5.28                                      | 13.66                                      | 14.76                                             | 14.90                                             |
| NM_004031        | IRF7        | IRF7        | interferon regulatory factor 7 | 2.30                                      | 2.31                                       | 4.20                                              | 2.17                                              |
| NM_138433        | KLHDC7B     | KLHDC7B     | kelch domain containing 7B | 5.74                                      | 10.45                                      | 2.77                                              | 13.77                                             |
| NM_001017402     | LAMB3       | LAMB3       | laminin, beta 3 | 2.12                                      | 3.48                                       | 5.68                                              | 2.89                                              |
| NM_005562        | LAMC2       | LAMC2       | laminin, gamma 2 | 2.73                                      | 4.65                                       | 2.05                                              | 4.42                                              |
| NM_015541        | LRG1        | LRG1        | leucine-rich repeats and immunoglobulin-like domains 1 | 2.29                                      | 2.49                                       | 4.45                                              | 2.27                                              |
| NM_004994        | MMP9        | MMP9        | matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase) | 11.45                                     | 27.25                                      | 13.49                                             | 44.92                                             |
| NM_018406        | MUC4        | MUC4        | mucin 4, cell surface associated | 2.49                                      | 4.72                                       | 2.34                                              | 2.76                                              |
| NM_181782        | NCOA7       | NCOA7       | nuclear receptor coactivator 7 | 2.08                                      | 2.89                                       | 2.14                                              | 3.31                                              |
| NM_004289        | NF2L3       | NF2L3       | nuclear factor, Erythroid 2-like 3 | 3.08                                      | 7.27                                       | 5.00                                              | 8.99                                              |
| NM_020529        | NFKBIA      | NFKBIA      | | 2.97                                      | 7.63                                       | 5.41                                              | 5.74                                              |
at 4 °C. Thereafter, 30 μl of Protein A Magnetic Beads were added and the samples were incubated for an additional 2 h at 4 °C. The precipitates were washed three times in lysis buffer, separated on a magnetic rack and resuspended in 3× Laemmli Sample Buffer (with DTT, without β-mercaptoethanol). ICAM1/CD54 and the co-immunoprecipitated proteins were detected by Western blot.

Soluble ICAM1 detection
To detect soluble ICAM1, the cell culture supernatants from TNF-α-treated, minocycline-treated, and TNF-α and minocycline-treated M13SV1-Cre cells and MDA-MB-435-pFDR1 cells were collected and concentrated using Vivaspin-2 centrifugal concentrators (100,000 MWCO; Sartorius, Göttingen, Germany). The total protein concentration of the concentrated supernatants was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific GmbH, Schwerte, Germany) according to the manufacturer’s instructions. An equal amount of supernatant from each sample was resuspended in Laemmli sample buffer. Soluble ICAM1 was detected by Western blot using an anti-ICAM1/CD54 antibody (clone 14C11, mouse monoclonal; ThermoFisher Scientific GmbH, Schwerte, Germany).

Statistical analyses
The statistical significance of the data presented in Fig. 2 was calculated using an unpaired, two-tailed Student’s t-test. The mean differences of the data presented in Figs. 4, 5, 6 and 7 were tested by ANOVA F-tests. Afterwards, multiple comparisons were performed using Scheffé post-hoc tests. Statistical analyses were performed using SPSS Version 23.0.0.2 and p-values < 0.05 were considered significant.

Language editing
The manuscript was language edited by American Journal Experts (Durham, NC, USA).

Table 2 Up-regulated genes (FC ≥ 2) in TNF-α treated cells under normoxic and hypoxic conditions (Continued)

| Genbank Accession | GeneSymbol | GeneName                                      | Normoxia M13SV1-Cre + TNF-α vs. M13SV1-Cre | Normoxia M13SV1-Cre + TNF-α vs. M13SV1-Cre | Hypoxia MDA-MB-435-pFDR1 + TNF-α vs. MDA-MB-435-pFDR1 | Hypoxia MDA-MB-435-pFDR1 + TNF-α vs. MDA-MB-435-pFDR1 |
|-------------------|------------|----------------------------------------------|--------------------------------------------|--------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|
| NM_004556         | NFKBIE     | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | 3.86                                       | 4.40                                       | 3.41                                                   | 3.14                                                   |
| NM_002526         | NTSE       | 5′-nucleotidase, ecto (CD73)                 | 4.37                                       | 9.64                                       | 7.12                                                   | 3.26                                                   |
| NM_002526         | NTSE       | 5′-nucleotidase, ecto (CD73)                 | 3.37                                       | 8.28                                       | 3.62                                                   | 2.54                                                   |
| NM_030952         | NUAK2      | NUAK family, SNF1-like kinase. 2             | 2.24                                       | 5.48                                       | 5.71                                                   | 2.87                                                   |
| NM_001005377      | PLAUR      | plasminogen activator, urokinase receptor    | 4.00                                       | 2.12                                       | 14.94                                                  | 2.74                                                   |
| NM_002852         | PTX3       | pentraxin 3, long                            | 4.37                                       | 6.10                                       | 16.59                                                  | 17.75                                                  |
| NM_006509         | RELB       | v-rel avian reticuloendotheliosis viral oncogene homolog B | 5.02                                       | 8.99                                       | 4.61                                                   | 4.41                                                   |
| NM_002960         | S100A3     | S100 calcium binding protein A3              | 2.01                                       | 2.88                                       | 4.90                                                   | 7.84                                                   |
| NM_001002236      | SERPINA1   | serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 | 2.50                                       | 2.73                                       | 5.44                                                   | 2.35                                                   |
| NM_015482         | SLC22A23   | solute carrier family 22, member 23          | 2.08                                       | 2.23                                       | 3.21                                                   | 7.09                                                   |
| NM_001024465      | SOD2       | superoxide dismutase 2, mitochondrial        | 2.48                                       | 5.20                                       | 16.52                                                  | 19.28                                                  |
| NM_014220         | TM4SF1     | transmembrane 4 L six family member 1        | 2.08                                       | 3.23                                       | 5.34                                                   | 3.43                                                   |
| NM_017870         | TMEM132A   | transmembrane protein 132A                  | 2.63                                       | 3.35                                       | 3.74                                                   | 3.53                                                   |
| NM_006290         | TNFAIP3    | tumor necrosis factor, alpha-induced protein 3 | 14.81                                      | 16.94                                      | 19.45                                                  | 9.77                                                   |
| NM_145725         | TRAF3      | TNF receptor-associated factor 3             | 2.21                                       | 3.61                                       | 2.02                                                   | 2.50                                                   |
| NM_006398         | UBD        | ubiquitin D                                 | 19.59                                      | 58.56                                      | 100.44                                                  | 226.21                                                  |
| NM_005429         | VEGFC      | vascular endothelial growth factor C         | 2.76                                       | 4.16                                       | 74.91                                                  | 33.90                                                   |
Results

Analysis of the gene expression pattern of M13SV1-Cre cells and MDA-MB-435-pFDR1 cells with TNF-α treatment in normoxic and hypoxic conditions

We recently demonstrated that the fusion of human M13SV1-Cre breast epithelial cells and human MDA-MB-435-pFDR1 breast cancer cells under normoxic and hypoxic conditions is positively triggered by TNF-α [18]. To analyse the changes in the gene expression profile of both cell lines induced by TNF-α (100 ng/ml) under normoxic and hypoxic conditions, cDNA-microarray studies were performed. In total, 51 genes were found to be significantly up-regulated in TNF-α-treated cell lines under both normoxia and hypoxia (Fig. 1a, b; Table 2), including adhesion molecules such as ICAM1, ICAM2, and ICAM4, components of the extracellular matrix, including COL27A1, LAMB3, LAMC2, and MUC4, chemokines, growth factors, and interleukins, such as CCL2, VEGFC and IL32, and proteases including ADAMTS9 and MMP9 (Fig. 1; Table 2). In particular, CCL2, MMP9 and ICAM1 have already been associated with cell fusion [9, 28]. In contrast, 15 genes were down-regulated in TNF-α-treated cells under normoxic and hypoxic conditions, such as ABCC6 and FOS (Fig. 1c, d; Table 3).

Validation of microarray data by qPCR and conventional PCR

To validate the microarray data, Claudin-1 (CLDN1), ICAM1, and MMP9 expression was analysed by qPCR and ADAMTS9, CCL2, IL4I1, IL7R, TNFAIP3 and VEGFC expression was analysed by conventional PCR. CLDN1 did not pass the filter criteria but the microarray data revealed a marked up-regulation of this protein in TNF-α-treated (100 ng/ml) MDA-MB-435-pFDR1 cells under normoxic and hypoxic conditions (Additional file 1). The qPCR and conventional PCR data partially matched the microarray data (Fig. 2a, d). The microarray data revealed an up-regulation of ICAM1 expression in TNF-α-stimulated (100 ng/ml) M13SV1-Cre cells under normoxic conditions (Fig. 1; Table 2), which was opposite to the qPCR data showing similar ICAM1 mRNA levels in untreated and TNF-α-treated M13SV1-Cre cells under normoxic conditions (Fig. 2b). In contrast, ICAM1 was up-regulated in TNF-α-stimulated M13SV1-Cre cells under hypoxia (Fig. 1; Table 2), which

| Genbank Accession | GeneSymbol | GeneName | Normoxia M13SV1-Cre + TNF-α vs. M13SV1-Cre | Hypoxia M13SV1-Cre + TNF-α vs. M13SV1-Cre | Normoxia MDA-MB-435-pFDR1 + TNF-α vs. MDA-MB-435-pFDR1 | Hypoxia MDA-MB-435-pFDR1 + TNF-α vs. MDA-MB-435-pFDR1 |
|-------------------|------------|----------|--------------------------------------------|--------------------------------------------|------------------------------------------------|------------------------------------------------|
| NM_001079528     | ABCC6      | ATP-binding cassette, sub-family C (CFTR/MPR), member 6 | −2.72                                      | −2.26                                      | −2.51                                           | −2.36                                           |
| NM_020764        | CASKIN1    | CASK interacting protein 1 | −2.03                                      | −2.26                                      | −2.52                                           | −7.21                                           |
| NR_024482        | CECD5-AS1  | CECD5 antisense RNA 1 | −2.10                                      | −2.94                                      | −3.66                                           | −6.97                                           |
| NM_001885        | CRYAB      | crystallin, alpha B | −2.76                                      | −4.62                                      | −2.03                                           | −6.23                                           |
| NM_001352        | DBP        | D site of albumin promoter (albumin D-box) binding protein | −2.36                                      | −4.44                                      | −2.20                                           | −4.92                                           |
| NM_005252        | FOS        | FB1 murine osteosarcoma viral oncogene homolog | −2.45                                      | −2.72                                      | −3.24                                           | −2.60                                           |
| NM_001136007     | FXYD3      | FXYD domain containing ion transport regulator 3 | −2.32                                      | −2.06                                      | −19.31                                          | −14.74                                          |
| NM_001136008     | FXYD3      | FXYD domain containing ion transport regulator 3 | −2.44                                      | −4.64                                      | −10.82                                          | −19.67                                          |
| XM_006715716     | HOXA6      | homeobox A6 | −2.75                                      | −4.61                                      | −2.38                                           | −2.30                                           |
| NM_030915        | LBH        | limb bud and heart development | −2.03                                      | −31.03                                     | −2.93                                           | −3.35                                           |
| NR_024485        | LOC100130093 | uncharacterized LOC100130093 | −2.28                                      | −3.05                                      | −3.11                                           | −3.30                                           |
| NM_014033        | METTL7A    | methyltransferase like 7A | −4.74                                      | −4.34                                      | −2.93                                           | −6.66                                           |
| BCI28044         | MYO15B     | myosin XVB pseudogene | −2.42                                      | −2.81                                      | −2.15                                           | −2.70                                           |
| NM_030625        | TET1       | tet methylcytosine dioxygenase 1 | −2.90                                      | −3.02                                      | −2.01                                           | −2.70                                           |
| NM_003881        | WISP2      | WNT1 inducible signaling pathway protein 2 | −3.96                                      | −4.69                                      | −3.99                                           | −5.53                                           |

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could be validated by qPCR (Fig. 2b). The TNF-α-induced increase in CLDN1 and MMP9 expression in M13SV1-Cre cells under normoxia and hypoxia (Fig. 1; Table 2) were validated by qPCR, whereas MMP9 mRNA expression was markedly up-regulated in TNF-α-stimulated cells (Fig. 2c). For MDA-MB-435-pFDR1 cells, only the microarray data for CLDN1 and ICAM1 could be validated by qPCR; the qPCR data for MMP9 did not match the microarray data (Figs. 1, 2c; Table 2). Markedly increased MMP9 expression levels were determined for TNF-α-stimulated MDA-MB-435-pFDR1 cells (Fig. 1; Table 2), which is opposite to qPCR rather showing comparable MMP9 mRNA expression levels in untreated and TNF-α treated cells (Fig. 2c).

CCL2, IL7R1, and VEGFC expression levels were partially up-regulated in TNF-α-stimulated MDA-MB-435-pFDR1 cells, which agreed with the microarray data (Fig. 1, 2b; Table 2). In contrast, the microarray data revealed an up-regulation of IL4I1 in TNF-α-stimulated MDA-MB-435-pFDR1 cells, which could not be validated by conventional PCR (Fig. 2d). Instead, only a weak PCR product was observed in TNF-α-stimulated MDA-MB-435-pFDR1 cells under normoxic conditions, whereas under hypoxia no PCR product was detected (Fig. 2d). TNF-α induced an increase in IL4I1, IL7R and TNFAIP3 expression in M13SV1-Cre cells under normoxic conditions (Fig. 2d) and in hypoxic conditions there was only a slight increase in VEGFC levels in response to TNF-α treatment (Fig. 2d). Hence, the M13SV1-Cre microarray data were only partially validated by conventional PCR (Figs. 1, 2d; Table 2).

**Validation of the microarray data by western blot**

To further validate the microarray and PCR data, Western blot analyses were conducted for CCL2, CLDN1, ICAM1 and MMP9. TNF-α-induced (100 ng/ml) CCL2 expression was observed in MDA-MB-435-pFDR1 cells with higher CCL2 expression under normoxia compared with hypoxia (Fig. 3a). In contrast, no CCL2 expression was found in M13SV1-Cre human breast epithelial cells (Fig. 3a), which agrees with conventional PCR data (Fig. 2b). The expression of CLDN1 was only observed in M13SV1-Cre cells concomitant with
slightly increased CLDN1 levels in the presence of TNF-α (Fig. 3a), which is similar to qPCR data (Fig. 2). Interestingly, no CLDN1 protein expression was detected in MDA-MB-435-pFDR1 cells (Fig. 3a) even though qPCR data revealed increased CLDN1 mRNA levels in TNF-α-treated cells (Fig. 2b). In agreement with the microarray data (Fig. 1; Table 2), but opposite to the qPCR data (Fig. 2a), increased ICAM1 protein expression was identified in TNF-α-treated M13SV1-Cre cells (Fig. 3a). Increased TNF-α-induced MMP9 levels were only observed in M13SV1-Cre cells (Fig. 3a), which agreed with the qPCR data (Fig. 2c). In addition to Western blot analysis, MMP9 expression was further analysed by zymography, which also showed increased MMP9 expression in TNF-α-treated M13SV1-Cre cells (Fig. 3b). Zymography analysis revealed a slight up-regulation of MMP9 expression in TNF-α-stimulated MDA-MB-435-pFDR1 cells (Fig. 3b), which confirmed the qPCR data showing a weak MMP9 PCR product in TNF-α-treated cells under normoxic conditions (Fig. 2c).

**Fusion of M13SV1-Cre cells and MDA-MB-435-pFDR1 cells is impaired by the inhibition of MMP9**

To investigate whether CLDN1, ICAM1 and MMP9 might be involved in the TNF-α-induced fusion of M13SV1-Cre and MDA-MB-435-pFDR1 cells, the expression of these proteins was blocked. The knockdown of CLDN1 with siRNA in M13SV1-Cre cells did not impair the TNF-α-induced fusion of the cells (Fig. 4a) even though the siRNA-mediated downregulation of CLDN1 expression in M13SV1-Cre was stable for at least 3 days (Fig. 4b). Interestingly, compared with M13SV1-Cre cells that were transfected with scrambled siRNA, M13SV1-Cre cells transfected with CLDN1 siRNA had a slightly increased fusion rate (Fig. 4a).

The inhibition of ICAM1 function with a blocking ICAM1 antibody was not correlated with a decreased TNF-α-induced rate of the fusion of M13SV1-Cre and MDA-MB-435-pFDR1 cells (Fig. 4c). In contrast, the inhibition of MMP9 function using the specific inhibitor SB-3CT was associated with a dose-dependent, impaired, TNF-α-induced cell fusion rate of M13SV1-Cre and MDA-MB-435-pFDR1 cells (Fig. 4d).

**The TNF-α-induced fusion of M13SV1-Cre and MDA-MB-435-pFDR1 cells is inhibited by minocycline**

The tetracycline based antibiotic minocycline was tested because of its capability to down-regulate MMP9 expression in cells [29, 30]. First, XTT proliferation studies were performed to determine the optimal minocycline concentration and a range between 5 μg/ml and 24 μg/ml was tested. Prolonged cultivation of M13SV1-Cre and MDA-MB-435-pFDR1 cells under hypoxia and in the presence of 10 μg/ml minocycline was associated with numbers of dead cells.
that were too high to perform further cell fusion quantification studies. 

The incubation of cells with 5 μg/ml or 10 μg/ml minocycline was correlated to a dose-dependent decreased TNF-α-induced fusion rate (Fig. 5), whereas the spontaneous fusion rate of the cells remained unaffected in the presence of different minocycline concentrations (Fig. 5).

**Effect of minocycline on CLDN1, ICAM1, and MMP9 expression**

Quantitative PCR and Western blot data showed that CLDN1 and ICAM1 expression in TNF-α-treated and TNF-α and minocycline-treated M13SV1-Cre cells were comparable (Fig. 6a, b). In contrast, the qPCR results showed significantly reduced MMP9 expression in M13SV1-Cre cells co-treated with TNF-α and minocycline as compared with TNF-α-treated cells (Fig. 6c), which was further validated by zymography (Fig. 6c). MMP9 protein expression was slightly increased in TNF-α + minocycline co-treated M13SV1-Cre cells compared with TNF-α-stimulated cells (Fig. 6c). This (reproducible) result remains ambiguous as did the finding that significantly higher MMP9 mRNA levels were observed in minocycline treated M13SV1-Cre cells (Fig. 6c) although increased MMP9 protein expression levels were not detected (Fig. 6c).

In contrast to M13SV1-Cre breast epithelial cells, neither CLDN1 nor MMP9 protein expression was detected in MDA-MB-435-pFDR1 cells (Fig. 6a, c). Because very weak MMP9 bands were observed in MDA-MB-435-pFDR1 cells by zymography (Fig. 6c), and MMP9 and CLDN1 were detected by qPCR (Fig. 6a, c) we assume that MMP9 and CLDN1 protein expression levels were below the detection threshold of the antibodies that were used. Minocycline treatment increased MMP9 mRNA expression in both M13SV1-Cre cells and MDA-MB-435-pFDR1 cells (Fig. 6c), which, however, did not get translated into increased expression of the subsequent proteins (Fig. 6c). Compared with M13SV1-Cre breast epithelial cells, increased ICAM1 expression levels were found in
TNF-α- and TNF-α + minocycline-treated MDA-MB-435-pFDR1 cells (Fig. 6b). Interestingly, significantly higher ICAM1 mRNA and protein levels were detected in the TNF-α + minocycline-treated MDA-MB-435-pFDR1 cells than in the TNF-α-stimulated cells (Fig. 6b).

Co-immunoprecipitation assays were performed to investigate whether MMP9 bound to ICAM1 and whether this would be correlated to increased soluble ICAM1 (sICAM1) levels because of MMP9-dependent proteolytic degradation [31]. As shown in Fig. 6d, MMP9 could be co-immunoprecipitated with ICAM1 in M13SV1-Cre cells but not in MDA-MB-435-pFDR1 cells. Higher ICAM1 and MMP9 levels were co-immunoprecipitated in TNF-α-stimulated M13SV1-Cre cells, whereas lower levels were detected in cells co-treated with TNF-α and minocycline (Fig. 6d), which is in agreement with the Western blot data (Fig. 6c). In contrast, because of rather low MMP9 expression levels in MDA-MB-435-pFDR1 cells, MMP9 was not co-immunoprecipitated. To prove, whether the bindings of ICAM1 were proteolytically degraded by MMP9, the supernatants from cell culture were collected. Western blot analysis revealed no differences in the relative amount of sICAM1 monomers and dimers between the control cells and those treated with TNF-α or TNF-α + minocycline.

Both minocycline and the MMP9 inhibitor SB-3CT impaired TNF-α-mediated cell fusion

The fusion rate of cells co-treated with both minocycline and SB-3CT (and TNF-α) was comparable to that of cells treated with minocycline and minocycline + TNF-α, respectively, and no additive effect was observed (Fig. 7). Interestingly, the cultivation of cells in the presence of both minocycline and the blocking ICAM1 antibody yielded in an increased cell fusion rate, which was more intense in the presence of TNF-α (Fig. 7). Even though the data were not significant, the findings were reproducible in independent experiments, indicating that the increased fusion rate of cells in the presence of minocycline and anti-ICAM1 (and TNF-α) is a true effect.

Discussion

In a previous study we demonstrated that the pro-inflammatory cytokine TNF-α is a potent trigger of cell fusion between human M13SV1-Cre breast epithelial cells and human breast cancer cell lines under both normoxic and hypoxic conditions [18]. Here, we investigated the impact of TNF-α on the gene expression profiles of M13SV1-Cre and MDA-MB-435-pFDR1 cells to identify which of the differentially-regulated genes might be involved in cell fusion. Of the identified target genes, only the inhibition of MMP9 was correlated with a decreased TNF-α-induced cell fusion rate.

Here, we used the MDA-MB-435 cancer cell line, whose origin is debated to be either breast cancer or melanoma. A systematic analysis of the gene expression profiles of 60 human cancer cell lines first revealed that MDA-MB-435 cells express genes that are highly expressed in most melanoma-derived cell lines, suggesting that this cell line originated from melanoma rather than breast cancer [32]. This assumption was further supported by comparative genomic hybridisation (VGH) and microsatellite polymorphism analyses showing that MDA-MB-435 cells and M14 cells share similarities [33]. In contrast, MDA-MB-435 and M14 cells markedly differed in their DNA methylation profiles; more hypermethylated CpG islands were detected in MDA-MB-435 cells than in M14 cells and MDA-MB-435 cells were not grouped with melanoma cells after a hierarchical cluster analysis [34]. Likewise, the phenotypic and molecular characterisation of MDA-MB-435 cells further revealed similarities to other breast cancer cell lines, such as MDA-MB-231, SUM1315 or HBL100 cells, which belong to the claudin-low intrinsic subtype of breast cancer [35].

PCR data showed that the microphthalmia-associated transcription factor (MITF), which is a lineage survival oncogene amplified in malignant melanoma cell lines, was expressed in MDA-MB-435 cells [36, 37]. However, MITF protein was not expressed in MDA-MB-435 cells [38] but mRNA expression was detected in other breast cancer cell lines, such as MCF-10A, MCF-7, SKBR3, and U87 and D54 glioblastoma cell lines [37]. The co-expression of neuronal, epithelial and melanocytic
markers in breast cancer, melanoma and glioblastoma cell lines might be related to lineage infidelity, which seems to be a common phenomenon in cancer cell lines [37]. In agreement with MCF-7 breast cancer cells, but in contrast to melanoma cells, the expression of breast epithelium-specific and epithelial-specific markers, such as \( \beta \)-casein, \( \alpha \)-lactalbumin, epithelial membrane antigen (EMA), and keratin-19 as well as the induction of \( \beta \)-casein expression and production of milk lipids by \( \beta \)-heregulin and vitamin E was clearly observed in MDA-MB-435 cells [38]. Moreover, MDA-MB-435 xenograft studies revealed that the vast majority of the cells were positive for cytokeratin and EMA, suggesting that MDA-MB-435 is a breast epithelial cell line that has gained melanocyte lineage characteristics [38].

Our data indicate that the TNF-\( \alpha \)-induced fusion of M13SV1-Cre and MDA-MB-435-pFDR1 cells depends on MMP9. Blocking MMP9 with a specific inhibitor or the inhibition of MMP9 expression using minocycline was associated with a decreased cell fusion frequency. These data agree with that of several studies that have already demonstrated that TNF-\( \alpha \) and/or MMP9 play a role in the fusion of different cell types [16–19, 39–41]. For instance, Song et al. showed that TNF-\( \alpha \) enhanced the fusion of oral squamous carcinoma cells and endothelial cells via a VCAM-1/VLA-4-dependent pathway [19]. MMP9 was not investigated in this particular study and it is unclear whether it is involved in the fusion of these cells. TNF-\( \alpha \)-induced VCAM-1 expression in endothelial cells is crucial for close cell-cell contact and is a prerequisite for the subsequent plasma membrane fusion step. Here, TNF-\( \alpha \) induced ICAM1 expression in both cell types, but no reduced cell fusion rate was observed in the presence of an ICAM1 blocking antibody. It is likely indicating that the TNF-\( \alpha \)-induced fusion of M13SV1-Cre and MDA-MB-435-pFDR1 was independent of ICAM1. Nonetheless, ICAM1 was recently identified to augment myoblast adhesion and fusion through homophilic trans-interactions and Rac-mediated actin remodelling [28]. Therefore, the

![Image](image-url)
fusogenic property of ICAM1-ICAM1 interactions was restricted to myogenic cells, as forced expression of ICAM1 by fibroblasts did not augment their fusion to ICAM1-positive myoblasts/myotubes [28].

MMP9 is involved in macrophage fusion. The IL-4 induced fusion of macrophages was reduced in the presence of MMP9 function-blocking antibodies and similar effects were also observed in MMP9 null-macrophages, clearly showing the impact of MMP9 in macrophage fusion [40]. Likewise, MMP9, E-cadherin and DC-STAMP were upregulated by both IL-4 signalling and DAP12 signalling in macrophages and DAP12 overexpression induced macrophage fusion [39]. Conversely, DAP12 deficiency was associated with an impaired frequency of macrophage fusion because of low MMP9 levels [39]. Low MMP9 expression levels, concomitant with a decreased macrophage fusion rate, were also found in MCP-1/CCL2-null mice [16]. Moreover, the impaired fusion capacity of MCP-1/CCL2-null macrophages is rescued by exogenous TNF-α and TNF-α-induced MMP9 expression [16], which agrees with our data. The finding that TNF-α could induce fusion via an MMP9-dependent mechanism is further supported by data showing that the fusion of osteoclasts in bone explants, which were stimulated by human breast cancer cells through TNF-α secretion, could be blocked by the inhibition of MMP9 [41].

Even though these findings indicate a correlation between the induction of MMP9 expression and cell fusion, the detailed mechanism of how MMP9 is involved in the merging of plasma membranes still remains unclear. Cell fusion is a multi-step process that can be subdivided into i) priming, ii) chemotaxis, iii) adhesion, iv) fusion, and v) post-fusion. IL-4 and DAP12 signalling program cells into a fusion-competent status [4, 39], whereas MCP-1/CCL2 signalling is associated with chemotaxis [4, 16]. Because TNF-α induces cell fusion, it might be capable of programming cells into a fusion-competent status. Because of its proteolytic activity, MMP9 may degrade extra-cellular matrix components, promote interaction with cell membranes or may induce signal molecules necessary for cell fusion [4, 40]. We wondered whether MMP9 may degrade membrane-bound ICAM1, which may allow the plasma membranes of two cells to come into close contact. Tsai and colleagues recently demonstrated that TNF-α induced an MMP9-dependent release of soluble ICAM1 release in osteoblast-like MC3T3-E1 cells [31]. However, increased levels of soluble ICAM1 were not observed in TNF-α-treated cells nor was the TNF-α induced fusion of the cells inhibited by an ICAM1 blocking antibody.

Whether CCL2 might be involved in the TNF-α-induced fusion of M13SV1-Cre and MDA-MB-435-pFDR1 cells remains unclear. Markedly increased CCL2 expression levels were only observed in TNF-α-stimulated MDA-MB-435-pFDR1 cells. CCL2 might conceivably be involved in the induction of chemotaxis in M13SV1-Cre cells.

The finding that MMP9 plays a role in the TNF-α-induced fusion of M13SV1-Cre and MDA-MB-435-pFDR1 cells was further supported by minocycline studies, which revealed markedly lower MMP9 expression levels and a reduced fusion rate in the presence of TNF-α. This finding agrees with in vitro and in vivo studies that show that both tumour growth and osseous metastasis of breast cancer cells was effectively reduced by minocycline because of its inhibition of MMP9 expression [42–44]. It is well-known that besides its bacteriostatic efficacy against both gram-positive and gram-negative bacteria, minocycline also possesses anti-inflammatory properties (for review see: [25]). The mechanisms involved in the anti-inflammatory activity of minocycline include its inhibitory effects on the activity of enzymes such as iNOS, the MMPs or COX2, the inhibition of apoptosis and the inhibition of immune cell activation and proliferation [25]. Several lines of evidence indicated that minocycline exerted its inhibitory
electrophoresis; siCAM1: Soluble intercellular cell adhesion molecule 1; 
SRPα: Signal-regulatory protein alpha; TNF-α: Tumour necrosis factor-α; 
TNFAP3: Tumour necrosis factor-alpha induced protein 3; VEGFC: Vascular 
endothelial growth factor C; ZBAPF: Centre for Biomedical Education and Research

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Availability of data and materials

The datasets used and/or analysed during the current study are available 
from the corresponding author upon reasonable request.

Authors’ contributions

JW performed the experiments, analysed the data, wrote and corrected the 
manuscript. MA prepared samples for cDNA microarray data analysis; 
analysed and validated the cDNA microarray data. KSZ read and corrected 
the manuscript. TD designed the experiments and wrote and corrected the 
manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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