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RESEARCH ARTICLE

Quantification of cell fusion events human breast cancer cells and breast epithelial cells using a Cre-LoxP-based double fluorescence reporter system

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Abstract The biological phenomenon of cell fusion plays an important role in several physiological processes, like fertilization, placentation, or wound healing/tissue regeneration, as well as pathophysiological processes, such as cancer. Despite this fact, considerably less is still known about the factors and conditions that will induce the merging of two plasma membranes. Inflammation and proliferation has been suggested as a positive trigger for cell fusion, but it remains unclear, which of the factor(s) of the inflamed microenvironment are being involved. To clarify this we developed a reliable assay to quantify the in vitro fusion frequency of cells using a fluorescence double reporter vector (pFDR) containing a *LoxP*-flanked HcRed/DsRed expression cassette followed by an EGFP expression cassette. Because cell fusion has been implicated in cancer progression four human breast cancer cell lines were stably transfected with a pFDR vector and were co-cultured with the stably Cre-expressing human breast epithelial cell line. Cell fusion is associated with a Cre-mediated recombination resulting in induction of EGFP expression in hybrid cells, which can be quantified by flow cytometry. By testing a panel of different cytokines, chemokines, growth factors and other compounds, including exosomes, under normoxic and hypoxic conditions our data indicate that the proinflammatory cytokine TNF-α together with hypoxia is a strong inducer of cell fusion in human MDA-MB-435 and MDA-MB-231 breast cancer cells.

Keywords Cell fusion · Breast cancer · Cre-LoxP recombination · Flow cytometry

Introduction

Even though it is well recognized that the biological phenomenon of cell fusion plays a fundamental role in a variety of physiological and pathophysiological events (for review see: [1–3], considerably less is known about the factors and conditions that facilitate the merging of the plasma membranes of two individual cells. What is known so far is that some of the cell fusion mediating molecules, such as EFF-1 or syncytin-1, are of viral or retroviral origin. For instance, the EFF-1 molecules, which facilitates cell fusion in *C. elegans* via homotypic zippering is structurally homologous to viral class II fusion proteins [4]. On the contrary, syncytin-1, which is mandatory for trophoblast fusion, thereby giving rise to syncytiotrophoblasts [5, 6], rather belongs to viral class I fusion proteins [7]. It is generally considered that syncytin-bearing retroviruses were captured by their host independently between 12 million and 85 million ago and account for the different structures of placentas among different species [8].

In addition to viral cell fusion-related proteins nature has developed other strategies to facilitate cell fusion. For

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instance, so-called podosome-like structures (PLS) are mandatory for myoblast fusion, which originate by actin reorganization in an Arp2/3-dependent manner (for review see: [9]). Osteoclasts originate from macrophages by cell fusion, whereby native macrophages first develop to fusion-competent macrophages in an IL-4, M-CSF and RANKL-dependent manner (for review see: [9]). Fusion-competent macrophages are characterized by expression of variety of surface molecules, like E-cadherin, DC-STAMP/OC-STAMP, CD44, CD200, SIRPα and the tetraspanins CD9 and CD81, as well as soluble factors, such as CCL2 or proteinases including MMP9 and MT1MMP (for review see: [9]). Thus, the biological phenomenon of cell fusion is not limited to the fusion process itself (the merging of the plasma membranes), but is rather a highly regulated multistep process that can be subdivided into five parts: (1) priming, (2) chemotaxis, (3) adhesion, (4) fusion and (5) post-fusion [10]. Moreover, from this knowledge it can be concluded that the ability of certain cell types, like macrophages, to fuse with other cells could be turned on and off. But what are the mediators that will turn on the cell fusion program? A plethora of tissue regeneration studies provided evidence that bone marrow-derived stem cells (BMDCs) as well as cells of the myelomonocytic lineage can adopt the phenotype of damaged foreign tissue cells by cell fusion [11–15]. In this context, data of Johansson et al. and Davies et al. are of interest showing that the fusion frequency of BMDCs was markedly increased in response to (chronic) inflammation [16, 17]. Thus, in turn suggests that the local (chronically) inflamed tissue, comprising of (pro)-inflammatory cytokines, chemokines, growth factors, proteases, etc. generates a fusion-friendly milieu enabling for example., recruited BMDCs to restore degenerated organ cells. This assumption would also account for malignant solid tumors, which resembles chronically inflamed tissue [18, 19]. It is well recognized that tumor cells could fuse with normal cells, like macrophages and stem cells, thereby giving rise to hybrid cells exhibiting novel properties, such as an enhanced metastatic capacity and/or an increased drug resistance, that will foster tumor progression (for review see: [20, 21]. For instance, Rachkovsky and colleagues [22] demonstrated that hybrid cells derived from macrophages and weakly malignant Cloudman S91 melanoma cells possessed an enhanced metastatic potential. Fusion of bone marrow-specific and lung-specific metastatic sublines of the human MDA-MB-231 breast cancer cell line gave rise to hybrid cells exhibiting a dual metastasis organotropism phenotype [23]. Recently, Xu et al. [24] provided evidence that epithelial-to-mesenchymal transition (EMT) and acquisition of stem cell-like properties were involved in the spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. An increased drug resistance was observed for hybrid cells derived from methotrexate resistant 168FAR and 5-fluorouracil-resistant 44FTO cancer cells, which not only were resistant to both chemotherapeutic compounds, but also to melphalan—a drug to which both parental cancer cell lines were susceptible for [25]. Fusion of human M13SV1-EGFP-Neo breast epithelial cells and human HS578T-Hyg and MDA-MB-435-Hyg breast cancer cells gave rise to various hybrid cell clones, of which some exhibited an increased resistance towards chemotherapeutic drugs, like doxorubicin, etoposide or paclitaxel [26]. Carloni and colleagues [27] provided evidence that the occurrence of cell fusion in a metastatic model of colon carcinoma caused the appearance of 5-fluorouracil and oxaliplatin double resistant tumor cells.

To analyze and to quantify the impact of cytokines, chemokines or growth factors on the fusion frequency of human breast cancer cells with a human breast epithelial cell line exhibiting stem cell properties [26, 28, 29] a *Cre-LoxP*-based cell fusion assay was developed. Thereby, human breast cancer cells were stably transfected with a fluorescence double reporter (FDR) vector containing a *loxP*-flanked HcRed/DsRed cassette followed by an EGFP cassette [30]. After co-cultivation of these breast cancer cells displaying a red fluorescence with Cre-expressing M13SV1 breast epithelial cells possessing stem cell properties Cre-mediated recombination will occur in hybrid cells resulting in excision of the HcRed/DsRed cassette concomitant with a stable EGFP expression and fluorescence [30], which can be easily quantified by flow cytometry.

Materials and methods

Cell culture

Human MDA-MB-435 (HTB-129; LGC Standards GmbH, Wesel, Germany), MDA-MB-231 (HTB-26; LGC Standards GmbH, Wesel, Germany), MCF-7 (HTB-22; LGC Standards GmbH, Wesel, Germany) and HS578T (HTB 126; LGC Standards GmbH, Wesel, Germany) breast cancer cells were maintained in standard media (MDA-MB-435 and MDA-MB-231: DMEM; MCF-7 and HS578T: RPMI 1640) supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin/streptomycin (PenStrep). Media and Penstrep were purchased from Sigma-Aldrich (Taufkirchen, Germany), whereas FCS was obtained form Biochrom GmbH (Berlin, Germany). The human M13SV1 breast epithelial cell line exhibiting stem cell properties [29] was kindly provided by James E. Trosko (Michigan State University, East Lansing, MI, USA) and was cultivated in MSU-1 basal media (Biochrom GmbH, Berlin,

Germany) supplemented with 10 % fetal calf serum (FCS) (Biochrom GmbH, Berlin, 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, and 10 nM β-estrogen (all chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany). HEK293 cells (CRL-1573; LGC Standards GmbH, Wesel, Germany) were maintained in DMEM (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10 % FCS (Biochrom GmbH, Germany) and 1 % PenStrep (Sigma-Aldrich, Taufkirchen, Germany). All cells were kept in a humidified atmosphere at 37 °C and 5 % CO_2.

Nucleofection of cells

HS578T, MDA-MB-231 and MCF-7 breast cancer cells were stably transduced with the fluorescence double reporter (FDR) vector pFDR-2 [30] by nucleofection (Lonza, Cologne, Germany) in accordance to the manufacturer's instructions. The pFDR-2 vector contains a *loxP*-flanked *HcRed-Neo*-Stop cassette followed by an *EGFP* reporter gene [30]. In addition to a neomycin (G418) resistance, pFDR-2 also contains a puromycin resistance gene. Deletion of the *HcRed-Neo*-Stop cassette by Cre-mediated recombination results in expression of EGFP [30]. Transfected cells were first selected for G418 resistance (400 µg/ml; Biochrom GmbH, Berlin, Germany). Subsequently, resistant clones were additionally purified by flow cytometry (FACScalibur; Becton Dickenson, Heidelberg, Germany) for sorting HcRed-positive cells using the FL4-H channel.

Lentiviral transduction of cells

MDA-MB-435 breast cancer cells and M13SV1 breast epithelial cells were stably transduced by a lentivirus-based approach either using the pFDR1 vector (MDA-MB-435) or the Cre-IRES-PuroR vector (M13SV1). The pFDR1 vector is similar to the pFDR2 vector, except that the *loxP*-flanked *HcRed-Neo*-Stop cassette was replaced by a *loxP*-flanked DsRed cassette [30]. The Cre-IRES-PuroR plasmid was a gift from Darrell Kotton [Addgene plasmid #30205 [31]. Lentiviral particles were produced by nucleofection of HEK293 cells with one of the above-mentioned plasmids as well as pMD2.G (gift from Didier Trono (Addgene plasmid #12259)] and psPAX2 [gift from Didier Trono (Addgene plasmid #12260)]. After 3 days the supernatant containing lentiviral particles was collected and was added to MDA-MB-435 cells and M13SV1 cells, respectively. After 48 h puromycin (Sigma-Aldrich, Taufkrichen, Germany) was added to cells (M13SV1-Cre-IRES-Puro: 1 µg/ml; MDA-MB-435-pFDR-1: 2 µg/ml). Individual M13SV1-Cre-IRES-Puro cell clones were picked and were cultivated separately prior to PCR and Western Blot analysis to detect Cre expression. By contrast, MDA-MB-435-pFDR-1 cells were further purified by flow cytometry (FACScalibur; Becton Dickenson, Heidelberg, Germany) for sorting DsRed-positive cells using the FL2-H channel. All transfected cells were maintained in their respective basal media, supplemented with 10 % FCS, 1 % PenStrep and the appropriate antibiotic concentration in a humidified atmosphere at 37 °C and 5 % CO_2.

Western blot analysis

Cells (2×10^5) were harvested, washed once with PBS, and were lysed in 3× Laemmli Sample Buffer (5 min, 95 °C) [32]. Samples were stored at −20 °C before being separated on a 10 % SDS polyacrylamide gel and transferred to Immobilon-P PVDF nitrocellulose membrane (EMD Millipore, Darmstadt, Germany) under semi-dry conditions. Membranes were blocked overnight with 10 % (w/v) non-fat dry milk powder in TBS-T. The following antibodies were used in this study: Cre recombinase (rabbit polyclonal), HIF-1α (rabbit polyclonal), TNFR1 (rabbit monoclonal), TNFR2 (rabbit polyclonal), anti-β-actin (rabbit monoclonal), anti-elf4E (rabbit polyclonal) and anti-rabbit-IgG-HRP-linked (all antibodies were purchased from New England Biolabs GmbH, Frankfurt am Main, Germany). The exosome marker antibodies anti-HSP70 (rabbit monoclonal) and anti-CD81 (rabbit monoclonal) were purchased from SBI (BioCat GmbH, Heidelberg, Germany). Bands were visualized using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Bonn, Germany) in accordance to the manufacturer's instructions and were detected with Aequoria Macroscopic Imaging System (Hamamatsu Photonics Germany, Herrsching am Ammersee, Germany).

Exosome preparation

Exosomes from the supernatant of MDA-MB-435 and HS578T human breast cancer cells were purified using Total Exosome Isolation Kit (Invitrogen, Karlsruhe, Germany) in accordance to the manufacturer's instructions. Cells were cultured for 12 h under serum-free conditions prior to collection of the exosome-enriched supernatant. Purified exosomes were analyzed for CD81 and HSP70 expression (western blot) and by scanning electron microscopy.

Scanning electron microscopy of exosomes

Polyethylene sponges have been immersed in 1 ml of 8 % tannin and 2.5 % buffered glutaraldehyde (Na-cacodylate 0.1 M buffer pH 7.0). Five to ten drops of exosome suspension were pipetted on the sponges and fixed for another 2 h at 4 °C in the same solution. After fixation the exosome containing sponges were washed twice for 15 min in Na-cacodylate buffer and stored overnight at 4 °C. Then they were post fixed with 1 % osium tetroxide in Na-cacodylate buffer for 30 min again washed twice for 15 min with buffer and stored overnight at 4 °C. Following the sponges with exosomes were dehydrated in graded acetone, critical point dried, and sputter coated with gold–palladium. SEM was carried out with at Zeiss, Sigma VP SEM (Zeiss, Oberkochen, Germany) using an In Lens SE detector at 20 kV acceleration voltage.

Cre protein transduction

Cre protein transduction was performed as described [30] with slight modifications. In brief, HS578T-pFDR-2 (2.5×10^3) and MDA-MB-435-pFDR-1 (5×10^3) breast cancer cells, respectively, were seeded in chamber slides and were grown overnight. Subsequently, 2–6 μM of HTN Cre-fusion protein [30] was added. Cre-mediated recombination (indicated by expression of EGFP) was visualized by confocal laser scanning microscopy (Leica TCS SP5; Leica Bensheim, Bensheim, Germany).

Cytokines, chemokines, growth factors, reagents

The following compounds were used in this study: CCL21 (Biochrom GmbH, Berlin, Germany), IL-11 (Biomol, Hamburg, Germany), IL-4 and FGF (Invitrogen, Karlsruhe, Germany), IL-8 (Biotrend, Cologne, Germany), NF-κB inhibitor (CalBiochem, Merck Millipore, Schwalbach, Germany), SDF-1α, IL-1α, IL-21, MCP-1, Gro-α and Galectin-1 (ImmunoTools, Friesoythe, Germany), TNF-α (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), and IL-1β, IL-6, INF-γ, EGF and TGF-α (Sigma-Aldrich, Taufkrichen, Germany). Chronic wound fluid (CWF) was kindly provided by Dr. Oliver Thamm (Colgne-Mehrheim) and was isolated in accordance to [33].

Quantification of cell fusion events under normoxic and hypoxic conditions

To quantify the frequency of cell fusion events pFDR-transfected breast tumor cells were co-cultured with M13SV1-Cre-PuroR breast epithelial cells in a ratio of 3:1 per well of a 96-well plate for 72 h in a humidified atmosphere at 37 °C and 5 % CO_2. In dependence of the experimental setting appropriate concentrations of the above-mentioned cytokines, chemokines, growth factors and reagents were added. Hypoxia was induced in accordance to the protocol of Voss et al. [34] with slight modifications. In brief, cells were cultured in a stepwise oxygen-deprivation (24 h 10 % O_2, 24 h 5 % O_2, 24 h 1 % O_2) in hypoxia chambers (Billups-Rothenberg, Del Mar, CA, USA) with constantly 5 % CO_2 and remainder being nitrogen. It should be noted that no consistent standard protocol for induction of hypoxia in tumor cells in vitro was developed so far. 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). The relative fold change was calculated in relation to untreated co-cultured cells (ratio of 3:1), which was set to 1. Each condition was assayed in triplicates. Freshly harvested pFDR-transfected breast cancer cells and M13SV1-Cre-PuroR breast epithelial cells (ratio of 3:1) served as a negative control to adjust the flow cytometer.

Blocking experiments

Cells were cultured under common co-culture conditions (see above) within the presence of different concentrations of TNF-α. To block TNFR1 and TNFR2 signaling the following blocking antibodies were added to the cells: anti-TNFR1 (5 μg/ml; mouse monoclonal; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) and anti-TNFR2 (1 μg/ml; mouse monoclonal; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany).

Statistical significance

Statistical significance was calculated using a two-tailed paired student's *t* test.

Results

Measuring cell fusion by Cre-LoxP recombination

The Cre-LoxP recombination technique has been successfully used in different studies to visualize cell fusion events [11, 35, 36]. The advantage of this approach is that recombination only occurs in hybrid cells, thus minimizing the risk of false-positive events, for example., due to intercellular dye exchange [37] between cells or that cells that do adhere to each other, but not yet have fused, might be recognized as one. Moreover, a much more important advantage of this approach is that cell fusion events could be counted (and thus quantified), which, however, depends on a suitable detection system, like the fluorescence double

reporter (FDR) vector [30], allowing to discriminate between non-fused and fused cells. Stably transfected cells display a bright red fluorescence that can be easily detected by confocal laser scanning microcopy (or any other fluorescence microscopy) and/or flow cytometry (Fig. 1a, c). After Cre-mediated recombination the loxP-flanked HcRed/DsRed cassette is excised resulting in a stable EGFP expression and fluorescence (Fig. 1a). In Fig. 1a, pFDR-expressing HS578T and MDA-MB-435 breast cancer cells were treated with 2 μM recombinant HTN Cre-fusion protein [30], which contains a TAT-leader domain enabling such proteins to pass the plasma membrane. An efficient Cre-mediated recombination was already observable after 6 h as demonstrated by a markedly green fluorescence of the cells (Fig. 1a). Identical data were obtained for MCF-7-pFDR2 and MDA-MB-231-pFDR breast cancer cells (data not shown).

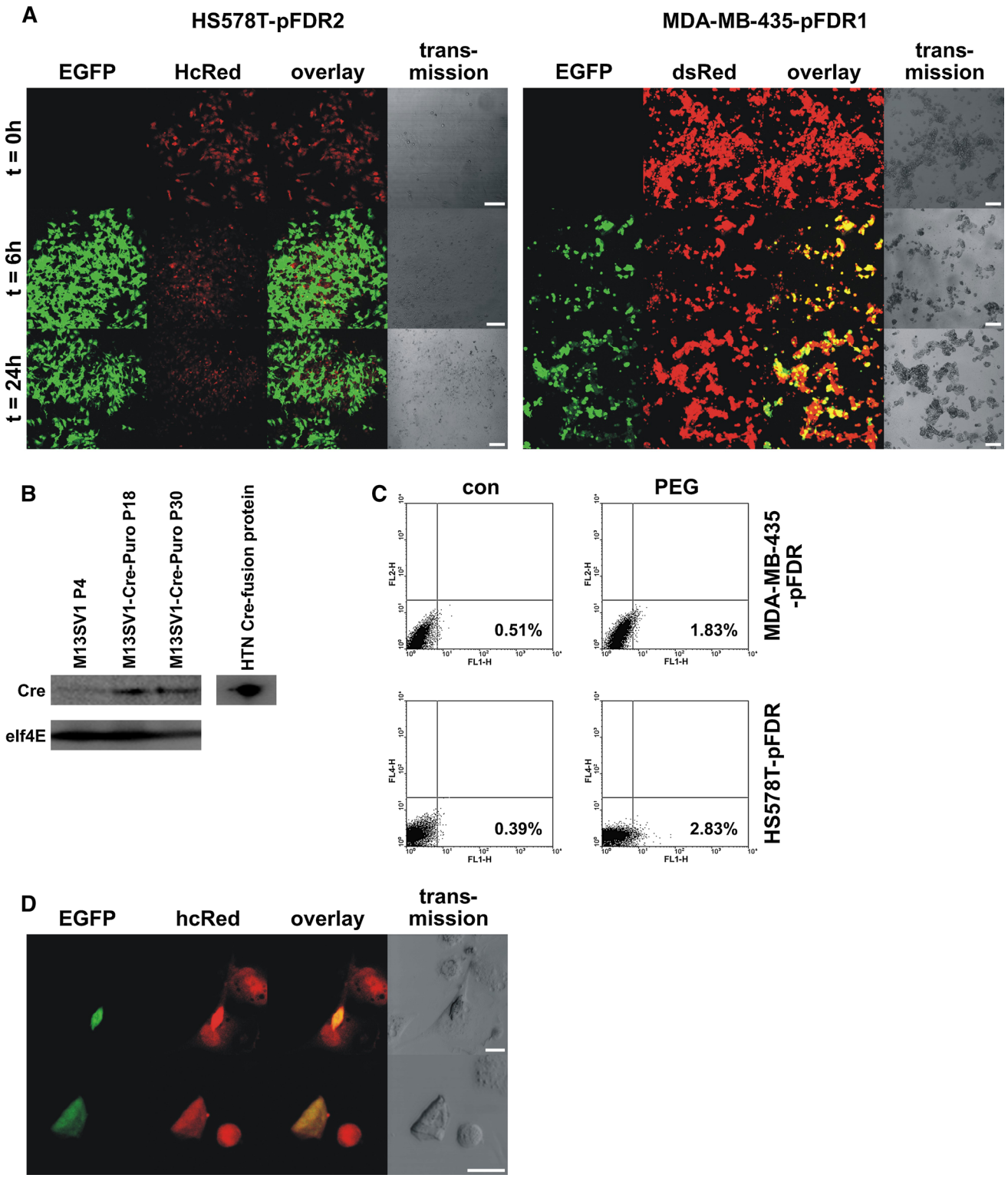
To prove whether this experimental setting is suitable for quantifying cell fusion events, stably Cre-expressing M13SV1 breast epithelial cells (Fig. 1b) were fused with stably pFDR-transfected breast cancer cells by PEG and were analyzed 24–48 h later by flow cytometry (Fig. 1c). Our data clearly showed an increased amount of EGFP-expressing cells indicating that *Cre-loxP* recombination has occurred in fused pFDR-expressing breast cancer cells and Cre-expressing M13SV1 breast epithelial cells. Likewise, we were able to identify EGFP-positive cells in co-cultured pFDR-expressing breast cancer cells and Cre-expressing M13SV1 breast epithelial cells (Fig. 1d) clearly showing that spontaneous fusion between these cells do occur, which, for HS578T and MDA-MB-435 breast cancer cells, is in view with former studies [26, 28]. Various ratios of stably pFDR-transfected breast cancer cells and Cre-expressing M13SV1 breast epithelial cells were tested before starting co-culture experiments with supplemented reagents. Best results were obtained with a ratio of 3:1 (data not shown).

Differential effects of cytokines, chemokines and growth factors on cell fusion

Several cytokines, chemokines, growth factors as well as compounds like chronic wound fluid (CWF) [33] or exosomes were tested for their ability to promote or inhibit cell fusion. Therefore, pFDR-transfected breast cancer cells and Cre-expressing M13SV1 breast epithelial cells were co-cultured within the presence of different concentrations of distinct factors/compounds for 72 h prior to flow cytometry-based analysis. The frequency of fusion events of treated co-cultured cells was calculated in relation to the spontaneous fusion frequency of untreated co-cultured cells, which was set to 1. To avoid false-positive results relative fold changes between ≥ 0.5 and ≤ 1.5 were excluded from statistical analysis. Figure 2 gives a brief overview of the tested factors/compounds and their impact on cell fusion, whereby only the results of one concentration is displayed. A more detailed overview is given in supplemented data 1.

Among the tested factors/compounds the proinflammatory cytokine TNF-α was found to be the most pro-fusogenic factor (Fig. 2a). An increased frequency of fusion events was observed for MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells both under normoxic and hypoxic conditions (Fig. 2a). Interestingly, the fusion frequency of TNF-α treated cells under hypoxic conditions was much higher in comparison to normoxia [e.g., MDA-MB-435-pFDR 100 ng/ml TNF-α normoxia: 1.64 ± 0.45 ($p < 0.05$) vs. hypoxia: 2.91 ± 0.23 ($p < 0.001$)] suggesting that both hypoxia and TNF-α might induce a pro-fusogenic phenotype. This assumption would be in view co-culture data indicating that both TNF-α and hypoxia caused a significantly increased fusion rate of HS578T-pFDR cells (Fig. 2a). Interestingly, under normoxic conditions TNF-α did not have an impact on the fusion frequency of HS578T-pFDR cells (Fig. 2a). In addition to TNF-α, a pro-fusogenic effect was also observed for IL-1β, but only in MDA-MB-231-pFDR breast cancer cells (Fig. 2a). Interestingly, a co-stimulation of MDA-MB-435-pFDR, HS578T-pFDR and MDA-MB-231-pFDR breast cancer cells with both TNF-α and IL-1β completely abrogated the pro-fusogenic effect of either TNF-α or IL-1β (Fig. 2a). Compared to MDA-MB-435-pFDR, HS578T-pFDR and MDA-MB-231-pFDR breast cancer cells, no pro-fusogenic effect of TNF-α and hypoxia was observed for MCF-7-pFDR breast cancer cells.

A pro-fusogenic effect was observed for CWF on the fusion of MDA-MB-435-pFDR and HS578T-pFDR breast cancer cells under normoxic conditions, but not under hypoxic conditions (Fig. 2b). By contrast, the fusion capacity of MDA-MB-231-pFDR and MCF-7-pFDR breast cancer cells remained unchanged in the presence of CWF (Fig. 2b). Stimulation of stably pFDR-transfected human breast cancer cells and M13SV1-Cre-PuroR human breast epithelial cells with different growth factors did not alter the cells fusion capacity under normoxic conditions (Fig. 2c) as well as hypoxic conditions (supplemental data 1). By contrast, treatment of HS578T-pFDR breast cancer cells and M13SV1-Cre-PuroR cells with both CCL21 and SDF-1α resulted in an increased cell fusion frequency under normoxic, but not hypoxic conditions (Fig. 2d). Interestingly, MCP-1 significantly impaired the fusion of MDA-MB-435-pFDR and M13SV1-Cre-PuroR cells under hypoxic conditions, but not under normoxic conditions (Fig. 2d). In contrast to HS578T-pFDR cells neither CCL21 nor SDF-1α had an impact on the fusion capacity of MDA-MB-435-pFDR cells (Fig. 2d).



Additionally, we further investigated whether the fusion of MDA-MB-435-pFDR and HS578T-pFDR breast cancer cells and M13SV1-Cre-PuroR breast epithelial cells could be triggered by exosomes, for which a possible role in cell fusion events has been suggested [38]. Exosomes were prepared from the supernatant of non-transfected MDA-MB-435 and HS578T breast cancer cells and were analyzed by scanning electron microscopy (Fig. 3a–c) and

◀**Fig. 1** Proof of principle of Cre-LoxP-mediated recombination in HS578T-pFDR2 and MDA-MB-435-pFDR1 breast cancer cells. Human breast cancer cells were stably transfected with either pFDR2 (HcRed—HS578T) or pFDR1 (DsRed—MDA-MB-435), whereas M13SV1 human breast epithelial cells were stably transfected with a Cre expression vector (Cre-PuroR). **a** To monitor Cre-mediated recombination in HS578T-pFDR2 and MDA-MB-435-pFDR cells were seeded in chamber slides and were subsequently treated with a recombinant HTN Cre-fusion protein that can pass the cells plasma membrane. The switch from *red* fluorescence towards a *green* fluorescence, which is equal to Cre-mediated recombination, was detected by confocal laser scanning microscopy clearly showing an efficient and stable Cre-mediated recombination within 6 h. *Bar* 20 μm. **b** Western blot analysis of stable Cre expression in different passages of M13SV1-Cre-PuroR cells. The recombinant HTN Cre-fusion protein served as a positive control. Shown are representative western blot data. **c** Quantification of cell fusion events by Cre-LoxP recombination in PEG-fused pFDR-expressing breast cancer cells and M13SV1-Cre-PuroR cells. EGFP-positive hybrid cells are located in the *lower right* quadrant. **d** Examples of spontaneous fused HS578T-pFDR2 and M13SV1-Cre-PuroR hybrid cells analyzed by confocal laser scanning microscopy. *Bar* 10 μm

western blot for expression of the exosome markers HSP70 and CD81 (Fig. 3d). Interestingly, an inhibitory effect was observed for MDA-MB-435- and HS578T-derived exosomes on the fusion of HS578T-pFDR cells, but not MDA-MB-435-pFDR cells, under normoxic conditions (Fig. 3e).

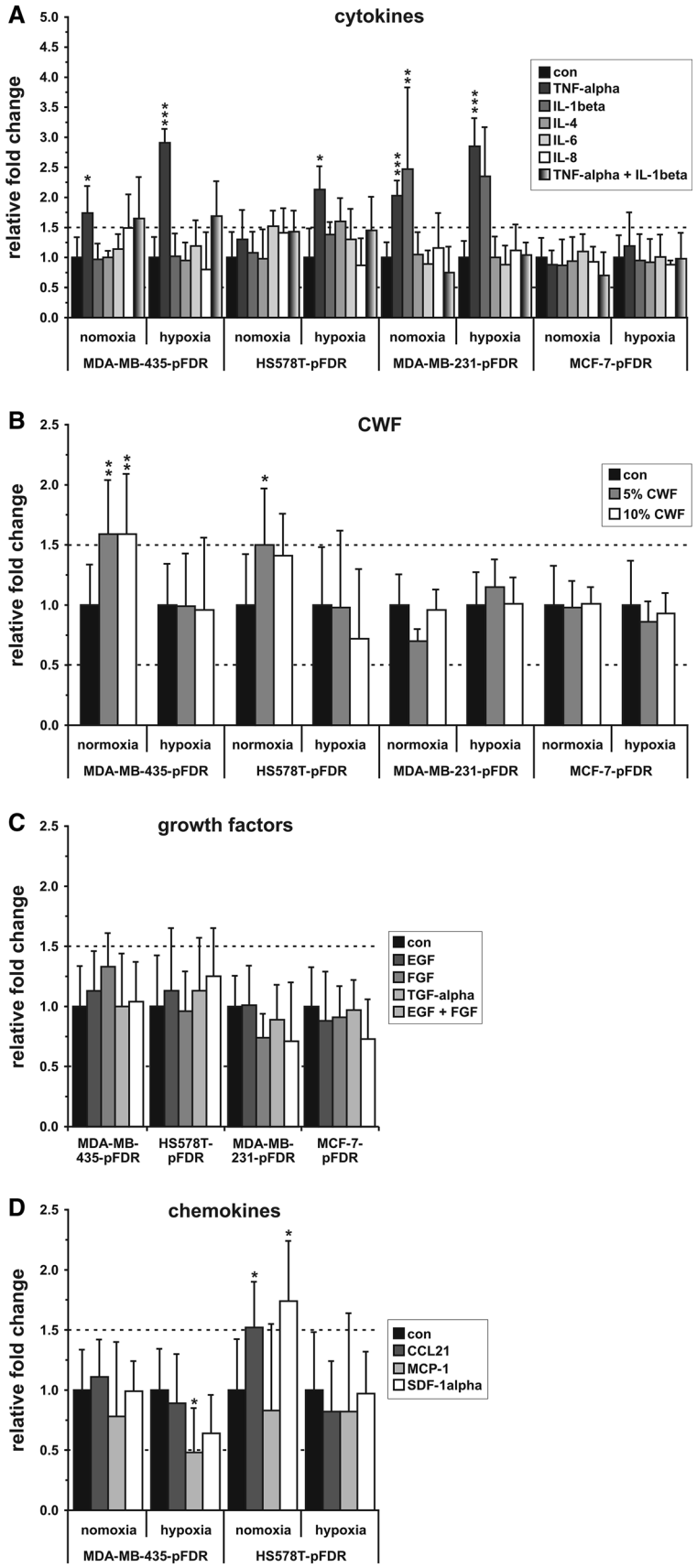
TNF-α mediates cell fusion in a dose-dependent manner

Because TNF-α was identified to be the most potent pro-fusogenic factor, this cytokine and its receptors TNFR1 and TNFR2 were analyzed more in detail. Co-culture experiments revealed that TNF-α supports the fusion MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells in a dose-dependent manner (Fig. 4a). In contrast to this, a dose-dependent pro-fusogenic effect of TNF-α on the fusion of HS578T-pFDR breast cancer cells and M13SV1-Cre-PuroR cells was only observed under hypoxic conditions (Fig. 4a). As expected, and in accordance to data shown in Fig. 2, the fusion capacity of MCF-7-pFDR and M13SV1-Cre-PuroR cells remained unaltered within different TNF-α concentrations (Fig. 4a). To prove whether the differential fusion capacity of human MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells and M13SV1-Cre-PuroR breast epithelial cells in dependence of TNF-α might be related to a differential TNFR1 and TNFR2 expression Western Blot studies were conducted, whereby cells were stimulated with 100 ng/ml TNF-α for up to 72 h and were kept under both normoxic and hypoxic conditions. In general, under normoxic conditions TNF-α stimulation was correlated with an increased expression of both TNF-α receptors in all cells (Fig. 4b). Interestingly, in both breast cancer cell lines a maximum of TNFR1 expression was observed after 48–72 h of TNF-α stimulation, whereas in M13SV1-Cre-PuroR cells increased TNFR1 levels were already detectable after 24–48 h (Fig. 4b). In accordance to TNFR1 a sustained TNF-α stimulation was also associated with TNFR2 up-regulation. In M13SV1-Cre-PuroR and MDA-MB-435-pFDR stably increased TNFR2 levels were detected after 24 h of TNF-α stimulation (Fig. 4b). By contrast, a markedly increased TNFR2 expression was first noticed after 72 h in MDA-MB-231-pFDR cells (Fig. 4b).

Interestingly, basal TNFR2 expression levels were higher in all cells being cultivated under hypoxic conditions, whereas basal TNFR1 expression levels were decreased (Fig. 4b). Moreover, all cell lines responded differently to TNF-α stimulation. In M13SV1-Cre-PuroR and MDA-MB-435-pFDR cells a slightly increased TNFR1 expression was observed after 48 h of TNF-α stimulation (Fig. 4b). However, compared to normoxic conditions the TNF-α-mediated increase in TNFR1 expression under hypoxic conditions was rather moderate (Fig. 4b). Interestingly, a sustained stimulation of MDA-MB-231-pFDR breast cancer cells with TNF-α under hypoxic conditions was correlated with a time-dependent decreased TNFR1 expression (Fig. 4b). In contrast to normoxic conditions, TNFR2 expression levels of all cells remained stable within the presence of TNF-α and hypoxia (Fig. 4b).

Blockade of TNF receptor signaling impairs cell fusion of human breast cancer cells

To further prove the putative correlation between TNF-α stimulation and an increased fusion frequency of the cells co-culture experiments were conducted with MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells within the presence of blocking TNFR1 and TNFR2 antibodies. Data are summarized in Fig. 5 clearly showing that in both cell lines and both under normoxic and hypoxic conditions the TNF-α-induced cell fusion was virtually completely abrogated when TNFR1 was blocked (Fig. 5a, b). Interestingly, addition of a TNFR2-blocking antibody also impaired the TNF-α-induced fusion of MDA-MB-435-pFDR cells (Fig. 5a), whereby the inhibitory effect was much higher and significant in cells cultivated under hypoxic conditions (Fig. 5a). On the contrary, the TNF-α-induced fusion frequency of MDA-MB-231-pFDR breast cancer cells remained unaltered in the presence of the TNFR2-blocking antibody (Fig. 5b). Whether this might be related to the differential time course of TNFR expression between MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells (Fig. 4b) is not clear.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MDA-MB-231-pFDR
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hypoxia
MDA-MB-435-pFDR
HS578T-pFDR

◀**Fig. 2** Summary of cell fusion data. Stably pFDR-transfected human breast cancer cells were co-cultured with M13SV1-Cre-PuroR human breast epithelial cells for 72 h in a ratio of 3:1 within the presence of different cytokines, chemokines, growth factors and chronic wound fluid. Shown are representative data of one applied concentration (100 ng/ml) and normoxia and hypoxia. All tested compounds, including different concentrations, are summarized in supplemental data 1. The spontaneous fusion rate of co-cultured cells was set to 1. **a** Cytokines, **b** CWF, **c** growth factors, **d** chemokines. Note that TNF-α and hypoxia potently induces cell fusion in MDA-MB-435-pFDR, HS578T-pFDR and MDA-MB-231-pFDR breast cancer cells. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

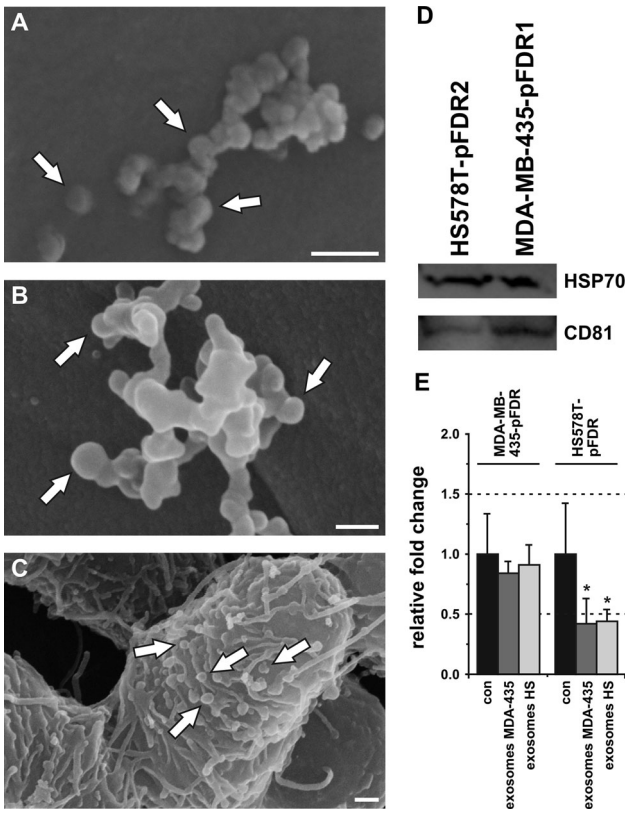

Fig. 3 Impact of exosomes on cell fusion. To study the impact of exosomes on cell fusion exosomes were prepared from the supernatant of non-transfected MDA-MB-435 and HS578T breast cancer cells. Exosome preparations were analyzed by scanning electron microscopy (**a**–**c**) and western blot (**d**). *Arrows* point to putative exosomes of MDA-MB-435 (**a**; *bar* 30 nm) and HS578T (**b**; *bar* 100 nm) cells. **c** HS578T breast cancer cells containing exosomal structures on its surface (*bar* 200 nm). **d** MDA-MB-435- and HS578T-derived exosomes are positive for the exosome markers HSP70 and CD81. Shown are representative western blot data. **e** Interestingly, the fusion of HS578T-pFDR breast cancer cells and M13SV1-Cre-PuroR was significantly impaired by exosomes. Statistical significance: $*p < 0.05$

Discussion

In the present study we investigated the impact of various cytokines, growth factors, chemokines, or exosomes as well as normoxia and hypoxia on the fusion of human breast cancer cell lines and a human breast epithelial cell line using a *Cre-LoxP*-based assay. A transition from a red to a green fluorescence in cells was defined as a positive recombination and as a readout for quantification purposes. Even though we assume that recombination in cells occurred due to a previous cell fusion events between breast epithelial cells and breast cancer cells horizontal gene transfer (HGT) could be another cell fusion-independent mechanism [39], which could cause a *Cre*-mediated recombination in pFDR-transfected breast cancer cells. Thereby, pFDR-transfected breast cancer cells may have taken up apoptotic bodies derived from M13SV1-Cre cells containing (fragmented) DNA and/or mRNA by phagocytosis [40–42]. This assumption would be in view with a previous work of Bergsmedh and colleagues [43] in which a *Cre-LoxP-based* system was applied to study HGT. HGT is a potent mechanism how non-tumorigenic cells, like endothelial cells or fibroblasts, could functionally acquire tumor DNA [42, 44–46]. Moreover, data of Ehnfors and colleagues [46] showed that tumor cell DNA is not only taken up, but also replicated by fibroblasts and endothelial cells suggesting that the phenotype of recipient cells could be altered due to uptake of foreign DNA. Recent findings revealed that in vitro cellular transformation and tumorigenesis could be induced in murine NIH3T3 fibroblasts by treating them with serum of colon cancer patients of supernatant of SW480 human cancer cells [45]. In vivo studies showed that immunocompetent rats subjected to colon carcinogenesis with 1,2-dimethylhydrazine had an increased rate of colonic tumors when injected in the dorsum with human SW480 colon cancer cells as a source of circulating oncogenic DNA [45].

Thus the question remains whether the turn from red to green fluorescence was attributed to a cell fusion-independent, a cell fusion-dependent process or a combination of both. Comparison of the amount of the cell debris fraction in the obtained FACS data, which also contains apoptotic bodies, only showed a correlation for TNF-α and CWF-treated MDA-MB-435-pFDR breast cancer cells (supplemental data 2A, B) suggesting that the Cre-mediated recombination was attributed to HGT in a cell fusion-independent manner. On the contrary, comparison of the amount of cell debris in relation to the fold change of MDA-MB-435-pFDR green fluorescing cells in the TNFR-blocking studies revealed no correlation (supplemental data 2E). For instance, the amount of cell debris of IgG1 and TNF-α-treated MDA-MB-435-pFDR cells/M13SV1-Cre-Puro cells was markedly increased under normoxic, but not hypoxic conditions, albeit for both conditions a significantly increased relative fold change of green fluorescing cells was observed (Fig. 5a). Likewise, a marked increase in the amount of cell debris was observed for MDA-MB-435-pFDR cells/M13SV1-Cre-Puro cells

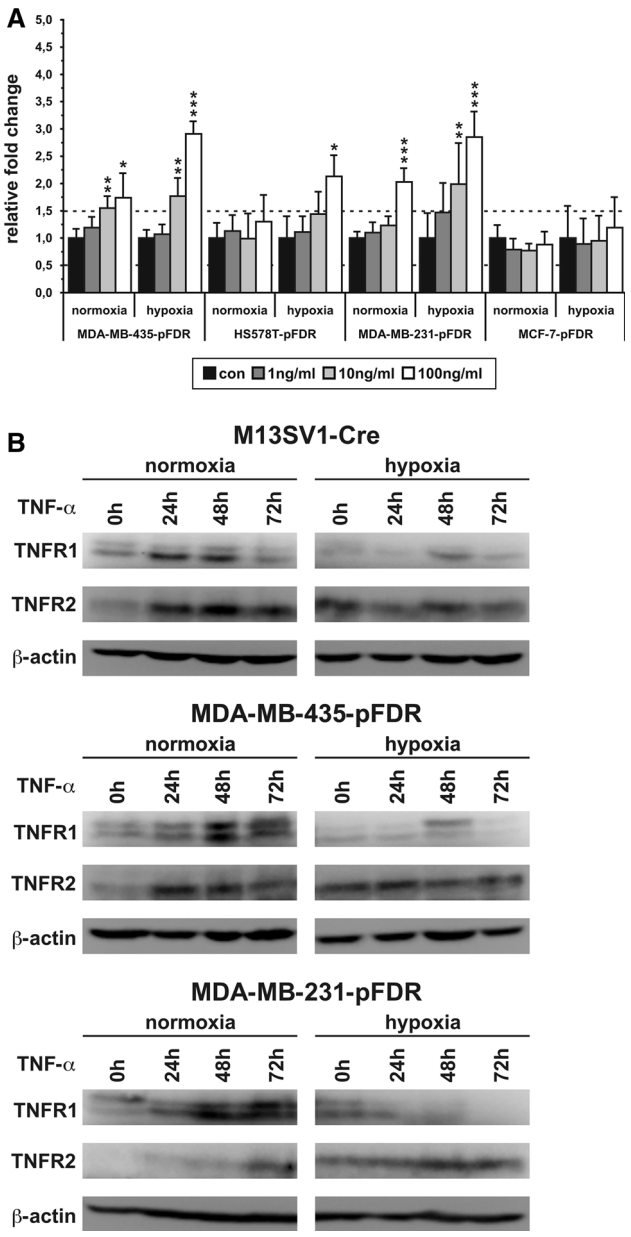

Fig. 4 TNF-α induces cell fusion in a dose-dependent manner in human breast cancer cell lines. Various concentrations of TNF-α were tested with regard to its influence of cell fusion. **a** Data clearly a dose-dependent effect on TNF-α on the fusion of MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells, which was additionally triggered when cells were cultured under hypoxic conditions. In accordance to MDA-MB-435-pFDR and MDA-MB-231-pFDR cells TNF-α also induced fusion of HS578T-pFDR breast cancer cells, but only under hypoxia. **b** Western blot analysis of TNFR1 and TNFR2 expression of M13SV1-Cre-PuroR breast epithelial cells and MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells under normoxic and hypoxic conditions and in time dependence of TNF-α stimulation (100 ng/ml). Shown are representative western blot data. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

treated with TNF-α and anti-TNFR2 under hypoxic conditions, but not for normoxic conditions (supplemental data 2E). Quantification of EGFP-expressing MDA-MB-435-pFDR cells treated with TNF-α and anti-TNFR2 under normoxic and hypoxic conditions revealed similar relative fold changes (Fig. 5). Because of this we conclude that the observed Cre-mediated recombination processes were mainly attributed to cell fusion events and not to HGT in a cell fusion-independent manner. This conclusion is further supported by the fact that for all other conditions and compounds, which resulted in an increased number of EGFP-positive cells, the amount of cell debris/apoptotic bodies remained unaltered in relation to controls (supplemental data 2). Moreover, we have already demonstrated that hybrid cell clones, derived from spontaneous fusion events between human M13SV1 breast epithelial cells and HS578T as well as MDA-MB-435 breast cancer cells exhibited a nearly doubled mean chromosomal number concomitant with an overlap of parental alleles (located on different chromosomes) [26, 28, 47]. Nonetheless, the impact of HGT in a cell fusion-independent manner can not be ruled out completely.

It is well recognized that various physiological and pathophysiological events depend on the biological phenomenon of cell fusion, including fertilization, placentation, muscle fiber formation, wound healing and tumor progression (for review see: [2, 3]). Even though some cell fusion mechanisms concomitant with cell fusion-associated proteins have already been deciphered considerably less is still known how the merging of two plasma membranes is managed. The fusion of two cells is not simply limited to the merging of the plasma membranes. Cell fusion is a highly regulated process that can be subdivided into five parts: (1) priming, (2) chemotaxis, (3) adhesion, (4) fusion and (5) post-fusion [10]. Moreover, the finding that only some cell types possess the capability to fuse with other cells and only under distinct conditions indicates that the cell fusion program can be switched-on and switched-off. Conjointly, cells are able to discriminate between themselves and other cells. Using a self-contact-inducing microfabricated substrate Sumida and colleagues demonstrated that self-contacts of normal epithelial cells are rapidly eliminated by membrane fusion between two opposing plasma membranes of a single cell, most likely via an E-cadherin and actin–myosin network-dependent mechanism [48]. E-cadherin is also involved in macrophage fusion, thereby giving rise to osteoclasts, whereby up-regulation of E-cadherin is induced by IL-4 [49]. IL-4 alone or in combination with M-CSF and RANKL has been associated with induction of a fusion-competent phenotype in macrophages [50]. Because many tumors, including gliomas, express high levels of the IL-4 receptor it was suggested that IL-4 might promote glioma cell–cell fusion [40]. However, in this study IL-4 did not foster the fusion of human breast cancer cells and human breast epithelial cells. This can be either attributed to fact that the tested cell

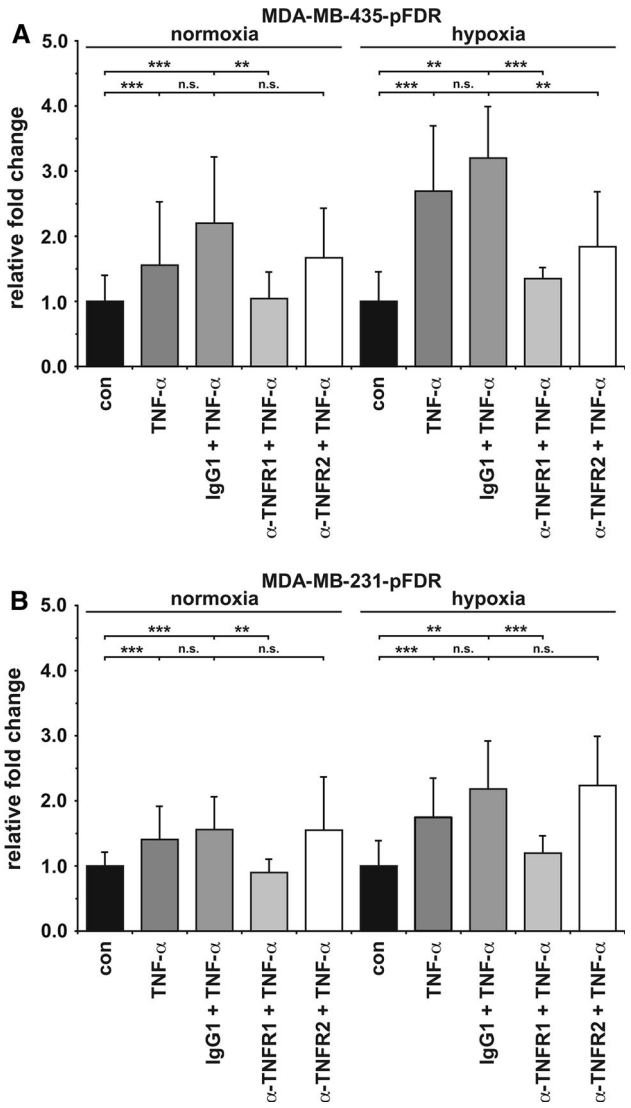

Fig. 5 Blocking of TNFR1 signaling effectively blocks the TNF-α-induced fusion of MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells. Cells were co-cultured under normoxic and hypoxic conditions in the presence of TNF-α (100 ng/ml) and monoclonal antibodies specifically blocking TNFR1 and TNFR2, respectively. Data clearly show that blocking of TNFR1 signaling completely inhibited the TNF-α-induced fusion of both breast cancer cell lines irrespective of normoxic or hypoxic conditions. **a** Data for MDA-MB-435-pFDR cells, **b** data for MDA-MB-231-pFDR cells. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

lines did not express the IL-4 receptor. On the contrary, it cannot be ruled out that, in case of being IL-4R-positive, the tested cell lines responded differently to IL-4 than macrophages. Moreover, studies on macrophages revealed that priming of macrophages towards a fusion-friendly phenotype did not only depend on IL-4 signaling alone, but also on cell–cell interactions, for example., via TREM-2/DAP12 [51]. Recently, Shabo et al. [52] demonstrated that DAP12 is expressed in breast cancer cells, which was further associated with a high tumor grade, liver and skeletal metastases and a general poor prognosis. However, the impact of DAP12 on tumor cell fusion processes remains unclear [52].

Our data indicate that three out of four tested human breast cancer cell lines responded to TNF-α treatment under hypoxic conditions with an increased fusion rate. TNF-α is well-known proinflammatory cytokine secreted by macrophages in response to pathogen invasion and/or tissue degeneration [53]. As mentioned above, inflammation is the strong trigger for cell fusion [16, 17]. Johansson et al. [17] reported that chronic inflammation resulting from severe dermatitis or autoimmune encephalitis led to robust fusion of BMDCs with Purkinje neurons and formation of hundreds of binucleate heterokaryons per cerebellum, a 10- to 100-fold higher frequency than previously reported. Similar data were provided for the fusion of BMDCs with intestine progenitors, whereby in addition to inflammation also proliferation was identified to be a positive trigger for cell fusion [16]. It is well recognized that the tumor microenvironment resembles chronically inflamed tissue [18, 19]. Thus, secretion of proinflammatory cytokines, like TNF-α, by invaded macrophages might be a putative trigger for tumor cell fusion. However, at present it remains unclear how TNF-α may foster cell fusion. Conceivably, and in analogy to macrophages, we conclude that TNF-α may prime (breast) tumor cells and their fusion partners, like breast epithelial cells, to adopt a more fusogenic phenotype. Blocking experiments revealed that the pro-fusogenic effect of TNF-α chiefly depended on TNFR1 signaling, which is known to activate several signal transduction cascades and induction of gene expression in an AP-1-, NF-κB- and MAPK-dependent manner [54]. Treatment of MDA-MB-231 breast cancer cells with TNF-α resulted in a dose-dependent up-regulation of MMP9 expression [55]. MMP9 is also up-regulated in IL-4 and/or M-CSF/RANKL induced pro-fusogenic macrophages and seems to be involved in the fusion process of the cells, since decreased MMP9 levels were associated with an impaired macrophage fusion rate [56]. Moreover, addition of exogenous TNF-α caused an increase in the production of MMP9 and an increased macrophage fusion frequency [56]. Thus, TNF-α might be a pro-fusogenic factor due to induction of MMP9 expression in human breast cancer cells and breast epithelial cells. However, it has to be considered that TNF-α stimulation does not only lead to MMP9 expression, but rather to an extensively altered gene expression pattern in the breast cancer cells including genes that are involved in cell fusion. Thus, it remains to be elucidated how TNF-α alters the gene expression profile of MDA-MB-435-pFDR and MDA-MB-231-pFDR breast cancer cells and M13SV1-Cre-PuroR breast epithelial cells and which of the differentially regulated genes, either alone or in combination, may contribute to cell fusion.

In addition to TNF-α an increased fusion rate was also observed for MDA-MB-231-pFDR cells stimulated with proinflammatory cytokine IL-1β. This finding is in view the data indicating that inflammation is a positive trigger for cell fusion. However, in accordance to TNF-α it remains unknown how IL-1β promote cell fusion of MDA-MB-231-pFDR breast cancer cells and M13SV1-Cre-PuroR breast epithelial cells. Like TNF-α also IL-1β can induce MMP9 expression in BT474 breast cancer cells [57] suggesting that IL-1β may promote MDA-MB-231-pFDR cell fusion via a MMP9-dependent mechanism. However, Ma and colleagues demonstrated that also EGF potently induced MMP9 expression in BT474 breast cancer cells [57]. Because our data revealed no impact of EGF on the fusion of stably pFDR-transfected human breast cancer cells with M13SV1-Cre-PuroR breast epithelial cells it can be concluded that a putative induction of MMP9 expression is not sufficient for promoting cell fusion.

Even though both TNF-α and IL-1β may promote the fusion of MDA-MB-231-pFDR breast cancer cells when applied as single agents this pro-fusogenic effect was absent when both cytokines were added simultaneously to the cells. Likewise, IL-1β potently blocked the TNF-α-induced fusion of MDA-MB-435-pFDR and HS578T-pFDR breast cancer cells. At present this observation remains ambiguous since both inflammatory cytokines commonly act synergistically together during inflammatory conditions. Moreover, this finding is opposing to data suggesting that inflammation is a positive trigger for cell fusion. On the other hand, the (chronically) inflamed (tumor) microenvironment does not only consist of TNF-α and IL-1β, but is rather a complex mixture of different cell types and of (proinflammatory) cytokines, chemokines, growth factors, matrix metalloproteinases and more [58], which all have an impact on the gene expression profile of the cells within. Thus, it cannot be ruled out that addition of a third or fourth factor to TNF-α and IL-1β will reverse the inhibitory IL-1β effect.

Our data further indicate that the pro-fusogenic effect of TNF-α was markedly increased when cells were cultured under hypoxic conditions. Comparison of MDA-MB-435-pFDR, HS578T-pFDR and MDA-MB-231-pFDR breast cancer cells and M13SV1-Cre-PuroR breast epithelial cells under normoxic and hypoxic conditions yielded in similar spontaneous fusion rates suggesting that hypoxia per se is not a pro-fusogenic condition, but most likely acts synergistically together with TNF-α. It is well recognized that hypoxia leads to the activation of the hypoxia-inducible factors (HIF) HIF-1α/HIF-2α and HIF-1β [59], which induces the transcription of numerous hypoxia-responsive genes including growth factors, cytokines, chemokines and matrix metalloproteinases that regulate angiogenesis, cell proliferation and survival, apoptosis as well as EMT, invasion and metastasis [59, 60]. Moreover, in tumor cells, inflammatory signals like TNF-α and hypoxia not only activate both NF-κB and HIF-1α—both transcription factors also activate each other—thus providing a positive feedback loop concomitant with an intensified expression of NF-κB- and HIF-1α-related target genes. Because TNF-α causes a NF-κB-dependent gene expression in cells [54], the positive feedback loop of NF-κB and HIF-1α might be an explanation for the increased cell fusion frequency of MDA-MB-453-pFDR and MDA-MB-231-pFDR cells and M13SV1-Cre-PuroR cells in the presence of TNF-α under hypoxic conditions. Data of Tang and colleagues revealed a hypoxia-induced expression of RANK and RANKL in a HIF-1α-dependent mechanism in human MDA-MB-231 and MCF-7 breast cancer cell lines [61]. Moreover, RANKL was able to induce migration in human MDA-MB-231 breast cancer cells [62] suggesting that RANKL may act in an autocrine and paracrine loop. In the context of cell fusion the interplay of RANK/RANKL is of interest since it facilitates macrophage fusion in a NF-κB-dependent mechanism [63]. Whether this may point to a putative involvement of RANK/RANKL in the TNF-α- and hypoxia-induced fusion of MDA-MB-231-pFDR and MDA-MB-435-pFDR breast cancer cells is not yet clear, but should be investigated in future studies.

In summary, here we have shown that the fusion of human breast cancer cell lines and a human breast epithelial cell line could be quantified by a Cre-LoxP-based recombination using a fluorescence double reporter vector. To us, this will help to identify factors, conditions and molecules that will favor the fusion of tumor cells concomitant with fostering tumor progression. Targeting these cell fusion-related structures would be a new aim to treat tumors in the future, thereby inhibiting the origin of tumor promoting cancer hybrid cells.

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References

1. Dittmar T (2002) Modern technologies in immune diagnosis. Infect Immun Pharmacol 1–2:17–19
2. Dittmar T, Zänker KS (2011) Cell fusion in health and disease, vol 1. Adv Exp Med Biol. Springer, Dordrecht
3. Dittmar T, Zänker KS (2011) Cell fusion in health and disease, vol 2. Adv Exp Med Biol. Springer, Dordrecht
4. Perez-Vargas J, Krey T, Valansi C, Avinoam O, Haouz A, Jamin M, Raveh-Barak H, Podbilewicz B, Rey FA (2014) Structural basis of eukaryotic cell–cell fusion. Cell 157(2):407–419. doi:10.1016/j.cell.2014.02.020

5. Huppertz B, Bartz C, Kokozidou M (2006) Trophoblast fusion: fusogenic proteins, syncytins and ADAMs, and other prerequisites for syncytial fusion. Micron 37(6):509–517
6. Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC Jr, McCoy JM (2000) Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature 403(6771):785–789
7. Podbilewicz B (2014) Virus and cell fusion mechanisms. Annu Rev Cell Dev Biol 30:111–139. doi:10.1146/annurev-cellbio-101512-122422
8. Cornelis G, Heidmann O, Bernard-Stoecklin S, Reynaud K, Veron G, Mulot B, Dupressoir A, Heidmann T (2012) Ancestral capture of syncytin-Car1, a fusogenic endogenous retroviral envelope gene involved in placentation and conserved in Carnivora. Proc Natl Acad Sci USA 109(7):E432–441. doi:10.1073/pnas.1115346109
9. Aguilar PS, Baylies MK, Fleissner A, Helming L, Inoue N, Podbilewicz B, Wang H, Wong M (2013) Genetic basis of cell–cell fusion mechanisms. Trends Genet 29(7):427–437. doi:10.1016/j.tig.2013.01.011
10. Zhou X, Platt JL (2011) Molecular and cellular mechanisms of mammalian cell fusion. Adv Exp Med Biol 713:33–64. doi:10.1007/978-94-007-0763-4_4
11. Alvarez-Dolado M, Martinez-Losa M (2011) Cell fusion and tissue regeneration. Adv Exp Med Biol 713:161–175. doi:10.1007/978-94-007-0763-4_10
12. Kozorovitskiy Y, Gould E (2003) Stem cell fusion in the brain. Nat Cell Biol 5(11):952–954
13. Vassilopoulos G, Russell DW (2003) Cell fusion: an alternative to stem cell plasticity and its therapeutic implications. Curr Opin Genet Dev 13(5):480–485
14. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422(6934):897–901
15. Willenbring H, Bailey AS, Foster M, Akkari Y, Dorrell C, Olson S, Finegold M, Fleming WH, Grompe M (2004) Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nat Med 10(7):744–748
16. Davies PS, Powell AE, Swain JR, Wong MH (2009) Inflammation and proliferation act together to mediate intestinal cell fusion. PLoS ONE 4(8):e6530. doi:10.1371/journal.pone.0006530
17. Johansson CB, Youssef S, Koleckar K, Holbrook C, Doyonnas R, Corbel SY, Steinman L, Rossi FM, Blau HM (2008) Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation. Nat Cell Biol 10(5):575–583
18. Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? Lancet 357(9255):539–545. doi:10.1016/S0140-6736(00)04046-0
19. Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 315(26):1650–1659
20. Dittmar T, Nagler C, Niggemann B, Zänker KS (2013) The dark side of stem cells: triggering cancer progression by cell fusion. Curr Mol Med 13(5):735–750
21. Duelli D, Lazebnik Y (2003) Cell fusion: a hidden enemy? Cancer Cell 3(5):445–448
22. Rachkovsky M, Sodi S, Chakraborty A, Avissar Y, Bolognia J, McNiff JM, Platt J, Bermudes D, Pawelek J (1998) Melanoma x macrophage hybrids with enhanced metastatic potential. Clin Exp Metastasis 16(4):299–312
23. Lu X, Kang Y (2009) Efficient acquisition of dual metastasis organotropism to bone and lung through stable spontaneous fusion between MDA-MB-231 variants. Proc Natl Acad Sci USA 106(23):9385–9390
24. Xu MH, Gao X, Luo D, Zhou XD, Xiong W, Liu GX (2014) EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. PLoS ONE 9(2):e87893. doi:10.1371/journal.pone.0087893
25. Miller FR, Mohamed AN, McEachern D (1989) Production of a more aggressive tumor cell variant by spontaneous fusion of two mouse tumor subpopulations. Cancer Res 49(15):4316–4321
26. Dittmar T, Schwitalla S, Seidel J, Haverkampf S, Reith G, Meyer-Staeckling S, Brandt BH, Niggemann B, Zanker KS (2011) Characterization of hybrid cells derived from spontaneous fusion events between breast epithelial cells exhibiting stem-like characteristics and breast cancer cells. Clin Exp Metastasis 28(1):75–90
27. Carloni V, Mazzocca A, Mello T, Galli A, Capaccioli S (2013) Cell fusion promotes chemoresistance in metastatic colon carcinoma. Oncogene 32(21):2649–2660. doi:10.1038/onc.2012.268
28. Dittmar T, Nagler C, Schwitalla S, Reith G, Niggemann B, Zanker KS (2009) Recurrence cancer stem cells—made by cell fusion? Med Hypotheses 73(4):542–547
29. Chang CC, Sun W, Cruz A, Saitoh M, Tai MH, Trosko JE (2001) A human breast epithelial cell type with stem cell characteristics as target cells for carcinogenesis. Radiat Res 155(1 Pt 2):201–207
30. Nolden L, Edenhofer F, Haupt S, Koch P, Wunderlich FT, Siemen H, Brustle O (2006) Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase. Nat Methods 3(6):461–467. doi:10.1038/nmeth884
31. Somers A, Jean JC, Sommer CA, Omari A, Ford CC, Mills JA, Ying L, Sommer AG, Jean JM, Smith BW, Lafyatis R, Demierre MF, Weiss DJ, French DL, Gadue P, Murphy GJ, Mostoslavsky G, Kotton DN (2010) Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. Stem Cells 28(10):1728–1740. doi:10.1002/stem.495
32. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
33. Koenen P, Spanholtz TA, Maegele M, Sturmer E, Brockamp T, Neugebauer E, Thamm OC (2015) Acute and chronic wound fluids inversely influence adipose-derived stem cell function: molecular insights into impaired wound healing. Int Wound J 12(1):10–16. doi:10.1111/iwj.12039
34. Voss MJ, Moller MF, Powe DG, Niggemann B, Zanker KS, Entschladen F (2011) Luminal and basal-like breast cancer cells show increased migration induced by hypoxia, mediated by an autocrine mechanism. BMC Cancer 11:158. doi:10.1186/1471-2407-11-158
35. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425(6961):968–973
36. Sprangers AJ, Freeman BT, Kouris NA, Ogle BM (2012) A Cre-Lox P recombination approach for the detection of cell fusion in vivo. J Vis Exp 59:e3581. doi:10.3791/3581
37. Heyder C, Gloria-Maercker E, Hatzmann W, Zänker KS, Dittmar T (2006) Circulating cancer cells: Flow cytometry, video microscopy and confocal laser scanning microscopy. In: Hayat MA (ed) Handbook of immunohistochemistry and in situ hybridization of human carcinomas, vol 4., Molecular genetics, gastrointestinal carcinoma, and ovarian carcinoma, vol 4Elsevier Academic Press, Burlington, pp 77–88
38. Record M (2014) Intercellular communication by exosomes in placenta: a possible role in cell fusion? Placenta 35(5):297–302. doi:10.1016/j.placenta.2014.02.009
39. Sinkovics JG (2011) Horizontal gene transfers with or without cell fusions in all categories of the living matter. Adv Exp Med Biol 714:5–89. doi:10.1007/978-94-007-0782-5_2

40. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ (2005) Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 5(11):899–904
41. Yan B, Wang H, Li F, Li CY (2006) Regulation of mammalian horizontal gene transfer by apoptotic DNA fragmentation. Br J Cancer 95(12):1696–1700. doi:10.1038/sj.bjc.6603484
42. Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, Holmgren L (2001) Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci USA 98(11):6407–6411
43. Bergsmedh A, Ehnfors J, Spetz AL, Holmgren L (2007) A Cre-loxP based system for studying horizontal gene transfer. FEBS Lett 581(16):2943–2946. doi:10.1016/j.febslet.2007.05.045
44. Nagler C, Hardt C, Zänker KS, Dittmar T (2011) Co-cultivation of murine BMDCs with 67NR mouse mammary carcinoma cells give rise to highly drug resistant hybrid cells. Cancer Cell Int 11:21
45. Trejo-Becerril C, Perez-Cardenas E, Taja-Chayeb L, Anker P, Herrera-Goepfert R, Medina-Velazquez LA, Hidalgo-Miranda A, Perez-Montiel D, Chavez-Blanco A, Cruz-Velazquez J, Diaz-Chavez J, Gaxiola M, Duenas-Gonzalez A (2012) Cancer progression mediated by horizontal gene transfer in an in vivo model. PLoS ONE 7(12):e52754. doi:10.1371/journal.pone.0052754
46. Ehnfors J, Kost-Alimova M, Persson NL, Bergsmedh A, Castro J, Levchenko-Tegnebratt T, Yang L, Panaretakis T, Holmgren L (2009) Horizontal transfer of tumor DNA to endothelial cells in vivo. Cell Death Differ 16(5):749–757. doi:10.1038/cdd.2009.7
47. Ozel C, Seidel J, Meyer-Staeckling S, Brandt BH, Niggemann B, Zanker KS, Dittmar T (2012) Hybrid cells derived from breast epithelial cell/breast cancer cell fusion events show a differential RAF-AKT crosstalk. Cell Commun Signal 10(1):10. doi:10.1186/1478-811X-10-10
48. Sumida GM, Yamada S (2013) Self-contact elimination by membrane fusion. Proc Natl Acad Sci USA 110(47):18958–18963. doi:10.1073/pnas.1311135110
49. Moreno JL, Mikhailenko I, Tondravi MM, Keegan AD (2007) IL-4 promotes the formation of multinucleated giant cells from macrophage precursors by a STAT6-dependent, homotypic mechanism: contribution of E-cadherin. In: J Leukoc Biol, vol 82. United States, pp 1542–1553. doi:10.1189/jlb.0107058
50. Helming L, Gordon S (2009) Molecular mediators of macrophage fusion. Trends Cell Biol 19:514–522. doi:10.1016/j.tcb.2009.07.005
51. Helming L, Tomasello E, Kyriakides TR, Martinez FO, Takai T, Gordon S, Vivier E (2008) Essential role of DAP12 signaling in macrophage programming into a fusion-competent state. Sci Signal 1:ra11. doi:10.1126/scisignal.1159665
52. Shabo I, Olsson H, Stal O, Svanvik J (2013) Breast cancer expression of DAP12 is associated with skeletal and liver metastases and poor survival. Clin Breast Cancer 13(5):371–377. doi:10.1016/j.clbc.2013.05.003
53. Xing Z, Jordana M, Kirpalani H, Driscoll KE, Schall TJ, Gauldie J (1994) Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor-alpha, macrophage inflammatory protein-2, interleukin-1 beta, and interleukin-6 but not RANTES or transforming growth factor-beta 1 mRNA expression in acute lung inflammation. Am J Respir Cell Mol Biol 10(2):148–153
54. Wu Y, Zhou BP (2010) TNF-alpha/NF-kappaB/Snail pathway in cancer cell migration and invasion. In: Br J Cancer, vol 102. England, pp 639–644. doi:10.1038/sj.bjc.6605530
55. Kim S, Choi JH, Kim JB, Nam SJ, Yang JH, Kim JH, Lee JE (2008) Berberine suppresses TNF-alpha-induced MMP-9 and cell invasion through inhibition of AP-1 activity in MDA-MB-231 human breast cancer cells. In: Molecules, vol 13. Switzerland, pp 2975–2985. doi:10.3390/molecules13122975
56. Skokos EA, Charokopos A, Khan K, Wanjala J, Kyriakides TR (2011) Lack of TNF-alpha-induced MMP-9 production and abnormal E-cadherin redistribution associated with compromised fusion in MCP-1-null macrophages. In: Am J Pathol, vol 178, United States, pp 2311–2321. doi:10.1016/j.ajpath.2011.01.045
57. Ma L, Lan F, Zheng Z, Xie F, Wang L, Liu W, Han J, Zheng F, Xie Y, Huang Q (2012) Epidermal growth factor (EGF) and interleukin (IL)-1beta synergistically promote ERK1/2-mediated invasive breast ductal cancer cell migration and invasion. In: Mol Cancer, vol 11, England, p 79. doi:10.1186/1476-4598-11-79
58. Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420(6917):860–867
59. Eltzschig HK, Carmeliet P (2011) Hypoxia and inflammation. N Engl J Med 364(7):656–665. doi:10.1056/NEJMra0910283
60. Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. Nature 454(7203):436–444. doi:10.1038/nature07205
61. Tang ZN, Zhang F, Tang P, Qi XW, Jiang J (2011) Hypoxia induces RANK and RANKL expression by activating HIF-1alpha in breast cancer cells. In: Biochem Biophys Res Commun, vol 408, United States, pp 411–416. doi:10.1016/j.bbrc.2011.04.035
62. Tang ZN, Zhang F, Tang P, Qi XW, Jiang J (2011) RANKL-induced migration of MDA-MB-231 human breast cancer cells via Src and MAPK activation. Oncol Rep 26(5):1243–1250. doi:10.3892/or.2011.1368
63. Yu M, Qi X, Moreno JL, Farber DL, Keegan AD (2011) NF-kappaB signaling participates in both RANKL- and IL-4-induced macrophage fusion: receptor cross-talk leads to alterations in NF-kappaB pathways. J Immunol, vol 187, United States, pp 1797–1806. doi:10.4049/jimmunol.1002628